



Relatório Técnico-Científico Intercalar Anual

ValorNatural – Valorização de Recursos Naturais através da Extração de
Ingredientes de Elevado Valor Acrescentado para Aplicações na
Indústria Alimentar

Sistema de Incentivos à Investigação e Desenvolvimento Tecnológico (SI & DT)
Programas Mobilizadores

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1. Identificação

Nº de projeto:	24479
Acrónimo do projeto:	VALORNATURAL
Título do projeto:	Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar
Data de início de projeto:	01/09/2018
Duração do projeto:	36 meses
Período de reporte do relatório:	01/09/2018 a 31/08/2019
Nº de relatório periódico:	2
“Web site” ou “microsite” do projeto:	www.valornatural.pt

2. Apresentação do consórcio

Promotor	PPS
TecPan - Tecnologia e Produtos para Pastelaria e Panificação, Lda.	1,3,4,5,8
Novavet - Produtos Agro - Pecuários, Lda.	1,5,8
Afonso, Lopes & C ^a , Lda.	1,5,8
Deifil Technology, Lda.	1,4,8
M. Ferreira & Filhas, Lda.	1,3,4,5,8
Paralab - Equipamentos Industriais e de Laboratório, S.A.	1,6,8
Vera Mata soluções Perfumadas, Lda.	1,4,8
Ponto Agrícola - Unipessoal, Lda.	1,3,8
Arménio Adérito Vaz	1,5,8
Instituto Politécnico de Bragança	1,3,4,5,6, 8
Universidade do Porto	1,5,6,8
Instituto de Ciência e Inovação em Engenharia Mecânica e Engenharia Industrial	1,6,8
Centro Nacional de Competências dos Frutos Secos	1,2,4,8
Instituto de Soldadura e Qualidade	1,8
Cooperativa Agrícola de Alfândega da Fé, CRL.	1,3,8

3. Sumário do projeto e seus objetivos globais

O uso de aditivos é uma prática comum no setor alimentar para aumentar o seu tempo de vida útil e/ou tornar os produtos mais apelativos. Existem mais de 2500 substâncias autorizadas para este fim, na maioria conservantes. Contudo, os efeitos nocivos associados ao seu consumo são cada vez mais conhecidos e suportados por estudos que alertam para o facto de muitos deles terem recebido, inadequadamente, o estatuto GRAS. Este cenário e as discrepâncias de legislação (EU vs. EUA), têm levado indústria e consumidores a aumentar a sua preferência por ingredientes naturais. Assim, o uso de aditivos sintéticos está cada vez mais limitado e, dada a tendência de mercado e conhecimento privilegiado do consórcio, prevê-se que muitos deles venham a ser proibidos.

Para viabilizar a produção industrial de ingredientes naturais é crucial desenvolver soluções que colmatem fragilidades da cadeia produtiva, nomeadamente: a) promover a produção sustentada de fontes naturais ricas em compostos de interesse e a utilização de bio resíduos; b) otimizar metodologias de extração, procurando eficiência e facilidade de scale-up; c) desenvolver equipamentos de extração/refinação versáteis e eficientes; d) desenvolver/aplicar técnicas de estabilização para aumentar a durabilidade, compatibilidade no processamento e eficácia nos produtos finais; e) otimizar metodologias de incorporação em produtos alvo. O projeto aborda adicionalmente aspetos ligados aos aromas e à conservação de matrizes naturais recorrendo a metodologias inovadoras.

Em suma, a solução proposta pelo consórcio, criado tendo em consideração áreas-chave e complementares, visa mobilizar uma cadeia de valor assente no desenvolvimento de ingredientes naturais nas classes dos corantes, aromas e bioativos, para utilização alternativa aos aditivos sintéticos.

Resumem-se de seguida os objetivos de cada PPS, de acordo com respetivo plano de trabalhos:

- A **PPS1 (gestão de projeto)** tem como promotor responsável o IPB e tem como principal objetivo garantir que as metas e entregáveis do projeto sejam alcançados com a qualidade desejada, e dentro do prazo e custos previstos. Sendo assim, tem como principais eixos de trabalho: a gestão técnica operacional e executiva do projeto.

- A **PPS 3 (corantes naturais)** tem como principal responsável o IPB e tem como objetivo central a obtenção de ingredientes naturais com efeito corante, a partir de flores comestíveis e bio resíduos de frutos, considerados desperdícios - o intuito é conferir-lhes uma utilização alternativa e com interesse económico. Tem como principais eixos de trabalho: a obtenção de ingredientes naturais com capacidade corante, a realização de estudos de estabilidade e metodologias de estabilização, o desenvolvimento de aplicações dos ingredientes corantes e a demonstração de protótipos em ambiente industrial operacional.
- A **PPS 4 (aromas e modelos de aromas)** tem como promotor responsável a OWNYA/Vera Mata e tem como objetivo o desenvolvimento de aromas naturais e modelos de aromas para utilização na indústria da panificação destinados à intensificação do aroma dos seus produtos, e para serem utilizados como estratégia de marketing olfativo. Sendo assim, tem como principais eixos de trabalho: a extração de aromas naturais com propriedades organolépticas de interesse para a indústria de panificação, o desenvolvimento de modelos de aromas para a indústria de panificação, a produção de protótipos industriais e a validação das aplicações de *marketing* olfativo.
- A **PPS 5 (bioativos naturais)** tem como principal responsável o IPB, e como objetivo central o desenvolvimento de produtos lácteos com micosteróis para efeitos hipocolesterolémicos, similares aos efeitos exibidos pelos produtos que incorporam fitoesteróis e, a fortificação de farinhas com vitamina D2 para o aumento da absorção de cálcio. Tem como principais eixos de trabalho: a obtenção de ingredientes naturais com bioatividade, realização de estudos de estabilidade, desenvolvimento de metodologias de estabilização, desenvolvimento de aplicações dos ingredientes bioativos e demonstração de protótipos em ambiente industrial operacional.
- A **PPS 6 (inovação em processos de extração, refinação e técnicas de conservação)** tem como líder a FEUP/LSRE, e tem como objetivo o desenvolvimento de processos inovadores associados à extração e refinação de ingredientes a partir de matérias-primas naturais, e ao desenvolvimento de novos processos de conservação. Sendo assim, tem como eixos de trabalho: inovação em processos de extração, em processos de refinação e em técnicas de conservação.
- A **PPS 8 (disseminação de informação e exploração de resultados)** tem como promotor responsável a TecPan, e tem como garantir que a comunicação do projeto e o impacto pretendido para o mesmo sejam alcançados com sucesso, bem como assegurar a

divulgação e exploração de resultados. Tem como principais eixos de trabalho: comunicação geral do mesmo, ações de vigilância tecnológica e inteligência competitiva, iniciativas de inovação aberta, gestão de propriedade industrial e análise de viabilidade de exploração económica dos resultados.

4. Sumário dos trabalhos realizados desde o início do projeto até ao final do período a que o relatório intercalar reporta, com especial enfoque ao período de reporte

4.1 PPS1 - Gestão de projeto

4.1.1 Diagrama de Gantt comparando o trabalho previsto na candidatura com o realizado



■ Realizado

Adiamentos em tom mais escuro

No que diz respeito à **PPS 1** decorreu tudo dentro do esperado, não se verificando nenhuma alteração ao cronograma estipulado.

4.1.2 Deliverables e milestones previstos desde o início do projeto até ao final do período a que o relatório intercalar reporta.

Nota: A numeração das Atividades e Tarefas nas secções seguintes inclui a nomenclatura utilizada no Anexo Técnico seguida da nomenclatura utilizada no Formulário de Candidatura. Por exemplo, a Atividade 1.1 no Anexo Técnico corresponde à Atividade 1 no Formulário de Candidatura, pelo que a denominação será 1.1 (1).

Deliverables

Nº do Deliverable	Nº da Tarefa	Título do Deliverable	Tipo de Deliverable	Data de entrega prevista no Anexo B	Data de entrega efetiva	Se não entregue, qual a data prevista para entrega	Nível de Divulgação	Comentários
1.2.1	1.2.1 (2.1)	Manual e documentação do projeto	Documento	30-09-2018	22/10/2018 (última atualização a 23-05-2019)	-	Confidencial	-
1.2.2	1.2.3 (2.3)	Relatório de execução semestral	Relatório	28-02-2018	27-03-2019	-	Confidencial	-
1.2.2	1.2.3 (2.3)	Relatório de execução semestral	Relatório	31-08-2019	4-10-2019	-	Confidencial	-

Milestones

Nº do Milestone	Nº da Tarefa	Título do Milestone	Meio de Verificação	Data de entrega prevista no Anexo B	Data de entrega efetiva	Alcançado?	Se não alcançado, qual a data prevista	Comentários
1.2.1	1.2.3 (2.3)	Relatório de execução anual	Documento	31-08-2019	04-10-2019	Sim		

4.1.3 Potenciais constrangimentos que possam dificultar a execução do projeto e medidas propostas para a sua mitigação

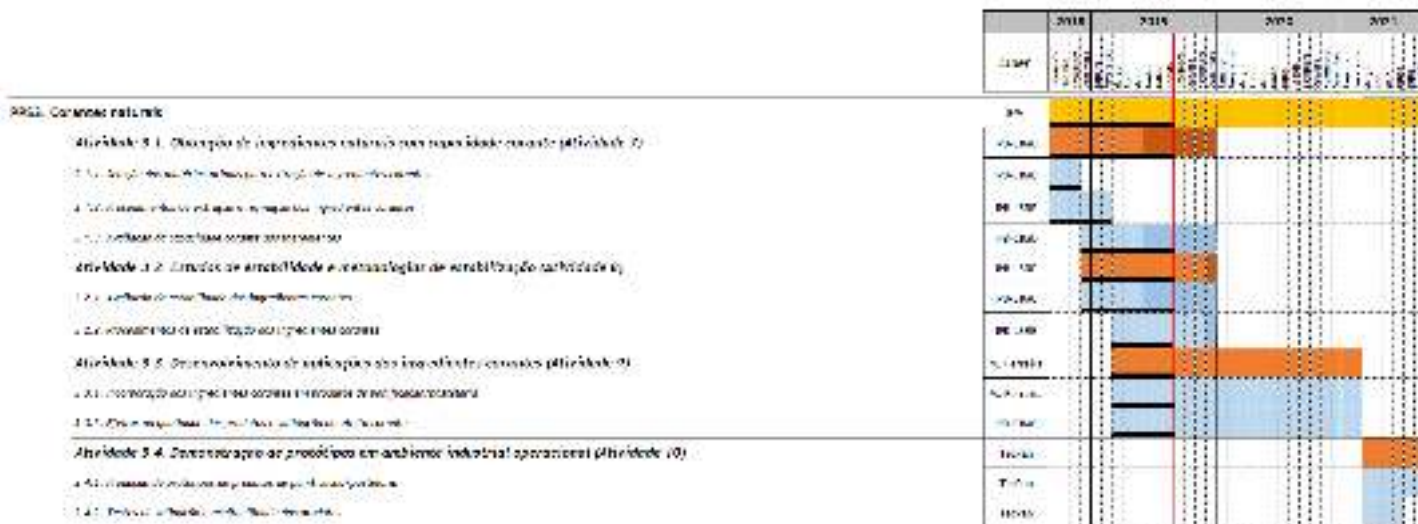
Não foram identificados riscos relevantes.

4.1.4 Promoção e divulgação de resultados

Não aplicável.

4.2 PPS3 - Corantes naturais

4.2.1 Diagrama de Gantt comparando o trabalho previsto na candidatura com o realizado



■ Realizado

Adiamentos identificados a cor mais escura.

O fim das tarefas 3.1.3, 3.2.1 e 3.2.2 foi adiado para dezembro de 2019 pois a gama de matrizes foi alargada, pelo que a respetiva capacidade corante, estabilidade, e processos de estabilização encontram-se ainda em estudo.

4.2.2 Deliverables e milestones previstos desde o início do projeto até ao final do período a que o relatório intercalar reporta.

Deliverables

Nº do Deliverable	Nº da Tarefa	Título do Deliverable	Tipo de Deliverable	Data de entrega prevista no Anexo B	Data de entrega efectiva	Se não entregue, qual a data prevista para entrega	Nível de Divulgação	Comentários
3.1.1	3.1.1 (7.1)	Folheto com procedimentos de colheita das matérias-primas	Folheto descritivo	30-11-2018	30-11-2018	-	Público	-

3.1.2	3.1.1 (7.1)	Base de dados com as matérias-primas mais ricas em moléculas corantes	Base de dados	30-11-2018	30-11-2018	-	Confidencial	-
3.1.3	3.1.2 (7.2)	Relatório com as especificações técnicas dos corantes a desenvolver	Relatório	30-11-2018	30-11-2018	-	Confidencial	-
3.1.4	3.1.2 (7.2)	Relatório das condições de extração ótimas para obtenção das moléculas corantes	Relatório	28-02-2019	28-02-2019	-	Confidencial	-
3.1.5	3.1.2 (7.2)	Relatório dos procedimentos de refinação dos ingredientes corantes	Relatório	28-02-2019	28-02-2019	-	Confidencial	-
3.1.6	3.1.3 (7.3)	Publicação dos ingredientes com maior capacidade corante e sem toxicidade	Publicação	31/05/2019	31/05/2019	-	Público	-
3.2.1	3.2.1 (8.1)	Folheto descritivo das condições que garantem a maior estabilidade dos ingredientes corantes	Folheto descritivo	31/05/2019	31/05/2019	-	Público	-

Milestones

Nº do Milestone	Nº da Tarefa	Título do Milestone	Meio de Verificação	Data de entrega prevista no Anexo B	Data de entrega efetiva	Alcançado?	Se não alcançado, qual a data prevista	Comentários
3.1.1	3.1.1 (7.1)	Recolha das matérias-primas para extração de ingredientes corantes	Base de dados	30-11-2018	30-11-2018	Sim	-	
3.1.2	3.1.3 (7.3)	Obtenção de ingredientes corantes	Relatório	31/05/2019	31/05/2019	Sim	-	-

4.2.3. Potenciais constrangimentos que possam dificultar a execução do projeto e medidas propostas para a sua mitigação

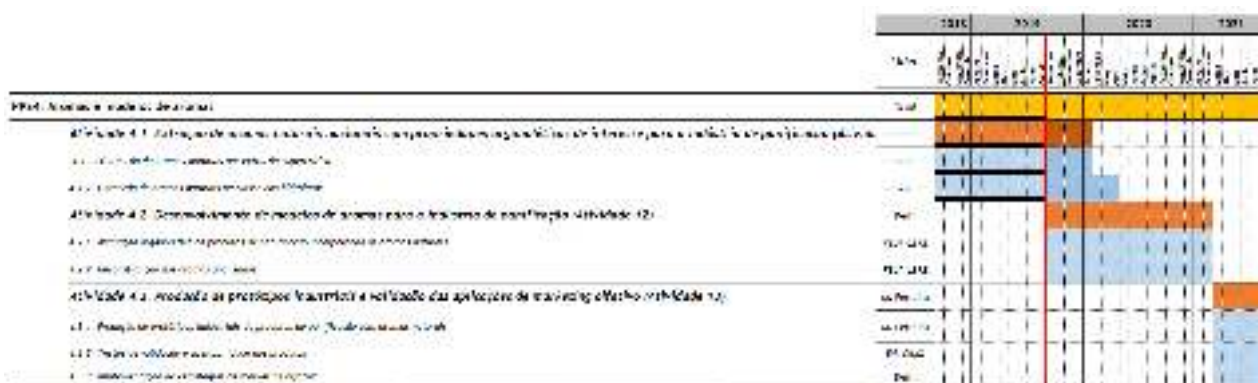
Risco	Probabilidade de ocorrência (alta, média, baixa)	Impacto (alto, médio, baixo)	Medida para mitigação
Processo para a obtenção de frações altamente puras em moléculas corantes, nomeadamente: (iso)goufreninas II e III, derivados de cianidinas, delphinidinas, gera custos elevados e/ou desperdícios significativos.	Médio	Alto	Uso de frações enriquecidas nas classes das moléculas de interesse corante.
Problemas de estabilidade e consequente degradação das moléculas corantes ao longo do tempo, perdendo a sua capacidade de coloração, influenciando a sua utilização nos alimentos finais.	Alto	Alto	Controlo dos parâmetros que afetam a estabilidade dos pigmentos e otimização de técnicas de estabilização para proteção das moléculas corantes ao longo do tempo.
Problemas de estabilidade das moléculas corantes nos produtos finais de panificação/pastelaria	Alto	Alto	Otimização pela avaliação do efeito corante em diferentes tempos e condições.
Os extratos enriquecidos em moléculas corantes não são facilmente dispersos nas formulações de panificação e pastelaria, resultando numa coloração heterogénea.	Médio	Baixo	Adaptação das formulações de panificação e pastelaria em coordenação com as empresas de panificação/pastelaria envolvidas.
Problemas de estabilidade e consequente degradação das moléculas corantes ao longo do tempo, perdendo a sua capacidade de coloração, influenciando a sua utilização nos alimentos finais.	Alto	Alto	Controlo dos parâmetros que afetam a estabilidade dos pigmentos e otimização de técnicas de estabilização para proteção das moléculas corantes ao longo do tempo.
Problemas de estabilidade das moléculas corantes nos produtos finais de panificação/pastelaria	Alto	Alto	Otimização pela avaliação do efeito corante em diferentes tempos e condições.
Os extratos enriquecidos em moléculas corantes não são facilmente dispersos nas formulações de panificação e pastelaria, resultando numa coloração heterogénea.	Médio	Baixo	Adaptação das formulações de panificação e pastelaria em coordenação com as empresas de panificação/pastelaria envolvidas.
Efeito corante nos produtos de pastelaria/panificação não ser o esperado por questões de variação da cor dependendo das características do produto.	Média	Alto	Estabilização dos corantes de modo a garantir uma maior afinidade com o produto e, consequentemente, uma menor variação da cor.
Alteração das propriedades dos produtos pela adição dos extratos corantes, afetando a sua qualidade.	Baixa	Médio	Reformulação dos extratos corantes de forma a contornar os problemas detetados.

4.2.4 Promoção e divulgação de resultados

Tipologia de Ação de promoção e divulgação de Resultados	Número de Ações
Press-Release	1
Publicações não científicas	4
Publicações científicas	17
Participação em Feiras e Exposições	1
Participação em Conferências	14

4.3 PPS4 - Aromas e modelos de aromas

4.3.1 Diagrama de Gantt comparando o trabalho previsto na candidatura com o realizado



■ Realizado

Adiamentos identificados a cor mais escura.

As tarefas 4.1.1 e 4.1.2 da atividade 4.1 sofreram um ligeiro atraso na sua execução (nova data de fim: 31/01/2020). Esta situação deveu-se ao atraso na instalação/formação e testes do equipamento usado para identificação dos aromas, assim como no atraso nas contratações das pessoas envolvidas na execução experimental da referida atividade. Assim, durante o segundo semestre do projeto iniciaram-se os ensaios de caracterização das matérias-primas, nomeadamente dos frutos secos. Com o objetivo de avaliar o conteúdo de água presente nas amostras de amêndoa e avelã, com e sem pele, foram realizados os testes de humidade e análises de termogravimetria diferencial de forma a se

monitorizar a perda de massa das amostras de amêndoa e avelã, em função da temperatura, num ambiente de temperatura e atmosfera controlado. Para avaliar a morfologia dos frutos secos antes e após a extração supercrítica, também foram realizadas análises de microscopia eletrónica de varrimento de amostras de amêndoa com pele. Durante este período foram iniciados os ensaios (preliminares) na unidade de extração de CO₂ supercrítico com o intuito de perceber o funcionamento da unidade alterando os valores de temperatura e pressão, tendo sido realizados os estudos para a obtenção dos extratos da amêndoa com pele. Relativamente aos ensaios na unidade de soxhlet foram realizados os ensaios de extração das amostras da amêndoa com e sem pele. Para a obtenção de aromas naturais na classe de hidrolatos iniciou-se a pesquisa bibliográfica referente às condições experimentais necessárias para a sua extração. Assim, por forma a concluir as tarefas da atividade 4.1 assume-se a data prevista para entrega do entregável E 4.1.2, 31 de janeiro de 2020 e o E 4.1.3, 30 de abril de 2020. Pretende-se ter concluído nestas datas os ensaios de extração tanto dos frutos secos como das plantas aromáticas, assim como os ensaios para obter os aromas naturais na classe dos hidrolatos e sua caracterização.

4.3.2 Deliverables e milestones previstos desde o início do projeto até ao final do período a que o relatório intercalar reporta.

Deliverables

Nº do Deliverable	Nº da Tarefa	Título do Deliverable	Tipo de Deliverable	Data de entrega prevista no Anexo B	Data de entrega efetiva	Se não entregue, qual a data prevista para entrega	Nível de Divulgação	Comentários
4.1.1.	4.1.1 (11.1)	Relatório com as especificações de extração dos aromas por extração supercrítica	Relatório	28-02-2019	28-02-2019	-	Confidencial	-
4.1.2	4.1.1 (11.1)	Relatório com a caracterização química e organoléptica dos aromas produzidos por extração supercrítica	Relatório	31-08-2019	-	31-01-2020	Confidencial	Nota 1
4.1.3	4.1.2 (11.2)	Relatório com a caracterização química e organoléptica dos hidrolatos e procedimentos de concentração	Relatório	31-08-2019	-	30-04-2020	Confidencial	Nota 2

Nota 1. A data de entrega prevista no Anexo B não foi atingida. Pretende-se entregar o referido entregável em janeiro de 2020. Foram obtidos através de extração supercrítica os aromas da amêndoa com pele, faltando assim os aromas da avelã e noz para os frutos secos. Ainda não foram iniciados os ensaios relativos às plantas aromáticas.

Nota 2. A data de entrega prevista no Anexo B não foi atingida. Pretende-se entregar o referido entregável em abril de 2020. Ainda não se iniciaram os ensaios de obtenção de aromas naturais na classe dos hidrolatos.

Milestones

Nº do Milestone	Nº da Tarefa	Título do Milestone	Meio de Verificação	Data de entrega prevista no Anexo B	Data de entrega efetiva	Alcançado?	Se não alcançado, qual a data prevista	Comentários
4.1.1	4.1.1 (11.1)	Recolha das matérias primas para a obtenção dos aromas naturais	Relatório	31-11-2018	28-02-2019	Sim	-	Foram rececionados os frutos secos em fevereiro e as plantas aromáticas serão rececionadas em março de 2019 (altura da recolha).
4.1.2	4.1.2 (11.2)	Obtenção dos aromas naturais	Relatório	31-08-2019	-	Não	31-01-2020	Nota 1.

Nota 1. Foram obtidos os aromas naturais para amêndoa com pele. Pretende-se obter os restantes aromas (frutos secos e plantas aromáticas) em janeiro de 2020. Foram observados atrasos na instalação/formação do equipamento de caracterização assim como na contratação das pessoas envolvidas na execução experimental da atividade 4.1.

4.3.3 Potenciais constrangimentos que possam dificultar a execução do projeto e medidas propostas para a sua mitigação

Risco	Probabilidade de ocorrer (alta, média, baixa)	Impacto (alto, médio, baixo)	Medida para mitigação
Obtenção dos aromas naturais por extração supercrítica	Média	Alto	Manutenção do equipamento de extração supercrítico e reposição dos consumíveis. Pesquisa prévia das melhores condições (temperatura e pressão) para a extração dos aromas dos frutos secos e plantas.
Obtenção dos aromas naturais por extração em soxhlet	Média	Alto	Manutenção do equipamento de extração e reposição dos consumíveis. Pesquisa prévia do solvente de extração e tempo de extração a ser usado para a extração dos aromas dos frutos secos e plantas.
Preparação e definição do método de identificação e quantificação dos aromas naturais a partir dos extratos em GC-MS das plantas e frutos secos.	Média	Alto	Pesquisa bibliográfica prévia dos aromas a serem extraídos, metodologias de preparação de amostras e definição do melhor método de identificação e quantificação dos aromas naturais.
Obtenção dos aromas naturais por hidrodestilação (hidrolatos)	Média	Alto	Instalação da unidade de obtenção dos aromas naturais por hidrodestilação (hidrolatos). Investigação do método e parâmetros de ensaio.

4.3.4 Promoção e divulgação de resultados

Sem informação a reportar.

4.4 PPS5 - Bioativos naturais

4.4.1 Diagrama de Gantt comparando o trabalho previsto na candidatura com o realizado



■ Realizado

Adiamentos identificados a cor mais escura.

No que diz respeito à tarefa 5.1.3, os ensaios de bioatividade do ergosterol com data inicial de término a 31 de maio de 2019, foram concluídos em julho de 2019. Relativamente aos ensaios de bioatividade da vitamina D2, prevê-se a conclusão deste estudo a 31 de outubro de 2019. Não foi ainda dado início à tarefa 5.3.2, uma vez que está pendente dos resultados da tarefa anteriormente referida.

4.4.2 Deliverables e milestones previstos desde o início do projeto até ao final do período a que o relatório intercalar reporta.

Deliverables

Nº do Deliverable	Nº da Tarefa	Título do Deliverable	Tipo de Deliverable	Data de entrega prevista no Anexo B	Data de entrega efetiva	Se não entregue, qual a data prevista para entrega	Nível de Divulgação	Comentários
5.1.1	5.1.1 (14.1)	Folheto com procedimentos de recolha dos bio-resíduos de cogumelos	Folheto descritivo	30-11-2018	30-11-2018	-	Público	-

5.1.2	5.1.1 (14.1)	Relatório do procedimento de conversão do ergosterol em vitamina D2	Relatório	30-11-2018	30-11-2018	-	Confidencial	-
5.1.3	5.1.2 (14.2)	Relatório com as especificações técnicas dos bioativos a desenvolver	Relatório	30-11-2018	30-11-2018	-	Confidencial	-
5.1.4	5.1.2 (14.2)	Relatório das condições de extração ótimas para a obtenção das moléculas bioativas	Relatório	28-02-2019	28-02-2019	-	Confidencial	-
5.1.5	5.1.2 (14.2)	Relatório dos procedimentos de refinação dos ingredientes bioativos	Relatório	28-02-2019	28-02-2019	-	Confidencial	-
5.1.6	5.1.3 (14.3)	Publicação dos ingredientes com maior capacidade hipocolesterémica e sem toxicidade	Publicação	31-05-2019	31-05-2019	-	Confidencial	-
5.1.7	5.1.3 (14.3)	Publicação dos ingredientes com melhor capacidade de aumento da absorção de cálcio e sem toxicidade	Publicação	31-05-2019	-	31/10/2019	Público	Nota 1
5.2.1	5.2.1 (15.1)	Folheto descritivo das condições que garantem a maior estabilidade dos ingredientes bioativos	Folheto descritivo	31-05-2019	31-05-2019	-	Público	-

Nota 1: Tendo em conta a otimização do tempo de irradiação e das doses ideais que permitem uma maior conversão do ergosterol em vitamina D2 e a subsequente otimização das condições de extração e estabilização, estes procedimentos requereram maior tempo de análise. Até ao momento foi possível obter um extrato refinado enriquecido em vitamina D2, e conhecer a sua estabilidade em diferentes condições. Deste modo, iniciaram-se os ensaios de bioatividade, nomeadamente a capacidade de favorecer a absorção de cálcio. Assim, prevemos a finalização deste entregável em outubro de 2019, data em que será possível obter as quantidades ideais de incorporação nas farinhas, e que nos permitirá obter a publicação com toda a informação referente aos ingredientes com capacidade de aumentar a absorção de cálcio.

Milestones

Nº do Milestone	Nº da Tarefa	Título do Milestone	Meio de Verificação	Data de entrega prevista no Anexo B	Data de entrega efetiva	Alcançado?	Se não alcançado, qual a data prevista	Comentários
5.1.1	5.1.1 (14.1)	Recolha das matérias primas para extração dos ingredientes bioativos e conversão de ergosterol em vitamina D2	Base de dados	30-11-2018	30-11-2018	Sim	-	-
5.1.2	5.1.3 (14.3)	Obtenção de ingredientes com capacidade	Relatório	31-05-2019	31/07/2019	Sim	-	-

		hipocolesterémica e sem toxicidade						
5.1.3	5.1.3 (14.3)	Obtenção de ingredientes com capacidade de aumentar a absorção de cálcio e sem toxicidade	Relatório	31-05-2019	-	Não	31/10/2019	-

4.4.3 Potenciais constrangimentos que possam dificultar a execução do projeto e medidas propostas para a sua mitigação

Risco	Probabilidade de ocorrer (alta, média, baixa)	Impacto (alto, médio, baixo)	Medida para mitigação
Problemas de estabilidade do ergosterol/extrato nos produtos lácteos finais.	Alto	Alto	Otimização pela avaliação do efeito hipocolesterémico em diferentes tempos e condições.
Problemas de estabilidade da vitamina D2 ao longo do tempo.	Alto	Alto	Otimização de técnicas de estabilização para proteção do ergosterol e da vitamina D2 ao longo do tempo.
Problemas de estabilidade da vitamina D2 nas farinhas.	Alto	Alto	Otimização pela avaliação da capacidade de aumento da absorção de cálcio em diferentes tempos e condições.

4.4.4 Promoção e divulgação de resultados

Tipologia de Ação de promoção e divulgação de Resultados	Número de Ações
Publicações científicas	4
Participação em Feiras e Exposições	1
Participação em Conferências	2

4.5 PPS6 - Inovação em processos de extração, refinação e técnicas de conservação

4.5.1 Diagrama de Gantt comparando o trabalho previsto na candidatura com o realizado



■ Realizado

Adiamentos identificados a cor mais escura.

Na **PPS 6**, até à data deste relatório, mantiveram-se as atividades dentro do que se encontrava programado à exceção da tarefa 6.1.2, tal como justificado na secção relativa a descrição detalhada das tarefas executadas. Assim, assume-se como novembro de 2019 a data prevista para entrega do Entregável 6.1.2 (Dossiê técnico do sistema laboratorial de extração SFE-CO₂) e para se atingir o Marco 6.1.1 (Fim do projeto do sistema laboratorial de extração SFE-CO₂).

4.5.2 Deliverables e milestones previstos desde o início do projeto até ao final do período a que o relatório intercalar reporta.

Deliverables

Nº do Deliverable	Nº da Tarefa	Título do Deliverable	Tipo de Deliverable	Data de entrega prevista no Anexo B	Data de entrega efectiva	Se não entregue, qual a data prevista para entrega	Nível de Divulgação	Comentários
6.1.1	6.1.1 (18.1)	Lista dos requisitos de funcionamento do sistema laboratorial de extração SFE-CO ₂	Relatório	28-02-2019	28-02-2019	-	Confidencial	-
6.1.2	6.1.2 (18.2)	Dossier técnico do sistema laboratorial de extração SFE-CO ₂	Relatório	31-08-2019	-	31-11-2019	Confidencial	Ver secção 5.5.2
6.2.1	6.2.1 (19.1)	Lista dos requisitos de funcionamento do sistema laboratorial de extração e refinação	Relatório	31-05-2019	31-05-2019	-	Confidencial	-
6.3.1	6.3.1 (20.1)	Lista dos requisitos de funcionamento do sistema laboratorial de produção de hidratos de CO ₂	Relatório	31-05-2019	31-05-2019	-	Confidencial	-

Milestones

Nº do Milestone	Nº da Tarefa	Título do Milestone	Meio de Verificação	Data de entrega prevista no Anexo B	Data de entrega efetiva	Alcançado?	Se não alcançado, qual a data prevista	Comentários
6.1.1	6.1.2 (18.2)	Fim do projeto do sistema laboratorial de extração SFE-CO ₂	Relatório	31-08-2019	-	Não	31-11-2019	Ver secção 5.5.2

4.5.3 Potenciais constrangimentos que possam dificultar a execução do projeto e medidas propostas para a sua mitigação

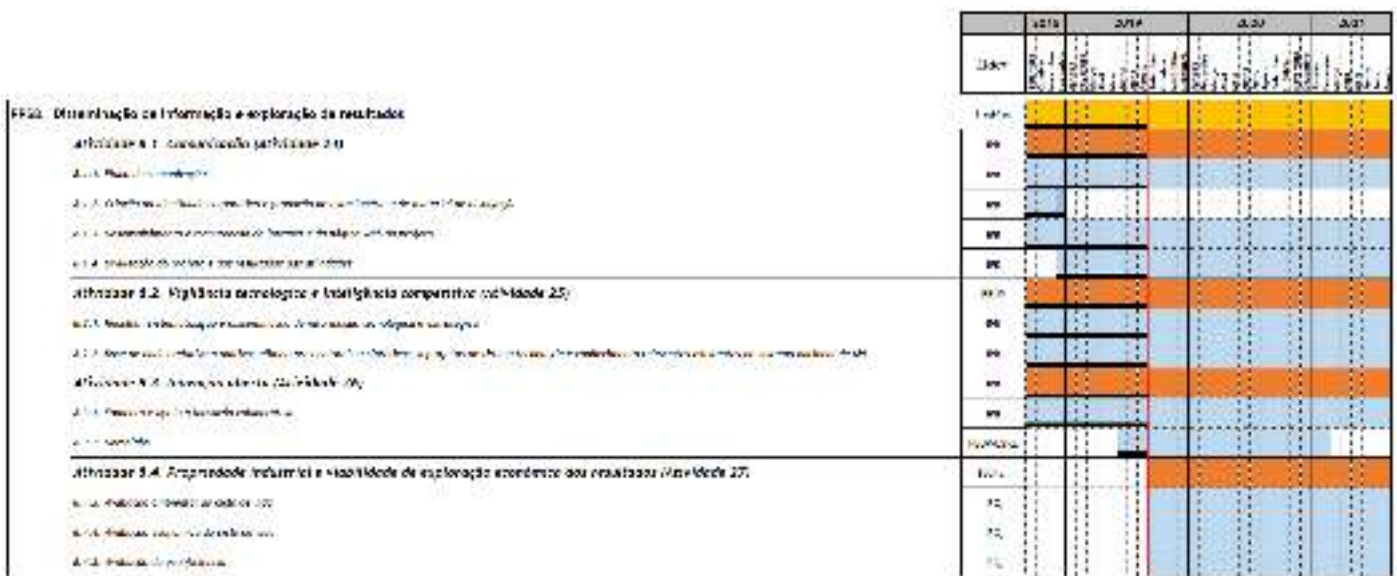
Risco	Probabilidade de ocorrer (alta, média, baixa)	Impacto (alto, médio, baixo)	Medida para mitigação
Fornecedores de equipamentos standard não possuem equipamentos em stock atrasando as entregas	Média	Alto	Antecipar os pedidos de orçamentação nas respetivas tarefas.
Fornecedor de equipamentos customizados atrasar a entrega por excesso de trabalho a realizar	Alta	Alto	Inquirir mais que um fornecedor sobre os prazos de entrega e questionar sobre possibilidades de atraso.

4.5.4 Promoção e divulgação de resultados

Sem informação a reportar.

4.6 PPS8 - Disseminação de informação e exploração de resultados

4.6.1 Diagrama de Gantt comparando o trabalho previsto na candidatura com o realizado



■ Realizado

Adiamentos identificados a cor mais escura.

No que diz respeito à **PPS 8** verificaram-se os seguintes desvios face ao que estava planeado: a base de dados relativa a aditivos alimentares e a projetos de I&I e a tecnologia e conhecimento relevantes (Entregável 8.2.3) tinha data prevista de conclusão no dia 28 de fevereiro de 2019, porém o prazo foi alargado de forma a concluírem-se as tarefas de programação, e esta foi terminada e tornada pública no dia 13 de setembro de 2019; a implementação de um portal *Web* de inovação colaborativa tinha data de entrega prevista no dia 31 de maio de 2019, porém sofreu um adiamento, de forma a conciliar o lançamento com a realização dos Demolabs, pelo que a data prevista de entrega será dia 4 de outubro de 2019; A realização da primeira edição do evento “Demolabs

ValorNatural” tinha data prevista de realização no dia 31 de agosto de 2019 e realizar-se-á dia 4 de outubro de 2019. Esta alteração pretende fazer coincidir as datas dos Demolabs com o fim de cada ano de execução do projeto, de forma a otimizar a divulgação de resultados. A data de início prevista em sede de candidatura para as tarefas 8.4.3, 8.4.4 e 8.4.5 era setembro de 2018. No entanto, tratou-se de um lapso, pois os novos processos tecnológicos apenas se encontrarão num estado de desenvolvimento que permita o começo dos trabalhos em setembro de 2019.

4.6.2 Deliverables e milestones previstos desde o início do projeto até ao final do período a que o relatório intercalar reporta.

Deliverables

Nº do Deliverable	Nº da Tarefa	Título do Deliverable	Tipo de Deliverable	Data de entrega prevista no Anexo B	Data de entrega efetiva	Se não entregue, qual a data prevista para entrega	Nível de Divulgação	Comentários
8.1.1	8.1.1 (24.1)	Plano de Comunicação	Documento	31/10/2018	30/10/2018	-	Público	-
8.1.2	8.1.2 (24.2)	Imagem e estacionário	Documento	31/12/2018	31/12/2018	-	Público	-
8.1.3	8.1.2 (24.2)	Materiais de promoção e divulgação	Publicação	31/12/2018	31/12/2018	-	Público	-
8.1.4	8.1.3 (24.3)	Website e Intranet	Website	31/12/2018	6/02/2019	-	Público	-
8.2.1	8.2.1 (25.1)	Publicação de boletins informativos semestrais	Publicação	-	28/01/2019, 21/03/2019, 24/05/2019	-	Público	-
8.2.3	8.2.2 (25.2)	Base de dados relativa a aditivos alimentares e a projetos de I&I e a tecnologia e a conhecimento relevantes	Base de dados	28/02/2019	19/09/2019	-	Público	-
8.3.1	8.3.1 (26.1)	Implementação de um portal Web de inovação colaborativa	Website	31/05/2019	-	4/10/2019	Público	-

Milestones

Nº do Milestone	Nº da Tarefa	Título do Milestone	Meio de Verificação	Data de entrega prevista no Anexo B	Data de entrega efetiva	Alcançado?	Se não alcançado, qual a data prevista	Comentários
8.1.1	8.1.3 (24.3)	Entrada em operação do Website, Intranet	Website	31/12/2018	31/12/2018	Sim	-	-

		e contas das redes sociais						
8.2.1	8.2.1 (25.1)	Entrada em funcionamento da plataforma de vigilância tecnológica e inteligência competitiva	Website	28/02/2019	28/02/2019	Sim	-	-
8.2.2	8.2.2 (25.2)	Informação aberta e acessível relativa a aditivos alimentares autorizados com base no código E	Website	28/02/2019	13/09/2019	Sim	-	-
8.3.1	8.3.1 (26.1)	Implementação da plataforma on-line de inovação colaborativa	Website	31/05/2019	-	Não	4/10/2019	-
8.3.2	8.3.2 (26.2)	Primeira edição dos <i>Demolabs</i>	Evento	31/08/2019	-	Não	4/10/2019	-

4.6.3 Potenciais constrangimentos que possam dificultar a execução do projeto e medidas propostas para a sua mitigação

Sem informação a reportar.

4.6.4 Promoção e divulgação de resultados

Tipologia de Ação de promoção e divulgação de Resultados	Número de Ações
Web Site	1
Participação em Feiras e Exposições	2
Outros (Redes sociais: <i>Facebook, Instagram, LinkedIn, Twitter</i>)	4

5. Apresentação dos desenvolvimentos obtidos no período de reporte a que o presente relatório respeita

5.1 PPS1 - Gestão de projeto

5.1.1 Apresentação dos resultados alcançados

No que diz respeito à **PPS 1** foi executada a gestão integrada das PPSs do projeto, conciliando e apoiando diversas áreas, tanto financeira e técnica como administrativa, para garantir o sucesso de cada uma delas. Foram desenvolvidos modelos de documentos, e guias de procedimentos que serão utilizados por todos os parceiros: manual de procedimentos, relatórios do projeto, registo de afetação de técnicos, comunicação de ações de publicitação e disseminação, relatório técnico-científico, e organização do dossier de projeto. Foram elaborados os relatórios semestral e anual relativos a todas as PPSs, com descrição dos resultados, entregáveis e marcos alcançados, da execução financeira de cada copromotor, e de desvios no cronograma.

Atividade: 1.1 (1) – Gestão técnica do projeto (Líder da Atividade: IPB)

Tarefa	1.1.1 (1.1) – Gestão técnica do projeto
Líder da tarefa	IPB
Participantes	Todos
Data de início	1-09-2018
Data de fim	31-08-2021

Descrição dos trabalhos realizados:

Gestão técnica do projeto.

Resultados:

De acordo com o Guia de Apoio ao Preenchimento do Formulário de Candidatura, esta tarefa combinou a gestão das despesas com técnicos oficiais de contas, custos indiretos e ainda outras despesas de suporte ou transversais às restantes tarefas e atividades do projeto.

Atividade: 1.2 (2) – Gestão operacional e executiva do projeto (Líder da Atividade: IPB)

Tarefa	1.2.1 (2.1) – Documentação do projeto
Líder da tarefa	IPB
Participantes	IPB
Data de início	1-09-2018
Data de fim	30-11-2018

Descrição dos trabalhos realizados:

Ao longo do período de tempo a que este relatório se reporta, foram desenvolvidos modelos de documentos e guias de procedimento que foram distribuídos por todos os membros do consórcio. Estes documentos são fundamentais, por exemplo, no que diz respeito aos relatórios intercalares e anuais, para controlo de versão de documentos, monitorização de entregáveis, deteção antecipada de eventuais problemas e para a comunicação no projeto.

Resultados:

Foi elaborado o Manual de Procedimentos, bem como diferentes modelos de documentos: modelos de relatórios do projeto, modelos de registo de afetação de técnicos, modelos para a comunicação de ações de publicitação e disseminação, modelo do relatório técnico-científico, e modelo de organização do dossier de projeto.

Tarefa	1.2.2 (2.2) – Gestão Administrativa e Financeira
Líder da tarefa	IPB
Participantes	IPB
Data de início	1-09-2018
Data de fim	31-08-2021

Descrição dos trabalhos realizados:

Através do serviço de secretariado administrativo e da secção de contabilidade foi executada a gestão administrativa e financeira adequada à realização de todas as atividades e tarefas definidas.

Resultados:

No que diz respeito à tarefa em questão foi necessário apoio nas reuniões periódicas, apoio administrativo ao Gestor e à Coordenadora do projeto, assim como ao Conselho de Administração (CA) e ao Conselho Consultivo (CC), apoio na comunicação entre parceiros e entre estes e a estrutura do Portugal 2020. No âmbito desta PSS também foi

executada a gestão das tarefas administrativas diárias, e a gestão financeira do projeto em geral.

Tarefa	1.2.3 (2.3) – Reporte
Líder da tarefa	IPB
Participantes	Todos
Data de início	1-09-2018
Data de fim	31-08-2021

Descrição dos trabalhos realizados:

Foram desenvolvidos os relatórios de execução semestrais, bem como o relatório anual do projeto.

Resultados:

No âmbito desta tarefa foram realizados os relatórios de execução semestral relativos às PPSs com execução durante o primeiro semestre, e o relatório anual onde foram descritas todas as atividades, tarefas, resultados alcançados, e possíveis desvios correspondentes ao primeiro ano. Estes foram disponibilizados à coordenação do projeto e a todos os membros do CA para serem revistos, e posteriormente discutidos nas reuniões periódicas, destinadas ao efeito. A equipa de gestão do projeto elaborou e distribuiu o relatório anual síntese aos membros do CC, de forma a poderem apresentar recomendações nas reuniões anuais de seguimento do projeto. A documentação elaborada, os modelos dos relatórios e as versões finais de todos os documentos foram sendo atualizadas na *Intranet* do projeto, à qual todos os copromotores têm acesso.

Tarefa	1.2.4 (2.4) – Gestão de conflitos e de propriedade industrial
Líder da tarefa	IPB
Participantes	Todos
Data de início	1-09-2018
Data de fim	31-08-2021

Descrição dos trabalhos realizados:

Durante o primeiro ano de execução a equipa de gestão apoiou a coordenação na resolução de questões operacionais que foram surgindo no decorrer das atividades previstas.

Resultados:

Os procedimentos para a tomada de decisões, definidos previamente, encontram-se descritos no Manual de Procedimentos, e são cruciais para lidar com questões que surgem no decorrer das Atividades e Tarefas respetivas.

5.1.2 Desvios e correções realizadas

No que diz respeito à **PPS 1** não foi necessário realizar qualquer tipo de alteração aos objetivos e atividades previamente estipulados para o período a que este relatório se refere.

5.1.3 Execução Financeira por promotor, apresentando os desvios, por rubrica, face ao previsto em candidatura

Tecpan – Tecnologia e Produtos para Pastelaria e Panificação, LDA.

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	29.774,70	6.131,52
a.xi) Despesas com TOC/ROC	694,50	0,00
b) Custos indiretos	7.443,67	1.532,88

Afonso, Lopes & CA, LDA.

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.xi) Despesas com TOC/ROC	104,28	0,00

Paralab – Equip. Industriais e de Laboratório S.A.

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.xi) Despesas com TOC/ROC	694,8	0,00
b) Custos indiretos	173,7	0,00

Instituto Politécnico de Bragança

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	21.651,91	7.808,83
a.x) Despesas com intervenção de Auditor técnico-científico	1.200,00	0,00
b) Custos indiretos	5.412,98	1.952,21

5.2 PPS3 – Corantes naturais**5.2.1 Apresentação dos resultados alcançados**

No âmbito da **PPS 3**, durante o primeiro semestre foi possível selecionar as matérias-primas mais ricas em compostos corantes e otimizar a sua obtenção a partir das mesmas. Foi ainda dado início a estudos de capacidade corante dos extratos, estabilidade, e processos de estabilização dos mesmos. No decorrer do segundo semestre de execução do projeto, foi dada continuação à avaliação da capacidade corante dos ingredientes desenvolvidos bem como da sua estabilidade. Os resultados obtidos tornaram possível a publicação dos ingredientes com maior capacidade corante e sem toxicidade, bem como a elaboração de um folheto descritivo das condições que garantem a maior estabilidade dos ingredientes corantes extraídos a partir das matrizes estudadas. Os corantes estabilizados foram introduzidos em diversos produtos de pastelaria e panificação (e.g. pastas de açúcar, creme de pasteleiro, croissants, waffles, biscoitos, cobertura de donuts, etc.), nos quais se avaliou a capacidade corante e os efeitos na qualidade dos produtos, nomeadamente no perfil nutricional e químico.

Atividade 3.1 (7) – Obtenção de ingredientes naturais com capacidade corante (Líder da Atividade: IPB-CIMO)

Tarefa	3.1.1 (7.1) - Seleção das matérias-primas para extração de ingredientes corantes
Líder da tarefa	IPB-CIMO
Participantes	Pragmático Aroma, Lda.; Ângelo Miguel Jorge de Oliveira “Produtor de medronho em Torre de Moncorvo”; Cooperativa Portuguesa do Medronho; Cooperativa Agrícola de Alfândega da Fé, CRL., Ponto Agrícola - Unipessoal, Lda.
Data de início	01/09/2018
Data de fim	30/11/2018

Descrição dos trabalhos realizados:

Seleção das matérias-primas para extração de ingredientes corantes. Desenvolvimento de procedimentos de colheita de várias espécies vegetais com vista à sua valorização através da extração dos seus ingredientes de valor agregado, nomeadamente compostos corantes (compostos fenólicos antociânicos e betacianinas) que podem ser posteriormente utilizados pela indústria alimentar em substituição de corantes sintéticos. As matrizes estudadas são na sua maioria provenientes de várias regiões de Portugal, no entanto, foram também estudadas matrizes provenientes da Alemanha e Tunísia.

Resultados:

As matrizes selecionadas e recolhidas para extração de ingredientes corantes de valor acrescentado foram: *Arbutus unedo* L. (Medronho, fruto, Torre de Moncorvo, Portugal); *Beta vulgaris* L. (Beterraba, colo tuberoso sem casca, Bragança, Portugal); *Carissa macrocarpa* (Eckl.) A.DC. (Ameixeira de Natal, fruto, Monastir, Tunísia); *Centaurea Cyanus* L. (Centaurea, pétalas, Castro Daire, Portugal/ Münster, Alemanha); *Dalia mignon* (Dália, pétalas, Castro Daire, Portugal); *Ficus carica* L. (Figo, casca externa da infrutescência, Bragança, Portugal); *Hibiscus sabdariffa* L. (Vinagreira, cálice, Alfândega da Fé, Portugal); *Prunus avium* L. (Cereja, fruto, Bragança, Portugal); *Prunus spinosa* L. (Abrunho, epicarpo, Bragança, Portugal); *Rosa damascena* ‘Alexandria’ e *R. gallica* ‘Francesa’ enxertada em *R. canina* (Rosa, pétalas, Castro Daire, Portugal); *Rubus umilfolius* Schott (Amora silvestre, fruto, Bragança, Portugal); *Sambucus nigra* L. (Sabugueiro, frutos, Bragança, Portugal); *Vaccinium myrtillus* L. (Mirtilo, fruto, Portugal).

Todas estas matrizes revelaram grandes potencialidades, no entanto, é nos frutos e flores que se encontram maiores quantidades de compostos de capacidade corante.

Tarefa	3.1.2 (7.2) - Procedimentos de extração e refinação dos ingredientes corantes
Líder da tarefa	IPB-LSRE
Participantes	IPB-CIMO; TecPan - Tecnologia e Produtos para Pastelaria e Panificação, Lda.; M. Ferreira & Filhas, Lda.; Pragmático Aroma, Lda.; Ângelo Miguel Jorge de Oliveira “Produtor de medronho em Torre de Moncorvo”; Cooperativa Portuguesa do Medronho; Cooperativa Agrícola de Alfândega da Fé, CRL., Ponto Agrícola - Unipessoal, Lda.
Data de início	01/09/2018
Data de fim	28/02/2019

Descrição dos trabalhos realizados:

Procedimentos de extração e refinação dos ingredientes corantes. Extração de ingredientes corantes das matérias-primas selecionadas com recurso a uma técnica convencional, a maceração assistida por calor, e através de dois métodos alternativos, a extração assistida por micro-ondas e ultrassons.

Resultados:

De entre as técnicas aplicadas, de um modo geral, as extrações assistidas por micro-ondas ou ultrassons possibilitaram uma maior recuperação de compostos das matrizes vegetais; no entanto, a eficiência de extração está muito relacionada com o tipo de matéria-prima em estudo. Foram otimizadas as condições de extração das moléculas corantes a partir das matrizes selecionadas. Os resultados encontram-se descritos em detalhe no Entregável nº 3.1.4 – Relatório das condições de extração ótimas para obtenção das moléculas corantes.

Tarefa	3.1.3 (7.3) – Avaliação da capacidade corante dos ingredientes
Líder da tarefa	IPB-CIMO
Participantes	TecPan - Tecnologia e Produtos para Pastelaria e Panificação, Lda.; M. Ferreira & Filhas, Lda.
Data de início	01/12/2018
Data de fim	31/12/2019

Descrição dos trabalhos realizados:

Avaliação da capacidade corante dos ingredientes.

Resultados:

A capacidade corante de alguns dos extratos obtidos a partir das matérias-primas selecionadas foi avaliada com sucesso (entregável E3.1.6), encontrando-se ainda em curso o estudo das restantes matrizes.

Atividade 3.2 (8) – Estudos de estabilidade e metodologias de estabilização (Líder da Atividade: IPB-LSRE)

Tarefa	3.2.1 (8.1) – Avaliação de estabilidade dos ingredientes corantes
Líder da tarefa	IPB-CIMO
Participantes	IPB-LSRE; TecPan - Tecnologia e Produtos para Pastelaria e Panificação, Lda.; M. Ferreira & Filhas, Lda.
Data de início	01/12/2018
Data de fim	31/12/2019

Descrição dos trabalhos realizados:

Avaliação da estabilidade dos ingredientes corantes.

Resultados:

A estabilidade dos ingredientes corantes obtidos até ao momento foi avaliada com sucesso (entregável E3.1.6), estando ainda em curso a avaliação de novos extratos.

Tarefa	3.2.2 (8.2) – Procedimentos de estabilização dos ingredientes corantes
Líder da tarefa	IPB-LSRE
Participantes	IPB-CIMO; TecPan - Tecnologia e Produtos para Pastelaria e Panificação, Lda.; M. Ferreira & Filhas, Lda.
Data de início	01/03/2019
Data de fim	31/12/2019

Descrição dos trabalhos realizados:

Procedimentos de estabilização dos ingredientes corantes.

Resultados:

Até ao momento, foram desenvolvidas várias formulações em pó para a estabilização de ingredientes corantes, através da otimização de procedimentos de spray-drying e usando vários materiais encapsulantes (maltodextrina e goma arábica) para as matrizes vegetais *Beta vulgaris* L., *Gomphrena globosa* L., *Lonicera caerulea* L., *Morus nigra* L., *Rubus fruticosus* Linnaeus e *Sambucus nigra* L. Foi também otimizado para a estabilização do extrato comercial de curcumina, extraído de *Curcuma longa* L. usando a técnica de spray-congealing com o material encapsulante cera de abelha. Todos estes procedimentos estão descritos no entregável E3.2.1.

Atividade 3.3 (9) – Desenvolvimento de aplicações dos ingredientes corantes (Líder da Atividade: M.Ferreira)

Tarefa	3.3.1 (9.1) – Incorporação de ingredientes corantes em produtos de panificação/pastelaria
Líder da tarefa	M. Ferreira & Filhas, Lda.
Participantes	IPB-CIMO; IPB-LSRE; TecPan - Tecnologia e Produtos para Pastelaria e Panificação, Lda.; Cooperativa Agrícola de Alfândega da Fé CRL; Ponto Agrícola Unipessoal Lda
Data de início	01/03/2019
Data de fim	28/02/2021

Descrição dos trabalhos realizados:

Incorporação dos ingredientes corantes em produtos de panificação/pastelaria.

Resultados:

Decorre ainda o estudo da capacidade corante em produtos de panificação/pastelaria, nomeadamente pastas de açúcar e creme de pasteleiro. A estabilidade da cor das pastas de açúcar será avaliada durante vários meses de armazenamento, com e sem exposição à luz. Relativamente ao creme de pasteleiro, encontra-se em análise a resistência dos diferentes corantes aplicados ao aquecimento e a outros fatores relevantes.

Tarefa	3.3.2 (9.2) – Efeitos na qualidade dos produtos e validação do efeito corante
Líder da tarefa	IPB-CIMO
Participantes	TecPan - Tecnologia e Produtos para Pastelaria e Panificação, Lda.; M. Ferreira & Filhas, Lda.
Data de início	01/03/2019
Data de fim	28/02/2021

Descrição dos trabalhos realizados:

Efeitos na qualidade dos produtos e validação do efeito corante.

Resultados:

Decorre ainda o estudo de validação do efeito corante dos extratos em produtos de panificação/pastelaria, bem como os efeitos provocados nos produtos onde foram aplicados.

Resultados passíveis de valorização económica:

Procedimentos otimizados para a estabilização dos extratos corantes obtidos de: i) *Beta vulgaris* L. usando a técnica de *spray-drying* e com o material encapsulante maltodextrina (20%); ii) *Gomphrena globosa* L. usando a técnica de *spray-drying* e com o material encapsulante maltodextrina (20%); iii) *Lonicera caerulea* L. usando a técnica de *spray-drying* e com o material encapsulante maltodextrina (60%) e maltodextrina:Goma Arábica (40:40 %); *Morus nigra* L. usando a técnica de *spray-drying* e com o material encapsulante maltodextrina (20%) e maltodextrina:Goma Arábica (10:10 %); *Rubus fruticosos* Linnaeus usando a técnica de *spray-drying* com o material encapsulante maltodextrina (20%) e maltodextrina: Goma Arábica (10:10 %); *Sambucus nigra* L. usando a técnica de *spray-drying* com o material encapsulante maltodextrina (20%); curcumina, extraído de *Curcuma longa* L. com 80% de pureza usando a técnica de *spray-congealing* com o material encapsulante cera de abelha (98,5%).

5.2.2 Desvios e correções realizadas

No âmbito da **PPS 3**, verificaram-se alterações ao nível das matrizes estudadas e respetivos compostos de interesse, tendo sido alargado o número de ambas. Consequentemente, relativamente à atividade 3.1, verificou-se a alteração no término da tarefa 3.1.3 de maio de 2019 para dezembro de 2019, e na atividade 3.2, a conclusão das tarefas 3.2.1 e 3.2.2 foi adiada também para dezembro de 2019.

5.2.3 Execução Financeira por promotor, apresentando os desvios, por rubrica, face ao previsto em candidatura

TecPan – Tecnologia e Produtos para Pastelaria e Panificação, LDA.

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	11.191,06	426,99
a.iii) Matérias-primas e componentes	2.800,00	0,00

b) Custos indiretos	3.497,76	106,75
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M. Ferreira & Filhas Lda.

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	1.496,26	0,00
a.iii) Matérias-primas e componentes	1.000,00	0,00
b) Custos indiretos	624,06	0,00

Ponto Agrícola Unipessoal LDA.

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	39.840,75	7.402,5
a.iii) Matérias-primas e componentes	5.400,00	5.000,00
b) Custos indiretos	11.307,93	3.100,63

Instituto Politécnico de Bragança

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	175.281,45	88.441,47
a.iii) Matérias-primas e componentes	72.843,08	29.980,48
a.v) Aquisição de instrumentos e equipamento científico	64.326,00	90.922,94
b) Custos indiretos	78.112,62	52.353,72

Cooperativa Agrícola de Alfandega da Fé CRL.

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	3.180,80	1.055,92
a.iii) Matérias-primas e componentes	3.000,00	0,00

b) Custos indiretos	1.545,20	263,98
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5.3 PPS4 – Aromas e modelos de aromas

5.3.1 Apresentação dos resultados alcançados

Relativamente à **PPS 4**, no primeiro semestre de execução do projeto, foi dado início à identificação das matérias-primas e aromas a obter. Foram selecionadas as plantas aromáticas e frutos secos que serão usados para a obtenção de aromas naturais por extração supercrítica com CO₂, assim como as especificações a serem definidas para o processo de extração. No segundo semestre de execução do projeto, foi dado início à receção, preparação, fracionamento e armazenamento das amostras previamente definidas como matérias-primas de estudo, nomeadamente as plantas aromáticas de alecrim e orégãos (desidratados e frescos), e os frutos secos como a amêndoa e avelã (com e sem pele). Todas as matérias-primas foram fracionadas antes do seu armazenamento, com a exceção das amostras frescas que foram congeladas e o seu fracionamento será realizado imediatamente antes da extração dos aromas. Posteriormente, foram iniciados os ensaios testes na unidade de extração de CO₂ supercrítico que permitiram a definição do design experimental (DCCR) com três pontos centrais, tendo como composto de investigação inicial, a amêndoa com pele. Paralelamente, foram iniciados os ensaios de extração na unidade experimental de soxhlet para os frutos secos, respetivamente amêndoa e avelã, com e sem pele. A identificação dos compostos extraídos através da unidade de extração de CO₂ supercrítica e soxhlet foi realizada através de cromatografia gasosa com detetor de massas (GC-MS), após saponificação dos extratos e preparação dos constituintes metil esterres. Desta forma, foi possível delinear a metodologia de ensaio apropriada aos extratos e evidenciar a presença de aromas naturais. Análises de humidade, termogravimetria (TGA) e microscopia eletrónica de varrimento (SEM) para os frutos secos forneceram resultados complementares de caracterização das amostras. Durante este período também foi realizada uma revisão bibliográfica com o objetivo de estudar as principais metodologias para a obtenção de aromas naturais na classe dos hidrolatos.

Atividade: 4.1 (11) - Extração de aromas naturais nacionais com propriedades organoléticas de interesse para a indústria de panificação (Líder da Atividade: FEUP-LSRE)

Tarefa	4.1.1 (11.1) - Obtenção de aromas naturais por extração supercrítica
Líder da tarefa	FEUP-LSRE
Participantes	IPB-CIMO; OWNYA/Vera Mata soluções Perfumadas, Lda.; Deifil Technology Lda, IPB/LSRE; CNCFS
Data de início	01/09/2018
Data de fim	31/01/2020

Descrição dos trabalhos realizados:

Em anexo a este relatório apresenta-se um documento descritivo do estado da arte, e dos materiais e métodos utilizados no desenrolar dos trabalhos (Anexo 1).

Resultados:

Os principais resultados são também apresentados no documento em anexo.

Tarefa	4.1.2 (11.2) - Obtenção de aromas naturais na classe dos hidrolatos
Líder da tarefa	FEUP-LSRE
Participantes	IPB-CIMO; OWNYA/Vera Mata soluções Perfumadas, Lda.; Deifil Technology Lda, CNCFS; IPB/LSRE
Data de início	01/09/2018
Data de fim	30/04/2020

Descrição dos trabalhos realizados:

Hidrodestilação (HD) é um método clássico utilizado no isolamento de óleos essenciais. A técnica consiste no aquecimento do material de extração em água (Tisserand e Young, 2014). Trata-se de uma metodologia alternativa a outros procedimentos de extração como extração com solvente orgânico, maceração, destilação por micro-ondas e extração supercrítica.

O esquema da Figura 1 ilustra o funcionamento do equipamento de hidrodestilação simples (aparelho de Clevenger).



Figura 1 - Esquema do aparelho de Cleverger, próprio para hidrodestilação simples.
 Fonte: Adaptado de Ferreira et al., 2017.

O balão de destilação deve ser aquecido através de uma manta de aquecimento, enquanto o condensador deve ser alimentado com água. A agitação da amostra é de interesse, de modo que evite a degradação do material denso e este se deposite no fundo do aparelho. Apesar de usual, a hidrodestilação apresenta alguns inconvenientes: termodegradação, hidrólise e solubilização em água de componentes que alteram o aroma de óleos essenciais (Machado et al., 2013). Geralmente, a hidrodestilação ocorre usando pressão atmosférica e, componentes de alto peso molecular não são facilmente extraídos nestas condições (Qin, 2003).

No entanto, na análise da composição do óleo de tomilho (*Thymus lotocephalus*), foram identificados 44 constituintes no extrato obtido por hidrodestilação, enquanto o extrato proveniente da extração supercrítica permitiu a identificação de 24 substâncias (Costa et al., 2012).

Com o objetivo de redução de perdas na composição do extrato, a destilação a vapor tem sido usada em substituição à água e, assim, são reconhecidas as subcategorias da hidrodestilação: destilação a vapor, destilação com água ou a combinação de ambas (Dilworth, Riley e Stennett, 2017).

Diferentemente da hidrodestilação, a destilação a vapor permite a separação de constituintes não voláteis, substâncias imiscíveis em água a temperaturas inferiores ao seu ponto de ebulição e preservação de componentes termolábeis. A extração de óleos essenciais dá-se a temperaturas próximas a 100 °C que resulta na redução do ponto de ebulição dos componentes individuais (Dilworth, Riley e Stennett, 2017).

Mezzomo et al. (2010), ao realizarem o estudo comparativo do rendimento da extração de óleo de amêndoa por diferentes métodos, obtiveram 0,17% (b.s.) na hidrodestilação, significativamente igual a extração por maceração e significativamente inferior aos métodos soxhlet e supercrítica. O mesmo foi indicado por Gomes, Mata e Rodrigues

(2007) para amostras de *rose geranium*. Os autores explicam que a metodologia é capaz de realizar apenas a extração da fração volátil do óleo, o que justifica os baixos rendimentos de extrato obtido quando comparado a outras metodologias. Por sua vez, Grosso et al. (2010) chegaram a valores de rendimento e natureza dos constituintes semelhantes quando realizada a extração de tomilho (*Thymus vulgaris* L.) em extração supercrítica e hidrodestilação.

Referências:

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- Mezzomo, N.; Martínez, J.; Ferreira, R. S. Supercritical fluid extraction of peach (*Prunus persica*) almond oil: Kinetics, mathematical modeling and scale-up. **The Journal of Supercritical Fluids**, v. 51, p. 10-16, 2009.
- Gomes, P. B.; Mata, V. G.; Rodrigues, A. E. Production of rose geranium oil using supercritical fluid extraction. **The Journal of Supercritical Fluids**, v. 41, p. 50-60, 2007.
- Grosso, C.; Figueiredo, A. C.; Burillo, J.; Mainar, A. M.; Urieta, J. S.; Barroso, J. G.; Coelho, J. A.; Palavra, A. M. F. Composition and antioxidant activity of *Thymus vulgaris* volatiles: comparison between supercritical fluid extraction and hydrodistillation. **Journal of separation science**, v. 33, p. 2211-2218, 2010.

Resultados:

Não há resultados a reportar.

Resultados passíveis de valorização económica:

O estudo de valorização económica será consolidado a partir da identificação, já iniciada, e futura quantificação dos constituintes dos óleos vegetais, na presença dos padrões cromatográficos. Seletividade e rendimento também são fatores alvo de investigação, os quais apresentam proporções distintas de acordo com o método de extração.

5.3.2 Desvios e correções realizadas

Na **PPS 4**, durante o segundo semestre do projeto, foram rececionadas as matérias-primas selecionadas para extração, faltando ainda receber as amostras de tomilho desidratado e fresco (serão recebidas durante o mês de setembro - altura da sua recolha). A unidade de extração de CO₂ supercrítico necessitou de manutenção para o seu funcionamento, tendo sido substituídas algumas peças, realizada a limpeza de várias válvulas e identificação de fugas no sistema. Foi também necessária a reposição de CO₂ a partir da aquisição de uma nova garrafa, para assim se atingir as pressões desejadas para extração dos aromas. No decorrer dos ensaios de extração e identificação dos compostos por cromatografia gasosa, verificou-se a necessidade de efetuar a encomenda de alguns reagentes necessários para a preparação das amostras e transesterificação dos extratos.

Verificou-se ainda que o número de pessoas mês (PM) atribuído inicialmente à Atividade 11 não é adequado, pelo que se optou por transferir PM's entre essa Atividade e a Atividade 12, não afetando o número de PMs totais do PPS4.

Os recursos humanos afetados por alteração são:

- Isabel Martins, Investigador Doutorado, 1 PM
- Julia Kessler, Bolseira Mestre, 1 PM
- José Miguel Loureiro, Professor Associado, 0.68 PMs

5.3.3 Execução Financeira por promotor, apresentando os desvios, por rubrica, face ao previsto em candidatura

TecPan – Tecnologia e Produtos para Pastelaria e Panificação, LDA.

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	2.063,93	0,00
a.iii) Matérias-primas e componentes	2.800,00	0,00
b) Custos indiretos	1.215,99	

Deifil Technology Lda.

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	2.142,00	1.033,20
a.iii) Matérias-primas e componentes	11.490,00	4.297,84
b) Custos indiretos	3.408,00	1 332,76

M. Ferreira & Filhas Lda.

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	477,28	0,00
b) Custos indiretos	119,32	0,00

Universidade do Porto

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	100.629,50	21.164,34
a.iii) Matérias-primas e componentes	9 863,19	1.103,76
a.v) Aquisição de instrumentos e equipamento científico	79 198,81	61.972,36
b) Custos indiretos	47.422,87	21.060,12

Centro Nacional de Competências dos Frutos Secos

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	4.917,92	1.522,02 €
b) Custos indiretos	1.229,48	380,51

5.4 PPS5 – Bioativos naturais

5.4.1 Apresentação dos resultados alcançados

No decorrer das atividades relativas à **PPS 5**, até ao momento foi possível reportar as condições ideais de recolha dos bio-resíduos de cogumelos na empresa e a sua preparação e armazenamento até à sua utilização para os ensaios posteriores. Foi também possível estudar as características técnicas dos bioativos (ergosterol e vitamina D2) presentes nos cogumelos e a sua estabilidade, relativamente ao seu comportamento quando expostos a diferentes fatores como diferentes valores de pH, temperaturas, exposição à luz, exposição a oxigénio, resistência à fermentação e à cozedura. Além disso foram também já otimizadas as condições de solubilidade dos bioativos de modo a selecionar os melhores solventes e matrizes alimentares para futura aplicação destes ingredientes. Foram otimizadas as condições de extração destes bioativos de forma a maximizar o rendimento. Relativamente ao ergosterol, após o estabelecimento das melhores condições de extração e estabilidade, foram já realizados estudos relativamente à sua capacidade hipocolesterémica em células CaCo2. Após verificar a sua capacidade na redução da absorção do colesterol pelas células, o extrato de *A. bisporus* foi incorporado em queijo curado. Neste momento estão em curso os ensaios de estabilidade do extrato após a incorporação no queijo e também a avaliação do seu tempo de prateleira. Relativamente à vitamina D2, estão em curso os ensaios de bioatividade em osteoblastos de forma a estabelecer a capacidade desta molécula em promover a absorção de cálcio.

Atividade 5.1 (14) – Obtenção de ingredientes naturais com bioatividade (Líder da Atividade: FEUP-LSRE)

Tarefa	5.1.1 (14.1) - Seleção das matérias-primas para obtenção de ingredientes bioativos
Líder da tarefa	IPB-CIMO
Participantes	FEUP-LSRE; Mogaricus Cogumelos – Sociedade Unipessoal Lda.
Data de início	01/09/2018
Data de fim	30/11/2018

Descrição dos trabalhos realizados:

Os bio-resíduos da indústria produtora de *Agaricus bisporus*, correspondendo a cerca de 15% da produção, são fornecidos pela empresa “Mogaricus”, Mogadouro, Portugal. Estes bio-resíduos são todas as partes dos cogumelos que apresentam irregularidades a nível fisiológico. Alguns exemplos são a parte de baixo e volva devido à sua textura dura, ou a matéria orgânica presente no efluente gerado nos processos de lavagem e branqueamento. Além disso, durante o cultivo e a colheita dos cogumelos, os espécimes com dimensões e forma irregulares são descartados. Aquando da colheita dos cogumelos na sua fase de maturação apropriada para consumo, todos estes bio-resíduos são colocados num recipiente refrigerado e transportados até ao laboratório onde são imediatamente congelados, liofilizados e armazenados a 4 °C ao abrigo da luz.

Resultados:

Foi possível elaborar um folheto descritivo com as condições ideais de recolha dos bio-resíduos de cogumelos.

Tarefa	5.1.2 (14.2) - Procedimentos de extração e refinação de ingredientes bioativos
Líder da tarefa	FEUP/LSRE
Participantes	IPB-CIMO; Afonso, Lopes & C ^a . Lda.; M. Ferreira & Filhas, Lda.; Novavet - Produtos Agro - Pecuários, Lda.; Mogaricus Cogumelos – Sociedade Unipessoal Lda.; Arménio Adérito Vaz
Data de início	01/09/2018
Data de fim	28/02/2019

Descrição dos trabalhos realizados:

A vitamina D2 (ergocalciferol) tem como precursor o ergosterol que é amplamente encontrado em cogumelos. O ergosterol é fotossensível e sob influência da irradiação ultravioleta (UV) ocorre a clivagem fotoquímica do anel B e em seguida, a pré-vitamina D2, sofre um rearranjo térmico levando à formação da vitamina D2. As doses de irradiação, duração da exposição e distância da fonte de luz UV serão otimizadas.

Resultados:

Foi possível estabelecer o procedimento de conversão do ergosterol em vitamina D2 através da irradiação ultravioleta. Foram estudadas as condições ótimas de extração dos bioativos (ergosterol e vitamina D2). Para ambos os bioativos, foi possível obter extratos ricos em ergosterol ou vitamina D2, com elevado rendimento, através da utilização da técnica de extração assistida por ultrassons otimizando-se as variáveis de tempo de extração, razão sólido-líquido, solvente mais eficaz e potência do equipamento.

No caso do ergosterol, o etanol 100% foi o solvente mais eficaz para a extração deste bioativo. As condições de operação do equipamento que permitem a maior quantidade de ergosterol extraída são: temperatura: ambiente, tempo: 15 min e potência: 375 W. Após a extração, segue-se uma filtração e posterior evaporação do solvente. O resíduo é de seguida redissolvido numa concentração conhecida e o ergosterol é quantificado através da técnica de cromatografia líquida de alta eficiência acoplada a um detetor de díodos a 280 nm. Estas condições ótimas de extração permitem a obtenção de 671.5 ± 0.5 mg de ergosterol/100 g de cogumelo seco.

No caso da vitamina D2, o hexano foi o solvente mais eficaz para a extração deste bioativo. As condições de operação do equipamento que permitem a maior quantidade de vitamina D2 extraída são: temperatura: 45 °C e tempo: 30 min. Após a extração, segue-se uma filtração e posterior evaporação do solvente. O resíduo é de seguida redissolvido numa concentração conhecida e a vitamina D2 é quantificada através da técnica de cromatografia líquida de alta eficiência acoplada a um detetor de díodos a 280 nm.

Tarefa	5.1.3 (14.3) – Avaliação dos efeitos bioativos dos ingredientes
Líder da tarefa	IPB-CIMO
Participantes	LSRE-FEUP; Afonso, Lopes & C ^a . Lda.; M. Ferreira & Filhas, Lda.; Novavet - Produtos Agro - Pecuários, Lda.; Arménio Adérito Vaz
Data de início	01/12/2018
Data de fim	31/10/2019

Descrição dos trabalhos realizados:

Após o estabelecimento das condições ideais de obtenção dos extratos de *A. bisporus* enriquecidos em ergosterol, estes foram avaliados pela sua capacidade de diminuição de absorção de colesterol em linhas CaCo2. Várias doses do extrato foram testadas de forma a verificar qual a dose que exerce a melhor capacidade e também qual a dose ideal a incorporar nos produtos lácteos (queijos). Foi também testada a capacidade hipocolesterémica do ergosterol puro, de forma a verificar se a molécula na sua forma pura exerceria uma capacidade mais forte. Como controlos, foram utilizadas soluções de colesterol, de forma a verificar a quantidade de colesterol naturalmente absorvida pelas células na ausência de agentes hipocolesterémicos.

Relativamente à vitamina D2, encontram-se em curso os ensaios de bioatividade em osteoblastos de forma a verificar a sua capacidade de promover a absorção de cálcio.

Resultados:

De acordo com os resultados obtidos, verificou-se que o extrato foi mais ativo, diminuindo a absorção de colesterol pelas células, devido à presença de outras moléculas que facilitam a passagem do extrato pelas células, competindo com o colesterol e impedindo a sua absorção. Comparativamente com as soluções de colesterol, verificou-se que na presença de extrato e de ergosterol puro, a passagem do colesterol para as células diminuiu em cerca de 15% e 12%, respetivamente.

Atividade 5.2 (15) – Estudos de estabilidade e metodologias de estabilização (Líder da Atividade: IPB-LSRE)

Tarefa	5.2.1 (15.1) – Estudos de estabilidade dos ingredientes bioativos
Líder da tarefa	IPB-CIMO
Participantes	IPB-LSRE; Afonso, Lopes & C ^a . Lda.; M. Ferreira & Filhas, Lda.; Novavet - Produtos Agro - Pecuários, Lda.; Arménio Adérito Vaz; FEUP-LSRE
Data de início	01/12/2018
Data de fim	30/05/2019

Descrição dos trabalhos realizados:

Os bioativos ergosterol e vitamina D2 foram testados relativamente à sua estabilidade face a diversos fatores importantes para a área alimentar, nomeadamente: variações de pH, temperatura, exposição à luz, presença de oxigénio.

Resultados:

De acordo com os resultados obtidos, o ergosterol revelou ser uma molécula estável à variação de pH, resistente a baixas e a altas temperaturas e ainda resistente à exposição à luz e ao oxigénio. Dada a sua estabilidade aos diferentes fatores analisados e, sendo também uma molécula com afinidade para matrizes lipofílicas, foi possível considerar viável a sua aplicação em produtos lácteos, nomeadamente queijos.

A vitamina D2 revelou ser lipossolúvel e relativamente estável após incorporação nos alimentos que têm carácter lipofílico. É considerada uma vitamina relativamente robusta, estável durante a cozedura até 200 °C.

Tarefa	5.2.2 (15.2) – Procedimentos de estabilização de ingredientes bioativos
Líder da tarefa	IPB-LSRE
Participantes	IPB-CIMO; Afonso, Lopes & C ^a . Lda.; M. Ferreira & Filhas, Lda.; Novavet - Produtos Agro - Pecuários, Lda.; Arménio Adérito Vaz; FEUP-LSRE

Data de início	01/03/2019
Data de fim	31/11/2019

Descrição dos trabalhos realizados:

Estão em curso procedimentos de estabilização do ergosterol, para possível aplicação em matrizes hidrofílicas (iogurtes). Os procedimentos passam pelo recurso a técnicas de spray-drying.

Resultados:

Estão em curso as atividades de estabilização, não havendo ainda resultados a reportar.

Atividade 5.3 (16) – Desenvolvimento de aplicações dos ingredientes bioativos (Líder da Atividade: Arménio Vaz)

Tarefa	5.3.1 (16.1) – Incorporação dos ingredientes biativos com micoesteróis em produtos lácteos, validação da bioatividade e efeitos na qualidade dos produtos
Líder da tarefa	Arménio Adérito Vaz
Participantes	IPB-CIMO; IPB-LSRE; FEUP-LSRE;
Data de início	01/03/2019
Data de fim	28/02/2021

Descrição dos trabalhos realizados:

Após ensaios de estabilidade e bioatividade, foi possível a incorporação do extrato *de A. bisporus* enriquecido em ergosterol, em queijo curado, para efeitos hipocolesterémicos.

Resultados:

Foi possível a incorporação de uma quantidade ativa, capaz de reduzir a absorção de colesterol presente nos queijos, sem afetar as características reológicas do mesmo. Neste momento encontram-se em curso os ensaios de estabilidade do extrato no queijo, bem como da avaliação do seu tempo de prateleira.

Tarefa	5.3.2 (16.2) – Fortificação de farinhas com vitamina D2, validação da bioatividade e efeitos na qualidade dos produtos
Líder da tarefa	Afonso, Lopes & C ^a . Lda.
Participantes	IPB-CIMO; IPB-LSRE; FEUP-LSRE; M. Ferreira & Filhas, Lda.; TecPan; NovaVet
Data de início	01/03/2019
Data de fim	28/02/2021

Descrição dos trabalhos realizados:

Ainda não foram iniciadas as atividades previstas nesta tarefa.

Resultados:

Não existem ainda resultados.

Resultados passíveis de valorização económica:

1. O estudo de Extratos ricos em ergosterol com capacidade hipocolesterémica a partir de bio-resíduos de *A. bisporus*;
2. Extratos ricos em vitamina D2 a partir de bio-resíduos de *A. bisporus*.
3. Queijos com extrato de *A. bisporus* com efeito hipocolesterémico.

5.4.2 Desvios e correções realizadas

Relativamente à **PPS 5**, foram efetuadas alterações no diagrama de Gantt relativamente às tarefas 5.1.3. em que os ensaios de bioatividade do ergosterol com data inicial de término a 31 de maio de 2019, foram concluídos em julho de 2019. Relativamente aos ensaios de bioatividade da vitamina D2, com data inicial de término prevista para 31 de maio de 2019, encontram-se neste momento em curso, com data de prevista de conclusão a 31 de outubro de 2019. Relativamente à fortificação de farinhas com vitamina D2 (tarefa 5.3.2), não foram ainda iniciados estes estudos.

5.1.3 Execução Financeira por promotor, apresentando os desvios, por rubrica, face ao previsto em candidatura

TecPan – Tecnologia e Produtos para Pastelaria e Panificação, LDA.

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	8.628,86	0,00
b) Custos indiretos	156,96	

Novavet – Produtos Agro-pecuários LDA.

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	19.891,80	2.963,40
b) Custos indiretos	4.972,95	740,85

Afonso, Lopes & CA, LDA.

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	49.592,41	0,00
a.v) Aquisição de instrumentos e equipamento científico	45.065,80	40.315,31
b) Custos indiretos	23.664,55	10.078,83

M. Ferreira & Filhas Lda.

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	381,82	0,00
a.iii) Matérias-primas e componentes	1.000,00	0,00
b) Custos indiretos	345,45	0,00

Instituto Politécnico de Bragança

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	162.752,01	62.618,30
a.iii) Matérias-primas e componentes	44.734,91	11.745,51
a.v) Aquisição de instrumentos e equipamento científico	5.807,95	5.910,92
b) Custos indiretos	55.073,72	20.068,68

Universidade do Porto

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	8.756,99	3.241,82
a.iii) Matérias-primas e componentes	10.000,00	0,00
b) Custos indiretos	4.689,25	810,45

Arménio Adérito Vaz

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	6.636,68	1.660,00
a.iii) Matérias-primas e componentes	5.000,00	2.000,00
b) Custos indiretos	2.909,17	915,00

5.5 PPS6 - Inovação em processos de extração, refinação e técnicas de conservação

5.5.1 Apresentação dos resultados alcançados

Na **PPS 6**, no âmbito da atividade 6.1, desenvolveu-se a solução construtiva do reservatório de extração. Este reservatório possui um circuito exterior de aquecimento que permite o controlo da temperatura durante o processo de extração; o design do reservatório de extração inclui uma cápsula interior para colocação da matéria-prima a extrair. Desenvolveram-se as soluções construtivas dos dois vasos separadores, com recolha facilitada de extrato e circuito externo para um fluido de aquecimento. Encontra-se em curso o processo de validação estrutural mecânica dos três vasos mencionados, para posterior processo de certificação dos mesmos. Está em curso o contacto com fornecedores, com intuito de selecionar os componentes necessários para a instalação. Iniciou-se o desenvolvimento de uma estrutura móvel para o sistema laboratorial, com a disposição dos componentes laboratoriais selecionados até a data. No âmbito da atividade 6.2 encontra-se em fase desenvolvimento a solução construtiva do misturador NETmix, assim como a solução construtiva do separador líquido-líquido. Em relação à atividade

6.3, procedeu-se ao desenvolvimento/projeto do sistema de produção de hidratos de CO₂ e, em desenvolvimento, o sistema de filtragem e dispersão.

Atividade: 6.1 (18) – Inovação em processos de extração (Líder da Atividade: INEGI)

Tarefa	6.1.1 (18.1) - Definição dos requisitos de funcionamento do sistema laboratorial de extração SFE-CO₂
Líder da tarefa	FEUP/LSRE-LCM
Participantes	INEGI; Paralab - Equipamentos Industriais e de Laboratório, S.A.; IPB-CIMO; Mogaricus Cogumelos – Sociedade Unipessoal Lda.
Data de início	01/09/2018
Data de fim	28/02/2019

Descrição dos trabalhos realizados:

Foi dado início ao processo de definição dos requisitos de funcionamento do sistema laboratorial de extração SFE-CO₂ nomeadamente dos seus principais componentes: fornecedor de CO₂, fornecedor de co-solvente, extrator, separadores, permutadores de calor, etc. Foram analisados os principais parâmetros operativos pretendidos para cada processo específico de extração: gamas de pressão, temperaturas, caudais mássicos, tempos de estágio, e existência de co-solvente.

Resultados:

No âmbito do projeto pretende-se construir um sistema laboratorial de extração SFE-CO₂ que seja versátil e facilmente adaptável a diferentes matérias-primas provenientes do setor agroalimentar (nomeadamente, a beterraba, a cereja, os cogumelos, entre outros). O sistema de extração será um equipamento inovador e versátil que deverá permitir a recirculação do dióxido de carbono durante a fase extração a pressão constante.

De modo a definir as especificações da instalação de extração em fluido supercrítico foi feita uma análise exaustiva às diferentes matérias-primas envolvidas no projeto. A Tabela 1 resume as diferentes condições de operação, nomeadamente a temperatura e pressão usadas no processo de extração usando CO₂ supercrítico.

Tabela 1 - Condições de operação do processo de extração com CO₂ supercrítico para diferentes matérias-primas.

Matéria-Prima	Nome Científico	Condições de Operação	Rendimento / % m/m	Ref.
Cereja	<i>Prunus avium</i> L.	T = 20 – 60 °C P = 25 – 250 bar Q = 20 – 40 L _{CO₂} /kg _{amostra} 0 – 20 % m/m EtOH	0.5 – 8	[1-3]
Sabugueiro	<i>Sambucus nigra</i> L.	T = 40 °C		[4]

		P = 200 bar		
Medronho	<i>Arbutus unedo</i> L.	T = 40 – 80 °C P = 150 – 300 bar Q = 30 kg _{CO₂} /kg _{amostra} 0 – 20 % m/m EtOH		[1]
Hibisco	<i>Hibiscus sabdariffa</i> L.	T = 40 – 80 °C P = 200 – 400 bar		[1]
Rosa	<i>Rosa damascena</i> 'Alexandria' <i>R. gallica</i> 'Francesa' enxertada em <i>R. canina</i>	T = 30 – 80 °C P = 250 – 450 bar Q = 0.4 – 1.6 kg _{CO₂} /kg _{amostra}	5.72	[1, 5]
Cogumelos	<i>Agaricus bisporus</i> L.	T = 40 °C P = 90 – 300 bar Q _{CO₂} = 3.4 kg/h 0 – 10 % v/v EtOH	0.5 – 2	[6]
Mirtilo	<i>Vaccinium myrtillus</i> L.	T = 40 °C P = 150 – 250 bar Q _{CO₂} = 0.4 – 0.5 kg/h	1.84 – 2.19	[7]

Tendo em conta que a matéria-prima de interesse são cogumelos da espécie *Agaricus Bisporus* L. (em especial, os compostos ergosterol e o ergocalciferol) indica-se, na Tabela 2 a composição dos cogumelos desta espécie e os métodos de análise que foram usados para a sua quantificação.

Tabela 2 - Composição da matéria-prima (*Agaricus Bisporus* L.).

Matéria-Prima	Composição (mg / 100 g _{dry weight})	Método de Análise	Ref.
Cogumelos (incluindo <i>Agaricus Bisporus</i> L.)	<ul style="list-style-type: none"> ▪ Ergosterol: 602 – 654 ▪ Ergosta-7,22-dienol: 14.6 – 15.2 ▪ Ergosta-7,5-dienol: 47.1 – 94.0 ▪ Fungisterol: 13.5 – 25.8 	GS-MS	[8]
Cogumelos (incluindo <i>Agaricus Bisporus</i> L.)	<ul style="list-style-type: none"> ▪ Ergosterol: 671.5 ± 0.5 (≈ 90 % da fração de esteróis) 	HPLC – UV	[9]

Assim, e de acordo com a pesquisa efetuada, ficou definido que o sistema de extração supercrítica deveria suportar temperaturas máximas até 80 °C e pressões até 450 bar. O caudal máximo de dióxido de carbono deverá estar na gama 0.1 – 5 litros/minuto e o extrator deverá ter uma capacidade compreendida entre os 0.5 e 5 litros. Esta gama de volumes foi definida com base na necessidade de se obter ca. 10 g de extrato para a sua caracterização e sabendo que cogumelos da espécie *Agaricus Bisporus* L. contêm cerca de 90 % m/m de água [10]. Estima-se que para um extrator com capacidade de ca. 2 litros, seja possível tratar numa amostra de 1 kg, sendo que 900 g correspondem a água e o restante a matéria-prima de base seca que contêm os compostos extraíveis. Assumindo

que 10 % desta matéria-prima seca é extraível, é possível obter aproximadamente 10 g de extrato.

Adicionalmente, a instalação deve permitir a recirculação do dióxido de carbono em ciclo fechado, de modo a atingir o limite de solubilidade dos compostos alvo no CO₂, ou seja permitir extrair o máximo de extrato durante a etapa de extração a pressão constante. Este sistema de extração deverá integrar um sensor em linha para monitorizar a concentração do extrato na corrente de CO₂ e, conseqüentemente, determinar o fim da etapa de extração. De referir que o método de espectroscopia por absorção UV/vis atualmente é utilizado com sucesso para pressões até 180 bar, sendo provável a possibilidade de operação a pressões superiores.

Para além disso, e com o objetivo de conservar todas as propriedades naturais da matéria-prima, será estudada a possibilidade de se utilizar a matéria-prima sem pré-tratamento, isto é, na sua forma hidratada. O uso da matéria-prima hidratada seria um passo inovador no *design* do processo de extração já que atualmente as matérias-primas passam por um processo de desidratação antes da etapa de extração. Na etapa de extração, a água seria arrastada pelo dióxido de carbono e, posteriormente, separada deste num separador com um sistema de purga para a remoção da água. Dado que o ergosterol (composto de interesse) é cerca de 1000 vezes mais solúvel em dióxido de carbono supercrítico do que em água, este composto seguirá na corrente de CO₂.

Após a secção de extração, o sistema terá dois separadores, com controlo independente de temperatura e pressão para permitir o fracionamento do extrato (produto extraído). Será estudado o efeito da injeção de co-solvente no extrator e/ou nos separadores com o objetivo de aumentar o rendimento de extração e/ou recuperar a totalidade do extrato.

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Tarefa	6.1.2 (18.2) - Projeto do sistema laboratorial de extração
Líder da tarefa	INEGI
Participantes	FEUP/LSRE-LCM; Paralab - Equipamentos Industriais e de Laboratório, S.A.
Data de início	01/12/2018
Data de fim	30/11/2019

Descrição dos trabalhos realizados e Resultados:

Em anexo a este relatório apresenta-se um documento descritivo dos trabalhos realizados e dos principais resultados (Anexo 2).

Tarefa	6.1.3 (18.3) – Montagem, implementação da programação e testes preliminares do sistema laboratorial de extração SFE-CO₂
Líder da tarefa	Paralab
Participantes	INEGI; FEUP-LSRE
Data de início	01/06/2019
Data de fim	31/05/2020

Descrição dos trabalhos realizados:

No decorrer da presente tarefa iniciou-se todo o processo de pesquisa de fornecedores e respetiva orçamentação para fabrico dos componentes não standard e standard. Deu-se ainda início aos trabalhos de desenvolvimento dos módulos de programação necessários a integrar na instalação alvo desta atividade.

Resultados:

À data ainda não existem resultados a reportar.

Atividade 6.2 (19) - Inovação em processos de refinação (Líder da Atividade: INEGI)

Tarefa	6.2.1 (19.1) - Definição dos requisitos de funcionamento do sistema laboratorial de extração e refinação
Líder da tarefa	FEUP/LSRE-LCM
Participantes	INEGI; Paralab - Equipamentos Industriais e de Laboratório, S.A.; IPB-CIMO; IPB-LSRE; Mogaricus Cogumelos – Sociedade Unipessoal Lda.
Data de início	01/09/2018
Data de fim	31/05/2019

Descrição dos trabalhos realizados:

No âmbito desta tarefa foi elaborado um diagrama detalhado da instalação de extração/refinação. Foram definidos os requisitos de funcionamento para o sistema de extração e refinação a escala laboratorial, nomeadamente as condições de operação, temperatura, pressão, caudais etc. Após uma análise pormenorizada do diagrama de fases do dióxido de carbono definiram-se as condições de operação do sistema de modo a evitar a formação de hidratos. Também foi necessário definir o caudal através do cálculo dos números de Reynolds para diferentes misturas de CO₂/H₂O.

Resultados:

Como principal resultado da tarefa tem-se o diagrama do processo (Figura 2). O sistema está constituído por dois reservatórios para o armazenamento dos solventes (água e dióxido de carbono líquido contendo extrato), um NETmix que irá promover a mistura entre as duas correntes, e um separador que permita um tempo de residência da mistura água-CO₂ para que se dê a segregação das duas fases, de seguida dois reservatórios para a recolha dos produtos da extração. Note-se que, após a extração líquido-líquido, é possível gaseificar o dióxido de carbono e, conseqüentemente, obter o extrato apolar na sua forma sólida e livre de solvente.

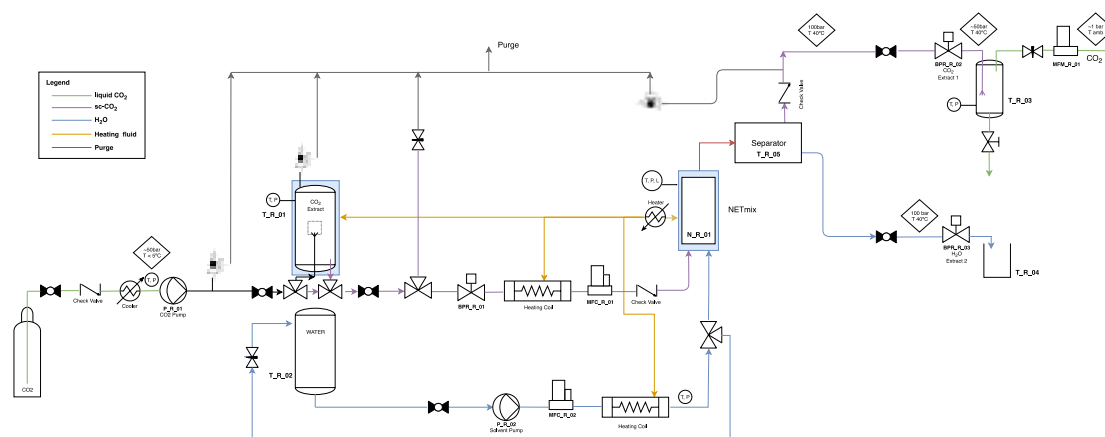


Figura 2 – Diagrama de processo do sistema de refinação.

Após a análise do diagrama de fases do dióxido de carbono, concluiu-se que, para evitar a formação de hidratos, se deverá limitar a temperatura mínima de operação a 15 °C e a pressão do dióxido de carbono deverá ser sempre superior a 80 bar (de modo a evitar a gaseificação deste solvente e consequente alteração da solubilidade do extrato no dióxido de carbono). Como limite superior de temperatura optou-se por uma temperatura máxima de operação a 40°C já que para uma mesma pressão, um aumento da temperatura irá originar uma diminuição da densidade do dióxido de carbono, e como consequência uma diminuição da solubilidade dos compostos no dióxido de carbono. A pressão de operação foi definida para uma gama de 80-100 bar (ver entregável E.6.2.1).

Relativamente ao NETmix, definiu-se um diâmetro hidráulico de ~1 mm para o NETmix, sendo que o diâmetro da câmara é de 6,75 mm. De forma a minimizar o efeito da parede no escoamento foi definido um mínimo de 8 colunas e que o comprimento do NETmix deve ser entre 3 e 5 vezes superior à sua largura (para a garantir uma mistura completa das fases). Através do cálculo dos números de Reynolds para diferentes misturas de CO₂/H₂O, foi definido que o Reynolds nos canais do NETmix deve ser superior a 300. É ainda de referir que o NETmix deverá possuir um permutador de calor com alhetas.

Tarefa	6.2.2 (19.2) - Projeto do sistema laboratorial de extração e refinação
Líder da tarefa	INEGI
Participantes	FEUP/LSRE-LCM; Paralab - Equipamentos Industriais e de Laboratório, S.A.
Data de início	01/12/2018
Data de fim	31/11/2019

Descrição dos trabalhos realizados:

Após a definição dos requisitos de funcionamento do sistema laboratorial iniciou-se a identificação de componentes e categorização em componentes standard e componentes customizados.

Nos componentes *standard* identificaram-se equipamentos como:

- Bombas de circulação;
- Válvulas de controlo/segurança dos caudais;
- Medidores de caudal;
- Reservatório de água;
- *Chiller*;
- Banho termostático (aquecimento);
- Tubagem e acessórios;
- Sensores e eletrónica.

Relativamente aos componentes customizados destacam-se:

- Misturador/Reator NETmix;
- Separador líquido-líquido;
- Reservatório de introdução de extrato;
- Serpentinhas de permuta;
- Tanque de drenagem.

Com esta divisão em mente procedeu-se ao projeto mecânico, químico e térmico dos respetivos equipamentos customizados iniciando-se pelo misturador NETmix, de tecnologia patenteada. Aqui foi posta em prática a tecnologia NETmix com o intuito de proporcionar uma mistura com elevada eficiência e elevada taxa de transferência de calor. Foram efetuados cálculos térmicos de modo a garantir a termostatização da mistura a 40°C e cálculos mecânicos de modo a suportar os 100 bar relativos que se verificam na zona da mistura, conjugando com a baixa pressão que se verifica no lado do fluido térmico/termostatizador. Os cálculos do projeto foram suportados recorrendo a simulação numérica, tanto estrutural como térmica.

Para o separador líquido-líquido foram iniciados os estudos químicos com o objetivo de otimizar a separação para posteriormente se proceder ao projeto mecânico e térmico. Para o estudo de otimização da separação recorreu-se não só a cálculos analíticos como

também a simulação numérica recorrendo a software de dinâmica de fluídos computacional com modelos multifásicos como o “VOF-Volume of Fluid”.

Para os restantes componentes customizados encontra-se ainda em aberto o projeto do reservatório de introdução de extrato, que necessitará de um estudo preliminar de automação/controlado e identificação dos objetivos concretos deste equipamento pois só assim se conseguirá projetar o equipamento final. Tanto as serpentinas de permuta como o tanque de drenagem encontram-se com o anteprojecto efetuado necessitando apenas de fechar todos os restantes componentes periféricos para poder concluir o projeto dos mesmos e iniciar o fabrico.

Até ao momento não foi identificada a necessidade de recorrer a mais equipamentos customizados pelo que todos os restantes componentes serão adquiridos como standard e a obtenção dos seus orçamentos já foi iniciada.

Por fim, tanto o projeto elétrico como o projeto da estrutura suporte da instalação estão a ser desenvolvidos em paralelo com o restante desenvolvimento.

Resultados:

Como resultados até à data pode-se apontar a conclusão dos requisitos funcionais do sistema, a conclusão do projeto do NETmix assim como a delimitação dos periféricos a adquirir.

Atividade 6.3 (20) - Inovação em técnicas de conservação (Líder da Atividade: INEGI)

Tarefa	6.3.1 (20.1) - Definição dos requisitos de funcionamento do sistema laboratorial de produção de hidratos de CO₂
Líder da tarefa	FEUP/LSRE-LCM
Participantes	INEGI; Paralab - Equipamentos Industriais e de Laboratório, S.A.; IPB-CIMO; Mogaricus Cogumelos – Sociedade Unipessoal Lda.; Deifil Technology Lda.
Data de início	01/09/2018
Data de fim	31/05/2019

Descrição dos trabalhos realizados:

No decorrer da tarefa foi elaborado um diagrama do processo de produção de hidratos com recurso a tecnologia NETmix e foram definidos os requisitos de funcionamento para o mesmo. O conceito de funcionamento desta instalação consiste na mistura de água com

uma corrente de dióxido de carbono gasoso que, às condições de pressão e temperatura predefinidas para a formação de hidratos, reagem numa reação do tipo exotérmica. O calor libertado tem de ser então removido pelo fluido de transferência de calor. A mistura entre estas duas correntes, promovida na rede do NETmix, irá permitir uma correta mistura melhorando a interface de contacto entre ambos os reagentes permitindo uma produção eficiente de hidratos. O intuito será o fornecimento de hidratos de dióxido de carbono que possam ser bons substitutos do gelo na conservação de alimentos.

Foi feita análise pormenorizada ao diagrama de fases do dióxido de carbono para promover a formação de hidratos.

Resultados:

Após a análise pormenorizada do diagrama de fases do dióxido de carbono. Concluiu-se que, para promover a formação de hidratos, se deverá limitar a temperatura mínima de operação a 0°C, evitando o congelamento da água e que a pressão do dióxido de carbono deverá ser sempre superior a 10 bar. Também se verificou que a zona de trabalho é limitada não apenas por limites mínimos de funcionamento como também por limites máximos. No caso da temperatura não se torna possível a formação de hidratos de dióxido de carbono acima dos 10°C. No que diz respeito à pressão, o valor máximo aceite serão, aproximadamente, os 45 bar. Resumindo, a gama de temperatura de funcionamento situa-se entre os 0°C e os 10°C. A nível de pressões de funcionamento trabalhar-se-á entre os 10 e os 45 bar (ver entregável E.6.3.1). A Figura 3 apresenta o diagrama do processo de produção de hidratos com recurso à tecnologia NETmix.

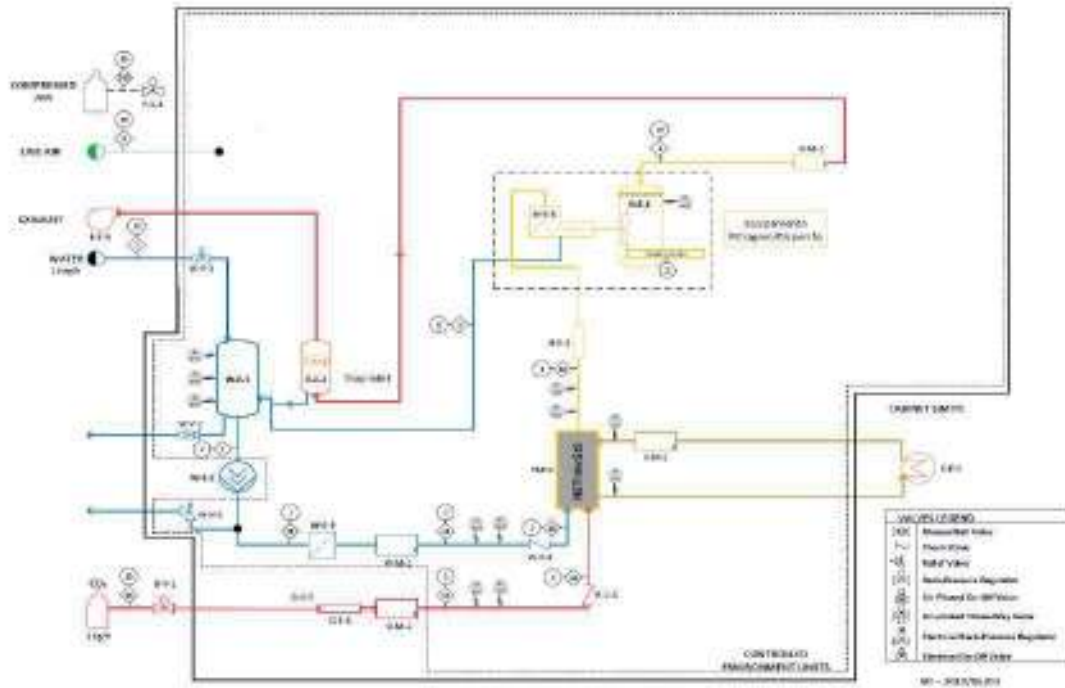


Figura 3 – Diagrama do processo de produção de hidratos com recurso à tecnologia NETmix.

Relativamente ao NETmix, definiu-se um diâmetro hidráulico de $\sim 0,5$ mm, sendo que o diâmetro da câmara é de 3,3 mm. De forma a minimizar o efeito da parede no escoamento foi definido um mínimo de 7 colunas e que o comprimento do NETmix deve ser entre 3 e 5 vezes superior à sua largura (para a garantir uma mistura completa das fases permitindo uma maior taxa de reação). Através do cálculo dos números de Reynolds para diferentes misturas de $\text{CO}_2/\text{H}_2\text{O}$, foi definido que o Reynolds nos canais do NETmix deve ser superior a 300. É ainda de referir que o NETmix deverá possuir um permutador de calor com alhetas. O caudal de produção deverá rondar 1 kg/h.

Tarefa	6.3.2 (20.2) - Projeto do sistema laboratorial de produção de hidratos de CO_2
Líder da tarefa	INEGI
Participantes	FEUP/LSRE-LCM; Parabol - Equipamentos Industriais e de Laboratório, S.A.
Data de início	01/12/2018
Data de fim	28/02/2020

Descrição dos trabalhos realizados:

Após a definição dos requisitos de funcionamento do sistema laboratorial iniciou-se a identificação de componentes e categorização em componentes standard e componentes customizados.

Nos componentes standard identificaram-se equipamentos como:

- Bomba de circulação;
- Válvulas de controlo/segurança dos caudais;
- Medidores de caudal;
- Reservatório de água;
- Chiller;
- Reservatório de purga CO₂;
- Tubagem e acessórios;
- Sensores e eletrónica.

Relativamente aos componentes customizados destacam-se:

- Reator NETmix;
- Visualizador;
- Sistema de filtragem e dispersão.

Com esta divisão em mente procedeu-se ao projeto mecânico, químico e térmico dos respetivos equipamentos customizados iniciando-se pelo reator NETmix, de tecnologia patenteada. Aqui foi posta em prática a tecnologia NETmix com o intuito de proporcionar uma mistura com elevada eficiência e elevada taxa de transferência de calor. Foram efetuados cálculos térmicos de modo a garantir que todo o calor libertado na reação exotérmica é removido e as condições de temperatura necessárias são garantidas, assim como cálculos mecânicos de modo a suportar os 30/40 bar relativos que se verificam na zona da mistura, conjugando com a baixa pressão que se verifica no lado do fluido de transferência de calor. Os cálculos do projeto foram suportados recorrendo a simulação numérica, tanto estrutural como térmica e fluídica.

O projeto do visualizador já se encontra fechado e tanto o projeto elétrico como o projeto da estrutura suporte da instalação estão a ser desenvolvidos em paralelo com o restante desenvolvimento.

Em contrapartida, encontra-se em fase de fecho de conceitos e início de projeto do sistema de filtragem e dispersão. Este sistema apresenta-se como um sistema complexo pois terá de responder a uma separação em contínuo de uma fase líquida e de uma fase sólida, instável às condições PTN. O principal conceito caíra, em princípio, num sistema de fuso que permitirá comprimir os hidratos sólidos e, simultaneamente, expelir a fase líquida.

Resultados:

Como resultados até à data pode-se apontar a conclusão dos requisitos funcionais do sistema assim como o projeto de todo o sistema à exceção do sistema de filtragem e dispersão e componentes associados.

Tarefa	6.3.4 (20.4) - Projeto e implementação do sistema laboratorial para caracterização das capacidades refrigerativas dos hidratos de CO₂
Líder da tarefa	INEGI
Participantes	FEUP/LSRE-LCM; Paralab - Equipamentos Industriais e de Laboratório, S.A.
Data de início	01/12/2019
Data de fim	31/08/2020

Descrição dos trabalhos realizados:

A data de início desta tarefa indicada no formulário eletrónico de candidatura coincidia com o início do projeto. Tratou-se de um lapso. Atendendo à sua natureza esta tarefa só poderá iniciar depois das anteriores (20.1, 20.2, 20.3) terem iniciado.

Resultados passíveis de valorização económica:

Como valorização económica destaca-se o desenvolvimento de processos de extração e refinação inovadores. Estão a ser desenvolvidos um sistema de extração SFE-CO₂ tendo como base conceitos inovadores e uso de novos materiais, assim como um sistema de extração líquido-líquido tendo como base a tecnologia NETmix, que irá permitir a refinação e separação de extratos a pressões e temperaturas moderadas.

5.5.2 Desvios e correções realizadas

No âmbito da **PPS 6**, assume-se à data um atraso na conclusão da tarefa 6.1.2 de 3 meses. Este atraso deve-se principalmente à dificuldade que se verificou em encontrar fornecedores para alguns dos componentes críticos deste sistema de extração supercrítica. O nível elevado da especificação, principalmente em termos de pressão, e a compatibilidade química do dióxido de carbono supercrítico com alguns elementos dos componentes a integrar (ex.: juntas de vedação de válvulas), limita muito o número de fornecedores possíveis assim como o número de modelos de componentes compatíveis. Tem sido feito um esforço nesta fase do projeto, por parte todos os intervenientes para

garantir a viabilidade não só técnica, mas também económica das soluções a integrar e o sucesso do projeto. Ainda assim, prevê-se que este atraso não tenha implicação em termos de duração da atividade 6.1 e da data de conclusão da tarefa 6.1.3 (“Montagem, implementação da programação e testes preliminares do sistema laboratorial de extração SFECO2”), uma vez que a PARALAB poderá avançar com a encomenda de alguns dos materiais a fabricar, tipicamente com prazos de entrega superiores, e avançar com a implementação de toda a programação necessária. Os desvios reportados não têm impacto nos objetivos e resultados previstos para o projeto.

5.5.3 Execução Financeira por promotor, apresentando os desvios, por rubrica, face ao previsto em candidatura

Paralab – Equip. Industriais e de Laboratório S.A.

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	233.083,90	80.025,81
a.iii) Matérias-primas e componentes	250.000,00	0,00
b) Custos indiretos	120.770,97	20.006,45

Universidade do Porto

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	173.948,21	45.075,25
a.iii) Matérias-primas e componentes	7.440,69	0,00
a.v) Aquisição de instrumentos e equipamento científico	2.559,31	2.080,74
b) Custos indiretos	45.897,05	11.789,00

INEGI – Instituto de Ciência e Inovação em Engenharia Mecânica e Engenharia Industrial

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	312.522,70	48.778,57
a.iii) Matérias-primas e componentes	43.000,00	0,00
a.v) Aquisição de instrumentos e equipamento científico	6.000,00	5.266,44
a.vi) Aquisição de software específico	27.000,00	2.134,60
b) Custos indiretos	97.130,67	14.044,90

Arménio Adérito Vaz

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.vii.1) Despesas com promoção e divulgação – feiras e exposições	2.250,00	0,00
a.viii) Viagens e estadas no estrangeiro	941,56	0,00
b) Custos indiretos	797,89	0,00

5.6 PPS8 - Disseminação de informação e exploração de resultados

5.6.1 Apresentação dos resultados alcançados

No âmbito da **PPS 8** procedeu-se ao desenvolvimento do Plano de Comunicação do projeto. Este tem como objetivo assegurar a divulgação, exploração de resultados relativos a todos os PPS, suscitar interesse nos diferentes públicos-alvo, bem como desenvolver um plano informativo para toda a comunidade. Durante este período de tempo também se procedeu ao desenvolvimento da identidade corporativa (imagem) do projeto, do estacionário (modelo de envelope e modelo de ofício), de diverso material de divulgação e promoção (posters A3, folhetos, desdobrável, *roll-up*), e da página *Web* e da *Intranet* do projeto. Foram ainda editadas várias edições da *Newsletter*, e disseminada na página *Web* do projeto (no contexto das atividades de vigilância tecnológica e inteligência

competitiva previstas para o projeto) informação relativa a notícias, projetos e tecnologias que estão relacionados com as temáticas em questão, e que poderão ser consultados por todos os utilizadores do *site*. No âmbito desta PPS foi também desenvolvido um portal como sistema de informação e comunicação que para além da plataforma de vigilância tecnológica e inteligência competitiva, apresenta funcionalidades como: a possibilidade registo de ideias, registo de provedores de soluções, sistema de comunicação entre os intervenientes e ainda sistema de reconhecimento da performance dos “fornecedores” de soluções. No decorrer deste mesmo período de tempo foi organizada a primeira edição do evento “Demolabs”, laboratórios de demonstração que têm como principais objetivos: disseminação e demonstração de resultados do projeto a entidades, nomeadamente empresas, externas ao consórcio, potenciar a geração de ideias que acrescentem valor aos resultados. Este evento irá realizar-se nas instalações do Brigantia Ecopark em Bragança no dia 4 de outubro de 2019.

Atividade 8.1 (24) – Comunicação (Líder da Atividade: IPB)

Tarefa	8.1.1 (24.1) – Plano de Comunicação
Líder da tarefa	IPB
Participantes	Todos
Data de início	1-09-2018
Data de fim	31-08-2021

Descrição dos trabalhos realizados:

Ao longo do período de tempo a que este relatório se reporta, foi desenvolvido o Plano de Comunicação do projeto, e foram criadas contas dedicadas ao mesmo nas redes sociais. O plano de Comunicação é um instrumento flexível e dinâmico, alvo de contínua monitorização, e que tem como objetivo a exploração e disseminação de resultados. Permitirá ajustar e repensar os diversos meios e técnicas disponíveis, com o intuito de se atingirem mais eficazmente todos os objetivos propostos.

Resultados:

Foi elaborado o Plano de Comunicação do projeto que está disponível para todos os copromotores.

Tarefa	8.1.2 (24.2) – Criação da identidade corporativa e produção de estacionário e de material de divulgação
Líder da tarefa	IPB
Participantes	Todos
Data de início	1-09-2018
Data de fim	31-12-2018

Descrição dos trabalhos realizados:

No âmbito desta tarefa foi desenvolvida a identidade corporativa (imagem), material de divulgação, e promoção, e procedeu-se à criação do material estacionário do projeto. A identidade corporativa do projeto ValorNatural foi desenvolvida pelos serviços de imagem do IPB, bem como o estacionário: modelo de envelope e modelo de ofício. Foi desenvolvido diverso material de divulgação e promoção do projeto. Através dos Serviços de Imagem do IPB foi possível a realização de um cartaz A3, um desdobrável, um folheto e um *roll-up*. Este material será utilizado para a promoção e divulgação do projeto que é de extrema importância para que se consiga atingir todos os públicos-alvo pretendidos, e contribuir assim para o alcance eficaz dos objetivos do projeto.

Resultados:

Imagem do projeto e material estacionário do projeto: modelo de envelope e modelo de ofício, cartaz A3, desdobrável, folheto, *roll-up*.

Tarefa	8.1.3 (24.3) – Desenvolvimento e manutenção da <i>Intranet</i> e da página <i>Web</i> do projeto
Líder da tarefa	IPB
Participantes	Todos
Data de início	1-09-2018
Data de fim	31-08-2021

Descrição dos trabalhos realizados:

Foi desenvolvida a página *Web* que tem como finalidade a comunicação com todas as partes interessadas externas ao projeto, a divulgação de assuntos relacionados com o tema do ValorNatural, e ainda a promoção de resultados do projeto. Para além da comunicação externa ao consórcio conseguida pelo *Website*, a *Intranet* operacionaliza a comunicação entre copromotores. Estas ferramentas, que foram desenvolvidas e serão mantidas pelo IPB, terão o contributo de todos os parceiros para serem otimizadas de forma a contribuírem para uma comunicação ágil e fluida.

Resultados:

No âmbito desta atividade foi possível desenvolver a página *Web* do projeto e a *Intranet*.

Tarefa	8.1.4 (24.4) – Divulgação do projeto e dos resultados das atividades
Líder da tarefa	IPB
Participantes	Todos
Data de início	1-12-2018
Data de fim	31-08-2021

Descrição dos trabalhos realizados:

Foram publicadas 3 edições da *Newsletters*, notícias no *site* e nas contas das redes sociais para o projeto. Os membros do consórcio participaram em eventos (feiras, congressos), com o objetivo de promover e divulgar os resultados do projeto, e foram ainda publicados artigos em revistas científicas de referência.

Resultados:

Consultar por favor a informação disponibilizada na secção 7 do presente relatório, relativa a promoção e divulgação de resultados.

Atividade 8.2 (25) – Vigilância tecnológica e inteligência competitiva (Líder da Atividade: FEUP)

Tarefa	8.2.1 (25.1) – Recolha, sistematização e disseminação de informação tecnológica e estratégica
Líder da tarefa	IPB
Participantes	Todos
Data de início	1-09-2018
Data de fim	31-08-2021

Descrição dos trabalhos realizados:

Durante o primeiro ano de execução do projeto foram editadas 3 edições da *Newsletter* ValorNatural. A informação que consta nestas edições é diversificada, atual e está sempre relacionada com o próprio projeto ou com os diferentes temas que ela abrange.

Resultados:

Foram editadas 3 edições da Newsletter do projeto ValorNatural.

Tarefa	8.2.2 (25.2) – Base de dados relativa a aditivos alimentares naturais e sintéticos, a projetos I&I e a tecnologia e conhecimento relevantes existentes no sistema nacional de I&I
Líder da tarefa	IPB
Participantes	Todos
Data de início	1-09-2018
Data de fim	31-08-2021

Descrição dos trabalhos realizados:

No âmbito desta tarefa, e através da página *Web* foi criada a possibilidade de cada utilizador aceder a informação relativa aos temas do projeto. Sendo assim, recorrendo ao Menu “Atualidades” é possível selecionar o conteúdo pretendido entre: Projetos, Notícias, Tecnologias e Publicações Científicas, no contexto das atividades de vigilância tecnológica e inteligência competitiva previstas para o projeto.

Resultados:

Foi desenvolvida a possibilidade de consultar informação relativa a Projetos, Notícias, Tecnologias e Publicações Científicas no Menu “Atualidades” na página *Web* do projeto.

Atividade 8.3 (26) – Inovação Aberta (Líder da Atividade: IPB)

Tarefa	8.3.1 (26.1) – Fomento e apoio à inovação colaborativa
Líder da tarefa	IPB
Participantes	Todos
Data de início	1-09-2018
Data de fim	31-08-2021

Descrição dos trabalhos realizados:

Relativamente à tarefa em questão foi desenvolvido um portal que reúne, para além da plataforma de vigilância tecnológica e inteligência competitiva, funcionalidades como: registo de ideias, registo de provedores de soluções, sistema de comunicação entre os intervenientes, sistema de reconhecimento da performance dos “fornecedores” de soluções.

Resultados:

Portal de inovação aberta.

Tarefa	8.3.2 (26.2) – Demolabs
Líder da tarefa	FEUP/LSRE-LCM
Participantes	IPB, INEGI, CNCFS, Deifil
Data de início	1-06-2018
Data de fim	28-02-2021

Descrição dos trabalhos realizados:

No âmbito desta tarefa foi organizada a primeira edição do evento “Demolabs”, laboratórios de demonstração que têm como principais objetivos: disseminação e

demonstração de resultados do projeto a entidades, nomeadamente empresas, externas ao consórcio, e potenciar a geração de ideias que acrescentem valor aos resultados.

Resultados:

Organização da primeira edição dos demolabs, a realizar dia 4 de outubro de 2019.

Tarefa	8.4.3 (27.3) – Avaliação ambiental do ciclo de vida
Líder da tarefa	ISQ
Participantes	INEGI, Deifil, TecPan, NovaVet, Afonso Lopes, M. Ferreira, Paralab, Ponto Agrícola, Arménio Vaz, CAAF
Data de início	01-09-2019
Data de fim	31-08-2021

Descrição dos trabalhos realizados:

A data de início prevista em sede de candidatura era setembro de 2018. No entanto, tratou-se de um lapso pois os novos processos apenas se encontrarão num estado de desenvolvimento que permita o começo dos trabalhos em setembro de 2019.

Tarefa	8.4.4 (27.4) – Avaliação económica do ciclo de vida
Líder da tarefa	ISQ
Participantes	INEGI, Deifil, TecPan, NovaVet, Afonso Lopes, M. Ferreira, Paralab, Ponto Agrícola, Arménio Vaz, CAAF
Data de início	01-09-2019
Data de fim	31-08-2021

Descrição dos trabalhos realizados:

A data de início prevista em sede de candidatura era setembro de 2018. No entanto, tratou-se de um lapso pois os novos processos apenas se encontrarão num estado de desenvolvimento que permita o começo dos trabalhos em setembro de 2019.

Tarefa	8.4.5 (27.5) – Avaliação da ecoeficiência
Líder da tarefa	ISQ
Participantes	INEGI, Deifil, TecPan, NovaVet, Afonso Lopes, M. Ferreira, Paralab, Ponto Agrícola, Arménio Vaz, CAAF
Data de início	01-09-2019
Data de fim	31-08-2021

Descrição dos trabalhos realizados:

A data de início prevista em sede de candidatura era setembro de 2018. No entanto, tratou-se de um lapso pois os novos processos apenas se encontrarão num estado de desenvolvimento que permita o começo dos trabalhos em setembro de 2019.

5.6.2 Desvios e correções realizadas

No âmbito da **PPS 8** a base de dados relativa a aditivos alimentares e a projetos de I&I e a tecnologia e conhecimento relevantes (Entregável 8.2.3) tinha data prevista de conclusão no dia 28 de fevereiro de 2019, porém o prazo foi alargado de forma a concluírem-se as tarefas de programação e esta foi terminada e tornada pública no dia 13 de setembro de 2019. No que diz respeito à implementação de um portal *Web* de inovação colaborativa (Entregável 8.3.1) tinha data de entrega prevista no dia 31 de maio de 2019, porém sofreu um adiamento, de forma a conciliar o lançamento com a realização dos Demolabs, pelo que a data prevista de entrega será dia 4 de outubro de 2019. A realização da primeira edição do evento “Demolabs” (Marco 8.3.2) tinha data prevista de realização no dia 31 de agosto de 2019 e realizar-se-á no dia 4 de outubro de 2019. Esta alteração pretende fazer coincidir as datas dos Demolabs com o fim de cada ano de execução do projeto, de forma a otimizar a divulgação de resultados. A data de início prevista em sede de candidatura para as tarefas 8.4.3, 8.4.4 e 8.4.5 era setembro de 2018. No entanto, tratou-se de um lapso, pois os novos processos tecnológicos apenas se encontrarão num estado de desenvolvimento que permita o começo dos trabalhos em setembro de 2019.

5.6.3 Execução Financeira por promotor, apresentando os desvios, por rubrica, face ao previsto em candidatura

TecPan – Tecnologia e Produtos para Pastelaria e Panificação, LDA.

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	2.992,52	1.028,96
a.vii.1) Despesas com promoção e divulgação – feiras e exposições	4.500,00	0,00
a.viii) Viagens e estadas no estrangeiro	2.100,00	0,00
b) Custos indiretos	2.398,13	257,24

Novavet – Produtos Agro-pecuários LDA.

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.vii.1) Despesas com promoção e divulgação – feiras e exposições	2.250,00	0,00
a.viii) Viagens e estadas no estrangeiro	750,00	0,00
b) Custos indiretos	750,00	0,00

Afonso, Lopes & CA, LDA.

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.vii.1) Despesas com promoção e divulgação – feiras e exposições	4.500,00	0,00
a.viii) Viagens e estadas no estrangeiro	2.100,00	0,00
b) Custos indiretos	1.650,00	0,00

Deifil Technology Lda.

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.vii.1) Despesas com promoção e divulgação – feiras e exposições	2.250,00	0,00
a.viii) Viagens e estadas no estrangeiro	750,00	0,00
b) Custos indiretos	750,00	0,00

M. Ferreira & Filhas Lda.

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.vii.1) Despesas com promoção e divulgação – feiras e exposições	2.250,00	0,00
a.viii) Viagens e estadas no estrangeiro	448,24	0,00
b) Custos indiretos	674,56	0,00

Paralab – Equip. Industriais e de Laboratório S.A.

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.vii.1) Despesas com promoção e divulgação – feiras e exposições	2.250,00	0,00
b) Custos indiretos	562,50	0,00

Ponto Agrícola Unipessoal LDA.

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.vii.1) Despesas com promoção e divulgação – feiras e exposições	1.500,00	0,00
a.viii) Viagens e estadas no estrangeiro	750,00	0,00
b) Custos indiretos	562,50	0,00

Instituto Politécnico de Bragança

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	164.199,52	54.452,43
a.vii.1) Despesas com promoção e divulgação – feiras e exposições	13.000,00	1.421,02
a.vii.2) Despesas com promoção e divulgação – outras despesas	10.000,00	141,45
b) Custos indiretos	46.799,88	14.003,73

Universidade do Porto

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	77.012,39	25.052,38
b) Custos indiretos	19.253,10	6.263,09

INEGI – Instituto de Ciência e Inovação em Engenharia Mecânica e Engenharia Industrial

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	26.910,72	0,00
a.viii) Viagens e estadas no estrangeiro	2.250,00	0,00
b) Custos indiretos	7.290,18	0,00

Centro Nacional de Competências dos Frutos Secos

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	20.156,55	162,66 €
a.vii.1) Despesas com promoção e divulgação – feiras e exposições	609,99	0,00
a.vii.2) Despesas com promoção e divulgação – outras despesas	1.308,75	0,00
a.viii) Viagens e estadas no estrangeiro	1.810,49	0,00
b) Custos indiretos	5.971,44	40,67

Instituto de Soldadura e Qualidade

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.i) Pessoal técnico do beneficiário	31.628,58	0,00
a.vii.1) Despesas com promoção e divulgação – feiras e exposições	1.600,00	0,00
a.viii) Viagens e estadas no estrangeiro	1.500,00	0,00
b) Custos indiretos	8.682,14	0,00

Cooperativa Agrícola de Alfandega da Fé CRL.

Despesas Elegíveis	Investimento Elegível contratado (€)	Investimento Elegível Realizado (€)
a.vii.1) Despesas com promoção e divulgação – feiras e exposições	1.500,00	0,00
b) Custos indiretos	375,00	0,00

6. Anexos

Anexo 1 – Trabalho realizado e resultados da tarefa 4.1.1

Anexo 2 – Trabalho realizado e resultados da tarefa 6.1.1

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E5.1.5 - Relatório dos procedimentos de refinação dos ingredientes bioativos

E5.1.6 – Publicação dos ingredientes com maior capacidade hipocolesterémica e sem toxicidade.

E5.1.7 – Publicitação dos ingredientes com melhor capacidade de aumento da absorção de cálcio e sem toxicidade

E5.2.1 – Folheto descritivo das condições que garantem a maior estabilidade dos ingredientes bioativos.

E6.1.1 Lista dos requisitos de funcionamento do sistema laboratorial de extração SFE-CO₂.

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E8.1.3 – Materiais de promoção e divulgação

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E8.2.3 – Base de dados relativa a aditivos alimentares e a projetos de I&I e a tecnologia e conhecimento relevantes

Anexo 1

Trabalho realizado e resultados da tarefa 4.1.1

ANEXO 1 - Descrição detalhada dos trabalhos realizados na Tarefa 4.1.1 (11.1)

Tarefa	4.1.1. Obtenção de aromas naturais por extração supercrítica
Líder da tarefa	FEUP-LSRE
Participantes	IPB-CIMO; OOWNYA/Vera Mata soluções Perfumadas, Lda.; Deifil Technology Lda. ; CNCFS; IPB/LSRE
Data de início	01/09/2018
Data de fim	31/08/2019

Descrição dos trabalhos realizados:

Estado da arte

A valorização de produtos comerciais a partir da incorporação de óleos essenciais, aromas, fragrâncias e extratos naturais têm apresentado um elevado interesse a nível mundial (Costa et al., 2015). Como principais áreas de aplicação destaca-se a área alimentar, cosmética e de produtos de higiene pessoal, com uma avaliação média de 20,75 bilhões US \$ em 2018 (Flavors and Fragrances Market Size, 2019). Como produtos naturais de maior interesse, destacam-se os oleaginosos e ervas aromáticas. Estes cultivos apresentam notáveis propriedades nutricionais e alto valor econômico (Bernardo-Gil, 2007).

Para a obtenção de extratos naturais e óleos essenciais, alguns métodos de extração podem ser utilizados, como a hidrodestilação (HD), extração sólido-líquido em soxhlet (SOX) e maceração (MAC). Apesar do alto rendimento, as técnicas possuem algumas limitações quanto ao custo energético, elevado uso de solvente, tempo de extração, retenção de solvente nos extratos, baixa seletividade e a utilização de elevadas temperaturas (Mezzomo et al., 2010). A utilização de elevadas temperaturas pode ser um fator bastante negativo no caso de compostos termolábeis.

Um método alternativo é a extração com fluido supercrítico, o CO₂. A tecnologia substitui os solventes orgânicos geralmente utilizados e, apresenta redução no potencial oxidativo dos extratos, alta seletividade, aumento da transferência de massa, tempos de extração e custo de operação reduzidos (Leo et al., 2005). Quimicamente inerte, o gás tem a capacidade de separar os constituintes de diferentes polaridades e massas moleculares. O CO₂ é não-tóxico, não-explosivo e não permanece como resíduo químico nos extratos.

Devido a estas características, a extração supercrítica é considerada uma tecnologia limpa e ideal para componentes termolábeis e naturais (Sánchez-Vicente, 2009; Mezzomo et al., 2009; Machado et al., 2013).

Tendo em conta os compostos com interesse no presente Projeto, os ensaios experimentais foram iniciados com os frutos secos, nomeadamente a amêndoa. A amêndoa apresenta, em média, 48 a 67% de óleo (Martínez et al., 2013), enquanto as avelãs possuem cerca de 56 a 61% de ácidos gordos (Oliveira et al., 2008).

A Tabela 1 apresenta os parâmetros experimentais obtidos na literatura, para extrações supercríticas de compostos de amêndoa e avelã. Através da referida tabela observa-se que, apesar de serem usadas diferentes metodologias de identificação dos compostos de extração, se obtém maiores rendimentos de extração a temperaturas de 40 a 50 °C e pressões de 300 a 400 bar. O aumento das interações dipolo-dipolo e ligações de hidrogênio entre solvente e soluto contribuem para uma maior solubilidade dos produtos de interesse no CO₂ e, estas interações podem ser favorecidas na presença de solventes orgânicos, mesmo em extrações supercríticas (Sánchez-Vicente et al., 2009).

Também se observam efeitos positivos em condições de alta densidade do gás. Termodinamicamente, a combinação de baixas temperaturas e altas pressões resultam em maiores densidades e, conseqüentemente, num maior transporte de matéria. No caso de se usar pressões superiores a 200 bar, o aumento da temperatura permite obter uma percentagem de extração superior, devido ao aumento da solubilidade do extrato no gás de extração, que possui uma maior influência no rendimento do que a densidade (Sánchez-Vicente et al., 2009).

Relativamente à seletividade dos extratos, é necessário um estudo aprofundado da literatura, uma vez que as metodologias de identificação são distintas.

Tabela 1. Parâmetros de ensaio da extração supercrítica apresentados na literatura para os frutos secos amêndoa e avelã.

Variedade	T (°C)	P (bar)	t (h)	Método de identificação	η (%)	Referência
Amêndoa	40	350	~16,7	-	~50,00 (40 °C/350 bar)	Marrone et al. (1998)
Amêndoa	50	330	-	HPLC e GC	15,50 to 64,30 (50 °C/330 bar)	Femenia et al. (2001)
Amêndoa	35-50	350-550	6,0	HPLC	50,00 (40 °C/420 bar)	Leo et al. (2005)
Amêndoa	40	150-250	10	-	19,08 (40 °C/+250 bar)	Mezzomo, Martínez e Ferreira, 2009 (2009)
Amêndoa	40-51	150-198	2,4	GC-MS	32,00 (51 °C/190 bar/5% EtOH)	Sánchez-Vicente et al. (2009)

<i>Amêndoa</i>	30-50	100-300	-	GC-FID	23,50 (50 °C/300 bar)	Mezzomo et al. (2010)
<i>Amêndoa</i>	40-80	200-400	6,0	GC-MS e HPLC-ELSD	36,57	Balvardi et al. (2015)
<i>Amêndoa</i>	40-55	100-200	~3,4	GC-FID	14,49	Nascimento et al. (2016)
<i>Avelã</i>	60	264	-	GC-RPLC	-	Blanch et al. (1998)
<i>Avelã</i>	35-48	180-200	4,0	GC-FID	-	Bernado-Gil et al. (2002)
<i>Avelã</i>	45-80	680	-	HPLC e GC- MS-FID	40,00	Crowe e White (2003)
<i>Avelã</i>	40-60	300-600	5,0	GC-FID	56,00-60,00	Özkal, Salgin e Yener (2005)
<i>Avelã</i>	40-60	300-500	3,0	-	65,00	Salgin e Salgin (2006)
<i>Avelã</i>	60-70	400-450	7,0	HPLC	-	Longo, Leo e Leone (2012)
<i>Avelã</i>	40-60	350-500	3,0	GC-FID	-	Manna, Bugnone e Banchemo (2015)

Da mesma forma, a Tabela 2 apresenta as condições de ensaio para extrações sólido-líquido em soxhlet para os frutos secos amêndoa e avelã.

Os dados apresentados para extrações sólido-líquido em soxhlet demonstram grande variabilidade nos parâmetros de ensaio, onde a polaridade dos solventes de extração é crescente em hexano < éter de petróleo < éter etílico < clorofórmio < acetato de etila < etanol < metanol.

Quanto maior a polaridade do solvente de extração, menor tende a ser a seletividade da extração (Petrovic et al., 2016), como observado nos resultados apresentados por Mezzomo et al. (2010) ao realizar a comparação do rendimento na extração do óleo de amêndoa com diferentes solventes, obtendo 44% de rendimento em etanol. Ainda que outras referências apresentem percentagens superiores com solventes de menor polaridade, as condições de ensaio não são passíveis de comparação.

Além disso, outros fatores podem influenciar o rendimento da extração, a exemplo da composição do cultivo, de acordo com a época do ano e genótipo (Amaral et al., 2006b).

Tabela 2. Parâmetros de ensaio da extração sólido líquido em soxhlet apresentados na literatura para os frutos secos amêndoa e avelã.

Variedade	Solvente extrator	Amostra (g)	t (h)	Método de identificação	η (%)	Referência
<i>Amêndoa</i>	Éter etílico	-	-	HPLC e GC	-	Femenia et al. (2001)
<i>Amêndoa</i>	Éter etílico e metanol	-	-	GC-MS	45,03	Takeoka e Dao (2003)
<i>Amêndoa</i>	Éter de petróleo	-	-	GC-FID	25,19-60,77	Askin et al. (2007)
<i>Amêndoa</i>	Metanol	6	0,5	-	122,2 mg para conteúdo fenólico	Isfahlan et al. (2010)

<i>Amêndoa</i>	n-hexano	-	-	GC-MS	48,00	Sánchez-Vicente et al. (2009)
<i>Amêndoa</i>	n-hexano, diclorometano, acetato de etila e etanol	5	6,0	GC-FID	44,00 EtOH	Mezzomo et al. (2010)
<i>Amêndoa</i>	Etanol e n-hexano	30	6,0	GC-FID	31,10	Nascimento et al. (2016)
<i>Avelã</i>	n-hexano	-	8,0	-	66,20	Bernado-Gil et al. (2002)
<i>Avelã</i>	Éter de petróleo	15	-	GLC-FID	64,00	Amaral et al. (2003)
<i>Avelã</i>	Éter de petróleo	-	-	GC-FID	82,79	Alasalvar, Amaral e Shahidi (2006)
<i>Avelã</i>	Éter de petróleo	15	-	HPLC	-	Amaral et al. (2006a)
<i>Avelã</i>	n-hexano	-	6,0	HPLC	67,75	Köksal et al. (2006)
<i>Avelã</i>	Éter de petróleo	5	-	GC-FID	61,60	Oliveira et al. (2008)
<i>Avelã</i>	n-hexano e metanol	25	10,0	-	18,60	Demirbas (2008)
<i>Avelã</i>	Éter de petróleo	10	6,0	GC e HPLC	56,37	Cristofori et al. (2008)
<i>Avelã</i>	n-hexano e clorofórmio	10	6,0	HPLC	-	Riethmüller et al. (2013)
<i>Avelã</i>	n-hexano	20	18,0	GC-FID	0,52	Manna, Bugnone e Banchemo (2015)

Algumas discussões acerca da influência do tamanho de partícula também foram realizadas (Leo et al., 2005; Salgin e Salgin, 2006; Bernardo-Gil e Casquilho, 2007; Grosso et al., 2010). Özkal, Salgin e Yener (2005) concluíram que no período de extração rápida, o mecanismo de migração do óleo até a superfície é determinante na transferência de massa e, tende a ser facilitado quanto menor for o tamanho da partícula, proporcionando maior área superficial de contato com o solvente extrator, seja ele orgânico ou supercrítico, onde são observadas a influência da solubilidade e da resistência da transferência de massa do soluto na fase fluida.

Tendo em conta todas as considerações mencionadas anteriormente e tendo em conta os objetivos do Projeto, foram iniciados os ensaios de extração supercrítica (SFE) preliminares e extração sólido-líquido em soxhlet. Os ensaios em SFE foram desenvolvidos para amostras de amêndoa com pele, enquanto os ensaios através da unidade de soxhlet foram realizados para ambos os frutos secos, amêndoa e avelã com e sem pele. As plantas aromáticas frescas e desidratadas iniciaram o seu estudo de extração de aromas num futuro próximo.

Material e métodos

Preparação das amostras:

Amostras de frutos secos (amêndoa e avelã, com e sem pele) e plantas (alecrim e orégãos, desidratados e frescos) foram recebidos e tratados distintamente. O tomilho será rececionado brevemente.

Os frutos secos e as plantas aromáticas desidratadas foram fracionadas usando um processador de alimentos Philips (modelo: Hr7762/90 Mini Chopper, Philips Walita, Países Baixos), sendo armazenados em sacos de polipropileno com vedação e acondicionados no frigorífico a 7 °C. As plantas aromáticas frescas foram acondicionadas em sacos de polipropileno a vácuo e mantidas a -15 °C até aquisição de um moinho criogênico para redução granulométrica, com o objetivo de evitar a degradação das amostras pela libertação de enzimas polifenoloxidasas.

Tendo em vista a influência do tamanho de partícula na eficiência de processos de extração, definiu-se o uso de partículas com *mesh* 14 (<1,41 mm) para os frutos secos, separadas através de uma peneira vibratória (D-42781, Retsch, Alemanha). As amostras foram secas usando uma estufa com circulação de ar (Venticell, MMM Medcenter, Alemanha) a 40 °C durante 25 horas e, 15 g (base humida.) foram pesadas usando uma balança analítica (AS60/220, Radwag, Polónia) e foram colocadas num cartucho individual de celulose para extração, a cada ensaio realizado. Para testes em extração supercrítica, usou-se três cartuchos de celulose e para a extração sólido-líquido apenas um. A diferença deve-se ao tamanho dos equipamentos utilizados.

As Figuras 1 e 2 apresentam os grãos e partículas de frutos secos e, plantas aromáticas frescas e desidratadas, respetivamente.



Figura 1. Frutos secos em grãos e particulados (<1,44 mm): (a) amêndoa com e sem pele e (b) avelã com e sem pele, respetivamente.

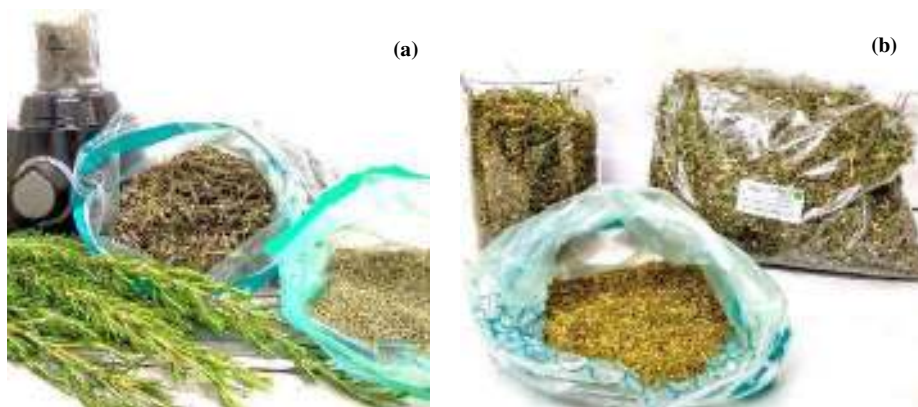


Figura 2. Plantas aromáticas: (a) alecrim (b) orégãos, frescas e desidratadas, respetivamente.

Extração supercrítica:

A obtenção dos aromas naturais por extração supercrítica foi realizada através de uma unidade de extração de CO₂ supercrítico desenvolvida por Gomes, Mata e Rodrigues (2007). Numa fase inicial foram realizados diversos ajustes no sistema, nomeadamente troca de peças e limpeza de válvulas, afim de melhorar o funcionamento da unidade de extração.

Foram realizados 11 ensaios preliminares, sendo que em 3 dos ensaios e com o objetivo de se obter um sistema semicontínuo, realizou-se a pressurização do sistema com alimentação de CO₂ na primeira e segunda hora de extração (tendo em conta que nesta altura nenhuma extração ultrapassou o tempo máximo de duas horas, com exceção do ensaio 10). Nos restantes testes, a pressurização ocorreu apenas na primeira hora de extração.

Os testes iniciais forneceram informações válidas para a definição do design experimental (DCCR), elaborado através do software Statistica 10 e apresentado na Tabela 3. Foram assim desenvolvidos 11 ensaios para cada amostra teste de frutos secos (4), variando a pressão de 80 a 100 bar, temperatura de 35 a 55 °C e o tempo de extração de 2 a 4 horas.

Tabela 3. Design experimental fatorial (DCCR) extração supercrítica.

Ensaio	P (bar)	T (°C)	t (h)	d (kg·m ⁻³) ¹
1	80	35	2	416,27
2	100	35	2	712,90
3	80	55	2	203,72
4	100	55	2	325,28
5	80	35	4	416,27
6	100	35	4	712,90
7	80	55	4	203,72

8	100	55	4	325,28
9	90	45	3	337,40
10	90	45	3	337,40
11	90	45	3	337,40

¹ Fonte: NIST Library, 2018.

Termodinamicamente, diferentes pressões e temperaturas combinadas originam mudanças na densidade do gás de extração, o CO₂, o que tem influência direta na transferência de massa dos compostos presentes nas amostras. Os resultados serão analisados estatisticamente, com uma significância de 5%.

Para a extração supercrítica das plantas aromáticas, uma análise bibliográfica e testes preliminares deverão ser realizados previamente para a definição dos parâmetros experimentais.

Extração sólido-líquido:

Com o intuito de obter dados comparativos à extração supercrítica, tendo em conta o rendimento e seletividade da extração, realizou-se extrações sólido-líquido para a amêndoa com e sem pele, em triplicado, usando um sistema de extração com refluxo intermitente soxhlet. Tendo em conta a pesquisa bibliográfica apresentada na Tabela 2, definiu-se os parâmetros de extração, conforme mostra a Tabela 4.

Tabela 4. Parâmetros de extração sólido-líquido usado uma unidade de extração Soxhlet.

Solvente extrator	Quantidade de amostra (g)	Tempo de extração (h)	Temperatura (°C)	Repetibilidade	Equipamento
n-hexano (P.A., Merck, Alemanha)	15	6	75	3	Soxhlet extrator 250 mL

A escolha do solvente n-hexano como solvente extrator deve-se ao facto de apresentar uma maior seletividade para a extração dos componentes apolares de interesse. De acordo com Petrovic et al. (2016), o hexano e o CO₂ apresentam maior seletividade quando comparados ao etanol, por exemplo.

Após extração, o óleo essencial foi concentrado num evaporador rotativo (RE 100, Bibby Scientific, Inglaterra) e seco usando uma estufa com circulação de ar (Venticell, MMM Medcenter, Alemanha).

Caracterização dos extratos

A identificação dos compostos presentes nos extratos obtidos por extração supercrítica e extração sólido-líquido foi realizada através de cromatografia gasosa (GCMS TQ8040, Shimadzu, Japão). A Tabela 5 mostra as três metodologias de análise testadas.

Tabela 5. Métodos cromatográficos testados em extratos de amêndoa transesterificados.

Método 1			Método 2			Método 3		
Taxa (°C/min)	T final (°C)	Tempo de espera (min)	Taxa (°C/min)	T final (°C)	Tempo de espera (min)	Taxa (°C/min)	T final (°C)	Tempo de espera (min)
-	100	5	-	100	5	-	100	5
5	160	10	2	160	10	5	180	5
10	220	10	10	220	15	5	220	5
-	-	-	-	-	-	2	250	5

Em todos os métodos a temperatura do forno se manteve em 100 °C, temperatura de injeção em 220 °C e o volume de injeção de 2 µL. Para o detetor de massas usou-se a temperatura de fonte de iões de 230 °C, temperatura de interface de 240 °C e tempo de corte do solvente de 7 min, com exceção do método 2 que iniciou em 5 min.

Os compostos identificados, tempos de retenção e similaridade foram comparados para definição da rampa de ensaio adequada.

Previamente às injeções, as amostras sofreram um tratamento de preparação de metil esterres, de modo a que se garantisse a parcial ou completa destruição de grupos epoxy, hidroperoxy, ciclopropenyl, ciclopropyl e possivelmente grupos hidroxyl (AOAC, 1965). Para a transesterificação dos extratos obtidos no SFE, seguiu-se metodologia proposta por Cristofori et al. (2008), com adaptações. Utilizou-se uma proporção de 2:1:2 de extrato e metanol a 2N KOH, seguida de 3 minutos de agitação intensa usando um vortex mixer (Zx³, Velp Scientifica, Itália) e adição de n-heptano (P.A., Merck, Alemanha). Um esquema genérico da reação é descrito na Figura 3, sendo o n-heptano o reagente adequado para a injeção da amostra na coluna cromatográfica como mencionado por Femenia et al. (2001), Bernardo-Gil et al. (2007) e Amaral et al. (2003).

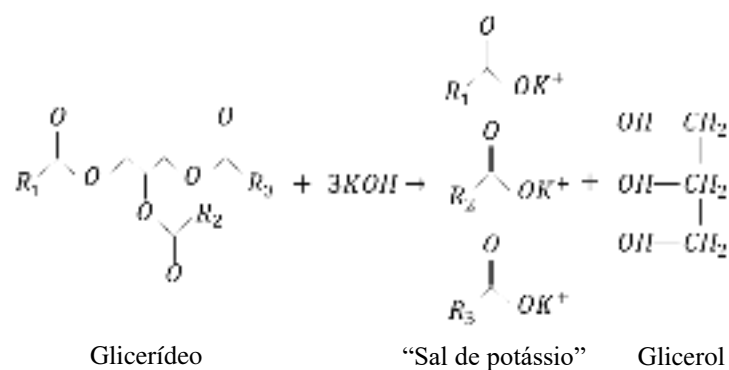


Figura 3. Reação de preparação de metil ésteres (transesterificação).

O extrato resultante da extração sólido-líquido deverá ser tratado, previamente à identificação dos compostos, através de uma nova metodologia para a separação dos ácidos gordos e glicerol, uma vez que o método aplicado aos extratos obtidos por extração supercrítica não foi eficiente. Espera-se aplicar os conceitos descritos por Metcalfe e Wang (1981) para a preparação de ácidos gordos metil éster transesterificados usando catalisador básico orgânico.

Humidade: As amostras de frutos secos foram submetidas à análise de humidade usando uma estufa com circulação de ar (Venticell, MMM Medcenter, Alemanha) a 105 °C até se obter um peso constante. Os ensaios foram realizados em triplicado.

Termogravimetria diferencial: Os ensaios termogravimétricos (TGA) foram realizados usando um equipamento de termogravimetria (TG 209F3, Netzsch, Estados Unidos). As amostras (6 a 8 mg) foram aquecidas a uma taxa constante de 10 °C·min⁻¹, de 25 a 700 °C, em atmosfera inerte (azoto) e oxidativa (ar).

Microscopia eletrónica de varrimento (SEM): A análise de microscopia eletrónica de varrimento foi aplicada à amostra, a amêndoa com pele, submetida à extração supercrítica. O objetivo foi visualizar possíveis modificações na morfologia dos pequenos grânulos do fruto seco oriundos da alta pressão exercida e consequente extração. As imagens foram obtidas em equipamento (Phenom ProX, ThermoFisher Scientific, Alemanha), com voltagem máxima do feixe de 10 kV, ampliação de 1500 a 3000 vezes e escala de 20 a 50 µm.

Resultados:

Extração supercrítica:

Os ensaios iniciais, realizados com amostras de amêndoa com pele, foram esclarecedores quanto ao funcionamento do equipamento de extração supercrítica. Todos os extratos foram obtidos no primeiro separador e a Tabela 6 apresenta os resultados obtidos para os 11 ensaios teste.

O rendimento total foi calculado a partir da razão entre a massa de óleo extraída e a massa da matéria-prima utilizada, em base seca (b.s.).

Com base nos resultados apresentados na literatura, o rendimento obtido foi consideravelmente inferior e pode ser justificado pelas baixas pressões e altas temperaturas usadas na extração, em função das limitações do equipamento. É possível observar que usando baixas densidades, há uma menor tendência para a transferência de massa pelo CO₂. Esta situação deveu-se ao facto de o sistema de pressurização se mostrar ineficaz em virtude do baixo volume de CO₂ líquido presente na garrafa, não atingindo assim a pressão inicial reconhecida como usual, 60 bar e, por isso chega ao extrator no estado gasoso, como observável no diagrama de fases do CO₂. Desta forma, uma nova garrafa de CO₂ foi adquirida como medida de mitigação.

Tabela 6. Parâmetros de análise e resultados obtidos nos ensaios iniciais de extração supercrítica.

Nº do ensaio	P (bar)	T (°C)	p (hCO ₂) ¹	t (h)	d (kg·m ⁻³) ²	η (%)
1	80	60	1+1	2	191,62	0,033
2	100	60	1	2	289,95	0,000
3	80	60	1+1	2	191,61	0,000
4	100	60	1	2	289,95	0,907
5	80	60	1+1	2	191,62	0,000
6	80	40	1	2	277,90	0,000
7	80	50	1	2	219,18	0,258
8	80	50	1	2	219,18	0,376
9	75	35	1	2	272,97	1,599
10	80	55	1	4	203,64	0,256
11	75	35	1	1	272,97	1,694

¹ tempos de pressurização de CO₂

² Fonte: NIST Library, 2018.

As baixas percentagens podem também estar associados à elevada temperatura usada na extração, necessária para que se atingisse pressões superiores a 75 bar, uma vez que mais

de 26% do conteúdo volátil da amostra de amêndoa são compostos termolábeis (Mezzomo et al., 2010).

Nos ensaios 2, 3, 5 e 6 não houve extração ou recuperação dos extratos, possivelmente devido à existência de fugas no sistema, uma vez que se tratam de aromas naturais e, portanto, são voláteis.

Os melhores resultados foram obtidos nos ensaios 9 e 11, independentemente do tempo de extração, usando uma pressão de 75 bar e temperatura de 35 °C. A densidade do componente extrator, o CO₂, apresenta um valor médio de 272,97 kg·m⁻³ o que, dentre das extrações realizadas, foi o maior valor. No entanto, novos ensaios são necessários afim de alcançar resultados confiáveis e reproduzíveis.

Extração sólido-líquido:

O resultado das extrações sólido-líquido realizadas em soxhlet são apresentadas na Tabela 7.

Tabela 7. Rendimentos médios da extração de óleo essencial de amêndoa, com e sem pele, em extrator sólido-líquido soxhlet.

Amostra	Média (%)	DP (%)
Amêndoa com pele	60,18	0,91
Amêndoa sem pele	62,89	0,69

Médias de $60,18 \pm 0,91\%$ e $62,89 \pm 0,69\%$ foram obtidas para amostras de amêndoa com e sem pele, respetivamente. Os valores são superiores aos mencionados na literatura, em condições semelhantes. Mezzomo et al. (2010) atingiram um rendimento de $44,00 \pm 2,00\%$ em extração realizada com etanol, por 6 horas, enquanto Nascimento et al. (2016) extraíram $31,10 \pm 0,30\%$ de óleo essencial de amêndoa após 6 horas de refluxo intermitente, em etanol. Os autores obtiveram valores semelhantes com o uso de n-hexano, $30,09 \pm 0,02\%$ de rendimento, enquanto Mezzomo et al. (2010) chegou aos $25,00 \pm 2,00\%$ com o mesmo solvente extrator. É previsto que a extração com etanol forneça rendimentos superiores ao n-heptano em função da sua polaridade, o que reduz a seletividade de compostos extraídos.

Resultados com rendimentos elevados também foram mencionados por Sánchez-Vicente et al. (2009) em extração com n-hexano, atingindo os 48,00%. Takeoka e Dao (2003), por sua vez, apresentaram o valor de $45,03 \pm 0,80\%$ de extração com metanol.

O rendimento elevado apresentado neste estudo pode estar associado à variedade do cultivo, ou ainda à preparação da amostra.

Para as plantas aromáticas frescas e desidratadas, não há resultados de extração a reportar.

Caracterização dos extratos:

Aproximadamente 40 picos foram obtidos, dentre os quais, 27 foram identificados de acordo com a biblioteca e *database* do software *GCMS Postrun system* (GCMS-TQ8040, Shimadzu, Japão). Os 27 compostos possivelmente identificados estão apresentados na Tabela 8, de acordo com o método aplicado. É descrito também o peso molecular, em gramas por mol, a fórmula molecular, a classificação, respetivos tempos de retenção, em minutos, e similaridade de cada substância, em percentagem.

Tabela 8. Compostos possivelmente identificados do extrato de amêndoa transesterificado, obtido em SFE, peso molecular ($\text{g}\cdot\text{mol}^{-1}$), fórmula molecular, classificação, tempo de retenção (min) e similaridade (%) da substância, de acordo com o método cromatográfico aplicado.

n °	Composto	PM ($\text{g}\cdot\text{mol}^{-1}$)	Fórmula molecular	Classificação	Método 1		Método 2		Método 3	
					Tr (min)	Sim. (%)	Tr (min)	Sim. (%)	Tr (min)	Sim. (%)
01	Menthyl acetate	198	C12H22O2	Monoterpeno	9,245	90	10,180		9,255	92
02	α -bourbonene	204	C15H24	Sesquiterpenoide	12,035	89	14,580	89	12,025	89
03	Tetradecane	198	C14H30	Hidrocarboneto alcano	12,465	96	-	-	12,455	95
04	Caryophyllene	204	C15H24	Sesquiterpeno	13,035	92	16,355	94	13,025	93
05	β -farnesene	204	C15H24	Sesquiterpeno	13,890	91	18,275	88	13,880	91
06	Pentadecane, 8-hexil	296	C21H44	Hidrocarboneto alcano	14,890	88	20,315	89	14,880	88
07	2-Tridecanone	198	C13H26O	Cetona	15,015	92	-	-	15,010	93
08	2,4-Di-Tert-Butylphenol	206	C14H22O	Ácido fenólico	15,310	92	21,200	93	15,315	93
09	1-ciclohexyl-3-ethoxy- butan-2-one	198	C12H22O2	Cetona	15,870	87	22,470	88	15,865	87
10	Nonane, 5-(1- methylpropyl)	184	C13H28	Ácido graxo metil éster	-	-	-	-	16,080	80
11	Caryophyllene oxide	220	C15H24O	Terpenoide	17,315	90	24,940	90	17,305	91
12	Veridiflorol	222	C15H26O	Sesquiterpenoide	17,655	91	25,655	91	17,625	91
13	Heptadecane 2,6,10,15- tetramethyl	296	C21H44	Sesquiterpenoide	17,830	93	26,610	94	17,780	93
14	Octadecane, 5-methyl	268	C19H40	Hidrocarboneto alcano	-	-	-	-	20,070	86
15	Heptadecane 8-methyl	254	C18H38	Hidrocarboneto alcano	21,125	89	32,015	89	20,170	89
16	Lignocerato de metilo	338	C24H50	Hidrocarboneto alcano	26,065	93	37,760	93	22,635	93
17	Phytone	268	C18H36O	Cetona	28,215	88	-	-	23,780	86
18	Methyl palmitate	270	C17H34O2	Ácido graxo metil éster	30,890	91	46,915	90	26,750	92
19	Palmitic acid	284	C18H36O2	Ácido graxo metil éster	32,340	89	49,340	89	29,050	90
20	Hexatriacontane	506	C36H74	Hidrocarboneto alcano	32,490	88	49,540	89	29,300	88
21	Phytol	296	C20H40O	Diterpeno	34,360	88	51,760	89	32,225	90
22	Methyl Stearate	298	C19H38O2	Ácido graxo metil éster	34,695	-	-	-	32,685	88

23	Heptadecanoic acid, 15-methyl ester	312	C ₂₀ H ₄₀ O ₂	Ácido graxo metil éster	36,100	90	53,635	90	34,300	90
24	Hexatriacontane	506	C ₃₄ H ₇₀	Hidrocarboneto alcano	36,285	93	53,820	93	34,485	93
25	Hexatriacontane	506	C ₃₄ H ₇₀	Hidrocarboneto alcano	42,175	92	59,870	92	40,545	93
26	Hexacontane	842	C ₆₀ H ₁₂₂	Hidrocarboneto alcano	-	-	-	-	47,730	90
27	Menthyl isovalerate	240	C ₁₅ H ₂₈ O ₂	Monoterpeno	-	-	-	-	50,140	87

Os cromatogramas obtidos para cada método são apresentados na Figura 4.

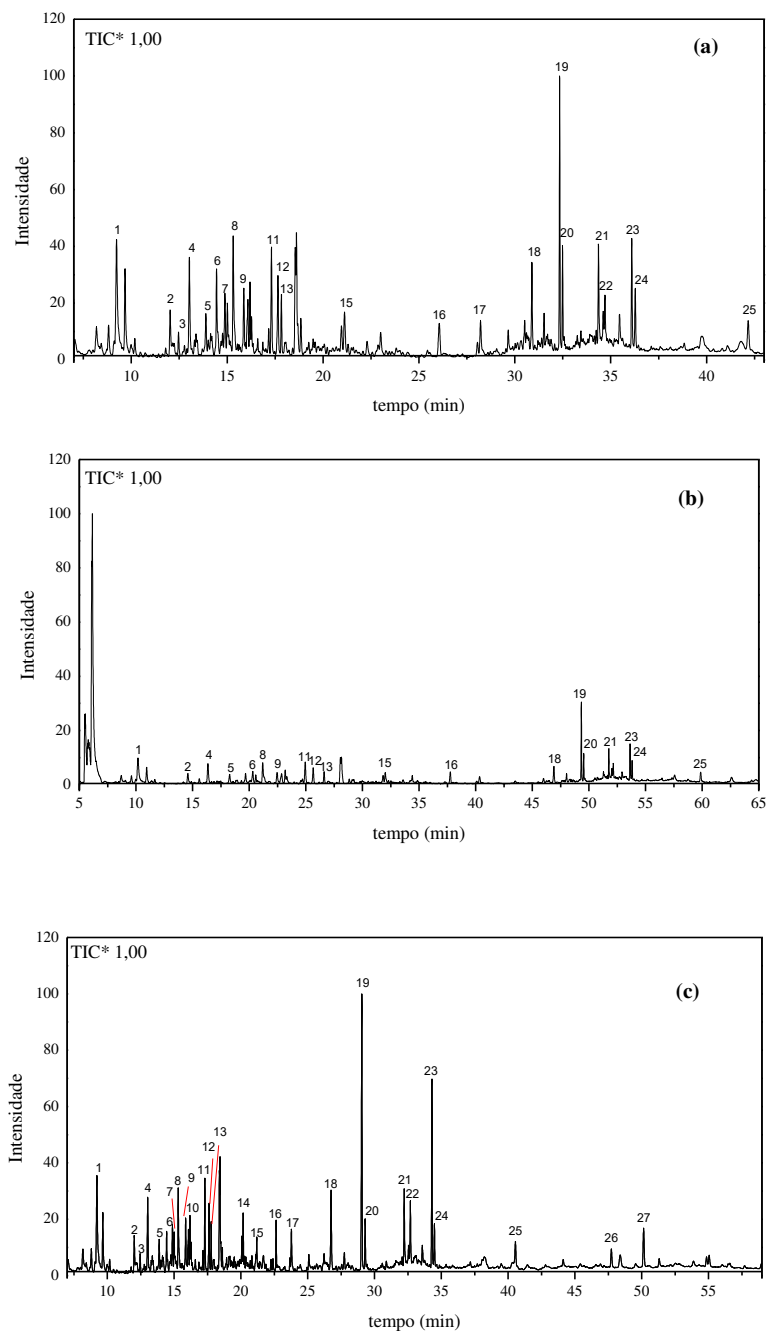


Figura 4. Cromatogramas do extrato de amêndoa, transesterificado e obtido em SFE, em três diferentes métodos de ensaio: (a) Método 1, (b) Método 2 e (c) Método 3.

Os compostos possivelmente identificados dizem respeito às seguintes classes: monoterpene (2), diterpene (1), sesquiterpene (2), sesquiterpenoide (3), terpenoide (1), cetona (3), hidrocarboneto alcano (9), ácidos graxos metil éster (5) e ácido fenólico (1). O método 3 apresentou o maior número de picos identificados e coerentes à matéria-prima em análise. A intensidade dos picos também foi notável em comparação com as restantes metodologias aplicadas.

De acordo com Machado et al. (2013), vários estudos apontam a presença de substâncias antioxidantes, antimicrobianas, anti-inflamatórias, anticancerígenas e anti-HIV em extratos naturais, sendo os principais compostos biológicos responsáveis por estas atividades os ácidos fenólicos, flavonoides, terpenos e sesquiterpenos. Estes biocompostos são identificados e quantificados, em valores representativos, quando extraídos via extração supercrítica.

Substâncias dos grupos terpeno e terpenoide são precursores dos esteróis e exibem inúmeros benefícios à saúde humana (Gómez-Coca, Pérez-Camino e Moreda, 2015). Chamados de fitoquímicos, são componentes voláteis e se apresentam principalmente na forma de caryophyllene, α e β -pinene, limonene e germacrene em extratos naturais (Verma et al., 2013). A presença de terpenos em frutos secos foi citada por Velickovic et al. (2016), tendo o caryophyllene oxide como composto em maior quantidade.

Cetonas, aldeídos, hidrocarbonetos aromáticos e hidrocarbonetos lineares também são componentes voláteis e foram reportados por Erten e Cadwallader (2017), Agila e Barringer (2012) e Vázquez-Araújo et al. (2008) em extratos de amêndoa torrada.

O ácido palmítico (palmitic acid), um ácido gordo metil éster, foi identificado com maior intensidade nos três métodos de análise. Inclui-se nessa classificação o nonane, 5-(1-methylpropyl), o methyl palmitate, o methyl stearate e o heptadecanoic acid, 15-methyl éster. Diferentemente do indicado pela literatura (Fernandes et al., 2017), o ácido gordo de maior representatividade em óleos essenciais não foi identificado, o ácido oleico (oleic acid). A identificação dos picos a partir do padrão analítico favorecerá a uma análise mais assertiva e confiável dos cromatogramas obtidos, assim como a quantificação dos respectivos componentes.

Outros ácidos gordos tais como lauric, mistiric, arachidic e linoleic são citados como constituintes de extratos de amêndoa (Fernandes et al., 2017; Roncero et al., 2016). Erten e Cadwallader (2017) apresentam também a identificação de 26 diferentes aromas ativos em óleo de amêndoa torrada e Sanahuja et al. (2011) confirmaram a presença de 22 compostos em extratos puros do fruto, inúmeros em acordo aos citados na Tabela 8.

Humidade: O conteúdo de água presente nas amostras de amêndoa e avelã é apresentado na Tabela 9.

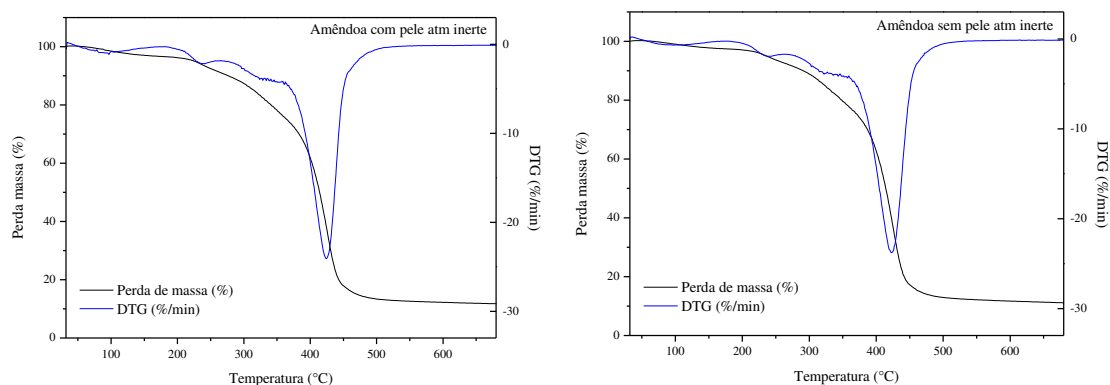
Tabela 9. Humidade média e desvio padrão das amostras de frutos secos.

Amostra	Humidade média (%)	± DP
Amêndoa com pele	4,39	0,13
Amêndoa sem pele	3,72	0,01
Avelã com pele	4,17	0,13
Avelã sem pele	1,67	0,04

Amostras com pele apresentam uma maior humidade quando comparadas com as amostras sem pele. A amêndoa foi o fruto seco com maior percentagem média de humidade, $4,39 \pm 0,13\%$, enquanto a amêndoa sem pele apresentou $3,72 \pm 0,01\%$. Conteúdo de água superior foi mencionado por Takeoka e Dao (2003) no estudo de amêndoa (*Prunus dulcis* (Mill.) D.A. Webb), atingindo os $11,39\%$. A diferença pode ser explicada em função do cultivo do fruto em análise.

A quantidade de água presente nas avelãs foi de $4,17 \pm 0,13\%$ e $1,67 \pm 0,04\%$, para os frutos com e sem pele, respetivamente. Oliveira et al. (2008) determinaram a humidade de diferentes variedades de avelã de Portugal e obtiveram valores médios de 3,00 a 5,60%. Já Köksal et al. (2006) chegaram a 2,49% e 5,25% de conteúdo de água em avelãs de dois diferentes cultivos da Turquia.

Análise gravimétrica: As curvas termogravimétricas são apresentadas na Figura 5.



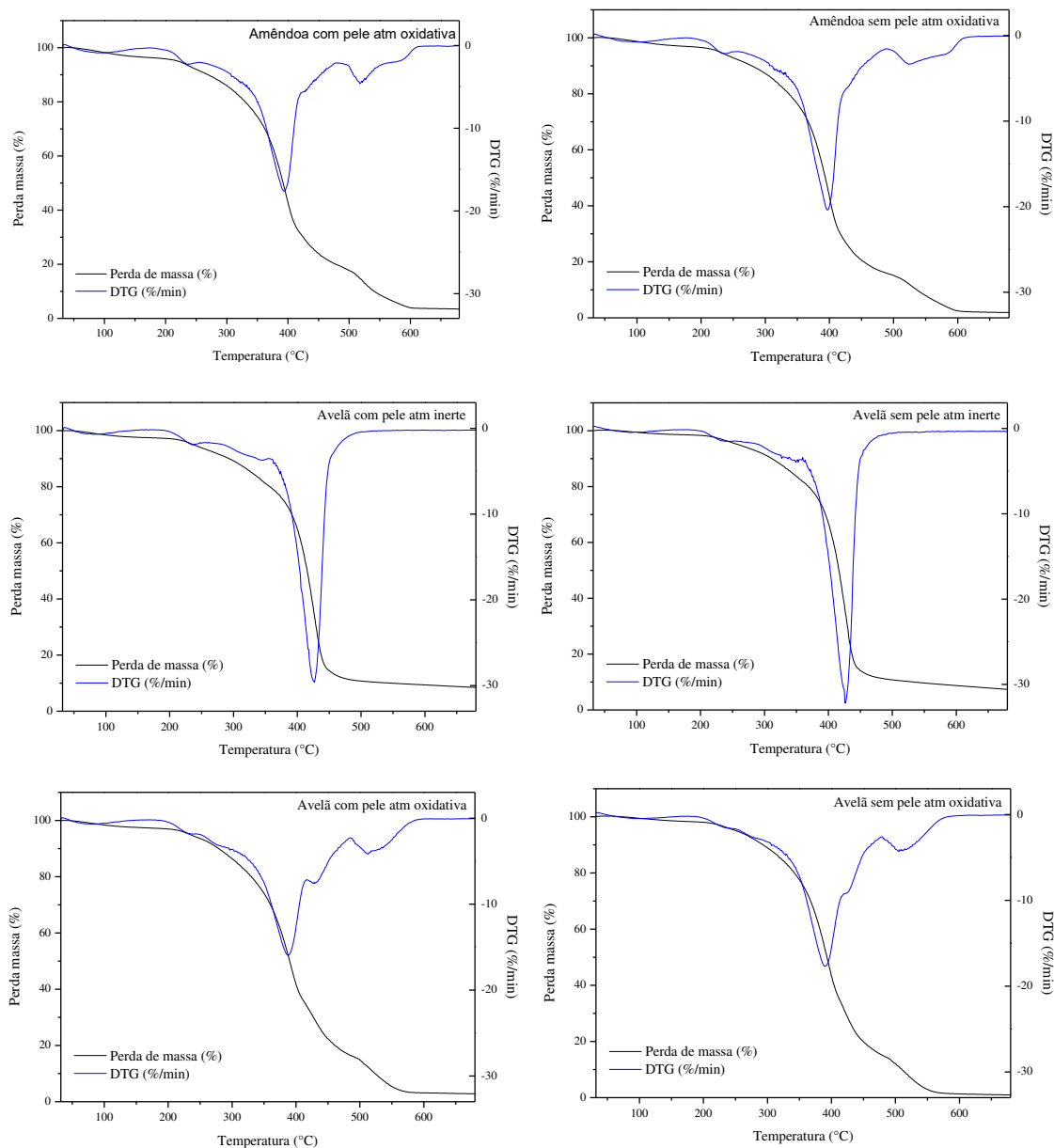


Figura 5. Curvas termogravimétricas obtidas para a amêndoa e avelã, com e sem pele, em atmosfera inerte e oxidativa.

Os resultados indicam perda de massa inicial a uma temperatura próxima dos 30 °C para todas as amostras analisadas, denominada região de decomposição I. A baixa decomposição térmica inicial pode ser atribuída à vaporização da água retida nos frutos secos, até 170 °C (Rezaei, Nasirpour e Tavani, 2016). A degradação se estende a temperaturas superiores a 500 °C e 600 °C, em atmosfera inerte e oxidativa, respetivamente. As regiões de decomposição térmica identificadas são apresentadas na Tabela 10.

Tabela 10. Dados termogravimétricos para amostras de amêndoa e avelã, com e sem pele, em atmosfera inerte e oxidativa.

Amostra	Atmosfera	Região	Faixa de temperatura (°C)	Perda de massa (%)	Massa residual (%)	
Amêndoa com pele	Inerte	I	32,6-99,0	1,58	11,66	
		II	99,0-179,0	1,93		
		III	179,0-267,0	5,71		
		IV	267,0-360,0	14,90		
		V	360,0-533,0	63,18		
	Oxidativa	I	32,0-101,0	1,83	3,41	
		II	101,0-170,0	1,77		
		III	170,0-254,0	4,69		
		IV	254,0-420,0	60,55		
		V	420,0-479,0	11,24		
		VI	479,0-562,0	12,55		
		VII	562,0-645,0	3,77		
	Amêndoa sem pele	Inerte	I	32,0-174,0	2,51	11,01
			II	174,0-267,0	5,22	
III			267,0-359,0	14,75		
IV			359,0-534,0	12,34		
Oxidativa		I	33,0-169,0	2,97	1,83	
		II	169,0-256,0	4,73		
		III	256,0-488,0	76,26		
		IV	488,0-695,6	14,06		

Amostra	Atmosfera	Região	Faixa de temperatura (°C)	Perda de massa (%)	Massa residual (%)
Avelã com pele	Inerte	I	33,0-100,0	1,49	8,31
		II	100,0-171,0	1,00	
		III	171,0-259,0	4,31	
		IV	259,0-360,0	13,66	
		V	360,0-546,0	69,46	
	Oxidativa	I	31,3-99,0	1,58	2,73
		II	99,0-167,0	1,06	
		III	167,0-247,0	3,53	
		IV	247,0-417,0	59,97	
		V	417,0-486,0	17,48	
		VI	486,0-606,0	13,27	
	Avelã sem pele	Inerte	I	31,8-100,0	0,53
II			100,0-169,0	0,86	
III			169,0-258,0	4,43	
IV			258,0-360,0	13,12	
V			360,0-517,0	71,55	
Oxidativa		I	31,3-99,0	0,54	0,92
		II	99,0-169,0	1,08	
		III	169,0-244,0	2,74	
		IV	244,0-418,0	63,40	
		V	418,0-480,0	17,21	
		VI	480,0-603,0	13,72	

De acordo com Maaloul et al. (2017), processos de pirólise resultantes da despolimerização das hemiceluloses e rompimento das ligações glicosídicas da celulose são características na faixa de temperatura de 220 a 310 °C em amêndoas, enquanto a degradação da celulose amorfa se dá a temperaturas de 310 a 410 °C. As regiões identificadas na Tabela 10 mostram que a decomposição é variável de acordo com a amostra e, apenas a amêndoa sem pele apresentou temperaturas superiores na região III quando comparada com os restantes frutos secos.

O comportamento descrito é observado nas curvas de DTG para as amêndoas com e sem pele em atmosfera inerte e oxidativa. Apenas em testes de atmosfera oxidativa a degradação da lignina ocorreu em temperaturas superiores a 450 °C, comportamento também indicado por Mechi et al. (2016), no entanto, em nitrogênio (inerte).

A massa residual para amostras de amêndoa, com e sem pele, foi semelhante em atmosfera inerte (11,66 a 11,01%, respetivamente), evidentemente superior ao resíduo identificado em atmosfera oxidativa (3,41 a 1,83%, respetivamente). Os valores são justificáveis dada a presença da curva DTG de decomposição final da lignina. A lignina possui degradação contínua de 100 a 900 °C, no entanto, a perda de massa se sobressai a temperaturas acima dos 370 °C (Rayón et al., 2015).

Curvas termogravimétricas semelhantes foram obtidas para a avelã, com e sem pele, em atmosfera inerte. Reações secundárias são observadas na presença de oxigênio, resultando em baixas quantidades residuais de matéria inorgânica: 2,73 e 0,92% com e sem pele, respetivamente. A diferença entre ambas é atribuída à maior quantidade celulósica presente em amostras com pele e, quando comparadas aos resultados apresentados para a amêndoa, indicam menor percentagem de celulose. Considerações semelhantes foram obtidas por Rayón et al. (2015) num estudo microestrutural, mecânico, termogravimétrico da amêndoa, avelã, damasco e pêsego. Os autores obtiveram as maiores perdas de massa a temperaturas de 210 a 330 °C (26,2 e 23,1% para amêndoa e avelã, respetivamente) e a 330 a 400 °C (25,6 e 29,2%, respetivamente). Para avelã com e sem pele, em atmosfera inerte, o conteúdo residual foi de 8,31 e 7,10%, respetivamente, superior ao apresentado em atmosfera oxidativa e inferior à amêndoa nas mesmas condições.

Tendo em vista a incorporação de aromas em produtos de panificação, é válido observar que, apesar de pequenas perdas de massa a temperaturas abaixo de 100 °C, além do conteúdo de água, o valor pode ser atribuído também ao conteúdo aromático volátil. Uma

vez que, a temperatura afeta as características físico-químicas de produtos naturais, formação de componentes voláteis e atributos sensoriais (Eric et al., 2014).

Microscopia eletrónica de varrimento (SEM): As amostras de amêndoa com pele, com granulometria < 1,44 mm, foram analisada susando um microscópio eletrónico de varimento com o objectivo de observar a morfologia das partículas antes e depois da extração supercrítica.

As amêndoas (*Prunus dulcis*) apresentam composição média de 48 a 67% em conteúdo lipídico, 12 a 22% em proteínas e 20% em carboidratos (Martínez et al., 2013; Ahmad, 2010; Özcan et al., 2011). Submetido ao processo de extração supercrítica, é suposto que se observe uma modificação na estrutura celular das partículas do fruto seco como resposta à transferência de massa proveniente das propriedades do CO₂, uma vez que o contato entre o material sólido e o solvente pressurizado tende a remover os compostos de interesse (Mezzomo, Martínez e Ferreira, 2009).

A Figura 6 mostra a superfície dos grânulos, antes (a e c) e depois (b e d) da extração supercrítica. São observadas cavidades circulares que contem óleo e estão disponíveis para extração. Visualmente, mesmo após a extração, as paredes das partículas parecem permanecer intactas. O mesmo foi observado por Femenia et al. (2001) e pode ser justificado pela possibilidade dos ácidos gordos percolarem a estrutura porosa sem a necessidade das paredes serem rompidas.

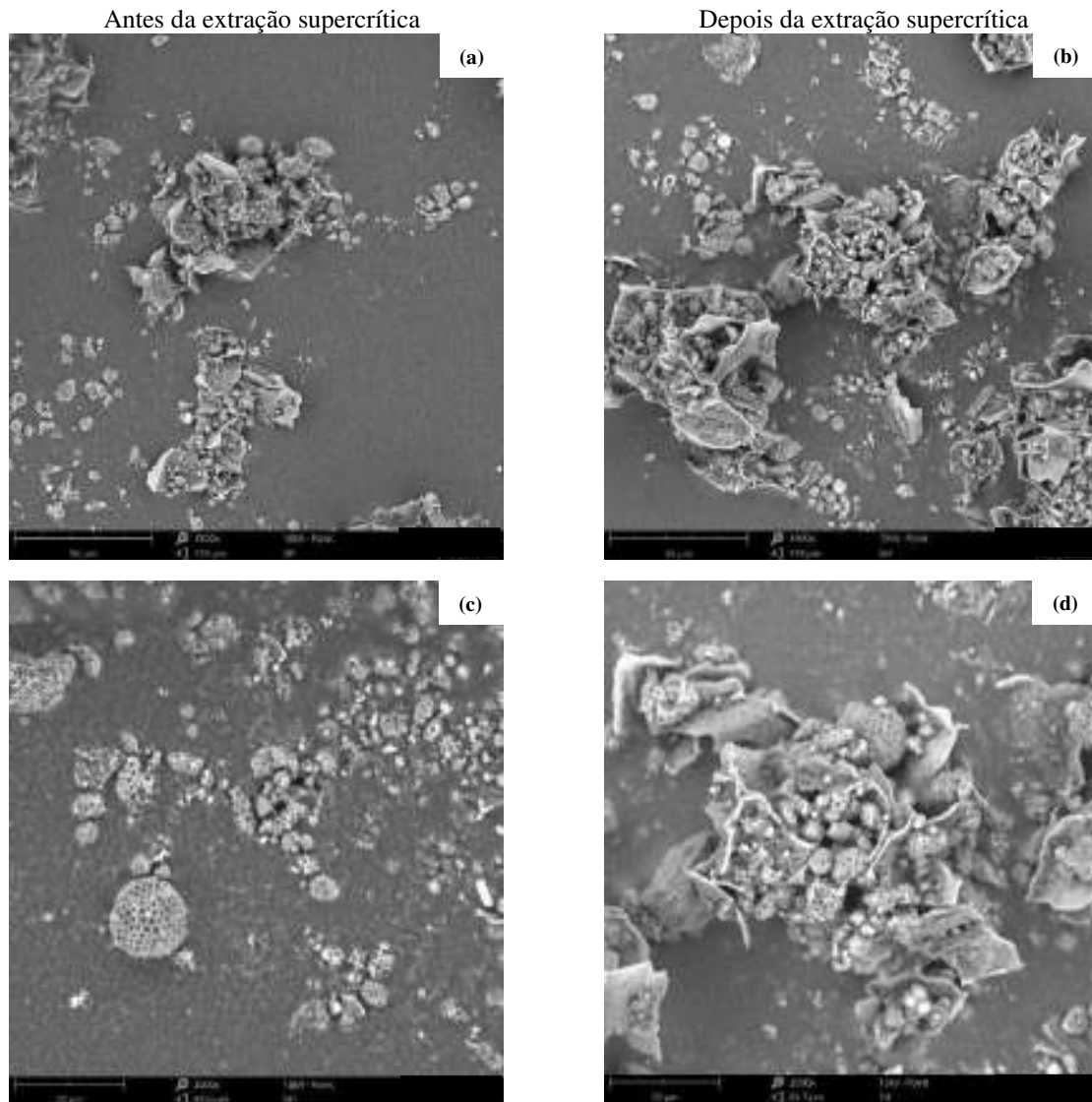


Figura 6. Imagens de micrografia eletrónica de varredura (SEM) de amostras de amêndoa triturada (< 1,44 mm) antes (a e c) e depois (b e d) da extração supercrítica com CO₂ com ampliações de 1500 x (a e b) e 3000 x (c e d).

Outro fator a ser considerado são as baixas pressões atingidas, responsável também pelo baixo rendimento obtido nas extrações supercríticas. Tal evidência implica na análise da diferença da distribuição das partículas na superfície e interior, de modo que é provável que a resistência à transferência de massa seja consideravelmente menor em áreas de contato direto com o solvente extrator (Marrone et al., 1998).

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Anexo 2

Trabalho realizado e resultados da tarefa 6.1.1

ANEXO 2 - Descrição detalhada dos trabalhos realizados na Tarefa 6.1.2 (18.2)

Tarefa	6.1.2 (18.2) - Projeto do sistema laboratorial de extração
Líder da tarefa	INEGI
Participantes	FEUP/LSRE-LCM; Paralab - Equipamentos Industriais e de Laboratório, S.A.
Data de início	01/12/2018
Data de fim	31/08/2019

Descrição dos trabalhos realizados:

No decorrer da presente tarefa analisou-se o estado da arte de sistemas de extração supercrítica, com análise das várias soluções laboratoriais existentes no mercado. Foram avaliadas as diferentes alternativas para a recirculação e/o reciclo do CO₂ no sistema, de modo de maximizar o rendimento do processo de extração. A Figura 1 mostra o diagrama atualizado do processo de extração.

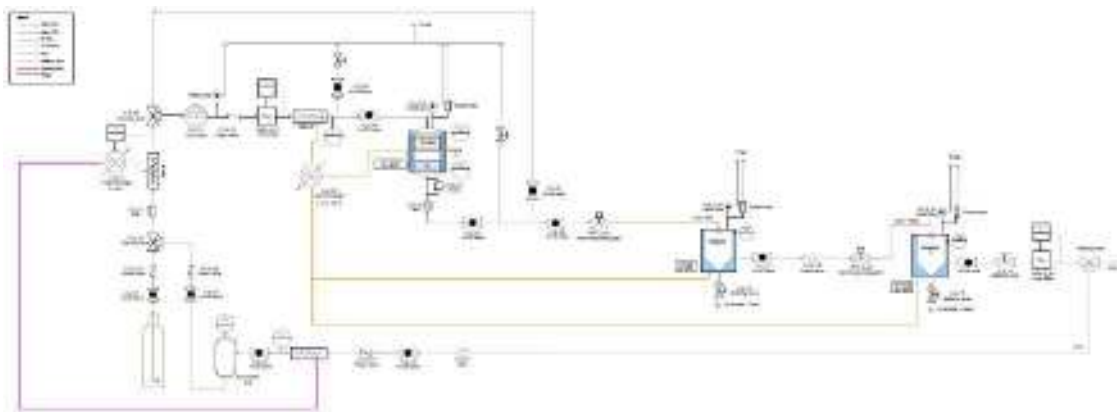


Figura 1 - Diagrama de processo do sistema de extração.

Desenvolveram-se várias soluções construtivas para o reservatório de extração e separadores, tendo por base, os requisitos para o sistema laboratorial.

Escolheu-se o design final para cada um dos vasos.

Desenvolveu-se uma cápsula, que permite a troca expedita de matéria-prima do interior do extrator, e que, permite o armazenamento de água gerada no processo.

Incorporou-se, em cada um dos vasos, um circuito exterior para um fluido de aquecimento.

Está em curso o processo de validação mecânica estrutural de cada uma das soluções construtivas escolhidas.

Está em curso, a alocação dos vários elementos mencionados e restantes componentes de compra necessários à instalação, numa estrutura compacta e móvel.

Está em curso a escolha de elementos standard (válvulas, vedantes, acessórios de instrumentação) e não standard, que sejam compatíveis com a aplicação e o seu design. Desenvolveram-se contactos com fornecedores e prestadores de serviços, com intuito de obter as soluções mais adequadas e orçamentação das mesmas.

Resultados:

Todos os conceitos desenvolvidos para o reservatório de extração, tinham por base um conceito de corpo cilíndrico oco com abertura pelos topos. As dimensões do corpo cilíndrico, semelhantes em todos os conceitos, foram definidas em função da resistência necessária, acessibilidade interior e volume requerido, procurando otimizar matérias-primas.

A principal diferença entre os conceitos residiu no modo de abertura das tampas. Verificou-se, que os conceitos inicialmente desenvolvidos, assentes numa desmontagem das tampas através de rosca ou elementos roscados, não permitiriam uma abertura expedita do reservatório. A elevada força gerada nas tampas conduziu a um grande comprimento de ligação roscado e a componentes amovíveis muito pesados para operação manual. A utilização de parafusos requeria o uso de ferramentas.

Os dois conceitos finais do reservatório, conseguiram compatibilizar uma abertura rápida com um diâmetro interior generoso, sem que isso se traduzisse em componentes com elevado atravancamento. Parte fundamental neste menor atravancamento das peças, foi o aço inoxidável escolhido, que além de oferecer uma maior resistência à corrosão que as ligas mais comumente utilizadas (AISI 304 e AISI 316) é substancialmente mais resistente mecanicamente.

No primeiro desses conceitos, a força vertical produzida na tampa pela pressão interior é suportada por dois pinos, conectados a uma peça roscada ao corpo cilíndrico do reservatório. A vedação entre a tampa e o interior do cilindro é realizado por um vedante de teflon (Figura 2). A abertura é realizada com o deslocamento horizontal dos dois pinos, permitindo a posterior remoção da tampa interior.

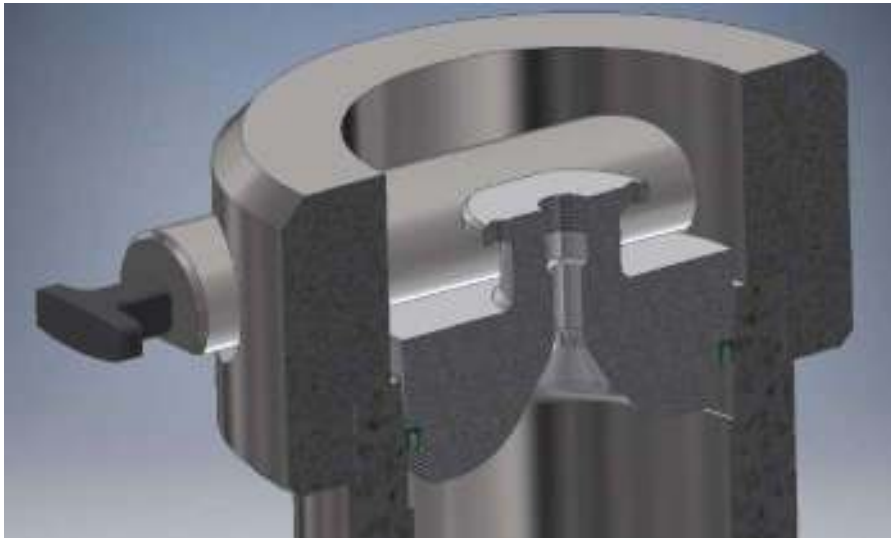


Figura 2- Conceito desenvolvido com fecho por pinos.

A solução construtiva final selecionada, baseia-se num fecho em baioneta. A forma geométrica dos dentes da tampa interior é replicada numa peça fêmea roscada ao corpo cilíndrico do reservatório. O suporte da força vertical durante a pressurização é realizado com o encosto vertical dos dentes da tampa aos dentes da peça roscada. Um elemento de mola com o pino garante o correto posicionamento entre os dentes. A abertura da tampa é feita através da rotação da mesma para uma posição de não interferência entre os dentes das duas peças, e posterior movimento vertical da tampa (Figura 3). A escolha deste conceito em detrimento do mencionado anteriormente deveu-se, principalmente, ao maior espaço na tampa para conexões a tubagens e acessórios.



Figura 3 – Conceito final, com fecho em baioneta.

Desenvolveu-se uma cápsula para colocar no interior do extrator, onde numa parte superior é colocada a matéria-prima a extrair e na parte inferior é acumulada a água durante a extração (Figura 4). A cápsula é solidarizada com a tampa superior e removida aquando desta. Depois de removida a cápsula é totalmente desmontável, em componentes de baixo peso, o que permite uma troca de matéria prima e limpeza do conjunto expedita, sobretudo quando comparada com as soluções de extração existentes no mercado. A entrada de fluxo de CO₂ é feita pela tampa superior, passando posteriormente pelo interior da cápsula e matéria-prima e saindo pela tampa inferior.

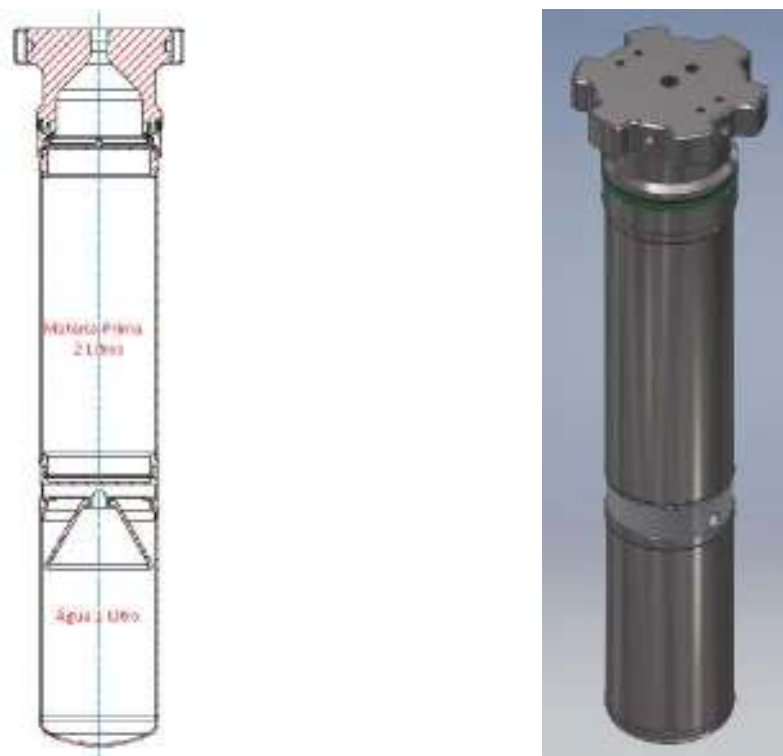


Figura 4 – Desenho 2D e 3D da cápsula de extração.

O design de abertura e fecho das tampas do extrator foi adotado para o primeiro separador, com um ajuste dimensional face às pressões inferiores de serviço deste vaso. A entrada e saída de fluxo de CO₂ são ambas realizadas pela tampa superior. Um tubo com furos radiais garante que o fluxo de entrada para uma parte mais inferior do vaso (Figura 5). A solução é proposta com um copo de recolha de extrato, contudo o design cónico da tampa inferior permite ao utilizador fazer a extração sem a utilização do copo, dependendo das quantidades de extrato em questão.



Figura 5 – Conceito desenvolvido separador 1.

A pequena volumetria e menor pressão de serviço do terceiro separador conduziram a um conceito bastante diferente, permitindo que o corpo cilíndrico do vaso sirva também como copo extração, existindo apenas abertura pela parte superior. A separação dos componentes é feita pelo desaperto da rosca do corpo cilíndrico, estando a tampa fixa.

Encontra-se em estudo, a possibilidade de revestir a cápsula do extrator e os vários componentes dos separadores com Teflon, com o intuito de facilitar o processo de limpeza entre processos de extração.

Desenvolveu-se um circuito de aquecimento exterior aos corpos cilíndricos dos três vasos mencionados, soldando um tubo de maior diâmetro e duas chapas de topo ao corpo dos reservatórios. A diferença de dimensão entre o diâmetro exterior dos vasos e o diâmetro interior do tubo soldado permite espaço ao fluxo de um filme de fluido de aquecimento. Um varão de baixo diâmetro conformado plasticamente contra o corpo dos reservatórios numa disposição helicoidal ao longo do comprimento destes, permitindo uma melhor distribuição do fluxo do fluido e, conseqüentemente, um aquecimento mais uniforme de todo o reservatório (Figura 6).



Figura 6 – Circuito de aquecimento dos reservatórios.

A validação estrutural dos reservatórios e garantia de segurança dos utilizadores são parte fundamental deste desenvolvimento. Com esse intuito, o projeto mecânico dos reservatórios procura cumprir a Europeia de Equipamentos sob Pressão 2014/68/EU seguindo a norma EN13445, harmonizada com essa mesma Diretiva. Garantindo assim, que os materiais de construção são permitidos pela Diretiva, seguindo as metodologias de cálculo adequadas, com margens de segurança adequadas e com uso de elementos limitadores de pressão. Pretende-se certificar os vasos, e para tal proceder a um ensaio hidroestático a uma pressão nunca inferior a 1,43 vezes a pressão de serviço.

Com objetivo de validar a geometria do reservatório sobre pressão foi realizada uma análise de elementos finitos, no software comercial Abaqus®. Afim de caracterizar o comportamento do material foi definido o modelo de elasticidade, com base no módulo de elasticidade ($E=200$ GPa) e coeficiente de poisson ($\nu=0.3$) e foram usados elementos tetraédricos lineares de 4 nós (C3D4) para discretizar a malha de elementos finitos.

Na Figura 8 pode observar-se a malha de elementos finitos para cada um dos elementos do reservatório. Uma vez tratar-se de uma zona que promove a concentração de tensões, a ligação roscada entre a tampa e o corpo do reservatório foi também modelada. Tendo

em conta as condições de simetria apresentadas pelo problema e afim de reduzir os custos computacionais apenas 1/8 do reservatório foi modelado, ajustando-se as condições de fronteira adequadas, restringido o deslocamento na direção normal às faces de simetria. Tendo em conta a pressão de teste prevista, foi aplicada uma pressão interna de 460 Bar.

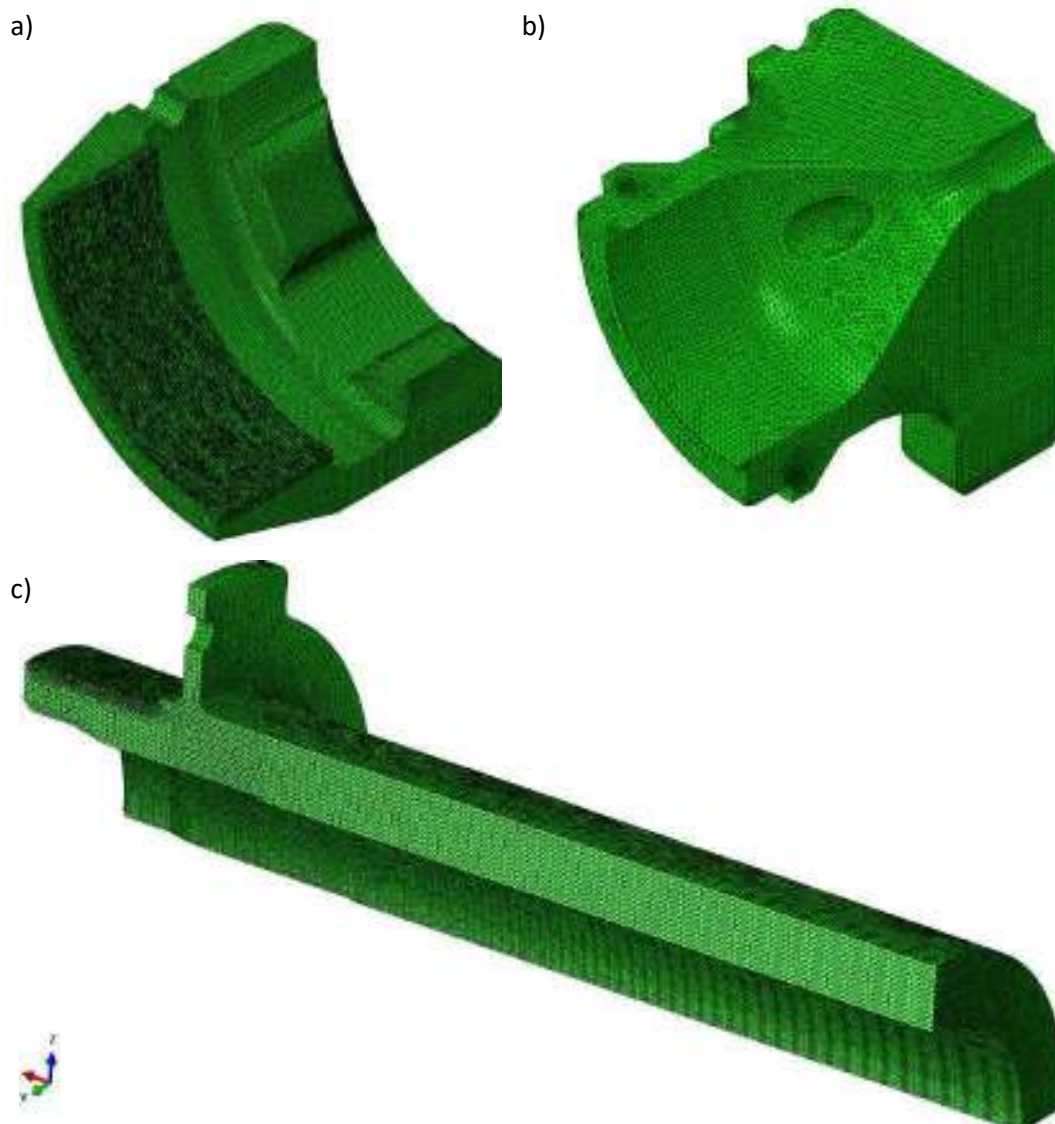


Figura 7 - Malha de elementos finitos: a) Tampa; b) Baioneta; c) Corpo reservatório.

O campo das tensões equivalentes é mostrado na Figura 8, verificando-se uma tensão máxima de 467 MPa na zona de contacto entre a baioneta e a tampa do reservatório, tratando-se um efeito muito local e potenciado pela malha de elementos finitos e modelação do contacto para essa localização. Quanto a região potencialmente mais crítica

(ligação roscada) verifica-se que as tensões obtidas são inferiores a 200 MPa (menos de metade da resistência mecânica do material), como apresentado na Figura 9.

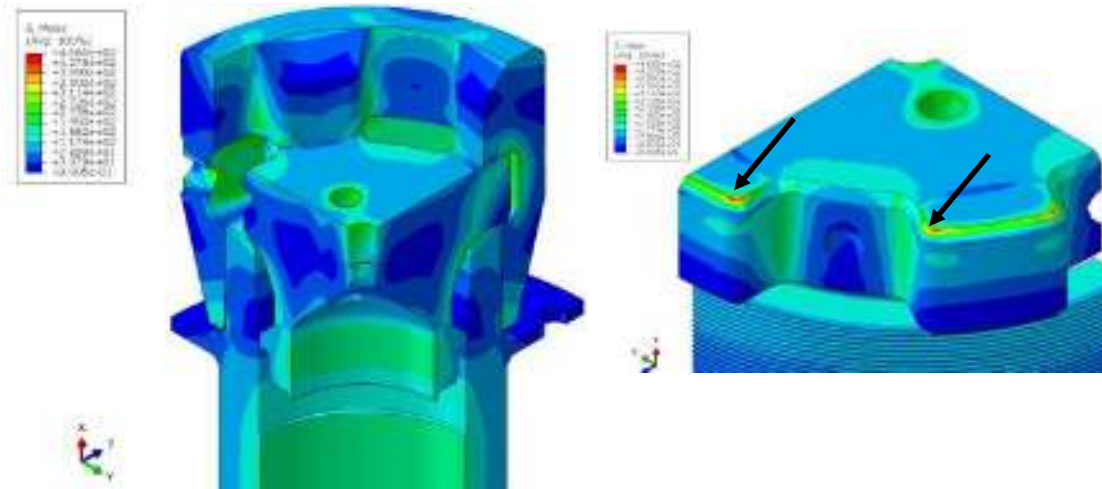


Figura 8 - Campo das tensões equivalentes obtido para o modelo global e na zona de contacto entre a tampa e baioneta.

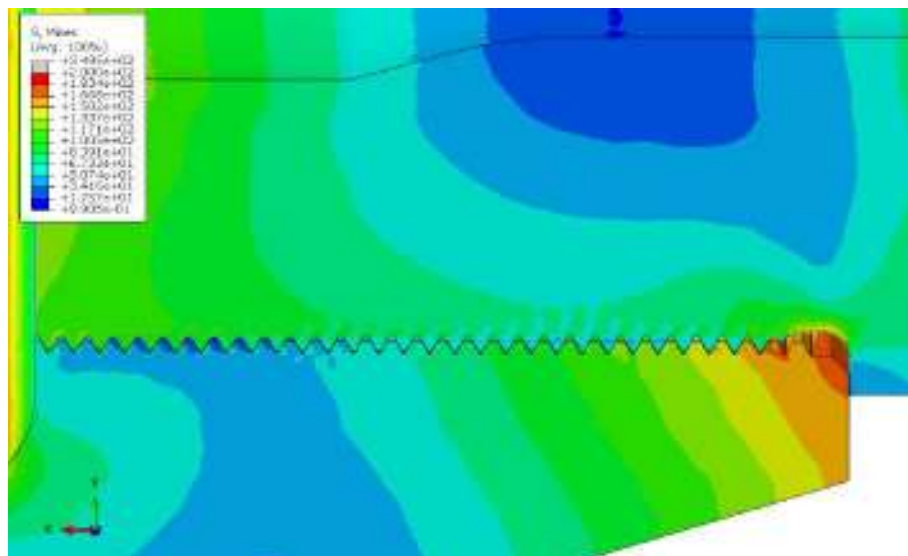


Figura 9 - Tensões equivalentes na ligação roscada entre a tampa e o corpo do reservatório.

Iniciou-se o desenvolvimento da estrutura de suporte da instalação, com a colocação na mesma dos produtos standard e não standard já definidos na mesma (Figura 10). Estudando o espaço e atravancamento necessário. O principal objetivo é desenvolver uma estrutura móvel e compacta, sem que isso, comprometa a sua acessibilidade e funcionalidade. A estrutura é composta por perfis de alumínio tubular de montagem modular. Este tipo de construção, comparativamente com uma estrutura de aço soldado, permite um baixo peso, boa resistência à corrosão, montagem expedita e sobretudo uma grande adaptabilidade a alterações futuras.

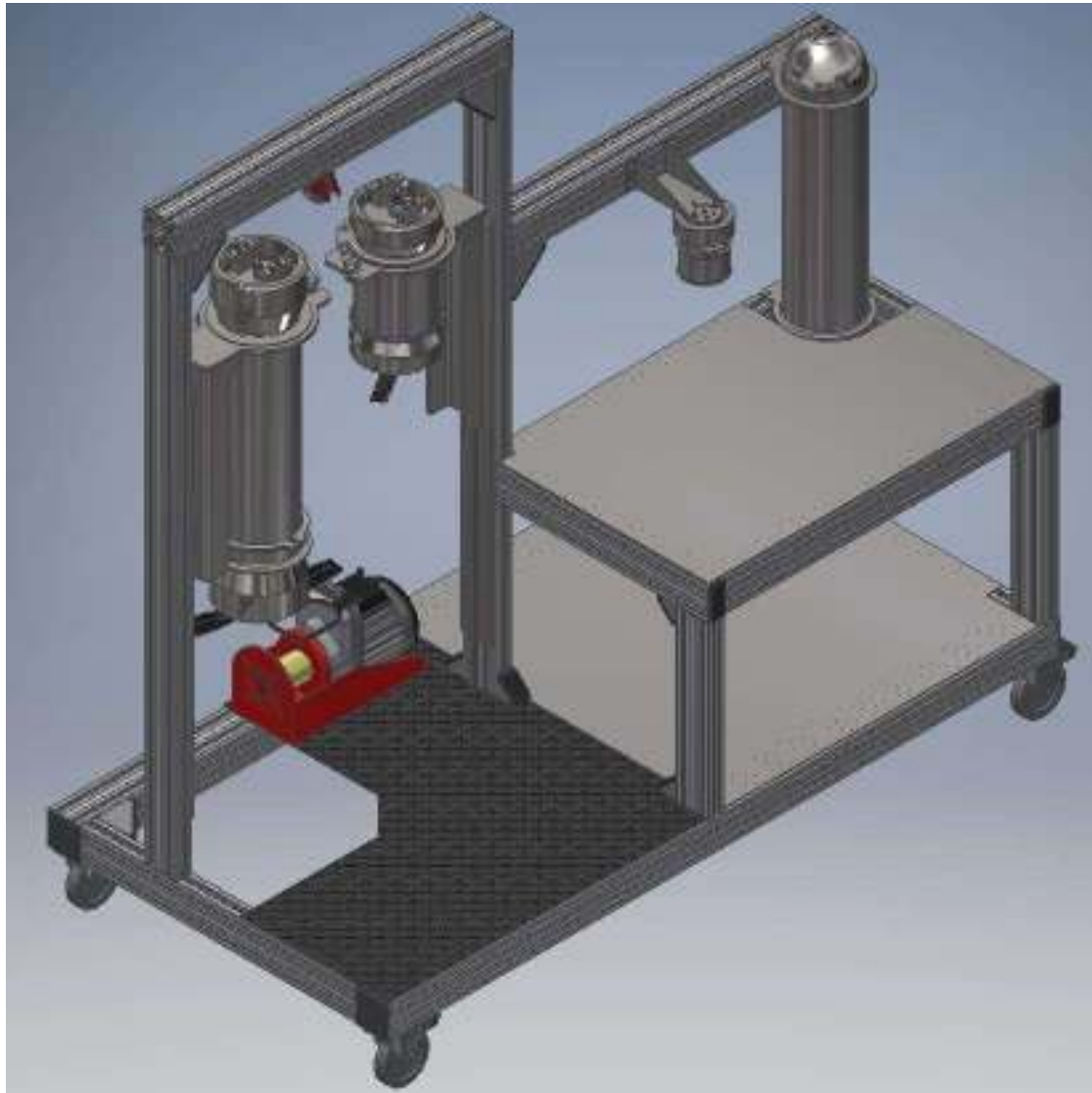


Figura 10 – Esquema da estrutura tubular da instalação.

Realizaram-se os desenhos técnicos 2D das diversas peças não standard, para discussão com possíveis parceiros de produção, a exequibilidade e orçamentação das mesmas (Figura 11).

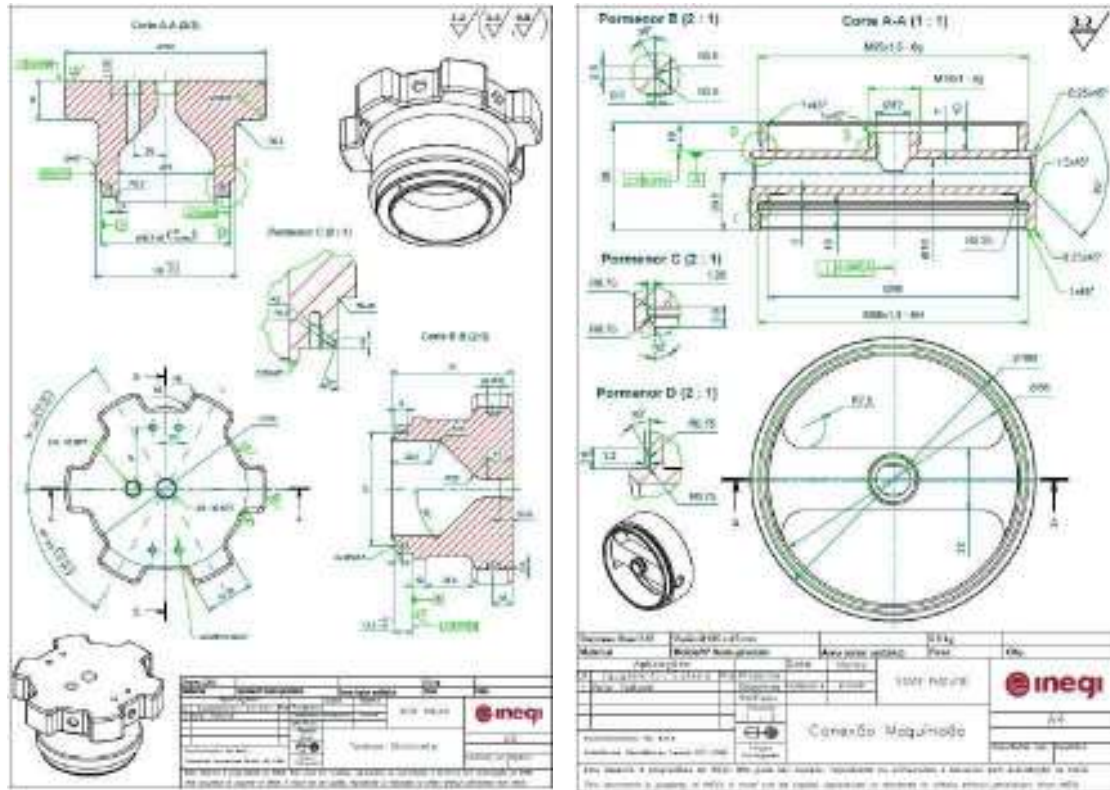


Figura 11 – Desenho técnico 2D de duas peças para maquinagem.

Anexo 3

Entregáveis



ValorNatural - Valorização de Recursos Naturais através da Extração de
Ingredientes de Elevado Valor Acrescentado para Aplicações na
Indústria Alimentar

Entregável nº 1.2.1

Versão do Documento: 5

Data de Submissão: 23/09/2019

Responsável: IPB

Nome do Documento: Manual de Procedimentos

Histórico de Revisão

Revisão	Data	Parceiros Envolvidos	Descrição
0.0	17/10/2018	IPB	Estruturação do documento
1.0	19/10/2018	IPB	Acréscimo de informação (Anexos do documento)
2.0	22/10/2018	IPB	Estruturação do documento (Anexos)
3.0	03/12/2018	IPB	Atualização de Anexos
3.1	7/02/2019	IPB	Atualização de informação relativa à utilização da <i>Intranet</i>
4.0	3/05/2019	IPB	Atualização da secção 4. Procedimentos para submissão de Pedidos de Pagamento
4.1	6/05/2019	IPB	Atualização da secção 6. Relatórios do Projeto
4.2	9/05/2019	IPB	Adição de anexo “Ficha de Potencial de Registo de Propriedade Industrial”
5.0	24/05/2019	IPB	Adição da secção 4. Pedidos de Alteração
6.0	23/09/2019	IPB	Atualização dos Anexos: Ficha de projeto, Modelo do Relatório Técnico-Científico, Modelo de Timesheets

Lista de Autores

Ana Saldanha (IPB)

José Santos (IPB)

Sumário

O Manual de Procedimentos tem como objetivo definir e reunir os procedimentos administrativos relativos ao projeto ValorNatural, bem como apoiar todos os beneficiários na gestão das suas funções. Sendo assim, o Manual de Procedimentos é um documento sujeito a atualizações, tendo em conta os procedimentos que deverão ser colocados em prática.

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1. Introdução

O Manual de Procedimentos define-se como um documento de carácter instrumental, e que pretende estabelecer as práticas e procedimentos no que diz respeito à gestão do Projeto ValorNatural (Anexo I - Ficha de Projeto). Este documento tem como objetivo apoiar todos os envolvidos na gestão, e no controlo das funções e competências que cada um assume.

O presente Manual é um documento dinâmico, e deverá ser alvo de atualização em função de alterações nos procedimentos de gestão a adotar.

2. Gestão do Projeto

Uma gestão ágil e equilibrada facilitará o cumprimento individual e coletivo dos objetivos propostos para o Projeto, e resultará no alcance dos resultados contratualizados. Neste sentido foi definida a estrutura de gestão descrita nos seguintes parágrafos.

O **Conselho de Administração (CA)** é a unidade coordenadora central de todas as atividades e responsável pela gestão de risco, é presidido pelo representante máximo da empresa promotora líder (Eng^a José Baltazar), sendo que ainda o constituem o Coordenador do Projeto (Prof^a Isabel Ferreira), os líderes de PPS (TecPan, IPB, OWNYA, FEUP), o Gestor de Projeto (Doutor José Santos) e o Gestor financeiro do Projeto (Dr. Eduardo Novais) (Figura 1). Todos os copromotores deverão estar representados neste órgão, que reunirá semestralmente, pelo que caso um determinado copromotor não desempenhe as funções mencionadas deverá eleger um representante. As decisões técnicas de alto nível, e ações a serem tomadas no caso de baixo desempenho dos beneficiários, serão tomados pelo CA. Assegurará ainda a identificação, proteção e exploração adequadas da Propriedade Intelectual (PI) gerada no Projeto. A estratégia de comunicação e divulgação, a ser monitorizada pela CA, deverá ser executada de forma a conciliar o fluxo aberto de informação e as oportunidades de exploração de PI que vierem a ser identificadas. A comunicação com organizações externas ao consórcio será gerida pelo CA, e pelo líder da atividade de comunicação.

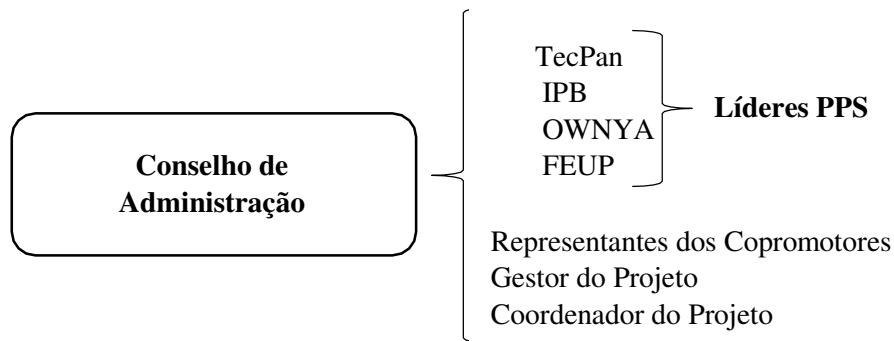


Figura 1. Diagrama representativo da organização do Conselho de Administração.

O **Conselho Consultivo (CC)** é composto por especialistas, nacionais e internacionais, de reconhecido mérito na temática do Projeto, e por representantes da comunidade empresarial. Este órgão terá a responsabilidade de aconselhar o consórcio quanto a aspetos estratégicos tais como, os planos definidos para atingir os resultados propostos, valorização económica dos mesmos, eventuais desvios na qualidade dos entregáveis previstos (Anexo II: Modelo para Entregável), bem como questões emergentes que poderão refletir-se na necessidade de alteração do projeto, direção das atividades de divulgação, e a via para a externalização dos resultados do projeto a nível nacional e internacional. O estatuto do CC é meramente consultivo, pelo que qualquer alteração sugerida ao projeto deve ser acordada pelo consórcio e aprovada pela estrutura do Portugal 2020. O CC **reunirá anualmente** com o CA ou, se necessário, com maior frequência. Posteriormente o Coordenador do Projeto, que pertence ao CA, comunicará as decisões relevantes à estrutura do Portugal 2020 (Figura 2).

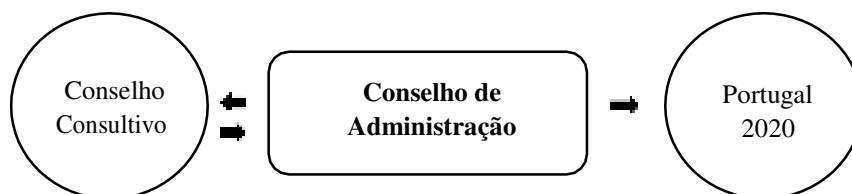


Figura 2. Diagrama representativo da relação do Conselho Consultivo, Conselho de Administração e Portugal 2020.

Descrevem-se de seguida as principais funções, para além do CA e do CC, definidas na estrutura de gestão do Projeto (Figura 3).

Os **líderes das atividades** são responsáveis pela execução das respetivas tarefas, tendo que reportar as mesmas ao líder da respetiva PPS. Em caso de qualquer desvio relativamente ao plano definido previamente, os líderes das atividades deverão reportar o sucedido aos líderes do PPS, para que em conjunto com o Coordenador, e os copromotores relevantes, possam discutir e acordar qual o melhor plano de ação. Caso o desvio implique a alteração na qualidade ou no prazo de qualquer entregável, a questão deve ser apresentada ao CA para aprovação sendo que, posteriormente o Coordenador informará o Portugal 2020 para aprovação formal e final.

Os **líderes dos PPS** são responsáveis pela monitorização e coordenação científica das atividades definidas, e são membros do CA do Projeto. Estes líderes necessitam de reportar ao Coordenador e ao CA o progresso dos trabalhos, o ponto da situação dos entregáveis e dos marcos estipulados, assim como reportar qualquer dificuldade encontrada. O alcance dos objetivos é fundamental, sendo que os líderes dos PPS devem apresentar propostas ao coordenador de reorientação dos trabalhos, reafectação de tarefas ou ainda alterações no orçamento.

O **Gestor de Projeto** faz parte da equipa de gestão do mesmo, bem como do CA. Tem como função desenvolver o manual de documentação e de procedimentos, ajudar na monitorização dos trabalhos e zelar pela conformidade dos entregáveis. A comunicação eficaz, eficiente e concisa entre copromotores também deverá ser assegurada pelo gestor.

O **Coordenador de Projeto** é o principal elo de comunicação entre todos os membros constituintes do Projeto e a estrutura do Portugal 2020, e faz parte do CA. As atividades de investigação, inovação, gestão, divulgação e a atempada submissão de entregáveis de alta qualidade em conformidade com o estipulado, também são competências que assume.

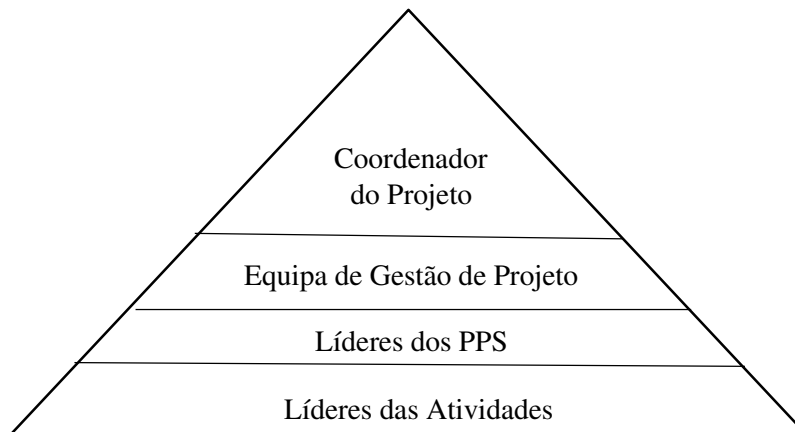


Figura 3. Diagrama representativo da organização do Projeto.

3. Gestão de Questões, Risco e Propriedade Intelectual

O Gestor de Projeto apoiará o Coordenador e os Copromotores na resolução de eventuais questões que possam surgir entre parceiros, e na gestão de riscos e de PI.

3.1 Tomada de Decisões

O Coordenador do Projeto, no âmbito do CA, facilitará o processo democrático de tomada de decisões, permitindo a todos os beneficiários apresentar o seu ponto de vista (Figura 4).

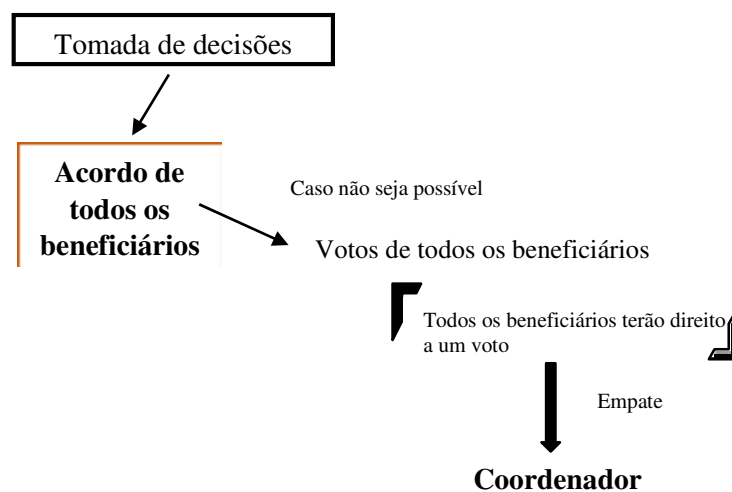


Figura 4. Diagrama representativo do processo de tomada de decisões.

3.2 Resolução de Questões

No caso de no decorrer do Projeto surgirem questões que não possam ser acordadas pelos beneficiários do consórcio, o CA poderá determinar a condução de uma investigação mais detalhada para apuramento dos factos, de forma a identificar a causa que originou o conflito e a fornecer uma análise objetiva aos beneficiários na forma de um relatório. Com base no relatório, será pedido aos beneficiários que tentem chegar a um acordo consensual ou que nomeiem um mediador externo. Na possibilidade da disputa envolver o Coordenador do projeto, os beneficiários devem acordar coletivamente a nomeação de um mediador alternativo, seja interno ou externo à parceria.

3.3 Gestão de Risco

A *Intranet* do projeto funciona para além de repositório de documentos e submissão dos mesmos, como calendário com os principais eventos e prazos, e como uma plataforma de comunicação. Neste âmbito será importante assinalar os Riscos identificados (na área Criar Conteúdo - (Escolher Grupo de Trabalho) - Risco) no grupo de trabalho a que a entidade pertence. O Consórcio conseguirá minimizar riscos significativos e implementar planos de recuperação adequados através de procedimentos de gestão apropriados, e da execução de planos de contingência adaptados. O CA é o órgão responsável pela gestão de risco no projeto, sendo que nas datas associadas aos marcos e entregáveis definidos no plano de trabalhos serão tomadas decisões “**STOP/GO**” em função das métricas de desempenho à data. Se se verificar a impossibilidade de cumprimento com uma data prevista para um determinado *milestone* ou entregável, será emitido um **STOP**. As medidas a adotar, a descrever nos planos de resposta aos riscos identificados, permitirão ao CA resolver e gerir aceitavelmente os riscos para cada PPS.

Os líderes dos PPS verificarão regularmente o progresso do Projeto de acordo com os planos acordados e compete-lhes informar o Coordenador de eventuais desvios (Figura 5). Estas ocorrências serão também formalmente revistas como ponto regular da agenda nas reuniões do CA. **Desvios graves ao cronograma do projeto** serão analisados em detalhe com o líder da PPS respetiva, e serão estabelecidas medidas e/ou ações corretivas, em conjunto com o Gestor do Projeto e a instituição beneficiária responsável pelo PPS.

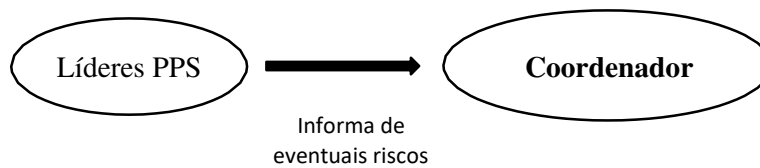


Figura 5. Diagrama representativo da comunicação de eventuais riscos.

No caso de as consequências dos desvios serem relevantes para o Projeto no seu todo e não puderem ser resolvidos pelo Coordenador bilateralmente com o beneficiário em questão, então o problema será transferido para o CA para resolução do mesmo (Figura 6).

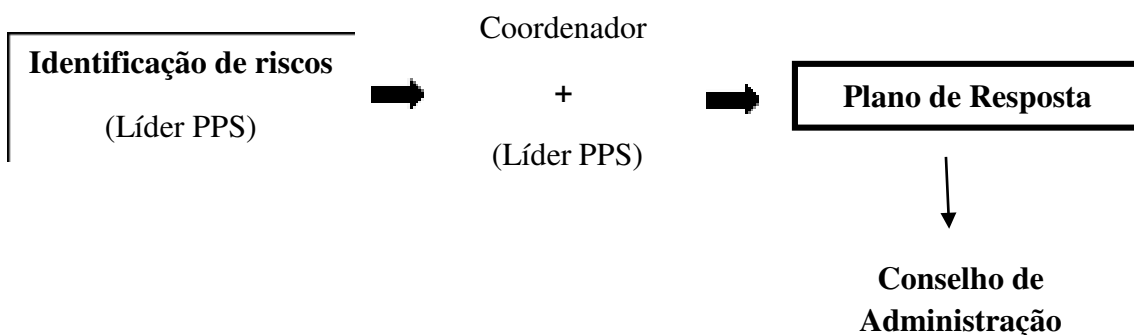


Figura 6. Diagrama representativo da identificação de desvios graves ao cronograma do projeto.

3.4 Gestão de PI

Os líderes das diferentes PPS identificarão oportunidades de proteção específicas para os resultados do Projeto, reportando-as ao CA para avaliação e definição dos respetivos planos de ação, devendo reportar em particular o seguinte:

- O resultado a explorar – funcionalidade, propósito, inovação, valor acrescentado para o conhecimento/mercado;
- O potencial valor comercial do resultado do projeto;
- Eventual utilização não-comercial;
- Impacto via desenvolvimento de novas metodologias;
- Parceiros envolvidos na exploração – qual o seu papel e atividades;

- O estado de desenvolvimento;
- Necessidade de trabalhos de investigação/desenvolvimento adicional e tipo de colaboração;
- Possíveis setores de exploração;
- Via de transferência de conhecimento a utilizar.

Para tal é disponibilizado um documento modelo (Anexo III) relativo a “Ficha de Potencial de Registo de Propriedade Industrial” que deverá ser remetido à equipa de gestão do projeto, preferencialmente através da Intranet.

4. Pedidos de alteração

Os pedidos de alteração solicitados pelos copromotores do projeto terão que ser submetidos através da conta PAS do beneficiário principal. Sendo assim, é necessário que cada copromotor proceda ao preenchimento dos documentos (“Pedido de Alteração Contratual - Minuta” e “Alteração na Equipa”) disponibilizados na *Intranet*, e posteriormente os envie ao IPB. O pedido de alteração terá que estar em conformidade com a informação reportada nos relatórios técnico-científicos e nos pedidos de pagamento posteriores à sua aprovação.

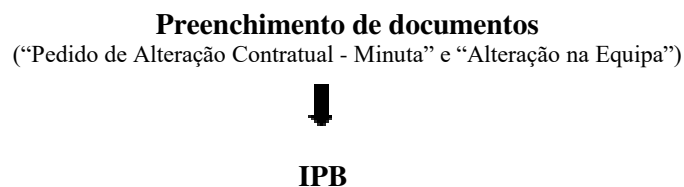


Figura 7. Diagrama representativo do procedimento para pedidos de alteração.

5. Procedimento para Submissão de Pedidos de Pagamento

Os pedidos de pagamento terão que ser efetuados em linha com o reportado no Relatório Técnico-Científico semestral (Anexo IV), modelo disponível na *Intranet*, e em eventuais pedidos de alteração. Cada pedido deve corresponder a pelo menos 10% da despesa elegível aprovada. O parceiro em questão introduz a informação no respetivo balcão do Projeto (PAS) e submete o pedido de pagamento pretendido (Figura 7). Porém, antes de submeterem o pedido de pagamento, cada parceiro deve enviar ao IPB o ficheiro Excel que se obtém no respetivo formulário da PAS por exportação da página “Movimentos”.

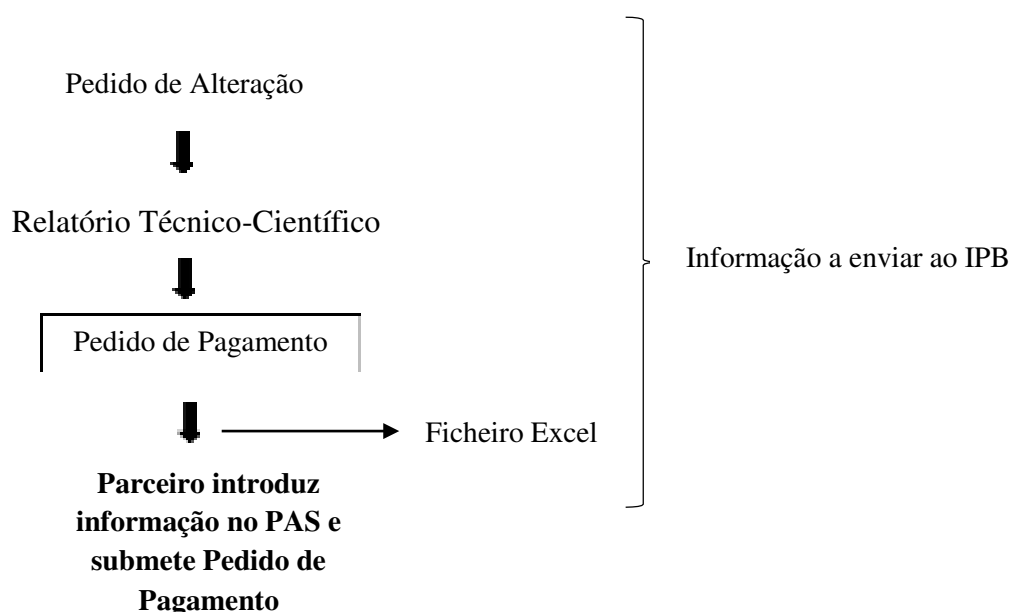


Figura 8. Diagrama representativo do procedimento para pedidos de pagamento.

6. Registo de Afetação de Técnicos

No que diz respeito à afetação de técnicos no âmbito do presente Projeto, é obrigação de cada beneficiário implementar um mecanismo que permita o registo da afetação a todas as atividades da empresa/instituição numa base diária, semanal ou mensal em papel ou tendo como base um sistema informatizado (Anexo V). Este registo (modelo disponibilizado na *Intranet*) deverá ser mantido para toda a equipa técnica aprovada, independentemente do método de imputação de custos (Figura 8). **As horas inseridas em pedidos de pagamento devem estar em conformidade com as horas constantes neste**

registo. Quando solicitados, os documentos deverão ser enviados para a equipa de gestão do Projeto através da *Intranet*, usando o formulário disponível no item “Contactos” que se encontra no Menu principal da plataforma.

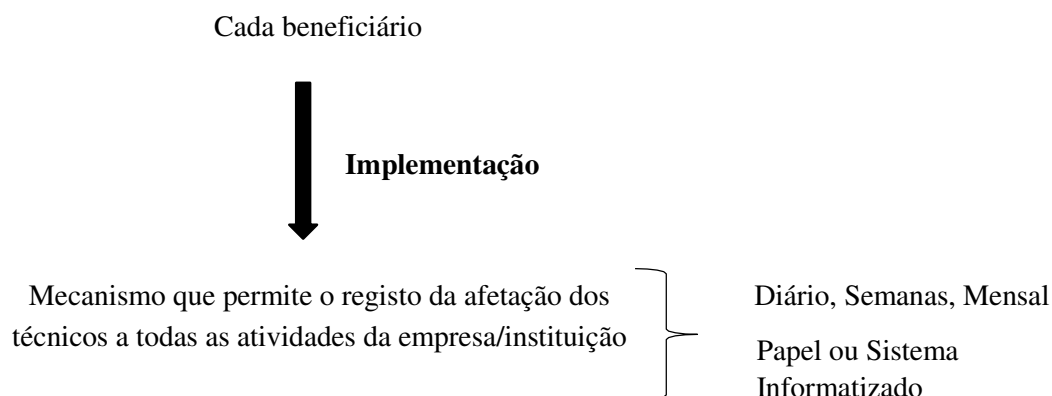


Figura 9. Diagrama representativo do procedimento para afetação de técnicos.

7. Relatórios de Projeto

Os relatórios de projeto, modelo disponível na *Intranet*, deverão demonstrar o progresso dos entregáveis tendo em conta o período estipulado para entrega, os marcos alcançados, o plano de trabalho detalhado para o próximo período, e informação relativa à interação entre os copromotores e com *stakeholders* relevantes. **Semestralmente**, o Gestor do Projeto submeterá um relatório de progresso que será avaliado e discutido nas reuniões do CA com a mesma periodicidade. De acordo com a análise efetuada aos relatórios será definido pelo CA um Plano de Ação, com o intuito de dar resposta às ameaças e oportunidades identificadas. Anualmente o Gestor de Projeto elaborará um relatório síntese ao Coordenador de Projeto para discussão na reunião anual do CC. **O Gestor e o Coordenador do Projeto serão responsáveis pela elaboração do relatório final** (Figura 9). Os entregáveis serão submetidos a um processo de revisão em duas fases, sendo revistos pelos líderes dos PPS e subsequentemente pelo Conselho de Administração. As versões finais dos documento gerados, tais como relatórios e entregáveis, deverão ser submetidas através da *Intranet*: menu “Criar Conteúdo”, escolher o grupo de trabalho em que quer submeter o documento e posteriormente escolher a opção “Ficheiro”. Quaisquer intenções de publicação de resultados devem ser aprovadas pelo CA.

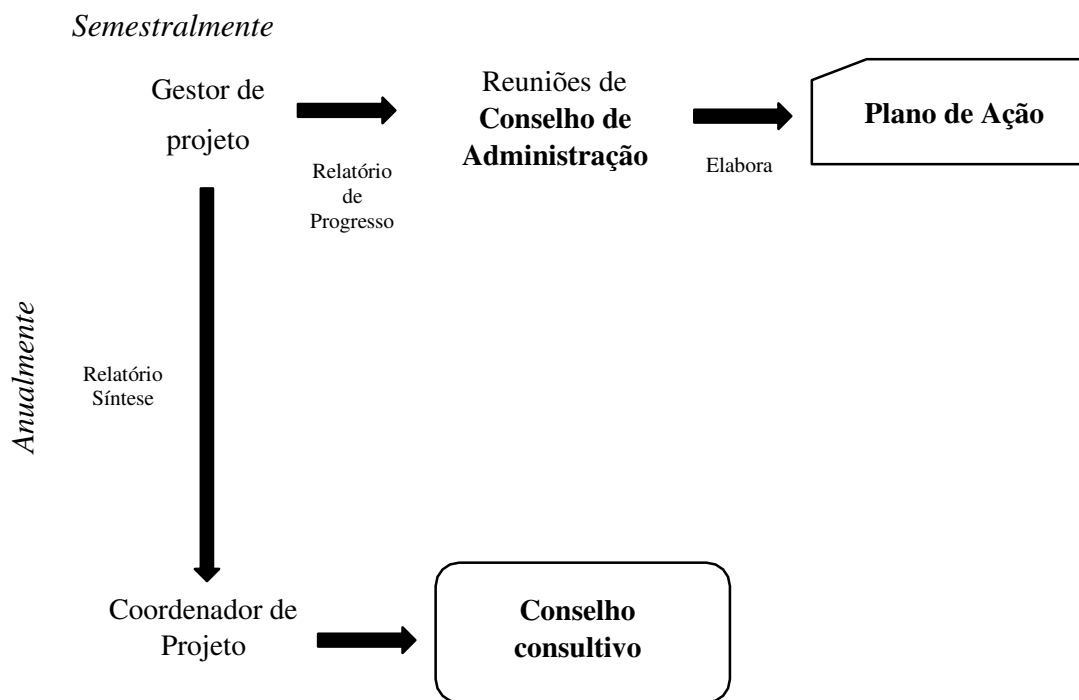


Figura 10. Diagrama representativo da entrega de relatórios do projeto semestralmente.

8. Ações de Publicitação e Disseminação

O documento “Guia de Apoio à Publicitação” (Anexo VI), modelo disponível na *Intranet*, tem como objetivo auxiliar os beneficiários do projeto no correto cumprimento das regras relativas à publicitação no âmbito do Portugal 2020. As ações de disseminação devem ser comunicadas atempadamente ao IPB através do documento “Comunicação de ações de disseminação” (Anexo VII), que deverá ser submetido na *Intranet* através do formulário disponível no item “Contactos” que se encontra no Menu principal da plataforma. O modelo é disponibilizado na *Intranet*, e inclui a seguinte informação:

- Tipologia da ação;
- Designação, âmbito e data da ação;
- Conteúdo da ação;
- Identificação de participantes.

O documento “Comunicação de Ações de Disseminação” terá de ser enviado ao IPB pelo menos com 15 dias de antecedência da ação de disseminação. O IPB informará

a ANI da mesma ação, com pelo menos 10 dias de antecedência (Figura 10). Em qualquer ação a realizar é obrigatório o uso de logotipos das entidades de financiamento (consultar “Guia de Apoio à Publicitação”, disponível na *Intranet*).

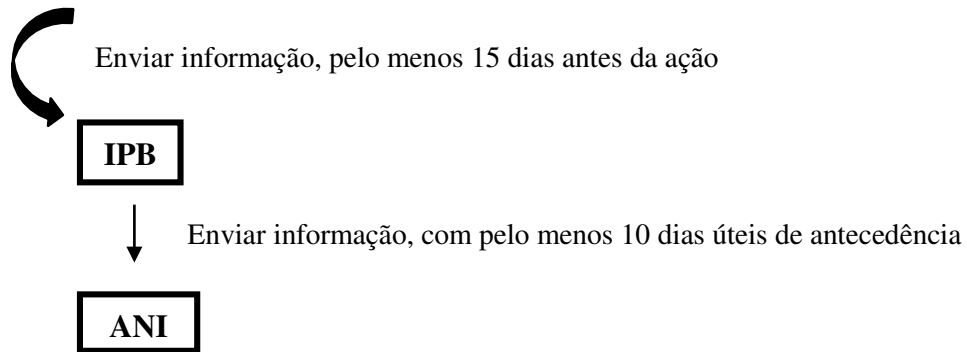


Figura 11. Diagrama representativo da comunicação de ações de publicitação e disseminação.

9. Deslocações no âmbito do Projeto

Aquando de uma deslocação no âmbito do Projeto é necessário o preenchimento do documento “Relatório de Missão” (Anexo VIII), que deverá ser submetido na *Intranet* através do formulário disponível no item “Contactos” que se encontra no Menu principal da plataforma.

O modelo está disponível na *Intranet*, e inclui a seguinte informação:

- Motivos de deslocação;
- Plano de trabalhos/Agenda;
- Resultados da deslocação.

10. Documentos a Consultar

- Guia do Beneficiário e outros documentos essenciais (<https://ani.pt/incentivos/execucao-de-projetos/>)
- Documento “Organização do Dossier” (Anexo IX) – disponível na *Intranet*.

11. Anexos

Anexo I – Ficha de Projeto

Designação do projeto | ValorNatural

Código do projeto | 24479

Objetivo principal | OT1 – Reforçar a investigação, o desenvolvimento tecnológico e a inovação

Região de intervenção | Norte

Entidade beneficiária | TecPan – Tecnologia e Produtos para Pastelaria e Panificação, Lda., Novavet – Produtos Agro-Pecuários, Lda., Afonso, Lopes & Filhas, Lda., M. Ferreira & Filhas, Lda., Paralab – Equipamentos Industriais e de Laboratório, S.A., OWNYA / Vera Mata Soluções Perfumadas, Lda., Ponto Agrícola – Unipessoal, Lda., Arménio Adérito Vaz, Inegi – Instituto de Ciência e Inovação em Engenharia Mecânica e Engenharia Industrial, FEUP – Faculdade de Engenharia da Universidade do Porto, IPB – Instituto Politécnico de Bragança, ISQ - Instituto de Soldadura e Qualidade, CAAF – Cooperativa Agrícola de Alfândega da Fé, CRL, Deifil Technology.

Data de aprovação | 10-05-2018

Data de início | 1-09-2018

Data de conclusão | 31-08-2021

Custo total elegível | 3.253.111,65€

Apoio financeiro da União Europeia | 2.506.560,76€

Objetivo, atividades e resultados esperados/atingidos:

No sentido de viabilizar a produção industrial de ingredientes naturais o projeto prevê o desenvolvimento de soluções que colmatem fragilidades da cadeia produtiva, nomeadamente: a) produção sustentada de fontes naturais ricas em compostos de interesse e a utilização de bio resíduos; b) otimização de metodologias de extração, procurando eficiência e facilidade de *scale-up*; c) desenvolvimento de equipamentos de extração/refinação versáteis e eficientes; d) desenvolvimento/aplicação de técnicas de estabilização para aumentar a durabilidade, compatibilidade no processamento e eficácia nos produtos finais; e) otimização de metodologias de incorporação em produtos alvo. O projeto abordará ainda aspetos ligados aos aromas e à conservação de matrizes naturais recorrendo a metodologias inovadoras. O projeto ValorNatural já marcou presença em dois eventos: na Qualifica 2019, que se realizou de 28 de janeiro a 3 de fevereiro, e na Alimentaria Horexpo Lisboa 2019, que se realizou de 24 a 26 de março. Nestes eventos foram apresentados os resultados obtidos à data, em particular no que diz respeito ao início da incorporação de extratos corantes em produtos de pastelaria/panificação, entre outro





Anexo II – Modelo de Entregável



ValorNatural – Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar.

Entregável nº

Versão do Documento:

Data de Submissão: nn/nn/nnnn

Responsável:

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Lista de Autores



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1. Identificação

<i>Deliverable</i>	[NÚMERO, NOME]
Tipo de deliverable	[relatório, <i>white paper</i> , documento, etc]
Nível de disseminação	[público, confidencial, outro]
PPS	[NÚMERO, NOME]



2. Informação

[TEXTO]

Anexo III – Modelo de Ficha de Potencial de Registo de Propriedade Industrial



Ficha de Potencial de Registo de Propriedade Industrial

ValorNatural – Valorização de Recursos Naturais através da Extração de
Ingredientes de Elevado Valor Acrescentado para Aplicações na
Indústria Alimentar

PPS

Data

No âmbito de cada PPS poderão ser identificadas oportunidades específicas de proteção do conhecimento e tecnologia gerados, devendo ser reportadas ao Conselho de Administração do Projeto para avaliação e definição de planos de ação. Neste sentido solicita-se a disponibilização da seguinte informação.

- 1. Qual o resultado a explorar (funcionalidade, propósito, inovação, valor acrescentado para o conhecimento/produtos/processos existentes, etc.)?**

- 2. Qual o valor comercial potencial do resultado do projeto?**

- 3. Utilização não-comercial ou impacto via desenvolvimento de novas metodologias, normas ou referenciais?**

- 4. Que parceiros estarão envolvidos na exploração: papel e atividades?**

- 5. Qual o estado de desenvolvimento?**

- 6. Necessidade de trabalhos de investigação ou desenvolvimento adicionais e tipo de colaboração necessária?**

- 7. Quais os possíveis setores de exploração ou com que fase da cadeia de valor acrescentado se relacionam (reguladores, empresários, consumidores, outras partes interessadas)?**

- 8. Que via de proteção de conhecimento/tecnologia será utilizada (patente em Portugal, patente internacional – PCT, patente na EU, patente nos EUA, modelo de utilidade, outros)?**

Anexo IV – Modelo de Relatório Técnico-científico



Relatório Técnico-Científico Intercalar Anual

ValorNatural – Valorização de Recursos Naturais através da Extração de
Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria
Alimentar

Sistema de Incentivos à Investigação e Desenvolvimento Tecnológico (SI & DT)
Programas mobilizadores



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**1. Identificação**

Nº de projeto:	24479
Acrónimo do projeto:	VALORNATURAL
Título do projeto:	Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar
Data de início de projeto:	01/09/2018
Duração do projeto:	36 meses
Período de reporte do relatório:	
Nº de relatório periódico:	
“Web site” ou “microsite” do projeto:	www.valornatural.pt

2. Apresentação do consórcio

Promotor	PPS
TecPan - Tecnologia e Produtos para Pastelaria e Panificação, Lda.	1,3,4,5,8
Novavet - Produtos Agro - Pecuários, Lda.	1,5,8
Afonso, Lopes & C ^a ., Lda.	1,5,8
Deifil Technology, Lda.	1,4,8
M. Ferreira & Filhas, Lda.	1,3,4,5,8
Paralab - Equipamentos Industriais e de Laboratório, S.A.	1,6,8
Vera Mata soluções Perfumadas, Lda.	1,4,8
Ponto Agrícola - Unipessoal, Lda.	1,3,8
Arménio Adérito Vaz	1,5,8
Instituto Politécnico de Bragança	1,3,4,5,6, 8
Universidade do Porto	1,5,6,8
Instituto de Ciência e Inovação em Engenharia Mecânica e Engenharia Industrial	1,6,8
Centro Nacional de Competências dos Frutos Secos	1,2,4,8
Instituto de Soldadura e Qualidade	1,8
Cooperativa Agrícola de Alfândega da Fé, CRL.	1,3,8

3. Sumário do projeto e seus objetivos globais

O uso de aditivos é uma prática comum no setor alimentar para aumentar o seu tempo de vida útil e/ou tornar os produtos mais apelativos. Existem mais de 2500 substâncias autorizadas para este fim, na maioria conservantes. Contudo, os efeitos nocivos associados ao seu consumo são cada vez mais conhecidos e suportados por estudos que alertam para o facto de muitos deles terem recebido, inadequadamente, o estatuto GRAS. Este cenário e as discrepâncias de legislação (EU vs. EUA), têm levado indústria e consumidores a aumentar a sua preferência por ingredientes naturais. Assim, o uso de aditivos sintéticos está cada vez mais limitado e, dada a tendência de mercado e conhecimento privilegiado do consórcio, prevê-se que muitos deles venham a ser proibidos.

Para viabilizar a produção industrial de ingredientes naturais é crucial desenvolver soluções que colmatem fragilidades da cadeia produtiva, nomeadamente: a) promover a produção sustentada de fontes naturais ricas em compostos de interesse e a utilização de bio resíduos; b) otimizar metodologias de extração, procurando eficiência e facilidade de scale-up; c) desenvolver equipamentos de extração/refinação versáteis e eficientes; d) desenvolver/aplicar técnicas de estabilização para aumentar a durabilidade, compatibilidade no processamento e eficácia nos produtos finais; e) otimizar metodologias de incorporação em produtos alvo. O projeto aborda adicionalmente aspetos ligados aos aromas e à conservação de matrizes naturais recorrendo a metodologias inovadoras.

Em suma, a solução proposta pelo consórcio, criado tendo em consideração áreas-chave e complementares, visa mobilizar uma cadeia de valor assente no desenvolvimento de ingredientes naturais nas classes dos corantes, aromas e bioativos, para utilização alternativa aos aditivos sintéticos.

Resumem-se de seguida os objetivos de cada PPS, de acordo com respetivo plano de trabalhos:

- A **PPS1 (gestão de projeto)** tem como promotor responsável o IPB e tem como principal objetivo garantir que as metas e entregáveis do projeto sejam alcançados com a qualidade desejada, e dentro do prazo e custos previstos. Sendo assim, tem como principais eixos de trabalho: a gestão técnica operacional e executiva do projeto.
- A **PPS 3 (corantes naturais)** tem como principal responsável o IPB e tem como objetivo central a obtenção de ingredientes naturais com efeito corante, a partir de flores comestíveis e bio resíduos de frutos, considerados desperdícios - o intuito é conferir-lhes uma utilização alternativa e com interesse económico. Tem como principais eixos de trabalho: a obtenção

de ingredientes naturais com capacidade corante, a realização de estudos de estabilidade e metodologias de estabilização, o desenvolvimento de aplicações dos ingredientes corantes e a demonstração de protótipos em ambiente industrial operacional.

- A **PPS 4 (aromas e modelos de aromas)** tem como promotor responsável a OWNYA/Vera Mata e tem como objetivo o desenvolvimento de aromas naturais e modelos de aromas para utilização na indústria da panificação destinados à intensificação do aroma dos seus produtos, e para serem utilizados como estratégia de marketing olfativo. Sendo assim, tem como principais eixos de trabalho: a extração de aromas naturais com propriedades organolépticas de interesse para a indústria de panificação, o desenvolvimento de modelos de aromas para a indústria de panificação, a produção de protótipos industriais e a validação das aplicações de *marketing* olfativo.

- A **PPS 5 (bioativos naturais)** tem como principal responsável o IPB, e como objetivo central o desenvolvimento de produtos lácteos com micosteróis para efeitos hipocolesterolémicos, similares aos efeitos exibidos pelos produtos que incorporam fitoesteróis e, a fortificação de farinhas com vitamina D2 para o aumento da absorção de cálcio. Tem como principais eixos de trabalho: a obtenção de ingredientes naturais com bioatividade, realização de estudos de estabilidade, desenvolvimento de metodologias de estabilização, desenvolvimento de aplicações dos ingredientes bioativos e demonstração de protótipos em ambiente industrial operacional.

- A **PPS 6 (inovação em processos de extração, refinação e técnicas de conservação)** tem como líder a FEUP/LSRE, e tem como objetivo o desenvolvimento de processos inovadores associados à extração e refinação de ingredientes a partir de matérias-primas naturais, e ao desenvolvimento de novos processos de conservação. Sendo assim, tem como eixos de trabalho: inovação em processos de extração, em processos de refinação e em técnicas de conservação.

- A **PPS 8 (disseminação de informação e exploração de resultados)** tem como promotor responsável a TecPan, e tem como garantir que a comunicação do projeto e o impacto pretendido para o mesmo sejam alcançados com sucesso, bem como assegurar a divulgação e exploração de resultados. Tem como principais eixos de trabalho: comunicação geral do mesmo, ações de vigilância tecnológica e inteligência competitiva, iniciativas de inovação aberta, gestão de propriedade industrial e análise de viabilidade de exploração económica dos resultados.



4. Sumário dos trabalhos realizados desde o início do projeto até ao final do período a que o relatório intercalar reporta, com especial enfoque ao período de reporte

4.1 PPSI - Gestão de projeto

4.1.1 Diagrama de Gantt comparando o trabalho previsto na candidatura com o realizado

4.1.2 Deliverables e milestones previstos desde o início do projeto até ao final do período a que o relatório intercalar reporta.

Nota: A numeração das Atividades e Tarefas nas secções seguintes inclui a nomenclatura utilizada no Anexo Técnico seguida da nomenclatura utilizada no Formulário de Candidatura. Por exemplo, a Atividade 1.1 no Anexo Técnico corresponde à Atividade 1 no Formulário de Candidatura, pelo que a denominação será 1.1 (1).

Deliverables

Nº do Deliverable	Nº da Tarefa	Título do Deliverable	Tipo de Deliverable	Data de entrega prevista no Anexo B	Data de entrega efetiva	Se não entregue, qual a data prevista para entrega	Nível de Divulgação	Comentários

Milestones

Nº do Milestone	Nº da Tarefa	Título do Milestone	Meio de Verificação	Data de entrega prevista no Anexo B	Data de entrega efetiva	Alcançado?	Se não alcançado, qual a data prevista	Comentários

4.1.3 Potenciais constrangimentos que possam dificultar a execução do projeto e medidas propostas para a sua mitigação

Risco	Probabilidade de ocorrer (alta, média, baixa)	Impacto (alto, médio, baixo)	Medida para mitigação



4.1.4 Promoção e divulgação de resultados

Tipologia de Ação de promoção e divulgação de Resultados	Número de Ações
Press-Release	
Publicações não científicas	
Publicações científicas	
Participação em Feiras e Exposições	
Participação em Conferências	

4.2 PPS3 - Corantes naturais

4.2.1 Diagrama de Gantt comparando o trabalho previsto na candidatura com o realizado

4.2.2 Deliverables e milestones previstos desde o início do projeto até ao final do período a que o relatório intercalar reporta.

Deliverables

Nº do Deliverable	Nº da Tarefa	Título do Deliverable	Tipo de Deliverable	Data de entrega prevista no Anexo B	Data de entrega efectiva	Se não entregue, qual a data prevista para entrega	Nível de Divulgação	Comentários

Milestones

Nº do Milestone	Nº da Tarefa	Título do Milestone	Meio de Verificação	Data de entrega prevista no Anexo B	Data de entrega efectiva	Alcançado?	Se não alcançado, qual a data prevista	Comentários



4.2.3 Potenciais constrangimentos que possam dificultar a execução do projeto e medidas propostas para a sua mitigação

Risco	Probabilidade de ocorrer (alta, média, baixa)	Impacto (alto, médio, baixo)	Medida para mitigação

4.2.4 Promoção e divulgação de resultado

Tipologia de Ação de promoção e divulgação de Resultados	Número de Ações
Press-Release	
Publicações não científicas	
Publicações científicas	
Participação em Feiras e Exposições	
Participação em Conferências	

4.3 PPS4 - Aromas e modelos de aromas

4.3.1 Diagrama de Gantt comparando o trabalho previsto na candidatura com o realizado

4.3.2 Deliverables e milestones previstos desde o início do projeto até ao final do período a que o relatório intercalar reporta.

Deliverables

Nº do Deliverable	Nº da Tarefa	Título do Deliverable	Tipo de Deliverable	Data de entrega prevista no Anexo B	Data de entrega efetiva	Se não entregue, qual a data prevista para entrega	Nível de Divulgação	Comentários

Milestones

Nº do Milestone	Nº da Tarefa	Título do Milestone	Meio de Verificação	Data de entrega prevista no Anexo B	Data de entrega efetiva	Alcançado?	Se não alcançado, qual a data prevista	Comentários
			Relatório				31-01-2020	

4.3.3 Potenciais constrangimentos que possam dificultar a execução do projeto e medidas propostas para a sua mitigação

Risco	Probabilidade de ocorrer (alta, média, baixa)	Impacto (alto, médio, baixo)	Medida para mitigação

4.3.4 Promoção e divulgação de resultados

Tipologia de Ação de promoção e divulgação de Resultados	Número de Ações
Press-Release	
Publicações não científicas	
Publicações científicas	
Participação em Feiras e Exposições	
Participação em Conferências	

4.4 PPS5 - Bioativos naturais

4.4.1 Diagrama de Gantt comparando o trabalho previsto na candidatura com o realizado

4.4.2 Deliverables e milestones previstos desde o início do projeto até ao final do período a que o relatório intercalar reporta.

Deliverables

Nº do Deliverable	Nº da Tarefa	Título do Deliverable	Tipo de Deliverable	Data de entrega prevista no Anexo B	Data de entrega efetiva	Se não entregue, qual a data prevista para entrega	Nível de Divulgação	Comentários

Milestones

Nº do Milestone	Nº da Tarefa	Título do Milestone	Meio de Verificação	Data de entrega prevista no Anexo B	Data de entrega efetiva	Alcançado?	Se não alcançado, qual a data prevista	Comentários

4.4.3 Potenciais constrangimentos que possam dificultar a execução do projeto e medidas propostas para a sua mitigação

Risco	Probabilidade de ocorrer (alta, média, baixa)	Impacto (alto, médio, baixo)	Medida para mitigação

4.4.4 Promoção e divulgação de resultados

Tipologia de Ação de promoção e divulgação de Resultados	Número de Ações
Publicações científicas	
Participação em Feiras e Exposições	
Participação em Conferências	

4.5 PPS6 - Inovação em processos de extração, refinação e técnicas de conservação

4.5.1 Diagrama de Gantt comparando o trabalho previsto na candidatura com o realizado

4.5.2 Deliverables e milestones previstos desde o início do projeto até ao final do período a que o relatório intercalar reporta.

Deliverables

Nº do Deliverable	Nº da Tarefa	Título do Deliverable	Tipo de Deliverable	Data de entrega prevista no Anexo B	Data de entrega efetiva	Se não entregue, qual a data prevista para entrega	Nível de Divulgação	Comentários

Milestones

Nº do Milestone	Nº da Tarefa	Título do Milestone	Meio de Verificação	Data de entrega prevista no Anexo B	Data de entrega efetiva	Alcançado?	Se não alcançado, qual a data prevista	Comentários



4.5.3 Potenciais constrangimentos que possam dificultar a execução do projeto e medidas propostas para a sua mitigação

Risco	Probabilidade de ocorrer (alta, média, baixa)	Impacto (alto, médio, baixo)	Medida para mitigação

4.5.4 Promoção e divulgação de resultados

Tipologia de Ação de promoção e divulgação de Resultados	Número de Ações
Publicações científicas	
Participação em Feiras e Exposições	
Participação em Conferências	

4.6 PPS8 - Disseminação de informação e exploração de resultados

4.6.1 Diagrama de Gantt comparando o trabalho previsto na candidatura com o realizado

4.6.2 Deliverables e milestones previstos desde o início do projeto até ao final do período a que o relatório intercalar reporta.

Deliverables

Nº do Deliverable	Nº da Tarefa	Título do Deliverable	Tipo de Deliverable	Data de entrega prevista no Anexo B	Data de entrega efetiva	Se não entregue, qual a data prevista para entrega	Nível de Divulgação	Comentários

Milestones

Nº do Milestone	Nº da Tarefa	Título do Milestone	Meio de Verificação	Data de entrega prevista no Anexo B	Data de entrega efetiva	Alcançado?	Se não alcançado, qual a data prevista	Comentários

4.6.3 Potenciais constrangimentos que possam dificultar a execução do projeto e medidas propostas para a sua mitigação

Risco	Probabilidade de ocorrer (alta, média, baixa)	Impacto (alto, médio, baixo)	Medida para mitigação

4.6.4 Promoção e divulgação de resultados

Tipologia de Ação de promoção e divulgação de Resultados	Número de Ações
Web Site	
Participação em Feiras e Exposições	
Outros (Redes sociais: Facebook, Instragram, LinkedIn, Twitter)	



5. Apresentação dos desenvolvimentos obtidos no período de reporte a que o presente relatório respeita

5.1 PPS1 - Gestão de projeto

5.1.1 Apresentação dos resultados alcançados

(Breve resumo dos resultados alcançados no período de tempo a que o relatório se refere)

Atividade:

Tarefa	
Líder da tarefa	
Participantes	
Data de início	
Data de fim	

Descrição dos trabalhos realizados:

Resultados:

Resultados passíveis de valorização económica:

5.1.2 Desvios e correções realizadas

5.1.3 Execução Financeira por promotor, apresentando os desvios, por rubrica, face ao previsto em candidatura

5.2 PPS3 – Corantes naturais

5.2.1 Apresentação dos resultados alcançados

(Breve resumo dos resultados alcançados no período de tempo a que o relatório se refere)

Atividade:

Tarefa	
Líder da tarefa	
Participantes	
Data de início	

Data de fim	
-------------	--

Descrição dos trabalhos realizados:

Resultados:

Resultados passíveis de valorização económica:

5.2.2 Desvios e correções realizadas

5.2.3 Execução Financeira por promotor, apresentando os desvios, por rubrica, face ao previsto em candidatura

5.3 PPS4 – Aromas e modelos de aromas

5.3.1 Apresentação dos resultados alcançados

(Breve resumo dos resultados alcançados no período de tempo a que o relatório se refere)

Atividade:

Tarefa	
Líder da tarefa	
Participantes	
Data de início	
Data de fim	

Descrição dos trabalhos realizados:

Resultados:

Resultados passíveis de valorização económica:

5.3.2 Desvios e correções realizadas

5.3.3 Execução Financeira por promotor, apresentando os desvios, por rubrica, face ao previsto em candidatura



5.4 PPS5 – Bioativos naturais

5.4.1 Apresentação dos resultados alcançados

(Breve resumo dos resultados alcançados no período de tempo a que o relatório se refere)

Atividade

Tarefa	
Líder da tarefa	
Participantes	
Data de início	
Data de fim	

Descrição dos trabalhos realizados:

Resultados:

Resultados passíveis de valorização económica:

5.4.2 Desvios e correções realizadas

5.4.3 Execução Financeira por promotor, apresentando os desvios, por rubrica, face ao previsto em candidatura

5.5 PPS6 - Inovação em processos de extração, refinação e técnicas de conservação

5.5.1 Apresentação dos resultados alcançados

(Breve resumo dos resultados alcançados no período de tempo a que o relatório se refere)

Atividade:

Tarefa	
Líder da tarefa	
Participantes	
Data de início	
Data de fim	

Descrição dos trabalhos realizados:



Resultados:

Resultados passíveis de valorização económica:

5.5.2 Desvios e correções realizadas

5.5.3 Execução Financeira por promotor, apresentando os desvios, por rubrica, face ao previsto em candidatura

5.6 PPS8 - Disseminação de informação e exploração de resultados

5.6.1 Apresentação dos resultados alcançados

(Breve resumo dos resultados alcançados no período de tempo a que o relatório se refere)

Atividade

Tarefa	
Líder da tarefa	
Participantes	
Data de início	
Data de fim	

Descrição dos trabalhos realizados:

Resultados:

Resultados passíveis de valorização económica:

5.6.2 Desvios e correções realizadas

5.6.3 Execução Financeira por promotor, apresentando os desvios, por rubrica, face ao previsto em candidatura



6 Anexos

Anexo V – Exemplo de Registo de Afetação de Técnicos

Timesheet

Copromotor:

Funcionário:

Mapa de horas trabalhadas																																							
	set18	out18	nov18	dez18	jan19	fev19	mar19	abr19	mai19	jun19	jul19	ago19	set19	out19	nov19	dez19	jan20	fev20	mar20	abr20	mai20	jun20	jul20	ago20	set20	out20	nov20	dez20	jan21	fev21	mar21	abr21	mai21	jun21	jul21	ago21			
Jornada diária (horas)	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7		
Nº de dias úteis trabaláveis (*)	20	22	21	20	22	20	21	20	22	18	23	21	21	23	20	21	22	20	22	21	20	20	23	21	22	21	21	20	20	20	23	21	21	21	20	22	22		
Horas trabaláveis potenciais	140	154	147	140	154	140	147	140	154	126	161	147	147	161	140	147	154	140	154	147	140	140	161	147	154	147	147	140	140	140	161	147	147	140	154	154			
PPS1 - Atividade 2																																							
PPS3 - Atividade 7																																							
PPS3 - Atividade 8																																							
PPS3 - Atividade 9																																							
PPS3 - Atividade 10																																							
PPS4 - Atividade 11																																							
PPS4 - Atividade 12																																							
PPS4 - Atividade 13																																							
PPS5 - Atividade 14																																							
PPS5 - Atividade 15																																							
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PPS5 - Atividade 17																																							
PPS6 - Atividade 18																																							
PPS6 - Atividade 19																																							
PPS6 - Atividade 20																																							
PPS8 - Atividade 24																																							
PPS8 - Atividade 25																																							
PPS8 - Atividade 26																																							
PPS8 - Atividade 27																																							
Subtotal	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Outros Projetos																																							
Outras Atividades																																							
Férias / baixas / licenças / faltas																																							
TOTAL	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

(*) exclui fins de semana e feriados

Data:

O Funcionário:

Aprovado:

Anexo VI – Guia de Apoio à Publicitação



Guia de Apoio à Publicitação

ValorNatural – Valorização de Recursos Naturais através da Extração de
Ingredientes de Elevado Valor Acrescentado para Aplicações na
Indústria Alimentar

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1. Introdução

O “Guia de Apoio à Publicitação” tem como principal objetivo auxiliar os copromotores do Projeto ValorNatural no correto cumprimento das regras de publicitação no âmbito do Portugal 2020. É importante que durante a execução do projeto o beneficiário publique e descreva detalhadamente a informação relativa ao apoio dos fundos da União Europeia (UE).

2. Ficha de Projeto a disponibilizar no Website

Os logotipos de cofinanciamento devem ser colocados na página inicial no *website* específico do Projeto. Porém, se o beneficiário já tiver um *site* que não seja específico para o Projeto em questão, deve ser disponibilizada na *homepage* uma ligação para uma página separada dentro do *site* ou *microsite*, com uma breve descrição do Projeto, objetivos, resultados e ainda destacar o apoio financeiro da União Europeia. O logotipo do cofinanciamento deverá estar na página inicial. Se a entidade tiver mais do que um projeto financiado, o *site* deverá ter uma página específica para cada projeto. A ficha de Projeto deve ser atualizada sempre que houver qualquer tipo de evolução.

3. Cartazes

Os beneficiários do projeto ValorNatural são cofinanciados pelo **Norte 2020**, sendo que têm a responsabilidade de promover o apoio da UE que lhe foi concedido num local que seja visível e facilmente identificado pelo público.

No caso de o apoio público, de forma individual a cada copromotor, ser inferior a 500.000 euros, durante a execução das operações relativas ao Projeto é obrigatória a afixação de um cartaz publicitário (por exemplo, na entrada do edifício). O cartaz deverá ser afixado a cores com tamanho recomendado A1 [594x841mm] e no mínimo A3 [297x420mm].

4. Documentos, Eventos, Workshops, Material Digital, Publicações

Os logotipos do cofinanciamento do Projeto terão que estar presentes nos diferentes documentos utilizados e nos diferentes eventos realizados. Sendo assim, terão que ser colocados da esquerda para a direita na seguinte ordem: Norte 2020, Portugal 2020 e União Europeia com a designação do FEDER por extenso. Por exemplo:

- Em brochuras, cartazes, livros, capas, convites e todas as publicações que forem impressas;
- Em anúncios publicitários do projeto que sejam impressos;
- Os *press-release* que forem elaborados deverão conter uma referência ao financiamento público, mencionando o Programa Operacional e o Fundo envolvido, os montantes de investimento e de apoio comunitário, bem como os objetivos finais do projeto;
- Na *homepage* dos sites referentes ao projeto;
- Nos slides das apresentações *powerpoint*;
- Em *e-newsletters*;
- Na abertura, antes da ficha técnica ou menção áudio, em filmes, DVD, vídeos;
- Em *spots* de rádio até 30 segundos;
- Em redes sociais na internet (*Facebook, Twitter, etc.*);
- Todos os documentos que forem utilizados para um evento relativo ao Projeto devem fazer referência ao apoio que lhe foi prestado, recorrendo aos logotipos de cofinanciamento;
- Certificados de Participação ou objetos promocionais, deverão ter presentes os logotipos do cofinanciamento, ou apenas o logotipo do Norte 2020 e a insígnia da UE sem descritivo.

Anexo VII – Formulário para Comunicação de Ações de Disseminação



Comunicação de Ações de Disseminação

ValorNatural – Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar.

1. Procedimento

- Enviar ao IPB a informação abaixo preenchida, pelo menos 15 dias antes da ação de disseminação;
- O IPB informará a ANI da mesma, com pelo menos 10 dias úteis de antecedência;
- É obrigatório o uso de logótipos das entidades de financiamento nas ações de disseminação do Projeto.

2. Informação a enviar ao IPB

2.1 Tipologia de ação

(Organização de conferência; Organização de Workshop; Demonstrações públicas de protótipos, linhas piloto; Publicações não científicas; Publicações científicas; Participação em Feiras e Exposições; Participação em Conferências-Poster; Participação em Conferências-Comunicação oral; Participação em Workshops; Outro – especificar)

2.2 Designação, âmbito e data de ação

3. Conteúdo de Ação

(Organização; poster; comunicação oral; stand em feira; participante; outro - especificar) (pode incluir imagens, printscreens, links, certificados,...)

4. Identificação dos participantes

PPS	
Tarefas Associadas	
Copromotor	
Membros participantes	

Anexo VIII – Modelo de Relatório de Missão



Relatório de Missão

ValorNatural – Valorização de Recursos Naturais através da Extração de
Ingredientes de Elevado Valor Acrescentado para Aplicações na
Indústria Alimentar

Nome do Evento:

Data de Partida:

País:

Data de Regresso:

Copromotor:

PPS:

Membro da equipa técnica:

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1. Motivos da Deslocação

(definir objetivo da Ação/evento; definir objetivo da deslocação)

2. Plano de Trabalhos/Agenda

(incluir o plano de trabalhos da missão, agenda, apresentações realizadas, etc.)

3. Resultados

(apresentar resultados de acordo com a missão, parceiros contactados, novos contactos estabelecidos, etc.)

4. Anexos

(Ex: Tríptico de brochura, programa da feira, certificado participação, fotografias onde seja visíveis os logotipos do projeto, website do evento, livro de abstract, programa onde esteja visível a intervenção relativa ao projeto, etc)



Anexo IX – Organização do Dossier de Projeto



ORGANIZAÇÃO DO DOSSIER DO PROJETO

ValorNatural – Valorização de Recursos Naturais através da Extração de
Ingredientes de Elevado Valor Acrescentado para Aplicações na
Indústria Alimentar

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Introdução

O **Dossier do Projeto** deve conter todos os documentos suscetíveis de comprovar as informações e declarações prestadas no âmbito do Projeto, e de fundamentar as opções de investimentos apresentadas, bem como todos os documentos comprovativos da realização das despesas de investimento. Poderá ser consultado a qualquer momento pelos organismos intervenientes no processo de análise, acompanhamento e fiscalização dos projetos cofinanciados no âmbito do P2020.

No caso de projetos financiados com fundos estruturais, este Dossier tem de ser mantido até três anos após a data de encerramento do respetivo programa financiador, podendo os contratos de concessão de incentivos definir prazos superiores.

A organização do Dossier é fundamental para a celeridade de todo o processo de acompanhamento e verificação.

PARTE I – Candidatura

Correspondente ao **Dossier de Candidatura** sobre o qual incidiu a decisão de homologação e aos elementos prestados para celebração do contrato de concessão de incentivos. Os documentos que devem constar são os seguintes:

- Cópia do Formulário de Candidatura;
- Cópia do cartão de pessoa coletiva ou fotocópia do cartão de empresário em nome individual;
- Cópia da Certidão Permanente da empresa, ou Fotocópia do Diário da República com publicação do contrato de sociedade, ou cópia da certidão de escritura do contrato de sociedade e cópia do registo de todas as alterações ocorridas no pacto social, se sociedade;
- Declaração de início de atividade, se Empresário em Nome Individual;
- Cópia da Certificação Eletrónica de PME;

- Cópia da autorização ao Organismo Intermédio para verificação *on-line* de situação regularizada perante a Administração Fiscal ou Certidão da Direção Geral de Contribuições e Impostos comprovativa de situação regularizada perante o Estado;
- Cópia da autorização ao Organismo Intermédio para verificação *on-line* de situação regularizada perante a Segurança Social ou Certidão do Instituto de Gestão Financeira da Segurança Social comprovativa de situação regularizada perante a Segurança Social;
- Declaração de início da atividade/alterações ou Declaração Anual ou outro documento comprovativo, com opção de contabilidade organizada;
- IES (Informação Empresarial Simplificada) referente aos três anos anteriores ao da entrega da candidatura ou Balanço e Demonstração de Resultados devidamente certificado por um TOC/ROC e responsáveis da empresa;
- Balanço Intercalar e respetiva certificação por ROC, para comprovação do indicador de autonomia financeira, se aplicável;
- Folhas de pagamento à Segurança Social, do último mês do ano fiscal anterior à apresentação da candidatura (quando aplicável);
- Documentos relativos aos detentores do capital do promotor (folha da Segurança Social relativos ao último mês do ano fiscal anterior à apresentação da candidatura e IES referente ao ano anterior);
- Documentos relativos às participadas pelo promotor (caso existam) em mais de 25% (folha da Segurança Social e modelo de IRC relativos ao último ano fiscal anterior à apresentação da candidatura);
- Declaração em como afeta o projeto à atividade e à localização geográfica, durante o período de vigência do contrato de incentivos, no mínimo, durante três anos após o encerramento do Projeto;
- Faturas pró-forma, orçamentos, catálogos e outros elementos que em sede de análise comprovem a intenção de investimento, numerados por ordem sequencial com a devida correspondência no mapa “Classificação dos Investimentos” do Formulário de Candidatura.

PARTE II – Contrato e Pedidos Pós-Contratação

- ✓ Contrato e Aditamentos;
- ✓ Cópia da Formalização de Pedidos Pós-Contratação;
- ✓ Memorando, referindo eventuais desvios na concretização do projeto e respetiva justificação relativamente a:
 - Investimentos não previstos na candidatura e previstos não concretizados;
 - Faseamento e Calendarização do investimento;
 - Recalendarização dos pedidos de pagamento de incentivo;
 - Financiamento do projeto.

PARTE III – Correspondência

Inclui toda a correspondência rececionada e enviada a todos os interlocutores oficiais da candidatura.

PARTE IV – Comprovantes do Investimento

- Organizar os documentos comprovativos das despesas do investimento realizadas, que vão servir de suporte documental aquando dos pedidos de pagamento de incentivo;
- Estes documentos devem constar do Dossier do Projeto e estarem dispostos de forma organizada e lógica, de modo a facilitar o trabalho de análise sempre que necessária e justificada a sua intervenção. Esta Parte deverá ser composta por:
 - Mapa de Despesas de Investimento;
 - Cópias dos comprovantes das despesas de investimento realizadas no âmbito do projeto, com aposição de um carimbo com menção ao código universal de projeto Norte 2020, a taxa de imputação e a rubrica de investimento, identificada com um

nº sequencial com correspondência ao Mapa de Despesas de Investimento. Cada comprovante, como a seguir se exemplifica, é composto por fatura(s), respetivo(s) documento(s) de quitação e outros documentos, devendo constituir um conjunto:

- Fatura(s) (com nº de lançamento para a contabilidade, data de lançamento, e classificação contabilística);
 - Recibo(s);
 - Nota(s) de débito/crédito;
 - Letra(s);
 - Transferência(s) bancária(s);
 - Cópia do(s) extrato(s) bancário(s) que evidencie o pagamento da(s) citada(s) fatura(s);
 - Quando o descritivo da fatura remeter para orçamento ou contrato que não conste da Parte I deste Dossier, deverão ser incluídas cópias das propostas e/ou contratos de suporte das transações efetuadas;
 - No caso de a cópia da fatura da despesa de investimento realizada já constar da Parte I do Dossier de candidatura, como suporte da intenção de investimento, deverá nesta Parte do Dossier (Parte IV), correspondente à realização do investimento, ser incluída nova cópia da referida fatura.
- Extratos de conta refletindo a contabilização das despesas de investimento e dos custos, quando previstos na lei, de fornecedores de imobilizado e do registo do incentivo concedido e pago;
- Balancete Analítico do mês anterior à apresentação da candidatura e do mês de conclusão do investimento;
- IES (Informação Empresarial Simplificada), Mapas de Amortizações e Reintegrações, Mapa de modelo oficial das mais e menos valias fiscais, cópia do relatório de gestão e do Conselho Fiscal (quando aplicável) respeitantes aos anos em que o investimento foi realizado, bem como do ano anterior ao início do mesmo. Juntar cópia da Ata de Aprovação das contas do exercício correspondente;

- Folhas de remuneração do mês de dezembro do ano anterior ao ano da candidatura e do mês mais recente devidamente autenticadas pela Segurança Social.

PARTE V – Comprovantes das Fontes de Financiamento do Projeto

Inclui os documentos comprovativos das fontes de financiamento utilizadas:

- Mapa de Financiamento do Projeto devidamente preenchido;
- Empréstimo Bancário: fotocópia do(s) contrato(s), extratos bancários da sua utilização e respetivo extrato contabilístico;
- Prestações Suplementares: fotocópia da ata de aprovação, comprovativos das entradas em caixa e/ou depósitos à ordem e extrato contabilístico da respetiva conta;
- Suprimentos Consolidados: fotocópia da ata de aprovação e declaração dos sócios de que os empréstimos por si concedidos não serão levantados durante a vida do projeto, bem como das entradas em caixa e/ou depósitos à ordem e extrato contabilístico da respetiva conta.

PARTE VI – Outros Documentos

- Outros elementos necessários à comprovação das condicionantes de aprovação da candidatura;
- Outros.



ValorNatural – Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar.

Entregável nº 1.2.2

Versão do Documento: 3

Data de Submissão: 23/09/2019

Responsável: Ana Saldanha

Nome do Documento: Relatórios de execução semestrais

Histórico de Revisão

Revisão	Data	Parceiros Envolvidos	Descrição
1.0	4/10/2019	IPB	Elaboração do Relatório Técnico-Científico Anual
2.0	23/09/2019	IPB	Atualização das imagens 1 e 2



Lista de Autores

Ana Saldanha

Sumário

No âmbito da PPS1 foi realizado o Relatório Técnico-Científico relativo ao primeiro semestre de execução do projeto ValorNatural, assim como o Relatório Técnico-Científico Intercalar Anual, combinando este o primeiro e segundo semestre de execução do projeto.

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1. Identificação

<i>Deliverable</i>	1.2.2
<i>Tipo de deliverable</i>	Relatório
Nível de disseminação	Confidencial
PPS	1

2. Informação

No âmbito da PPS 1, Gestão do Projeto, e da Tarefa número 1.2.3 foi executado o primeiro Relatório Técnico-Científico relativo ao primeiro semestre de execução do projeto (Imagem 1), assim como o Relatório Técnico-Científico Intercalar Anual onde foram reportados o primeiro e o segundo semestre de execução do projeto (Imagem 2). O relatório em questão refere-se aos objetivos alcançados nas PPS 1, PPS 3, PSS 4, PPS 5, PPS 6 e PPS 8. As diferentes PPS's apresentam diferentes objetivos, e de acordo com o tema de trabalho de cada uma, é importante reunir toda a informação relativa aos objetivos alcançados, potenciais constrangimentos identificados, divulgação de resultados e informação relativa à execução financeira de cada promotor no final do primeiro ano de execução do projeto.



Relatório Técnico-Científico Intercalar

ValorNatural – Valorização de Recursos Naturais através da Extração de
Ingredientes de Elevado Valor Acrescentado para Aplicações na
Indústria Alimentar

Sistema de Incentivos à Investigação e Desenvolvimento Tecnológico (SI & DT)
Programa Mobilizatório

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1. Identificação

Nº de Projeto:	24479
Acronímico de projeto:	VALORNATURAL
Título do projeto:	Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar
Data de início do projeto:	01/09/2018
Duração do projeto:	36 meses
Período de reporte do relatório:	01/09/2018 a 28/02/2019
Nº de relatório periódico:	1
"Web site" ou "microsite" do projeto:	www.valornatural.pt
Composição do consórcio:	TecPan, Novavet, Afonso, Lopes & C, M. Ferreira & Filhas, Parabol, Vera Mata, Ponto Agrícola, Arménio Aderito Vas, IPB, UP, INEGI, CNCFS, ISQ, Cooperativa Agrícola de Alfindoga da Fé, Deffil Technology,
PPS	1/3/4/5/6/8
Co-promotores envolvidos	Todos

Imagem 1. Imagem da capa de rosto, do Índice e da Identificação do Relatório Técnico-Científico Intercalar.


Relatório Técnico-Científico Intercalar Anual

ValorNatural – Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar

Sistema de Investimentos e Inovação e Desenvolvimento Tecnológico (SI&ITE)
Programa IM&Inov@ção

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Projeto nº 24479

1. Identificação

Nº de projeto:	24479
Acrónimo do projeto:	VALORNATURAL
Título do projeto:	Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar
Data de início de projeto:	01/09/2018
Duração do projeto:	36 meses
Período de reporte do relatório:	01/09/2018 a 31/08/2019
Nº de relatório periódico:	2
"Web site" ou "microsite" do projeto:	www.valornatural.pt

Imagem 2. Imagem da capa de rosto, do Índice e da Identificação do Relatório Técnico-Científico Anual.



ValorNatural – Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar.

Entregável nºE.3.1.1

Versão do Documento: 1

Data de Submissão: 30/11/2018

Responsável: IPB-CIMO

Nome do Documento: Folheto com procedimentos de colheita das matérias-primas (hibiscos e perpétua-roxa: bio-resíduos dos frutos de cerara, mirtilo e medronho)

Histórico de Revisão

Revisão	Data	Parceiros Envolvidos	Descrição



Lista de Autores

Maria Inês Dias

Sumário

Neste folheto está descrito o procedimento de colheita de várias espécies vegetais com vista à sua valorização através da extração dos seus ingredientes de valor agregado, nomeadamente compostos corantes (compostos fenólicos antociânicos e betacianinas) que podem ser posteriormente utilizados pela indústria alimentar em substituição de corantes sintéticos. As matrizes estudadas são na sua maioria provenientes de várias regiões de Portugal, no entanto, foram também estudadas matrizes provenientes da Alemanha e Tunísia.

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1. Identificação

<i>Deliverable</i>	E.3.1.1. Folheto com procedimentos de colheita das matérias-primas (hibiscos e perpétua-roxa: bio-resíduos dos frutos de cerara, mirtilo e medronho)
<i>Tipo de deliverable</i>	Folheto descritivo
Nível de disseminação	Público
PPS	PPS3. Corantes Naturais

2. Informação

Arbutus unedo L.



Nome Comum: Medronho

Origem geográfica: Torre de Moncorvo, Portugal

Parte da planta estudada: Fruto

Colheita: Amostra obtida de um produtor local. Os frutos foram colhidos num estado de maturação avançado, e imediatamente congelados após receção em laboratório. Foram depois submetidos a um processo de liofilização e triturados até a obtenção de um pó fino e homogéneo, posteriormente armazenado sob condições controladas de luz, temperatura e humidade até análise.

Beta vulgaris L.



Nome Comum: Beterraba

Origem geográfica: Bragança, Portugal

Parte da planta estudada: Colo tuberizado sem casca

Colheita: Amostra obtida comercialmente em fresco. Após receção em laboratório, removeu-se a casca externa e procedeu-se à extração dos compostos corantes no sumo a partir de diferentes processos de extração (ainda em desenvolvimento). Os diferentes extratos foram congelados, liofilizados e armazenados sob condições controladas de luz, temperatura e humidade até análise.

Carissa macrocarpa (Eckl.) A.DC.



Nome Comum: Ameixeira de Natal

Origem geográfica: Monastir, Tunísia

Parte da planta estudada: Fruto

Colheita: As amostras de fruto foram coletadas após a floração de outono. Após receção em laboratório os frutos foram secos a 40°C até secagem total, e posteriormente moídos para obtenção de um pó fino, armazenado posteriormente sob condições controladas de luz, temperatura e humidade até análise.

Centaurea Cyanus L.


Nome Comum: Centaurea

Origem geográfica: Castro Daire, Portugal/ Münster, Alemanha

Parte da planta estudada: Pétalas

Colheita: As amostras de pétalas portuguesas foram gentilmente cedidas pela empresa RBR Foods na forma seca. As flores provenientes da Alemanha foram compradas na Blumen Lennartz, que, após receção em laboratório se destacou as pétalas azuis tubulares do recetáculo e congeladas, para posterior liofilização. Ambas as amostras foram posteriormente moídas até obtenção de um pó fino e homogéneo, armazenados sob condições controladas de luz, temperatura e humidade até análise.

Dalia mignon


Nome Comum: Dália

Origem geográfica: Castro Daire, Portugal

Parte da planta estudada: Pétalas

Colheita: Amostras de pétalas gentilmente cedidas pela empresa RBR Foods na forma seca. Após receção em laboratório, moídas até obtenção de um pó fino e homogéneo, armazenados sob condições controladas de luz, temperatura e humidade até análise.

Ficus carica L.


Nome Comum: Figo

Origem geográfica: Bragança, Portugal

Parte da planta estudada: Casca externa da infrutescência

Colheita: Realizada entre os meses de Setembro e Outubro após maturação das infrutescências (estado de maturação avançado). Após receção em laboratório a infrutescência foi separada em casca externa e recetáculo carnoso (parte descartada). As cascas foram então congeladas, liofilizadas e moídas até obter um pó fino e homogéneo, armazenado sob condições controladas de luz, temperatura e humidade até análise.

***Gomphrena globosa* L.**


Nome Comum: Perpétua-roxa

Origem geográfica: Mezio, Portugal

Parte da planta estudada: Flores (brácteas e bractéolas).

Colheita: As inflorescências foram gentilmente cedidas pela Empresa Ervital na forma seca. Após receção em laboratório, as flores consistindo nas brácteas e bractéolas (partes pigmentadas das inflorescências) foram separadas através de um processo mecânico. Posteriormente moídas até obter um pó fino e homogéneo, armazenado sob condições controladas de luz, temperatura e humidade até análise.

***Hibiscus sabdariffa* L.**


Nome Comum: Vinagreira

Origem geográfica: Alfândega da Fé, Portugal

Parte da planta estudada: Cálice

Colheita: As amostras de flores foram gentilmente cedidas pela Empresa Pragmático Aroma Lda., em fresco. Após receção em laboratório, às flores foi-lhes destacada a parte do cálice para análise, congeladas, liofilizadas e posteriormente moídas até obtenção de um pó fino e homogéneo, armazenado sob condições controladas de luz, temperatura e humidade até análise.

***Prunus avium* L.**


Nome Comum: Cereja

Origem geográfica: Bragança, Portugal

Parte da planta estudada: Fruto

Colheita: Os frutos foram colhidos em Maio em fase de maturação avançado. Após receção em laboratório é retirado mecanicamente, o caroço do fruto, sendo congelados até posterior análise (ainda em desenvolvimento os melhores processos de extração dos compostos corantes).

***Prunus spinosa* L.**



Nome Comum: Abrunho

Origem geográfica: Bragança, Portugal

Parte da planta estudada: Epicarpo

Colheita: Os frutos de abrunho foram colhidos entre setembro e outubro. Após receção em laboratório o epicarpo foi destacado da parte carnuda do fruto e congelado. Posteriormente foi liofilizado e moído até obtenção de um pó fino e homogéneo, armazenado sob condições controladas de luz, temperatura e humidade até análise.

Rosa damascena* ‘Alexandria’ e *R. gallica* ‘Francesa’ enxertada em *R. canina



Nome Comum: Rosa

Origem geográfica: Castro Daire, Portugal

Parte da planta estudada: Pétalas

Colheita: Amostras de pétalas gentilmente cedidas pela empresa RBR Foods na forma seca. Após receção em laboratório, moídas até obtenção de um pó fino e homogéneo, armazenados sob condições controladas de luz, temperatura e humidade até análise.

***Rubus umilifolius* Schott**



Nome Comum: Amora silvestre

Origem geográfica: Bragança, Portugal

Parte da planta estudada: Fruto

Colheita: A colheita foi feita em setembro. Após receção em laboratório os frutos foram congelados, liofilizados e posteriormente triturados até obtenção de um pó fino e homogéneo, armazenado sob condições controladas de luz, temperatura e humidade até análise.

***Sambucus nigra* L.**


Nome Comum: Sabugueiro

Origem geográfica: Bragança, Portugal

Parte da planta estudada: Frutos

Colheita: A colheita foi realizada entre finais de outubro e inícios de novembro, com os frutos em estado de maturação avançado. Após receção em laboratório, as bagas de fruto são separadas manualmente dos talos e congeladas até posterior análise (ainda em desenvolvimento os melhores processos de extração dos compostos corantes).

***Vaccinium myrtillus* L.**


Nome Comum: Mirtilo

Origem geográfica: Castro Daire, Portugal

Parte da planta estudada: Fruto

Colheita: Amostras de fruto gentilmente cedidas pela empresa RBR Foods na forma seca. Após receção em laboratório, moídos até obtenção de um pó fino e homogéneo, armazenados sob condições controladas de luz, temperatura e humidade até análise.



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Entregável nº 3.1.2

Versão do Documento: 1

Data de Submissão: 30/11/2018

Responsável: IPB-CIMO

Nome do Documento: Base de dados com as matérias-primas mais ricas em moléculas corantes ((iso)gonfreninas II e III, derivados de cianidinas, derivados de delfinidinas)

Histórico de Revisão

Revisão	Data	Parceiros Envolvidos	Descrição



Lista de Autores

Eliana Pereira

Sumário

A presente base de dados descreve as matérias-primas mais ricas em moléculas corantes ((iso)gonfreninas II e III, derivados de cianidinas, derivados de delphinidinas, entre outros) a utilizar no desenvolvimento de corantes naturais.

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1. Identificação.....	5
2. Informação.....	6

2. Identificação

<i>Deliverable</i>	3.1.2. Base de dados com as matérias-primas mais ricas em moléculas corantes ((iso)gonfreninas II e III, derivados de cianidinas, derivados de delphinidinas)
<i>Tipo de deliverable</i>	Base de dados
<i>Nível de disseminação</i>	Confidencial
<i>PPS</i>	3. Corantes Naturais

3. Informação

Amostra	Classe	Composto
<i>Arbutus unedo</i>	Antocianinas	Cianidina 3- <i>O</i> -glucósido
		Cianidina 3- <i>O</i> -pentósido
		Delfinidina 3- <i>O</i> -glucósido
<i>Centaurea cyanus</i> L.	Antocianinas	Cianidina 3,5-di- <i>O</i> -glucósido
		Cianidina 3- <i>O</i> -(6"-malonilglucósido)-5- <i>O</i> -glucósido
		Cianidina 3- <i>O</i> -(6"-succinilglucósido)-5- <i>O</i> -glucósido
		Delfinidina-hexósido
		Cianidina-glucurónido
		Delfinidina-malonilhexósido
		Pelargonidina 3- <i>O</i> -(6"-succinilglucósido)-5- <i>O</i> -glucósido
		Pelargonidina-malonilhexósido
		Pelargonidina-3- <i>O</i> -sinapoilglucósido-5- <i>O</i> -glucósido
<i>Coriandrum sativum</i> clone A	Antocianinas	Cianidina-3- <i>O</i> -feruloil glucósido-5- <i>O</i> -glucósido
		Cianidina-3- <i>O</i> - <i>p</i> -coumaroil glucósido -5- <i>O</i> -glucósido
		Peonidina-3- <i>O</i> -sinapoilglucósido-5- <i>O</i> -glucósido
		Peonidina-3- <i>O</i> -feruloil glucósido-5- <i>O</i> -glucósido
		Peonidina-3- <i>O</i> - <i>p</i> -coumaroil glucósido -5- <i>O</i> -glucósido
		Cianidina-3- <i>O</i> -glucósido
<i>Crataegus monogyna</i>	Antocianinas	Cianidina-3- <i>O</i> -rutinósido
		Pelargonidina-3- <i>O</i> -glucósido
		Cianidina-3- <i>O</i> -pentósido
		Peonidina-3- <i>O</i> -glucósido
		Delfinidina di- <i>O</i> -glucósido
<i>Crocus sativus</i>	Antocianinas	Delfinidina-3- <i>O</i> -glucósido
		Cianidina-3- <i>O</i> -glucósido
		Petunidina di- <i>O</i> -glucósido
		Cianidina-hexósido
<i>Dahlia mignon</i>	Antocianinas	Cianidina-hexósido
		Pelargonidina-rutinósido
		Cianidina-acetilhexósido
		Metilapigeninidina-hexósido
		Cianidina-rutinósido
		Pelargonidina 3,5-di- <i>O</i> -glucósido
		Cianidina-acetilhexósido
		Pelargonidina-hexósido
<i>Ficus carica</i> L.	Antocianinas	cianidina 3-rutinósido
<i>Fragaria vesca</i> L. (silvestre)	Antocianinas	Cianidina-3-glucósido
		Pelargonidina-3-glucósido
		Peonidina-3-glucósido
		Cianidina-malonilglucósido

			Pelargonidina-malonilglucósido
			Peonidina-malonilglucósido
<i>Gomphrena globosa</i> L.	Betalainas	Betacianinas	Gonferina II
			Gonferina II
			Gonferina III
			Iso gonferina II
			Iso gonferina III
			17-Descarboxi-amarantina
<i>Hibiscus sabdariffa</i> L.	Antocianinas		Cianidina-3- <i>O</i> -sambubiósido
			Delfinidina-3- <i>O</i> -sambubiósido
			Delfinidina-3- <i>O</i> -glucósido
<i>Prunus avium</i> L.	Antocianinas		Cianidina-3- <i>O</i> -glucósido
			Cianidina-3- <i>O</i> -rutinósido
			Peonidina-3- <i>O</i> -rutinósido
<i>Prunus spinosa</i> L.	Antocianinas		Cianidina 3- <i>O</i> -glucósido
			Cianidina 3- <i>O</i> -rutinósido
			Peonidina 3- <i>O</i> -glucósido
			Peonidina 3- <i>O</i> -rutinósido
			Cianidina 3- <i>O</i> -pentósido
			Peonidina 3- <i>O</i> -pentósido
			Cianidina 3- <i>O</i> -acetilglucósido
			Peonidina 3- <i>O</i> -acetilglucósido
<i>Rosa canina</i> L.	Antocianinas		Cianidina 3- <i>O</i> -glucósido
<i>Rosa damascena</i>	Antocianinas		Cianidina 3,5-di- <i>O</i> -glucósido
			Cianidina-3- <i>O</i> -glucósido
<i>Rosa micrantha</i> Borrer	Antocianinas		Cianidina 3- <i>O</i> -glucósido
<i>Rubus ulmifolius</i> Schott	Antocianinas		Cianidina- <i>O</i> -di-hexósido
			Cianidina-3- <i>O</i> -glucósido
			Pelargonidina-3- <i>O</i> -glucósido
			Cianidina-3- <i>O</i> -xilósido
			Cianidina-3- <i>O</i> -dioxail-glucósido
<i>Ocimum basilicum</i> var. <i>purpurascens</i>	Antocianinas		Cianidina-3-(<i>p</i> -cumaroil-6'-Cafeoil)Soforósido isómero 1
			Cianidina-3-(<i>p</i> -cumaroil-6'-Cafeoil)Soforósido isómero 2
			Cianidina-3-(6- <i>p</i> -cumaroil)Soforósido-5-(6-Malonil)Glucósido
			Cianidina-3-(6- <i>p</i> -cumaroil)Glucósido-5-Glucósido
			Cianidina-3-(6- <i>p</i> -cumaroil-6'-Cafeoil)Soforósido-5-Glucósido isómero 1
			Cianidina-3-(6- <i>p</i> -cumaroil-6'-Cafeoil)Soforósido-5-(6-Malonil)Glucósido isómero 2
			Cianidina-3-(6- <i>p</i> -cumaroil-6'-Cafeoil)Soforósido-5-(6-Malonil)Glucósido isómero 1
			Cianidina-3-(6- <i>p</i> -cumaroil-6'-Cafeoil)Soforósido-5-Glucósido isómero 1
			Cianidina-3-(6,6'-di- <i>p</i> -cumaroil)Soforósido-5-Glucósido
			Cianidina-3-(6,6'-di- <i>p</i> -cumaroil)Soforósido-

			5-(6-Malonil)Glucósido
			Pelargonidina-3-(6,6'-di- <i>p</i> -cumaroil)Soforósido-5-Glucósido
			Cianidina-3-(6- <i>p</i> -cumaroil-X-Malonil-6'-Cafeoil)Soforósido-5-Glucósido
			Cianidina-3-(6- <i>p</i> -cumaroil-X-Malonil-6'- <i>p</i> -cumaroil)Soforósido-5-Glucósido
<i>Opuntia spp.</i>	Betaláinas	Betaxantinas	Isómero de Indicaxantina I
			Isómero de Indicaxantina II
		Betacianinas	Betanidina-5- <i>O</i> -β-soforósido
			Betanidina-5- <i>O</i> -β-glucósido (betanina)
			Isobetanina
			Gomfrenina I
			Betanidina



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Responsável: IPB-CIMO

Nome do Documento: Relatório com as especificações técnicas dos corantes a desenvolver

Histórico de Revisão

Revisão	Data	Parceiros Envolvidos	Descrição



Lista de Autores

Eliana Pereira

Sumário

O presente relatório descreve as especificações técnicas dos corantes em desenvolvimento a partir de matrizes naturais.

Índice

1. Identificação.....	5
2. Informação.....	6

1. Identificação

<i>Deliverable</i>	3.1.3. Relatório com as especificações técnicas dos corantes a desenvolver
Tipo de <i>deliverable</i>	Relatório
Nível de disseminação	Confidencial
PPS	3. Corantes Naturais

2. Informação

No âmbito deste projeto, os pigmentos naturais obtidos através de matrizes naturais foram, até à presente data, antocianinas e betalaínas.

As antocianinas caracterizam-se como um grupo de compostos fenólicos, com capacidade corante, amplamente difundidos e encontrados numa grande variedade de frutos, vegetais e flores, apresentando uma gama de cor entre o vermelho, azul e violeta. A coloração das antocianinas depende maioritariamente do pH, apresentando uma disparidade significativa de cores. Em meio aquoso, apresentam cor vermelha a pH 1-3, são incolores a pH 4-5, roxas a pH 6-7, azuis a pH= 7-8 e amarelas a pH 8-9. No entanto, a estabilidade destes compostos é também influenciada pela temperatura, humidade e concentração de sais do meio envolvente, ou até mesmo condições de stress e armazenamento.

As betalaínas são moléculas muito semelhantes às antocianinas, incluindo compostos com cores que oscilam na gama do vermelho-violeta (betacianinas) a amarelo-laranja (betaxantinas). Apresentam maior estabilidade que as antocianinas, sendo estáveis a uma vasta gama de pH e também capacidade regenerativa após tratamento térmico, no entanto, condições como a luz, a presença de oxigénio, a humidade e as condições de armazenamento podem afetar a sua estabilidade química.



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Entregável nº 3.1.4.

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Data de Submissão: 28/02/2019

Responsável: IPB-CIMO

Nome do Documento: Relatório das condições de extração ótimas para obtenção das moléculas corantes

Histórico de Revisão

Revisão	Data	Parceiros Envolvidos	Descrição



Lista de Autores

Maria Inês Dias



Sumário

Neste relatório estão descritas as condições de extração ótimas para obtenção de moléculas corantes a partir de várias espécies vegetais.



Índice

1. Identificação	5
2. Informação	6



1. Identificação

<i>Deliverable</i>	E.3.1.4. Relatório das condições de extração ótimas para obtenção das moléculas corantes
<i>Tipo de deliverable</i>	Relatório
<i>Nível de disseminação</i>	Confidencial
<i>PPS</i>	PPS3. Corantes Naturais

2. Informação

Arbutus unedo L.



Nome Comum: Medronho

Parte da planta estudada: Fruto

Compostos maioritários: Cianidina-3-*O*-glucósido

Condições de extração: Extração assistida por calor durante 5 min a 90°C, usando como solvente uma mistura etanol:água (80:20, v/v)

Carissa macrocarpa (Eckl.) A.DC.



Nome Comum: Ameixeira de Natal

Parte da planta estudada: Fruto

Compostos maioritários: Glicosilados de cianidina

Condições de extração: Extração assistida por calor durante 40 min a 90°C, usando como solvente etanol (100%).

Centaurea Cyanus L.



Nome Comum: Centaurea

Parte da planta estudada: Pétalas

Compostos maioritários: Cianidina-malonilglucoronido-hexósido

Condições de extração: Maceração sob agitação (150 rpm) durante 1h (com re-extração do resíduo) à temperatura ambiente (25°C), usando como solvente uma mistura metanol:água (80:20, v/v) com TFA (0,1%).

Dalia mignon



Nome Comum: Dália

Parte da planta estudada: Pétalas

Compostos maioritários: Cianidina-acetilhexósido

Condições de extração: Maceração sob agitação (150 rpm) durante 1h (com re-extração do resíduo) à temperatura ambiente, usando como solvente uma mistura metanol:água (80:20, v/v) com TFA (0,1%).

***Ficus carica* L.**


Nome Comum: Figo

Parte da planta estudada: Pele da infrutescência

Compostos maioritários: Cianidina-3-*O*-rutinosídeo

Condições de extração: Extração por ultrassons a 310 W durante 21 minutos, usando como solvente etanol (100%).

***Gomphrena globosa* L.**


Nome Comum: Perpétua-roxa

Parte da planta estudada: Flores (brácteas e bractéolas).

Compostos maioritários: Gonfrenina e isogonfrenina II e III

Condições de extração: Maceração sob agitação (150 rpm) durante 165 min a 25°C, usando como solvente água (100%) numa razão sólido/líquido de 5 g/L.

***Hibiscus sabdariffa* L.**


Nome Comum: Vinagreira

Parte da planta estudada: Cálice

Compostos maioritários: Delfinidina-3-*O*-sambubiósido

Condições de extração: Maceração sob agitação (150 rpm) durante 1h (com re-extração do resíduo) à temperatura ambiente, usando como solvente uma mistura etanol:água (80:20, v/v).

***Prunus avium* L.**


Nome Comum: Cereja

Parte da planta estudada: Fruto

Compostos maioritários: Cianidina-3-*O*-glucósido

Condições de extração: Maceração sob agitação durante 45 min, a 65°C, usando como solvente uma mistura etanol:água (65:35, v/v).

***Prunus spinosa* L.**


Nome Comum: Abrunho

Parte da planta estudada: Epicarpo

Compostos maioritários: Cianidina-3-rutinósido e peonidina-3-rutinósido

Condições de extração: Extração por ultrassons a 400 W durante 5 minutos, usando como solvente uma mistura etanol:água (47,98:52,02; v/v).

Rosa damascena* ‘Alexandria’ e *R. gallica* ‘Francesa’ enxertada em *R. canina


Nome Comum: Rosa

Parte da planta estudada: Pétalas

Compostos maioritários: Cianidina-dihexósido

Condições de extração: Maceração sob agitação (150 rpm) durante 1h (com re-extração do resíduo) à temperatura ambiente (25°C), usando como solvente uma mistura metanol:água (80:20, v/v) com TFA (0,1%).

***Rubus umilfolius* Schott**


Nome Comum: Amora silvestre

Parte da planta estudada: Fruto

Compostos maioritários: cianidina-3-*O*-glucósido e pelargonidina-3-*O*-glucósido

Condições de extração: Maceração sob agitação durante 20 min, a 56,78 °C, usando como solvente uma mistura etanol:água (46,07:53,93, v/v).

***Sambucus nigra* L.**


Nome Comum: Sabugueiro

Parte da planta estudada: Frutos

Compostos maioritários: Cianidina-3-*O*-sambubiósido

Condições de extração: Maceração sob agitação (150 rpm) durante 1h (com re-extração do resíduo) à temperatura ambiente (25°C), usando como solvente água (100%).

Vaccinium myrtillus L.

Nome Comum: Mirtilo

Parte da planta estudada: Fruto

Compostos maioritários: Malvidina-*O*-hexósido

Condições de extração: Maceração sob agitação (150 rpm) durante 1h (com re-extração do resíduo) à temperatura ambiente (25°C), usando como solvente uma mistura metanol:água (80:20, v/v) com TFA (0,1%).



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Entregável nº 3.1.5.

Versão do Documento: 1

Data de Submissão: 28/02/2019

Responsável: IPB-CIMO

Nome do Documento: Relatório dos procedimentos de refinação dos ingredientes corantes

Histórico de Revisão

Revisão	Data	Parceiros Envolvidos	Descrição



Lista de Autores

Maria Inês Dias

Sumário

O presente relatório descreve os procedimentos de refinação dos ingredientes corantes extraídos de matrizes vegetais.

Índice

1. Identificação	5
2. Informação	6

1. Identificação

<i>Deliverable</i>	3.1.5. Relatório dos procedimentos de refinação dos ingredientes corantes
Tipo de <i>deliverable</i>	Relatório
Nível de disseminação	Confidencial
PPS	PPS3. Corantes Naturais

2. Informação

No âmbito deste projeto, os corantes obtidos a partir de matrizes naturais foram, até à presente data, antocianinas e betalainas. O protocolo de refinação destes compostos corantes para as matrizes naturais *Carissa macrocarpa* (Eckl.) A.DC., *Vaccinium myrtillus* L., *Centaurea Cyanus* L., *Dalia mignon*, *Rosa damascena* ‘Alexandria’ e *R. gallica* ‘Francesa’ enxertada em *R. canina* e *Prunus avium* L. foi o seguinte:

- O extrato antociânico foi depositado num cartucho C-18 SepPak Vac 3 cc, previamente ativado com metanol e água; sendo que os açúcares e substâncias mais polares foram posteriormente removidos com a passagem de 10 mL de água e as antocianinas eluídas com 5 mL de uma solução de metanol:água (80:20, v/v) contendo 0,1% de TFA (ácido trifluoroacético). O extrato foi então concentrado sob vácuo, liofilizado, redissolvido em 1 mL de solução hidrometanólica a 20% e filtrada através de um filtro 0,22 µm para posterior análise em HPLC.

Para as amostras de *Arbutus unedo* L., *Ficus carica* L., *Prunus spinosa* L., *Gomphrena globosa* L., *Hibiscus sabdariffa* L., *Sambucus nigra* L. e *Rubus umilfolius* Schott não foi necessário a aplicação de nenhum protocolo de refinação ao extrato antociânico.



ValorNatural – Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar.

Entregável nº 3.16

Versão do Documento: 1

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Histórico de Revisão

Revisão	Data	Parceiros Envolvidos	Descrição



Lista de Autores

Isabel Ferreira

Cristina Caleja

Maria Inês Dias

Eliana Pereira

Sumário

Publicações relativas aos ingredientes com maior capacidade corante e que não apresentam toxicidade.

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1. Identificação

<i>Deliverable</i>	3.1.6
<i>Tipo de deliverable</i>	Publicação
Nível de disseminação	Público
PPS	3

2. Informação

As publicações relativas aos ingredientes com maior capacidade corante e sem toxicidade são:

Stability of a cyanidin-3-*O*-glucoside extract obtained from *Arbutus unedo* L. and incorporation into wafers for colouring purposes

Lopez C. J., Caleja C., Prieto M. A., Sokovic M., Calhella R. C., Barros L., Ferreira I. C. F. R.

Food Chemistry, 275, 426-438, 2019

Incorporation of natural colorants obtained from edible flowers in yogurts

Pires T. C. S. P., Dias M. I., Barros L., Barreira J. C. M., Santos-Buelga C., Ferreira I. C. F. R.

LWT-Food Science and Technology, 97, 668-675, 2018

Gomphrena globosa L. as a novel source of food-grade betacyanins: Incorporation in ice-cream and comparison with beet-root extracts and comercial betalains.

Roriz C. L., Barreira J. C. M., Morales P., Barros L., Ferreira I. C. F. R.

LWT, 92, 101-107, 2018

Optimization of heat- and ultrasound-assisted extraction of anthocyanins from *Hibiscus sabdariffa* calyces for natural food colorants

Pinela J., Prieto M. A., Pereira E., Jabeur I., Barreiro M. F., Barros L., Ferreira I. C. F. R.

Food Chemistry, 275, 309-321, 2019

Optimization of the Extraction Process to Obtain a Colorant Ingredient from Leaves of *Ocimum basilicum* var. *purpurascens*

Fernandes F., Pereira E., Prieto M. A., Calhella R. C., Ćiric A., Soković M., Simal-Gandara J., Barros L., Ferreira I. C. F. R.

Molecules, 24, 686, 2019

Recovery og bioactive anthocyanin pigments from *Ficus carica* L. peel by heat, microwave, and ultrasound based extraction techniques

Backes E., Pereira C., Barros L., Prieto M. A., Genena A. K., Barreiros M. F., Ferreira I. C. F. R.

Food Research International, 113, 197-209, 2018

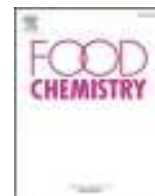
Ultrasound as a Rapid and Low-Cost Extraction Procedure to Obtain Anthocyanin-Based Colorants from *Prunus spinosa* L. Fruit Epicarp: Comparative Study with Conventional Heat-Based Extraction

Leichtweis M. G., Pereira C., Prieto M.A., Barreiro M. F., Braldi I. J., Barros L., Ferreira I. C. F. R.

Molecules, 24, 573, 2019



3. Anexos



Stability of a cyanidin-3-*O*-glucoside extract obtained from *Arbutus unedo* L. and incorporation into wafers for colouring purposes



Cecilia Jiménez López^a, Cristina Caleja^{a,b}, M.A. Prieto^{a,c}, Marina Sokovic^d, Ricardo C. Calhella^a, Lillian Barros^{a,*}, Isabel C.F.R. Ferreira^{a,*}

^a Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal

^b Laboratoire de Séparation et Réaction Engineering (LSRE), Associate Laboratory LSRE/LCM, IPB, Campus de Santa Apolónia, 1134, 5301-857 Bragança, Portugal

^c Nutrition and Bromatology Group, Faculty of Food Science and Technology, University of Vigo, Ourense Campus, E32004 Ourense, Spain

^d University of Belgrade, Department of Plant Physiology, Institute for Biological Research “Siniša Stanković”, Bulevar Despota Stefana 142, 11000 Belgrade, Serbia

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ABSTRACT

An extract from *Arbutus unedo* fruits, rich in anthocyanins, was studied as a natural colorant with bioactive properties (antioxidant, antimicrobial and cytotoxic). The aqueous stability of the extract was monitored using the anthocyanins' content as response (determined by HPLC-DAD) in function of time, temperature and pH. Aided by mechanistic/phenomenological models, the conditions that favours the stabilization of the extract were provided, highlighting the suitability of the colorant for pastry/bakery products. As a case study, the extract was incorporated into wafers and the changes on the nutritional profile, free sugars, fatty acids and antioxidant properties were monitored during 6 days of storage. The results provide information for: i) potential application of the rich extract in anthocyanins for producing a natural colorant with bioactive properties; and ii) shelf-life predictions. The extract incorporation did not cause changes in the nutritional components of wafers but added colorant and antioxidant properties.

1. Introduction

Anthocyanins are secondary metabolites of plants belonging to the group of phenolic compounds, colouring from fruits and flowers, to roots and seeds (Cavalcanti, Santos, & Meireles, 2011). Besides that, their beneficial effects to human health are well known: thanks to the presence of phenolic hydroxyl groups, they show antioxidant properties, preventing pathologies such as cardiovascular diseases, cancer and diabetes (Ge & Ma, 2013; Prior & Wu, 2006). Several studies also attribute to these compounds chemopreventive and chemotherapeutic capacity, due to their ability to inhibit tumour cells' growth (Ding et al., 2006). However, their stability also deserves a special mention due to a chemical structure very susceptible to degradation by several factors, as temperature, pH, presence of oxygen and even light (Cevallos-Casals & Cisneros-Zevallos, 2004; Garzón, 2008).

Although artificial colours began to dominate the market for paints and textiles, in the nineteenth century these pigments started to be highly used in food industry to improve the appearance of certain foods. Initially, these colorants began to be applied in wine, pasta and butter (Ibañez, Torre, & Irigoyen, 2003), but their use was not fully regulated. The use of artificial colorants by the industry raised the concern of

consumers, due to some reports on health problems (hyperactivity and allergic reactions) (Esatbeyoglu, Wagner, Schini-Kerth, & Rimbach, 2015). Nowadays, it is a subject very well regulated, both at continental and world level, through various committees created by WHO, FAO and European Communities' Commission (EFSA). The extensive use of artificial colorants to make food more attractive for consumers (Ibañez et al., 2003) has been related to harmful effects, which increased the interest for labels as “natural” or “without artificial additives” (Carocho, Barreiro, Morales, & Ferreira, 2014; Martins, Roriz, Morales, Barros, & Ferreira, 2016).

Following the legal and commercial requirements that are emerging, there is a trend of the food industry for natural ingredients (Esatbeyoglu et al., 2015). In the case of colorants, anthocyanins represent an attractive and natural alternative to the artificial ones. Anthocyanins stability is essential to ensure the quality and delivery of these bioactive components. However, natural extracts rich in anthocyanins are susceptible to degradation by several factors and the stability of these compounds during its application is a crucial step. The main identified factors affecting the stability are time (*t*), temperature (*T*) and pH (Komatsu et al., 2014; Zhu et al., 2002). Nonetheless, other factors such as light exposure, moisture content, metal ions content,

* Corresponding authors.

E-mail addresses: lillian@ipb.pt (L. Barros), iferreira@ipb.pt (I.C.F.R. Ferreira).

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etc., have also been pointed out as relatively important (Li, Taylor, & Mauer, 2011).

Arbutus unedo L. is a shrub belonging to the subfamily Vaccinioideae (or Arbutoideae, according to the different authors) of the Ericaceae family (Miguel, Faleiro, Guerreiro, & Antunes, 2014). The edible fruits are grown in this shrub and are popularly recognized for their beneficial properties, particularly in the treatment of some symptomatology's (Ziyyat et al., 2002). The antimicrobial and antioxidant properties of the fruits are described in the literature and are associated, according to some authors, with the presence of phenolic compounds (Guimarães et al., 2013; Miguel et al., 2014).

Therefore, the aims of this study were to evaluate: 1) the bioactive properties (antioxidant, antimicrobial and cytotoxic) of the colorant extract produced; and 2) the stability of the extract considering the main affecting variables of *t*, *T* and *pH* in aqueous solution, by analysing the anthocyanins content by HPLC-DAD. Once the extract was stabilized, it was incorporated into wafers as a case study of the potential application in pastry products. Nutritional profile, free sugars, fatty acids and antioxidant activity were monitored after the wafers were baked, and after 3 and 6 days of storage.

2. Material and methods

2.1. Preparation of an anthocyanins rich extract from *A. unedo* fruits

2.1.1. Source material

The fruits of *A. unedo* L. (strawberry tree) were provided by a local producer of Torre de Moncorvo, Portugal. The fruits (1 kg) were lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA) and reduced to powder (~20 mesh). The obtained powder was then mixed to guarantee the homogeneity of the samples, which were stored in a freezer (average -20 °C), and protected from light, until further analysis.

2.1.2. Heat assisted extraction

The extract rich in cyanidin-3-*O*-glucoside was obtained from *A. unedo* L. following the optimized process previously reported by Jiménez et al. (2018). Briefly, the samples (600 mg) were placed in a beaker with 80% of acidified ethanol (acidified with 0.05% of hydrochloric acid) in order to obtain the desired solid/liquid ratio (10 g/L). The beaker was placed in a thermostatic water bath under continuous electro-magnetic stirring using a CIMAREC i Magnetic Stirrer (Thermo Scientific, San Jose, CA, USA) with a fixed agitation speed (500 rpm) for 5 min at 90 °C. Then, the extract solution was filtered through Whatman n° 4 paper and evaporated at 35 °C to remove the ethanol, frozen and lyophilized, to obtain a dry extract. As reported by Jiménez et al. (2018), following this procedure, it was obtained a residual extract of ~60% of the total fruit dw, with a total anthocyanins content of ~500 µg/g of fruit dw (~800 µg/g of extracted material).

2.2. Evaluation of the bioactive properties of the anthocyanins rich extract

A brief summary of the work performed is presented in Fig. 1. Next, each step performed will be described in detail.

2.2.1. General

The extract rich in anthocyanins (125 g) was dissolved in acidified distilled water (500 mL, 0.05% HCl). Dry extracts were further diluted to different concentrations to be submitted to the in vitro bioassays: i) final concentration 20 mg/mL in 80% of ethanol (0.05% HCl) and further diluted to working solutions of 5–0.156 mg/mL, for antioxidant activity; ii) stock concentration of 8 mg/mL, re-dissolved in water (0.05% HCl) and further diluted to 400–1.5 µg/mL working solutions for cytotoxic evaluation; and iii) the extracts were diluted in appropriate media according to bacteria requirements and the pH was adjusted with 0.05% HCl and successive dilutions were carried out in the

wells (1–0.1 mg/mL of final concentration).

2.2.1.1. Evaluation of antioxidant properties. DPPH radical-scavenging activity and reducing power were evaluated using ELX800 microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) at 515 and 690 nm, respectively. β-Carotene bleaching inhibition and lipid peroxidation inhibition, using the thiobarbituric acid reactive substances – TBARS, were evaluated spectrophotometrically at 470 and 532 nm, respectively. The complete protocols were previously described by the authors (Sarmiento, Barros, Fernandes, Carvalho, and Ferreira, 2015).

2.2.1.2. Evaluation of cytotoxicity properties. The extract was re-dissolved in water at a concentration of 8 mg/mL. According with Guimarães et al. (2013), four human tumour cell lines were used: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). The cell growth inhibition was measured using sulforhodamine B assay, where the amount of pigmented cells is directly proportional to the total protein mass and, therefore, to the number of bounded cells.

For hepatotoxicity evaluation, a cell culture was prepared from a freshly harvested porcine liver which was acquired from certified slaughterhouses and was used in order to obtain the cell culture, designated as PLP2. A phase-contrast microscope was used to monitor the growth of the cell cultures. They were sub-cultured and plated in 96 well plates (density of 1.0×10^4 cells/well). Dulbecco's modified eagle's medium (DMEM) was used, with 10% of Fetal bovine serum (FBS), 100 U/mL of penicillin and 100 µg/mL of streptomycin. The growth inhibition was evaluated using the sulphorhodamine B (SRB) assay, as previously described (Guimarães et al., 2013).

2.2.1.3. Evaluation of antimicrobial properties. Following the procedure previously described by Soković, Glamočlija, Marin, Brkić, and van Griensven (2010), the antibacterial activity was evaluated against four Gram-negative bacteria: *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), *Salmonella enteritidis* (ATCC 13076), and four Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240), and *Listeria monocytogenes* (NCTC 7973).

Following the procedure previously described by Soković & van Griensven (2006), the antifungal activity was evaluated against eight fungi: *Aspergillus fumigatus* (ATCC 1022), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus versicolor* (ATCC 11730), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112), *Candida crusei* (clinical isolate) and *Penicillium verrucosum* (food isolate).

2.2.2. Response evaluation and statistical analysis

Antioxidant responses were evaluated by the EC_{50} values (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay) and expressed in µg/mL. Trolox was used as positive control in all the assays.

Cytotoxicity responses were determined by the GI_{50} values (sample concentration providing 50% of cytotoxic activity) and the results were expressed in µg/mL. Ellipticine was used as a positive control.

Antimicrobial responses were evaluated by the minimum inhibitory (MIC) and minimum bactericidal or fungicidal (MBC or MFC) concentrations and results were expressed in µg/mL. Streptomycin and ampicillin were used as positive controls for bacteria growth, while bifonazole and ketokonazole were used as controls for fungi growth.

In all cases the assays were carried out in triplicate. The results were expressed as mean values and standard deviation (SD).

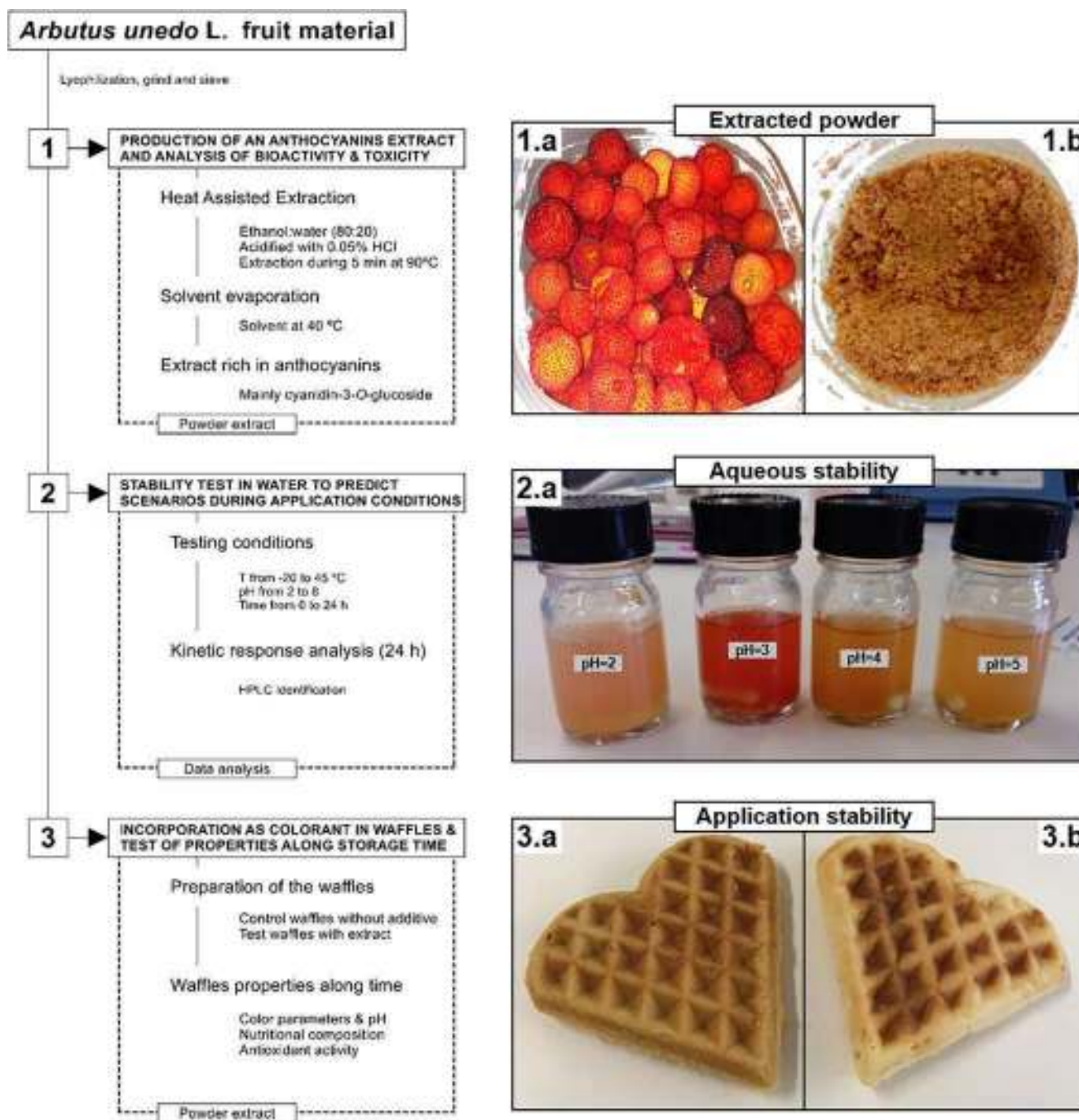


Fig. 1. Summarized diagram describing work performed: **Section 1** shows the extraction process of anthocyanin compounds, **Fig. 1.a** and **b** presents the *A. unedo* fruit and extract, respectively; **Section 2** shows the stability in aqueous solution systems considering its potential application in food matrices (**Fig. 2.a** shows the differences caused by the *pH* variable); and **Section 3** shows the visual results of *A. unedo* extract rich in cyanidin-3-O-glucoside incorporated into wafers (**Fig. 3.a** shows wafer with *A. unedo* extract and **3.b** control wafer).

2.3. Stability of the of the anthocyanins rich extract in aqueous solution systems

A brief summary of the work performed is presented in **Fig. 1**. Next, each step performed will be described in detail.

2.3.1. General procedure

The extract rich in anthocyanins (125 g) was dissolved in acidified distilled water (500 mL, 0.05% HCl). This solution was divided in 5 different flasks with 100 mL and adjusted with the addition of HCl (1 M) or NaOH (3%) to different *pH* values (2, 3.5, 5, 6.5 and 8). Then, the

samples were subdivided in amber vials containing 2 mL (50 vials per *pH* tested) and the samples were stored at different temperatures of 4, 25, 40, 55 and 70 °C for monitoring of the anthocyanin stability along the time (*t*). Samples were collected at different *t* values of the storage period depending on the *T* applied, for example at all *pH* values, at 4 °C only 3 *t* values were used (0, 72 and 140 h), meanwhile at 70 °C 12 *t* values were used (0, 2, 4, 8, 12, 20, 48, 72, 96, 140, 164, 190 h). A total of 250 individual experimental points were collected to understand the patterns behind the stability of the extract in aqueous solution. As response criteria, the anthocyanins content was determined through HPLC-DAD, at each period of time. All independent measures were

obtained in triplicate ($n = 3$).

2.3.2. Identification and quantification of anthocyanins by HPLC-DAD

Each individual experimental point was filtered through a 0.22 μm disposable LC filter disk before chromatographic analysis, which was performed with a HPLC-DAD-ESI/MSn (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA) system. The separation was achieved on a Waters Spherisorb S3 ODS-2 C18 (3 μm , 4.6 mm \times 150 mm, Waters, Milford, MA, USA) operating at 35 $^{\circ}\text{C}$. The solvents used were: (A) 0.1% TFA in water, and (B) 100% acetonitrile. The gradient elution followed these parameters: 10% B for 3 min, from 10 to 15% B for 12 min, 15% B for 5 min, from 15 to 18% B for 5 min, from 18 to 30% B for 20 min, from 30 to 35% B for 5 min, and from 35 to 10% B for 10 min. The resulting total run time was 60 min, followed by column reconditioning of 10 min, using a flow rate of 0.5 mL/min. Detection was carried out by DAD, using 520 nm as the preferred wavelength. Data acquisition was carried out with Xcalibur[®] data system (Thermo Finnigan, San Jose, CA, USA). A total of three anthocyanin compounds (Fig. A1, Supplementary material) were found and identified: delphinidin-3-*O*-glucoside (C1), cyanidin-3-*O*-glucoside (C2) and cyanidin-3-*O*-pentoside (C3), as previously described by Jiménez et al. (2018). For quantitative analysis, a 5-level calibration curve was obtained by injection of known concentrations (50–0.25 $\mu\text{g}/\text{mL}$) of cyanidin-3-*O*-glucoside ($y = 243287x - 1000000$; $R^2 = 0.9953$).

2.3.3. Responses evaluation and statistical analysis

The anthocyanins content was studied as function of mechanistic and phenomenological equations typically applied in similar processes.

2.3.3.1. Individual model for the analysis of the stability variable effects. Effect of the time on the stability response

For the t effect, a typical exponential function was applied:

$$e(t) = k \exp(-rt) \quad (1)$$

where k represents the starting point and r is the decay degradation rate of the reaction.

Effect of the temperature on the stability response

The Arrhenius equation establishes that the rate constant of a chemical reaction is a function of the absolute T according to the following relation:

$$e(T) = A \exp\left(-\frac{Ea}{RT}\right) \quad (2)$$

where the pre-exponential factor A represents the frequency of collisions among reacting molecules, Ea is the activation energy (kJ) and R the constant of gases (8.31 kJ/mol.K). In context, A and Ea can be considered as fitting parameters.

Effect of the pH on the stability response

The characteristic solution for the description of the pH effect is the exponential function, similar to the one used for t effect, that is usually found in many biological system responses (Komatsu et al., 2014; Prieto, Vázquez, & Murado, 2012b) and can be expressed as follows:

$$e(\text{pH}) = s \exp(-b\text{pH}) \quad (3)$$

where s represents the starting point and b is the degradation rate of the reaction.

2.3.3.2. Multivariable analysis. To be able to develop a multivariable analysis of these three variables, the logical approach is to insert the equations that take control of pH and T into Eq. (1) that governs the time variable by modifying its parameters, the starting value (k) and the degradation rate (r). Even if the three variables are fully independent, any event that may occur in the surrounding environment of a reaction must be always referred to the time variable. Therefore, a global possible description of the stability at the molecular level could be described by the following approach:

$$e(t, \text{pH}, T) = k \exp(-rt) \quad \text{where} \quad \begin{aligned} k(\text{pH}, T) &= k \times e(T) \times e(\text{pH}) \\ r(\text{pH}, T) &= r \times e(T) \times e(\text{pH}) \end{aligned} \quad (4)$$

where k represents the starting point and r is the degradation rate of the reaction caused by the effect of time as described Eq. (1) but modified by the governing equations of the effect of T (Eq. (2)) and pH (Eq. (3)).

2.3.3.3. Numerical methods and statistical analysis. Fitting procedures, coefficient estimates and statistical calculations were performed as previously described (Pinela et al., 2016). In brief, a) the coefficient measurement was performed using the nonlinear least-square (quasi-Newton) method provided by the macro ‘Solver’ in Microsoft Excel, which allows minimizing the sum of the quadratic differences between the observed and model-predicted values; b) the coefficient significance was evaluated using the ‘SolverAid’ to determine the parametric confidence intervals. Not statistically significant terms (p -value > 0.05) were dropped to simplify the model; and c) the model reliability was verified using the following criteria: i) the Fisher F -test ($\alpha = 0.05$) was used to determine whether the constructed models were adequate to describe the observed data; ii) the ‘SolverStat’ macro was used for the assessment of parameter and model prediction uncertainties; iii) the R^2 was interpreted as the proportion of variability of the dependent variable explained by the model.

2.4. Incorporation of the anthocyanins rich extract in wafers as natural colorant additives. Evaluation of colour, nutritional composition and antioxidant activity along storage time

A brief summary of the work performed is presented in Fig. 1. Next, each step performed will be described in detail.

2.4.1. Preparation of the wafers samples

A traditional formulation was followed to prepare the wafers: 165 g of wheat flour was thoroughly mixed with 100 g of sugar and 1 g of baking powder. Then, 130 g of butter were sequentially added to the mixture while mixing vigorously with a hand mixer at 450 W during 5 min and after 2 eggs and 20 mL of lemon juice were added. Two lots of wafers (18 per lot, 6 wafers for each storage time) were prepared: i) control wafers – without the addition of any substance; and ii) wafers with the *A. unedo* extract rich in cyanidin-3-*O*-glucoside (5.50 g). The wafers were baked in a wafers machine for 10 min. All samples were lyophilized, finely crushed and analysed, in triplicate, immediately after preparation and after three and six days of storage (at room temperature and packed in a sealed plastic bags covered with aluminium paper).

2.4.2. Evaluation of colour parameters and pH of the wafers along storage time

The colour of the samples was measured using a colorimeter (model CR-400, Konica Minolta Sensing Inc., Tokyo, Japan). The illuminate C was used and a diaphragm aperture of 8 mm and previously calibrated against a standard white tile. The CIE L^* (lightness), a^* (greenness/redness), b^* (blueness/yellowness) colour space values were registered using a data software ‘Spectra Magic Nx’ (version CM-S100W 2.03.0006) (Caleja et al., 2016). The samples’ pH was measured by inserting the pH-meter (HI 99161, Hanna Instruments, Woonsocket, Rhode Island, USA) in the waffle sample. The determinations were performed in triplicate for each sample.

2.4.3. Evaluation of the proximate composition, free sugars, fatty acids and antioxidant activity of the wafers along storage time

- The contents of protein, fat, carbohydrates and ash, were determined following the AOAC methods (AOAC International, 2016). Total energy was calculated following the equation: Energy

Table 1
Antioxidant, cytotoxic and antimicrobial activities of *Arbutus unedo* L. extract (mean \pm SD).

Antioxidant activity	<i>A. unedo</i> extract EC_{50} ($\mu\text{g/mL}$)	Control (Trolox) EC_{50} ($\mu\text{g/mL}$)		
DPPH scavenging activity	295 \pm 13	41 \pm 1		
Reducing power	447 \pm 4	41.7 \pm 0.3		
β -carotene bleaching inhibition	901 \pm 41	18 \pm 1		
Thiobarbituric acid reactive substances (TBARS)	257 \pm 4	23 \pm 1		
Cytotoxicity in tumour cell lines	<i>A. unedo</i> extract GI_{50} ($\mu\text{g/mL}$)	Control (Ellipticine) GI_{50} ($\mu\text{g/mL}$)		
NCI-H460 (non-small cell lung carcinoma)	> 400	1.0 \pm 0.1		
HeLa (cervical carcinoma)	350 \pm 10	1.91 \pm 0.06		
HepG2 (hepatocellular carcinoma)	> 400	1.1 \pm 0.2		
MCF-7 (breast carcinoma)	338 \pm 14	0.91 \pm 0.04		
Cytotoxicity in non-tumour cells	<i>A. unedo</i> extract GI_{50} ($\mu\text{g/mL}$)	Control (Ellipticine) GI_{50} ($\mu\text{g/mL}$)		
PLP 2 (porcine liver primary cells)	> 400	3.2 \pm 0.7		
Antibacterial activity	<i>A. unedo</i> extract		Control (Ampicillin)	
	MIC ($\mu\text{g/mL}$)	MBC ($\mu\text{g/mL}$)	MIC ($\mu\text{g/mL}$)	MBC ($\mu\text{g/mL}$)
<i>Gram negative bacteria</i>				
<i>Escherichia coli</i>	> 1000	> 1000	400	500
<i>Salmonella enteritidis</i>	150	300	300	600
<i>Salmonella typhimurium</i>	200	300	400	750
<i>Enterobacter cloacae</i>	> 1000	> 1000	250	500
<i>Gram positive bacteria</i>				
<i>Staphylococcus aureus</i>	300	600	250	450
<i>Bacillus cereus</i>	150	450	250	400
<i>Micrococcus flavus</i>	300	600	250	400
<i>Listeria monocytogenes</i>	300	600	400	500
Antifungal activity	<i>A. unedo</i> extract		Control (Ketoconazole)	
	MIC ($\mu\text{g/mL}$)	MFC ($\mu\text{g/mL}$)	MIC	MFC
<i>Aspergillus fumigatus</i>	150	450	250	500
<i>Aspergillus ochraceus</i>	200	450	1500	2000
<i>Aspergillus versicolor</i>	300	600	200	500
<i>Penicillium funiculosum</i>	450	600	200	500
<i>Penicillium ochrochloron</i>	300	600	2500	3500
<i>Candida crusei</i>	300	600	075	150
<i>Penicillium verrucosum</i>	450	600	200	300

EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay. GI_{50} values correspond to the sample concentration achieving 50% of growth inhibition in human tumour cell lines or in liver primary culture PLP2. MIC values correspond to the minimal sample concentration that inhibited the bacterial growth; MBC or MFC correspond to the minimum bactericidal or fungicidal concentrations, respectively.

(kcal) = $4 \times (\text{g proteins} + \text{g carbohydrates}) + 9 \times (\text{g lipids})$. Total protein content ($N \times 5.70$) was calculated as nitrogen content by the Kjeldahl method, while crude fat relied on the extraction of dried samples with petroleum ether using a Soxhlet apparatus. Finally, the ash content was determined by incineration at $550 \pm 15^\circ\text{C}$ (Barros et al., 2013).

- For free sugars analysis, 1.0 g of dried sample powder was spiked

with melezitose as internal standard (IS, 5 mg/mL), and extracted with 40 mL of 80% aqueous ethanol at 80°C for 30 min. The resulting suspension was centrifuged (Centurion K24OR refrigerated centrifuge, West Sussex, UK) at 15,000g for 10 min. The supernatant was concentrated at 60°C under reduced pressure and defatted three times with 10 mL of ethyl ether, successively. After concentration at 40°C , the solid residues were dissolved in water to a final volume of 5 mL and filtered through 0.2 μm Whatman nylon filters. Free sugars were determined in defatted samples by HPLC coupled to a refraction index (RI) detector following a procedure previously described (Caleja et al., 2016). The free sugars were identified by comparison with standards and further quantified considering the internal standard (melezitose) (g/100 g of wafers).

- The fat obtained after the Soxhlet extraction was subjected to a methylation process with 5 mL of methanol: sulfuric acid: toluene 2: 1: 1 (v: v: v), for 12 h in a water bath at 50°C and 160 rpm; then 3 mL of deionized water was added to obtain phase separation; FAME was recovered by adding 3 mL of diethyl ether, stirring on a Vortex shaker and passing the upper phase through a micro-column of anhydrous sodium sulfate, in order to eliminate the water. The sample was collected in a vial with Teflon and filtered with a 0.2 μm Whatman nylon filter before injection. The fatty acids were determined, by gas chromatography coupled to flame ionization detector (GC-FID), identified by comparison with standards (standard 47885, Sigma-Aldrich, St. Louis, Missouri, USA) and expressed as relative percentages of each fatty acid (Caleja et al., 2016).
- For evaluation of the antioxidant activity, the samples were submitted to DPPH and reducing power assays, described in a previous section (results expressed in EC_{50} values mg/mL).

2.4.4. Response evaluation and statistical analysis

The results were analysed using a Student's *t*-student test in order to determine the significant difference between less than three different samples, with $p = 0.05$, and this treatment was carried out using the SPSS v. 23.0 program.

3. Results and discussion

3.1. Bioactive properties of the anthocyanins rich extract

Our research group has previously developed some works using fruits of *A. unedo* due to their traditional uses in the northeast of Portugal. The studies described the antioxidant properties of those fruits as well as the presence of important antioxidant molecules such as tocopherols and carotenoids (Barros, Carvalho, Morais, & Ferreira, 2010; Guimaraes, Barros, Carvalho, & Ferreira, 2010). Later, Guimarães et al. (2013) carried out an extensive characterization of the phenolic compounds present in *A. unedo* fruits and compared the bioactive properties of two different extracts rich in non-anthocyanin phenolic compounds and anthocyanins, respectively (Guimarães et al., 2014). Furthermore, an extraction optimization from these fruits was performed in order to obtain a rich extract in cyanidin-3-*O*-glucoside (Jiménez et al., 2018). The extract obtained under the optimal point was evaluated in the present study regarding bioactive and colorant simultaneous capacities, as also the conditions that most favoured its stability.

3.1.1. Antioxidant properties

There are several techniques that can be used to evaluate the antioxidant activity of pure compounds or complex mixtures (as in the case of plant extracts). In our study, to evaluate the antioxidant activity of *A. unedo* extract, four complementary *in vitro* assays were selected: DPPH free radicals scavenging, reducing power, β -carotene bleaching inhibition and TBARS formation inhibition. The results are expressed in EC_{50} values ($\mu\text{g/mL}$) and summarized in Table 1. The anthocyanins extract obtained from *A. unedo* fruits showed high antioxidant activity in the

different assays, mainly in the ability to inhibit TBARS formation with the lowest EC_{50} value. In a recent study, an extract from *A. unedo* rich in catechin was prepared and a high antioxidant activity was also described (Takwa et al., 2018). The reducing power assay was the only method performed that gave a higher antioxidant activity result (EC_{50} value = $328 \pm 2 \mu\text{g/mL}$), in comparison with this study; for the remaining assays the present extract revealed a greater antioxidant activity.

3.1.2. Cytotoxic properties

The potential effects of natural phenolic compounds as anticancer agents *in vitro* as well as *in vivo* has been described in different studies (Carocho & Ferreira, 2013). Thus, the effects of the extracts on the growth of the four human tumour cell lines (MCF-7, NCI-H460, HeLa, and HepG2) were determined and the values of the GI_{50} (concentrations that caused 50% of the cell growth inhibition) are detailed in Table 1. The *A. unedo* extract rich in cyanidin-3-*O*-glucoside did not show positive results for NCI-H460, nor HepG2 cell lines ($GI_{50} > 400 \mu\text{g/mL}$), showing to be able to inhibit the growth of HeLa (cervical carcinoma) and MCF7 (breast carcinoma) cell lines in a moderate way. This extract did not demonstrate cytotoxicity in PLP2 cell lines ($GI_{50} > 400 \mu\text{g/mL}$). These results are in agreement with the work presented by Ziani et al. (2015), which studied the bioactive properties of infusions prepared from *A. unedo* flowers and leaves (rich in phenolic compounds, such as flavonoids, especially flavonols and phenolic acid esters), describing the absence of cytotoxic effects.

3.1.3. Antimicrobial properties

The antimicrobial activity was also studied against a panel of eight bacteria and eight fungi chosen due to their importance in public health. The results obtained for this activity are presented in Table 1 and were divided into antibacterial and antifungal activities. Results are expressed as MIC and MBC or MFC values ($\mu\text{g/mL}$). The results showed that *A. unedo* extract rich in cyanidin-3-*O*-glucoside, besides its high antioxidant activity, it also has a high antimicrobial activity. This activity was particularly high against *Salmonella enteritidis* and *Bacillus cereus*. Otherwise, the high antifungal activity of the extract was also confirmed, being *Aspergillus fumigatus* and *A. ochraceus* the most sensitive fungi, with the lowest MIC and MFC values. The antioxidant and antimicrobial capacities demonstrated by the *A. unedo* extract may be explained by the presence of significant levels of phenolic compounds, such as anthocyanins, as previously demonstrated by Mak, Chuah, Ahmad, and Bhat (2013).

3.2. Stability of the extracts in aqueous solution systems

The *A. unedo* extract rich in anthocyanins (mainly cyanidin-3-*O*-glucoside, Fig. A1) presents a total optimized content of $\sim 500 \mu\text{g/g}$ of fruit dw or $\sim 800 \mu\text{g/g}$ of R extract residue (knowing that the residual extract was $\sim 60\%$ of the total fruit dw). Such values can be located within the intermediate values described by other authors in similar plant-based samples. The anthocyanins content varies widely in similar fruit samples, from $\sim 20 \mu\text{g/g}$ of fruit dw (plum) (Timberlake and Henry, 1988) to $\sim 15000 \mu\text{g/g}$ of fruit dw (elderberry) (Clifford, 2000). The achieved results are within the same values as those well-known fruits with high content of anthocyanins such as *Nitraria tangutorum* Bobr. ($\sim 650 \mu\text{g/g}$ of fruit dw) (Sang, Sang, Ma, Hou, & Li, 2017) and *Aristotelia chilensis* L. (400 at $1500 \mu\text{g/g}$ of fruit dw) (Gironés-Vilaplana et al., 2014). Non-controllable variables such as soil properties, sun exposition, harvest time, etc., and controllable variables such as the extraction conditions (time, solvent, temperature, etc.) and techniques (ultrasound, maceration, microwave, etc.), may affect compounds concentration and could increase/decrease the yield efficiencies. However, the higher efficiencies in anthocyanins extraction from *A. unedo* fruits, in comparison with other sources emphasizes the need to perform more detailed evaluations of the stability of the compounds

obtained. From the stability point of view, the main factors includes t (Komatsu et al., 2014), pH (Su, Leung, Huang, & Chen, 2003), T (Demeule et al., 2002), oxygen level (Labbé, Têtu, Trudel, & Bazinet, 2008) and concentration of other compounds such as antioxidants level, metal ions and other compositional ingredients (Zhu, Zhang, Tsang, Huang, & Chen, 1997). Although all of these parameters are relevant, most of the authors agree that the essential ones are t , pH and T (Komatsu et al., 2014; Li, Taylor, Ferruzzi, & Mauer, 2012; Li et al., 2011). Therefore, the stability of anthocyanins content was studied regarding the functions t , pH and T in an aqueous solution system. For all these responses, the stability of the compounds was monitored by HPLC-DAD.

The mathematical analysis of the stability of anthocyanin compounds is first performed from an invariable perspective, fitting each set of conditions of T and pH to the time-dependent model of Eq. (1). Afterwards, the effects caused by the affecting variables T and pH over the time-dependent parametric values of Eq. (1) are depicted and established in mathematical terms by auxiliary functions. Then, the mathematical analysis is presented with a global performing fitting analysis from a multivariable perspective. Mathematical analysis using an invariable or multivariable perspective allowed to summarize the response behaviour into parametric information, which helps to perform easier comparisons and predictions. However, the development of models from a multivariable perspective facilitates the possibility of combining the effects of all variables simultaneously into a single master curve that is able to fit all the experimental data (Prieto, Vázquez, & Murado, 2012a). Such a solution allows to control most factors that affect the system, helping to reduce the over fitted resolutions that perturbs the comprehension of the real effects caused by the variables and therefore, the final parametric values are more reproducible.

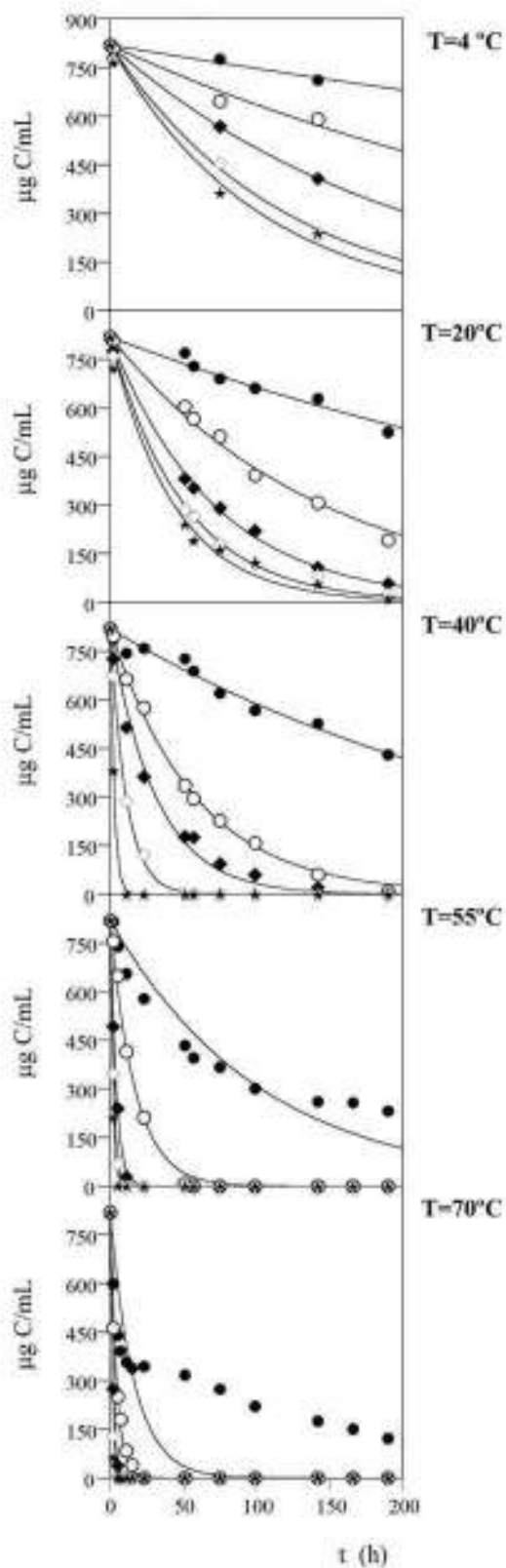
3.2.1. Individual time analysis of each of the conditions of temperature and pH

The individual time-dependent graphical analysis of the stability of the anthocyanin content in aqueous solution as function of the pH and T are presented in Section A of Figs. 2 and 3. Fig. 2 shows the stability results for the total anthocyanin content, meanwhile Fig. 3 shows the stability of the individual content of the identified anthocyanin compounds of C1 (delphinidin-3-*O*-glucoside), C2 (cyanidin-3-*O*-glucoside) and C3 (cyanidin-3-*O*-pentoside). Each graphical illustration shows the time degradation effects (0–190 h) of each T tested (4, 25, 40, 55, and 70 °C). Points are the experimental data of the different pH s tested (●2, ○3.5, ◆5, ◇6.5 and ★8) and lines (—) show the results predicted by Eq. (1). The parametric results and correlation coefficients obtained after fitting each kinetic degradation at each of the T and pH values tested by Eq. (1) are presented in Table 2. In all cases the statistical description was significant and the prediction of the anthocyanins content stability in solution by Eq. (1) showed highly consistent R^2 values. When observing the patterns of the kinetic parametric values k and r (Table 2), it can be observed that the kinetic parameter value of k remain constant at each value of T and pH , meanwhile the kinetic parameter r is the one that takes into account all the effects caused by the changes of T and pH .

3.2.2. Multivariable analysis for the global comprehension of the anthocyanins stability

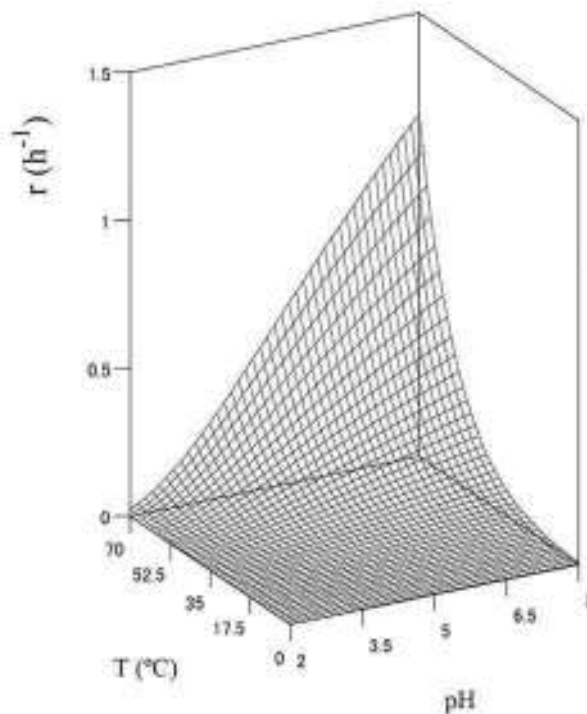
To be able to describe those effects in a multivariate form, the degradation rate parameter of Eq. (1) (r) assumes all the perturbation effects caused by the pH and T variables. The increase in pH and T units causes an exponential increase on the degradation rate of the stability of anthocyanin compounds. The T effect behaves following the Arrhenius equation presented in Eq. (2). However, the pH effect cannot be described by any standard physical-chemical function, because the main mechanisms behind the pH in basic chemical reactions or complex living organisms are dissimilar and heterogeneous. For this particular

A: INDIVIDUAL ANALYSIS

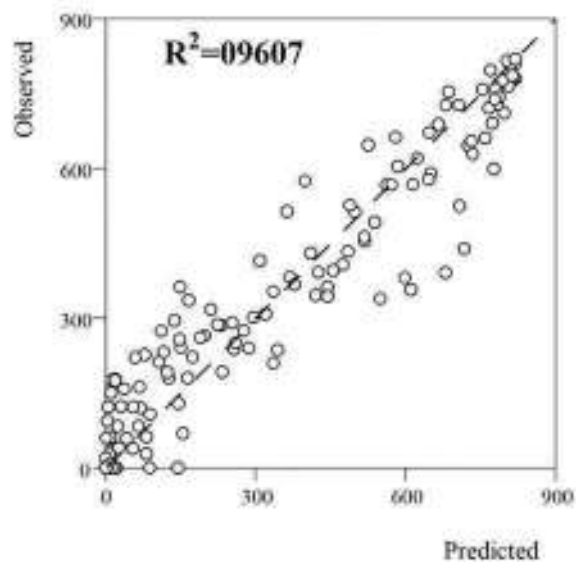


B: MULTIVARIABLE ANALYSIS

B1: Parametric pattern



B2: Experimental data and modelled



(caption on next page)

Fig. 2. Total anthocyanins stability as function of the t , T and pH in aqueous solution systems simulating food matrices. Section A shows the individual time-dependent graphical analysis of the stability results based on the anthocyanins content as function of the T and pH . Each graph shows the time degradation effects (0–190 h) of each T tested (5, 25, 40, 55 and 70 °C). Points are the experimental data of the different pH s tested (●2, ○3.5, ◆5, ◇6.5 and ★8) and lines (—) the results predicted by Eq. (1). The parametric results and correlation coefficients are presented in Table 2. Section B shows the global multivariable fitting results of applying Eq. (5) to describe the full multivariable data: B1 shows the parametric net surface pattern of the kinetic r as a function of their respective affecting variables (pH and T); and B2 shows the correlation between the experimental values and the predicted ones obtained with the multivariable model presented in Eq. (5).

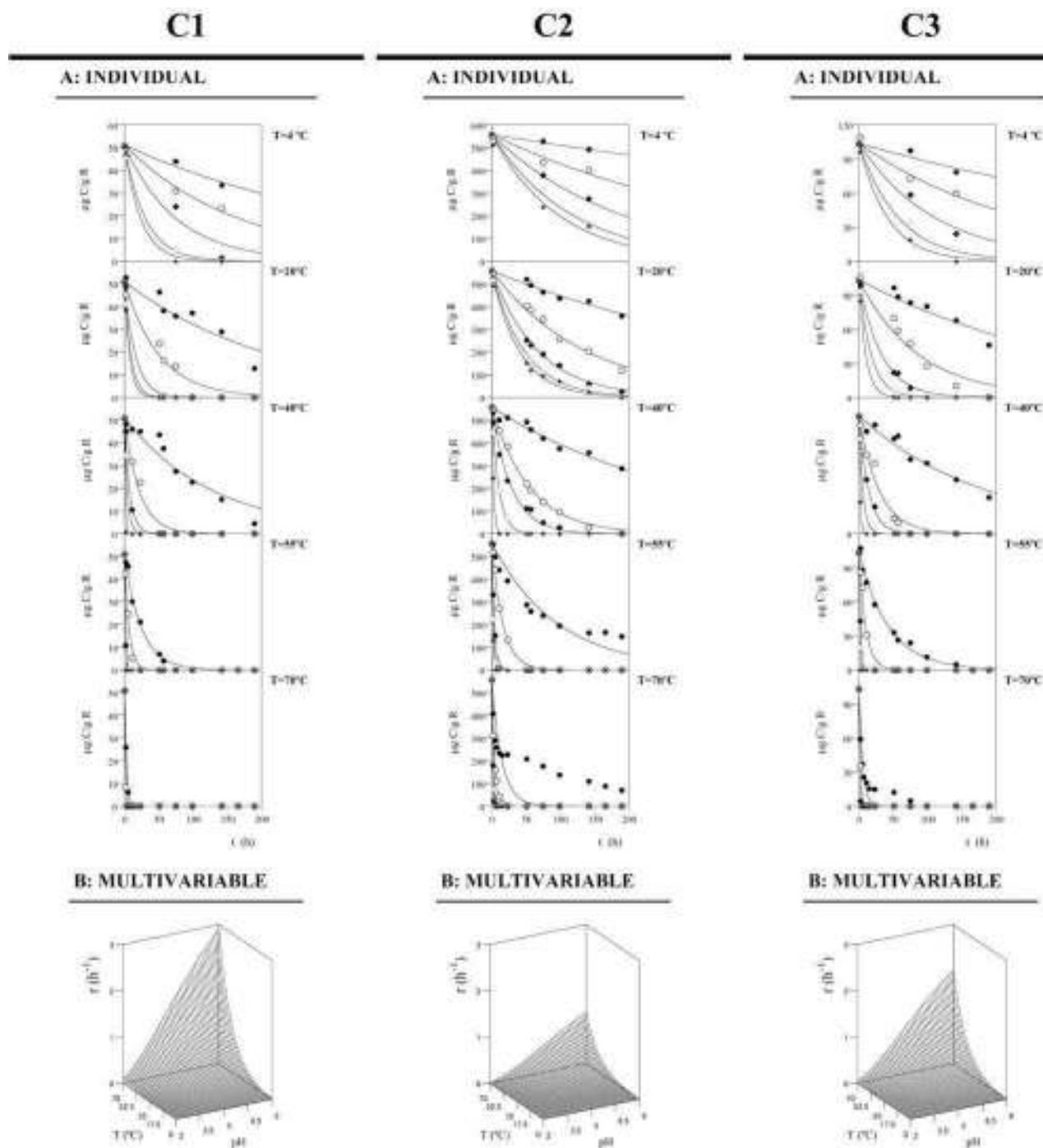


Fig. 3. Individual stability of delphinidin-3-O-glucoside (C1), cyanidin-3-O-glucoside (C2) and cyanidin-3-O-pentoside (C3) as function of the t , T and pH in aqueous solution systems simulating food matrices. Two sub-sections for each compound are represented as in Fig. 2. Section A shows the individual time-dependent graphical analysis of the stability results based on the anthocyanins content as function of the T and pH . Each graph shows the time degradation effects (0–190 h) of each T tested (5, 25, 40, 55 and 70 °C). Points are the experimental data of the different pH s tested (●2, ○3.5, ◆5, ◇6.5 and ★8) and lines (—) the results predicted by Eq. (1). The parametric results and correlation coefficients are presented in Table 2. Section B shows the global multivariable fitting results of applying Eq. (5) to describe the full multivariable data of the parametric net surface pattern of the kinetic r as a function of their respective affecting variables (pH and T).

Table 2
Results of the parametric and confidence intervals of the total anthocyanins, delphinidin-3-glucoside (C1), cyanidin-3-glucoside (C2) and cyanidin-3-pentoside (C3) content fitted to the time dependent model presented in Eq. (1) at different pH and T conditions when evaluating the stability during the process for obtaining the enriched extract.

T	pH	Total			R ²	C1			R ²	C2			R ²	C3			R ²
		$k(t)$		$r(t)$		$k(t)$		$r(t)$		$k(t)$		$r(t)$		$k(t)$		$r(t)$	
		($\mu\text{g/g R}$)	(h^{-1})	($\mu\text{g/g R}$)		(h^{-1})	($\mu\text{g/g R}$)	(h^{-1})		($\mu\text{g/g R}$)	(h^{-1})	($\mu\text{g/g R}$)		(h^{-1})			
4.0	2.0	818.9 ± 66.28	0.001 ± 0.001	0.909	51.4 ± 4.47	0.003 ± 0.001	0.870	557.0 ± 38.43	0.001 ± 0.001	0.890	103.7 ± 9.23	0.002 ± 0.000	0.890				
4.0	3.5	818.9 ± 79.46	0.003 ± 0.001	0.970	51.4 ± 5.10	0.006 ± 0.001	0.992	557.0 ± 53.59	0.003 ± 0.001	0.962	103.7 ± 10.11	0.004 ± 0.000	0.975				
4.0	5.0	818.9 ± 81.41	0.005 ± 0.002	0.994	51.4 ± 4.89	0.013 ± 0.001	0.952	557.0 ± 55.41	0.005 ± 0.001	0.995	103.7 ± 10.13	0.009 ± 0.000	0.976				
4.0	6.5	818.9 ± 81.48	0.008 ± 0.003	0.995	51.4 ± 5.13	0.033 ± 0.001	0.998	557.0 ± 55.31	0.009 ± 0.002	0.993	103.7 ± 10.19	0.017 ± 0.001	0.983				
4.0	8.0	818.9 ± 81.15	0.010 ± 0.003	0.991	51.4 ± 5.12	0.044 ± 0.001	0.997	557.0 ± 55.12	0.010 ± 0.002	0.990	103.7 ± 10.35	0.024 ± 0.001	0.998				
20.0	2.0	818.9 ± 79.25	0.002 ± 0.001	0.968	51.4 ± 4.62	0.005 ± 0.001	0.899	557.0 ± 53.90	0.002 ± 0.001	0.968	103.7 ± 9.53	0.003 ± 0.000	0.919				
20.0	3.5	818.9 ± 81.34	0.007 ± 0.002	0.993	51.4 ± 4.96	0.020 ± 0.001	0.965	557.0 ± 55.21	0.007 ± 0.002	0.991	103.7 ± 10.06	0.012 ± 0.000	0.970				
20.0	5.0	818.9 ± 81.77	0.014 ± 0.005	0.998	51.4 ± 5.12	0.064 ± 0.001	0.997	557.0 ± 55.56	0.015 ± 0.003	0.997	103.7 ± 10.34	0.031 ± 0.001	0.997				
20.0	6.5	818.9 ± 81.73	0.020 ± 0.006	0.998	51.4 ± 5.14	0.111 ± 0.002	1.000	557.0 ± 55.63	0.021 ± 0.005	0.999	103.7 ± 10.37	0.057 ± 0.002	1.000				
20.0	8.0	818.9 ± 81.36	0.023 ± 0.007	0.993	51.4 ± 5.14	0.143 ± 0.003	1.000	557.0 ± 55.42	0.025 ± 0.006	0.995	103.7 ± 10.37	0.102 ± 0.004	1.000				
40.0	2.0	818.9 ± 79.24	0.003 ± 0.001	0.967	51.4 ± 4.78	0.008 ± 0.001	0.930	557.0 ± 53.64	0.003 ± 0.001	0.963	103.7 ± 9.79	0.005 ± 0.000	0.944				
40.0	3.5	818.9 ± 81.75	0.017 ± 0.006	0.998	51.4 ± 5.05	0.047 ± 0.001	0.984	557.0 ± 55.62	0.018 ± 0.004	0.999	103.7 ± 10.17	0.034 ± 0.001	0.980				
40.0	5.0	818.9 ± 81.34	0.032 ± 0.010	0.993	51.4 ± 5.09	0.129 ± 0.003	0.991	557.0 ± 55.39	0.035 ± 0.008	0.994	103.7 ± 10.34	0.071 ± 0.003	0.997				
40.0	6.5	818.9 ± 81.83	0.092 ± 0.030	0.999	51.4 ± 5.11	0.237 ± 0.005	0.994	557.0 ± 55.67	0.098 ± 0.022	0.999	103.7 ± 10.23	0.177 ± 0.007	0.986				
40.0	8.0	818.9 ± 81.88	0.385 ± 0.126	1.000	51.4 ± 5.14	2.250 ± 0.046	1.000	557.0 ± 55.69	0.412 ± 0.092	1.000	103.7 ± 10.37	0.659 ± 0.027	1.000				
55.0	2.0	818.9 ± 77.79	0.010 ± 0.003	0.950	51.4 ± 5.11	0.041 ± 0.001	0.994	557.0 ± 53.18	0.010 ± 0.002	0.955	103.7 ± 10.29	0.023 ± 0.001	0.992				
55.0	3.5	818.9 ± 81.71	0.059 ± 0.019	0.998	51.4 ± 5.09	0.155 ± 0.003	0.991	557.0 ± 55.52	0.062 ± 0.014	0.997	103.7 ± 10.26	0.102 ± 0.004	0.989				
55.0	5.0	818.9 ± 81.84	0.254 ± 0.083	0.999	51.4 ± 5.14	0.791 ± 0.016	1.000	557.0 ± 55.60	0.268 ± 0.060	0.998	103.7 ± 10.28	0.498 ± 0.020	0.991				
55.0	6.5	818.9 ± 81.84	0.449 ± 0.147	0.999	51.4 ± 5.14	6.688 ± 0.137	1.000	557.0 ± 55.63	0.481 ± 0.107	0.999	103.7 ± 10.37	0.843 ± 0.035	1.000				
55.0	8.0	818.9 ± 81.82	0.699 ± 0.229	0.999	51.4 ± 5.14	12.894 ± 0.265	1.000	557.0 ± 55.66	0.740 ± 0.165	0.999	103.7 ± 10.37	4.784 ± 0.198	1.000				
70.0	2.0	818.9 ± 67.27	0.062 ± 0.017	0.821	51.4 ± 5.11	0.393 ± 0.008	0.994	557.0 ± 46.19	0.068 ± 0.013	0.829	103.7 ± 9.97	0.194 ± 0.008	0.961				
70.0	3.5	818.9 ± 81.55	0.237 ± 0.077	0.996	51.4 ± 5.14	0.922 ± 0.019	1.000	557.0 ± 55.56	0.253 ± 0.056	0.997	103.7 ± 10.34	0.572 ± 0.024	0.997				
70.0	5.0	818.9 ± 81.85	0.561 ± 0.184	0.999	51.4 ± 5.14	6.271 ± 0.129	1.000	557.0 ± 55.62	0.592 ± 0.132	0.999	103.7 ± 10.37	1.591 ± 0.066	1.000				
70.0	6.5	818.9 ± 81.89	0.927 ± 0.304	1.000	51.4 ± 5.14	5.635 ± 0.116	1.000	557.0 ± 55.52	0.836 ± 0.186	0.997	103.7 ± 10.37	4.967 ± 0.206	1.000				
70.0	8.0	818.9 ± 81.90	1.288 ± 0.422	1.000	51.4 ± 5.14	4.943 ± 0.102	1.000	557.0 ± 55.70	1.517 ± 0.338	1.000	103.7 ± 10.37	4.285 ± 0.178	1.000				

case, the *pH* effect follows a positive exponential relation as described by Eq. (3). Therefore, the general solution presented in Eq. (4) is reduced to a simpler solution in which only the kinetic parameter *r* is assuming the effects caused by the variables. In conclusion, the global multivariable model that controls the effect of *t*, *pH* and *T* on the stability in aqueous system can be established by substituting the *r* parameter of Eq. (1) with the equations leading the effect of the variables *T* (Eq. (2)) and the *pH* (Eq. (3)), as follows:

$$e(t, pH, T) = k \exp\left(-p \exp\left(-\frac{Ea}{RT} + bpH\right)t\right) \quad (5)$$

When substituting the *r* parameter by the multiplicative results of Eqs. (2) and (3), the new resulting expression share a pre-exponential factor (*A* and *s*) and their use in conjunction will be redundant therefore, a new factor is described and noted as *p*. All other parametric notations are as defined in the material and methods section. Section B of Figs. 2 and 3 show the global multivariable fitting results of applying Eq. (5) to describe the full multivariable data.

The parametric results for the different multivariable analysis are:

- The resulting parameters of the total anthocyanin content analysis were $k = 819.00 \pm 56.1 \mu\text{g/g R}$, $p = 59.32 \pm 11.2$, $Ea = 4.35 \times 10^{+9} \pm 0.98 \times 10^{+9} \text{ kJ}$ and $b = 10.05 \pm 3.5$ obtaining a R^2 values of 0.9577.
- For C1 were $k = 50.16 \pm 4.3 \mu\text{g/g R}$, $p = 57.10 \pm 9.7$, $Ea = 4.35 \times 10^{+9} \pm 1.21 \times 10^{+9} \text{ kJ}$ and $b = 8.91 \pm 2.3$ obtaining a R^2 values of 0.9396.
- For C2 were $k = 527.35 \pm 116.4 \mu\text{g/g R}$, $p = 59.38 \pm 7.8$, $Ea = 4.35 \times 10^{+9} \pm 0.46 \times 10^{+9} \text{ kJ}$ and $b = 10.23 \pm 3.2$ obtaining a R^2 values of 0.9590.
- For C3 were $k = 101.20 \pm 31.4 \mu\text{g/g R}$, $p = 57.95 \pm 7.6$, $Ea = 4.35 \times 10^{+9} \pm 0.44 \times 10^{+9} \text{ kJ}$ and $b = 9.42 \pm 3.5$ obtaining a R^2 values of 0.9534.

In all cases the statistical description was significant and the prediction of the anthocyanin content stability in solution by Eq. (5) showed highly consistent R^2 values. The multivariable analysis of the kinetic parameter *r* as a function of their respective affecting variables (*pH* and *T*) presented in the subsection B of Figs. 2 and 3, show the 3D graphical surface response for the degradation rate of the total anthocyanins content and for the individual content of the identified anthocyanin compounds (C1, C2 and C3). The conclusions are in accordance with previous results reported (Komatsu et al., 2014; Li et al., 2012; Li et al., 2011), revealing that at $T < 20^\circ\text{C}$ and $pH < 3.5$ the extract is more stable lowering as much as possible the degradation rate of anthocyanin compounds.

In the aqueous solution system, it proved to be highly dependent on the three variables studied. Compounds decayed completely over a period of time at different *pH* and *T*. This phenomenon was also detected by other authors that studied the stability of catechin and derivatives from other matrices such as green tea and cacao (Komatsu et al., 2014; Li et al., 2012; Zhu et al., 2002; Zhu et al., 1997). In similar terms with other authors (Li et al., 2012), results indicated that anthocyanin extracts in aqueous solution remained stable at a *pH* value lower than 3.5 and temperatures below 30°C for a period of 72 h. Even at high thermal process conditions (70°C) with a *pH* lower than 3.5, the anthocyanins content is detected during 1 h period without great losses. These results may limit the anthocyanin extract direct application, favouring acid foods as some chesses, fruit juice, vegetable/fruit products, mayonnaises and yogurts.

The key issue lies down behind the fact that anthocyanins undergo kinetic degradation during thermal processing and that the increase of the *pH* causes molecular changes in detriment of its colour properties (Komatsu et al., 2014; Ruiz-Rodríguez et al., 2011). The analytical solution of such a system through mathematical models is important and necessary, but not exempt of complexity due to the heterogeneous

responses of the variables involved. Achieving a successful mathematical model solution would allow to control most factors that affect the system, helping to standardize the key variables for producing stable plant-based extracts and therefore, to optimize the complete extraction process.

The analysis of the anthocyanin compounds stability from the strawberry tree fruits is crucial for predicting the shelf life behaviour of the compounds in various processing situations. In fact, food processing or other processes are factors that affect directly on the integrity of the molecules. Controlling the conditions of *t*, *pH* and *T* among others are essential aspects for keeping the process efficiency and for obtaining high quality products. Mathematical models were developed and multiple graphical plots were conducted to establish and illustrate the optimum values of the independent variables studied. Thus, the kinetic models could be used for calculating shelf-life and predicting compounds stability at given *pH* and *T* conditions for aqueous systems. The optimal stability conditions for anthocyanins content in aqueous solution remained intact at $pH < 3.5$ and $T < 30^\circ\text{C}$ for a period of at least 72 h. Moreover, using the optimal processing conditions, it is possible to produce functional extracts with high potential as nutraceuticals or as active ingredients in the design of functional foods, which can be also extended to other industrial fields such as pharmaceutical and cosmeceutical industries.

3.3. Incorporation of the anthocyanins rich extract as a natural colorant additive in wafers: Colour parameters, *pH*, nutritional composition and antioxidant activity of the samples along storage time

The bakery industry represents an important economic sector and is currently in great growth and constant innovation. Wafers are well-known cakes and consumed worldwide. Thus, their preparation using natural colorants instead of artificial ones, is a representative case study for the bakery sector. However, it is important to evaluate the colour changes caused in the food matrix within the storage time, as well as the effects in other parameters such as *pH*, nutritional composition and the preservative function, evaluated using antioxidant activity assays.

Part A of Table 3 shows the result values obtained for each of the evaluated colour parameter (L^* , a^* and b^*) in each one of the food samples during the storage period (6 days). The results presented in Table 3 (part A) demonstrate that the incorporation of *A. unedo* extract rich in cyanidin-3-O-glucoside caused slight changes in the wafers when compared to the control wafer, along the different storage time. In visual terms, the *A. unedo* extract provides a slightly golden colour to the wafer (Fig. 1, sub-Fig. 3.a) when compared to the control wafer (Fig. 1, sub-Fig. 3.b). These results are in accordance with those produced by other authors (Debonne, Van Bockstaele, Phillips, De Leyn, & Eeckhout, 2017), when evaluating the colorimetric parameters in pastry and baking products, revealing that it is necessary to consider that the colour of the crust is directly related to the temperature and cooking time and not entirely related to the products used.

- Parts B, C, D and E of Table 3 show the *pH* values, macronutrients content, individual sugar contents and energy of the control wafers and of the wafers with the *A. unedo* extract rich in cyanidin-3-O-glucoside during the self-life period of the product. As a bakery product which has been baked with temperature, moisture values (Table 3) were expected to be quite low. It is possible to verify that the carbohydrates are the most abundant macronutrients in wafers. The results show that the incorporation of the extract did not cause significant changes in relation to the control sample, for all the studied storage times. Three individual sugars were detected in the samples: fructose, glucose and sucrose (Fig. A1), with a high prevalence of the disaccharide. Significant differences were detected only for fructose and glucose after 3 and 6 days of storage, whereas the wafers incorporated with the extract always showed higher amounts of these sugars when compared to the control wafers. This

Table 3
Results of colour parameters, pH, nutritional composition, free sugars, fatty acids and antioxidant activity of wafer samples.

Parameters	0 Days			3 Days			6 Days		
	Control	AU	t-test	Control	AU	t-test	Control	AU	t-test
A) Colour parameters									
L^*	52.6 ± 0.6	52 ± 1	0.91	50.0 ± 0.4	54 ± 2	0.191	50.5 ± 0.4	54 ± 2	0.341
a^*	18.1 ± 0.5	16 ± 1	0.168	19.5 ± 0.1	15.3 ± 0.5	0.005	18.2 ± 0.5	15.6 ± 0.4	0.02
b^*	36.8 ± 0.7	35 ± 1	0.149	36.1 ± 0.6	38 ± 1	0.114	35.1 ± 0.5	36 ± 1	0.533
B) pH value									
pH	5.64 ± 0.09	5.3 ± 0.1	0.009	5.58 ± 0.04	5.7 ± 0.3	0.329	5.48 ± 0.06	5.9 ± 0.5	0.185
C) Nutritional composition									
Moisture (g/100 g fw)	11.42 ± 0.08	11.25 ± 0.09	0.058	11.5 ± 0.2	11.9 ± 0.1	0.054	11.4 ± 0.2	10.9 ± 0.1	0.018
Ash (g/100 g fw)	0.018 ± 0.01	0.018 ± 0.01	0.682	0.020 ± 0.01	0.017 ± 0.01	0.205	0.017 ± 0.01	0.018 ± 0.01	0.396
Fat (g/100 g fw)	21.7 ± 0.5	22.5 ± 0.1	0.044	22.6 ± 0.7	21.5 ± 0.9	0.17	22.4 ± 0.2	21.7 ± 0.2	0.026
Proteins (g/100 g fw)	9.6 ± 0.3	9.48 ± 0.09	0.388	8.47 ± 0.07	9.0 ± 0.2	0.005	9.45 ± 0.5	9.0 ± 0.1	0.096
Total sugars (g/100 g fw)	57.3 ± 0.4	56.8 ± 0.1	0.123	57.4 ± 0.6	57.6 ± 0.8	0.764	56.8 ± 0.4	58.3 ± 0.2	0.002
Energy (kcal/100 g fw)	463 ± 2	467 ± 1	0.027	467 ± 3	460 ± 4	0.099	466 ± 1	465 ± 1	0.203
D) Free sugars									
Fructose (g/100 g fw)	0.32 ± 0.01	0.64 ± 0.09	0.001	0.35 ± 0.01	0.76 ± 0.003	< 0.001	0.33 ± 0.01	0.69 ± 0.001	< 0.001
Glucose (g/100 g fw)	0.38 ± 0.01	0.51 ± 0.06	0.005	0.39 ± 0.02	0.58 ± 0.004	< 0.001	0.31 ± 0.02	0.53 ± 0.003	< 0.001
Sucrose (g/100 g fw)	28.1 ± 0.2	28.3 ± 0.9	0.702	28.4 ± 0.4	29.2 ± 0.7	0.06	28.17 ± 0.07	30.0 ± 0.7	0.004
Total (g/100 g fw)	28.8 ± 0.2	29 ± 1	0.216	29.1 ± 0.4	30.6 ± 0.7	0.011	28.82 ± 0.05	31.2 ± 0.7	0.001
E) Fatty acids									
C12:0 (%)	3.3 ± 0.1	3.22 ± 0.09	0.332	3.1 ± 0.2	3.21 ± 0.07	0.234	3.28 ± 0.08	3.3 ± 0.1	0.728
C14:0 (%)	1.65 ± 0.03	1.65 ± 0.02	0.898	1.59 ± 0.06	1.63 ± 0.03	0.322	1.68 ± 0.03	1.67 ± 0.03	0.851
C16:0 (%)	30.8 ± 0.4	30.8 ± 0.4	0.942	29.8 ± 0.3	30.5 ± 0.5	0.073	31.4 ± 0.4	31.4 ± 0.5	0.839
C18:0 (%)	30.9 ± 0.3	31.6 ± 0.1	0.042	30.8 ± 0.5	31 ± 1	0.736	29.9 ± 0.5	31.0 ± 0.5	0.055
C18:2n6 (%)	30.4 ± 0.2	29.7 ± 0.3	0.036	31.9 ± 0.5	31.3 ± 0.4	0.121	30.9 ± 0.2	29.8 ± 0.4	0.021
C18:3n3 (%)	1.28 ± 0.04	1.37 ± 0.02	0.053	1.30 ± 0.03	1.25 ± 0.09	0.459	1.376 ± 0.01	1.30 ± 0.09	0.236
SFA (%)	67.9 ± 0.2	68.5 ± 0.3	0.046	66.4 ± 0.5	67.1 ± 0.4	0.138	67.4 ± 0.2	68.5 ± 0.5	0.03
MUFA (%)	0.34 ± 0.03	0.38 ± 0.01	0.162	0.35 ± 0.01	0.39 ± 0.01	0.024	0.34 ± 0.04	0.38 ± 0.01	0.19
PUFA (%)	31.7 ± 0.2	31.1 ± 0.3	0.041	33.2 ± 0.5	32.5 ± 0.4	0.125	32.2 ± 0.2	31.1 ± 0.5	0.027
F) Antioxidant activity									
DPPH activity (mg/mL)	> 200	43.3 ± 0.6	< 0.001	> 200	43.5 ± 0.5	< 0.001	> 200	57 ± 1	< 0.001
Reducing power (mg/mL)	15.0 ± 0.4	14 ± 0.1	< 0.001	21.7 ± 0.8	14.42 ± 0.20	< 0.001	23.9 ± 0.3	15.9 ± 0.4	< 0.001

Control- wafer without extract and AU- wafers with *A. unedo* extract rich in cyanidin-3-*O*-glucoside. In each line and for each storage time a Student's *t*-test was used to determine the significant difference between two different samples, with $\alpha = 0.05$. L^* , a^* and b^* represent colour parameters. SFA- Saturated fatty acids; MUFA- Monounsaturated fatty acids; PUFA- Polyunsaturated fatty acids. Antioxidant activity was evaluated in terms of EC₅₀ values that correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay.

fact may be related with the presence of free sugars in the *A. unedo* extract.

- Table 3E shows the six most abundant fatty acids detected in wafer samples. Although fourteen fatty acids were identified in the studied samples (Fig. A1), eight were detected only in trace amounts (data not shown). The most abundant fatty acids were palmitic acid (C16:0), stearic acid (C18:0) and linoleic acid (C18:2n6). In this work, it was verified that the saturated fatty acids appear in higher quantity than the unsaturated counterparts, and the incorporation of the extract did not cause significant changes in the fatty acids profile when compared to the control wafers at any storage period. The amount of extract added was in a low amount and as such it would be expected that no changes are made to the fatty acid profile of the wafers, since the shelf life was very short and therefore over time there were also no changes.
- Finally, the antioxidant activity results (preservation potential of the extract rich in anthocyanins) based on the DPPH radical scavenging activity and reducing power of the wafers samples during the shelf-life period are presented in Table 3F. The incorporation of the extract obtained from *A. unedo* and rich in cyanidin-3-*O*-glucoside provides significantly beneficial properties to the tested food matrix as compared to the control wafers. This is in agreement with the results obtained in previous studies where chestnut flowers were incorporated into “económicos” cakes (Carocho et al., 2015), aqueous extracts of fennel and chamomile in cookies (Caleja, Barros, Antonio, Oliveira, & Ferreira, 2017) and in a study using a catechin-rich extract obtained from *A. unedo* incorporated into bread (Takwa

et al., 2018). In general, it was concluded that the incorporation of these natural ingredients introduces beneficial properties to the tested food products.

As demonstrated in other studies involving natural extracts with the aim of replacing artificial additives (Carocho et al., 2015; Caleja, et al., 2017), the key lies down behind the preservation of the beneficial properties of the extracts without altering the organoleptic characteristics of the original product. Therefore, as a function of the results presented in Table 3, the extract from *A. unedo* rich in cyanidin-3-*O*-glucoside is able to provide colorant properties with functional antioxidant properties without altering the main organoleptic characteristics of the food sample, demonstrating the potential of *A. unedo* fruit extracts for industrial applications.

4. Conclusions

There are no productive applications found at an industry level for the fruits of *Arbutus unedo* L. (Ericaceae family), because this plant is mainly used as an ornamental plant. Spontaneous growth by seedlings are found along the Mediterranean region easily. Most of the fruit production is discarded, because it only reaches a pleasant flavour during a short period of time and only minor traditional uses have been described (jams, wines and liqueurs). The edible reddish sweet fruit produced contains a diverse source of health promoting compounds, such as tocopherols, carbohydrates, sugars, and phenolic compounds. Additionally, given the evidence that the fruits contain a good amount

of anthocyanins, and also that this raw material is easy to obtain in good quantities in the areas where they are abundant, it may be interesting to use them as a new source of anthocyanins, which contributes, favours and promotes the use of these compounds as a natural colorant. Therefore, valorising, producing added-value extract rich in anthocyanins and understanding the stability patterns of this compounds with interest for the food technology field, from this underused fruit, could be of interest for the industrial sector and research community.

The present results provide information for: i) potential industrial application of extracts from *A. unedo* fruits, as alternative source of anthocyanins to be used as natural colorant ingredients with bioactive properties; ii) shelf-life predictions of the extract rich in anthocyanins (mainly cyanidin-3-O-glucoside) at specific conditions of temperature and pH. Overall, the incorporation of the *A. unedo* extract gave a more attractive colour to the wafers and improved the antioxidant activity, without causing significant changes in the nutritional profile of the wafers.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2018.09.099>.

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Incorporation of natural colorants obtained from edible flowers in yogurts

Tânia C.S.P. Pires^{a,b}, Maria Inês Dias^a, Lillian Barros^a, João C.M. Barreira^a,
Celestino Santos-Buelga^b, Isabel C.F.R. Ferreira^{a,*}

^a Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal

^b Grupo de Investigación en Polifenoles (GIP-USAL), Facultad de Farmacia, Universidad de Salamanca, Campus Miguel de Unamuno s/n, 37007 Salamanca, Spain

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ABSTRACT

The substitution of artificial dyes by natural colouring agents is among the top concerns of food industry to fulfil current consuming trends, justifying the prospection of novel natural sources of these compounds. Herein, the hydrophilic extracts from rose, cornflower and dahlia were tested as potential substitutes to E163 (anthocyanin extract). Besides comparing the colouring capacity, the potential occurrence of changes in the chemical composition of yogurts (nutritional parameters, free sugars and fatty acids) was also assessed throughout storage (up to 7 days) and compared with a “blank” (free of any additive) yogurt formulation. In general, yogurts prepared with flower extracts, presented similar nutritional value and free sugars profile to those prepared with E163 and to the “blank” yogurt. Nevertheless, rose extract turned out to be the most suitable alternative to E163 as these two groups of yogurts had similar nutritional composition, free sugars and fatty acids composition, besides presenting close scores in colour parameters.

1. Introduction

Fermented milk is a dairy product processed by lactic fermentation, which ends up by coagulating milk casein due to the acidification process (pH values around 4.6). Among different fermented dairy products, yogurt is certainly one of the most popular, being widely consumed all over the world due to its organoleptic and nutritional properties (Arioui, Ait Saada, & Cheriguene, 2017; Caleja et al., 2016).

Some yogurt formulations are prepared using specific additives, such as exemplified by colorants. However, the recent concerns about the safety of artificial colorants in food products, has encouraged the development and application of natural colorants, which are generally considered safer than artificial ones (Pop, Lupea, Popa, & Gruescu, 2010). Anthocyanins are authorised food colorants (E163 in EU) and have previously been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1982 and by the EU Scientific Committee for Food (SCF) in 1975 and 1997 (Pop et al., 2010; Rodríguez-Amaya, 2016). Anthocyanins are water-soluble pigments isolated from plants, being responsible for the blue, purple, and red colour of many plant tissues. These phenolic compounds are widely found in fruits (especially berries), as well as flowers and leaves, mainly linked to sugar units. Their sugar-free counterparts (anthocyanidins) are based on the flavylum cation, which might present different substitution patterns originating the diversity of anthocyanidins found in

nature (Hidalgo & Almajano, 2017). Among the 17 natural anthocyanidins, cyanidin, delphinidin, petunidin, peonidin, pelargonidin and malvidin, are the major forms in most species (Hidalgo & Almajano, 2017).

In what concerns the application of anthocyanins in food products, there are some previous reports describing the incorporation of rose (*Rosa damascena*) petals extracts in yogurt (e.g., Chanukya & Rastogi, 2016). Owing to the previously evidenced suitability of *R. damascena* as a colour ring agent in yogurt, we selected that species as one of the plant sources of anthocyanins to be incorporated in the yogurt formulations prepared in the lab. Likewise, we selected the flowers of *Centaurea cyanus* L. (cornflower), mainly due to its richness in cyanidin 3-O-(6-O-succinylglucoside)-5-O-glucoside (Takeda et al., 2005), but also in other bioactive phenolic compounds such as apigenin-glucuronide (Pires, Dias, Barros, & Ferreira, 2017) and *Dahlia mignon* (dahlia), which also presents a rich composition in different phenolic compounds like naringenin-3-O-glucoside, kaempferol-pentosyl-rhamnosyl-hexoside or apigenin-hexoside (Deguchi, Ohno, Hosokawa, Tatsuzawa, & Doi, 2013; Pires et al., 2018).

The selection and purchase of food products are greatly influenced by sensory expectations (Spence, Levitan, Shankar, & Zampini, 2010). Visual perception deliver so called quality cues, perceived prior to actual consumption and give hints of the quality attributes that are apparent during the consumption (Jantathai, Sungsi-in, Mukprasirt, &

* Corresponding author.

E-mail address: iferreira@ipb.pt (I.C.F.R. Ferreira).

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Duerrschmid, 2014; Spence et al., 2010). Colour plays an important role in the development of food preferences and sensory perception (Jantathai et al., 2014).

Nevertheless, colour is not important only in what concerns the product appearance. In fact, some colouring agents may have important functions beyond their primary effect. Anthocyanins, for instance, might have beneficial health effects due to their antioxidant, anti-inflammatory, anticancer, and anti-diabetic properties, thereby being of great interest to the food industry (Rodríguez-Amaya, 2016). However, it is also necessary to take into account that anthocyanins might degrade or react in food systems to form complex reaction products, leading to a mixture of products in addition to the parent anthocyanins (Rodríguez-Amaya, 2016). The intensity and stability of anthocyanins when used as food additives are influenced by pH, structure, concentration, co-pigmentation and metal complexing, as well as temperature, light, oxygen, acetaldehyde, ascorbic acid, sugars and their degradation products, sulphur dioxide, amino acids and catechins. Still, when low pH conditions are maintained, anthocyanins are relatively stable (EFSA, 2013; Rodríguez-Amaya, 2016).

Accordingly, the aim of the present study was to develop a new colouring strategy in yogurt products using natural anthocyanin rich extracts obtained from edible flower petals of *Dalia mignon*, *Centaurea cyanus* L. and *Rosa damascena* “Alexandria” mixed with *Rosa gallica* “French” draft in *Rosa canina*. These flowers were firstly characterized and quantified regarding the anthocyanin content, through an HPLC-DAD-ESI/MS system. Additionally, the chromatic stability was evaluated by performing the evaluation studies (nutritional parameters, free sugars, fatty acids, anthocyanin content, and colour parameter) in yogurt formulation at two different periods (preparation day and after 7 days of storage).

2. Materials and methods

2.1. Samples

Dried commercial samples of petals of *Dahlia mignon*, rose resulting from *R. damascena* 'Alexandria' and *R. gallica* 'Francesca' draft in *R. canina*, and *Centaurea cyanus* L. were provided by RBR foods (Castro D'aire, Portugal).

In order to prepare the extracts, samples were reduced to powder (20 mesh) and were extracted by maceration (25 °C, 150 rpm, 1 h) using a stirring plate (VELP scientific, Keyland Court, NY, USA) by adding 1 g of dry material to 50 mL of distilled water. Afterwards, the mixture was filtered through Whatman filter paper No. 4, frozen and lyophilized. The lyophilized extracts obtained were used as natural additives.

2.2. Anthocyanin compounds identification by HPLC-DAD-ESI/MS

The chromatographic data of anthocyanin compounds were acquired from a Dionex Ultimate 3000 system (Thermo Scientific, San Jose, CA, USA), coupled to diode array, using 520 nm as preference wavelength, and to a mass spectrometer (MS, Linear Ion Trap LTQ XL mass spectrometer, Thermo Finnigan, San Jose, CA, USA) operating in the positive mode (Gonçalves et al., 2017). Retention times, UV–Vis and mass spectra were compared with available standards and with literature data to identify the anthocyanin's. Calibration curves of the available anthocyanin standards were constructed based on the UV signal to perform quantitative analysis, in case of an unavailable commercial standards, the compounds were quantified via the calibration curves of the most similar available standards. The results were expressed as µg/g of dry extract.

2.3. Fortification of yogurts with natural and commercial colorant additives

2.3.1. Incorporation process

The base formulation yogurts (fat 3.8%; protein 5.0% and

carbohydrates 4.7%) were purchased at the local market. Five groups (three samples/group) of yogurts (70 g each) were prepared, with three replicates of each: i) control samples (BY); ii) yogurts with commercial colorant, E 163 (AY); iii) yogurts with rose petals extract (RY); iv) yogurts with *Centaurea cyanus* L. petals extract (CY); v) yogurts with *Dahlia mignon* petals extract (DY). All colorants were added to a portion of 70 g of yogurt and were prepared in duplicate. The E163 colorant was added at a 0.02% concentration; in the case of yogurts added with petals extracts, slightly higher concentrations of each extract (0.05% for dahlia extract; 0.15% for rose extract; 0.10% for centaurea extract) were added (the quantity was added until an evident change in colour was obtained).

2.3.2. Nutritional and chemical composition

The proximate composition was determined according to AOAC procedures (AOAC, 2016), including protein (991.02), crude fat (989.05) and ash (935.42) contents. Crude protein (N × 6.25) was determined by the Kjeldahl method; ash content was estimated by subjecting the sample to incineration at 600 ± 15 °C for 5 h, while crude fat was determined using a Soxhlet apparatus with petroleum ether as recycling solvent and total carbohydrate was estimated by difference. The total energy was calculated using the following equation: Energy (kcal) = 4 × (g protein + g carbohydrates) + 9 × (g fat).

Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI; Knauer, Smartline system 1000, Berlin, Germany), using melezitose as an internal standard. All the mentioned procedures were previously described by the authors (Barros, Pereira, & Ferreira, 2013; Dias et al., 2015).

The fatty acids were determined by gas chromatography coupled with a flame ionization detector (GC-FID/capillary column, DANI model GC 1000, Contone, Switzerland), a split/splitless injector and a Macherey–Nagel column. The identification of fatty acids was performed by comparing the relative retention times of FAME peaks from samples with commercial standards (Barros et al., 2013; Dias et al., 2015).

Anthocyanins were determined in the yogurt sample by extracting 3 g of dry yogurt with water at 25 °C, 150 rpm during 1 h, followed by filtration through a Whatman filter paper No. 4. The remaining residue was re-extracted with an additional portion of water mixture, stored at –20 °C and lyophilized for further analysis. The lyophilized extracts were analysed using the HPLC-DAD-ESI/MS system mentioned above.

2.4. Physico-chemical parameters

The colour was measured in triplicate for each sample using a colorimeter (model CR-400, Konica Minolta Sensing Inc., Tokyo, Japan). The CIE L^* , a^* and b^* colour space values were registered using a data software “Spectra Magic Nx” (version CM-S100W 2.03.0006), using the illuminant C and diaphragm aperture of 8 mm (Fernandes et al., 2012). The pH values of the samples was measured directly with a HI 99161 pH-meter (Hanna Instruments, Woonsocket, Rhode Island, USA).

2.5. Statistical analysis

All statistical tests were performed at a 5% significance level using IBM SPSS Statistics for Windows, version 22.0. (IBM Corp., Armonk, NY, USA).

Data were expressed as mean ± standard deviation, maintaining the significant numbers allowed by the magnitude of the corresponding standard deviation.

An analysis of variance (ANOVA) with type III sums of squares was performed using the general linear model (GLM) procedure to compare the parameters evaluated in the prepared yogurts. The dependent variables were analysed using 2-way ANOVA with the factors “yogurt formulation” (YF) and “storage” (SE). When a statistically significant

interaction was detected among the two factors, their effect was evaluated by checking estimated marginal means plots for all levels of each factor. On the contrary, if no statistical significant interaction was found, means were compared using Tukey's multiple comparison test, after verifying the homogeneity of variances through Levene's test.

In addition, a linear discriminant analysis (LDA) was used to have a better understanding about the YF overall effect. A stepwise technique was applied, considering the Wilks' λ test with the usual probabilities of F (3.84 to enter and 2.71 to be removed) for variable selection. Only variables with a statistically significant classification performance ($p < 0.050$) were maintained by the statistical model. The significant independent variables were selected following the stepwise method of LDA. This procedure is based in sequential forward selection and backward elimination steps, where the inclusion of a new variable requires verifying the significance of all previously selected variables (Zielinski et al., 2014). The main purpose was estimating the relationship between the single categorical dependent variables (yogurt formulations) and the quantitative independent variables (results obtained in the laboratorial assays). The LDA outputs allowed determining which independent variables contributed more to the differences in the average score profiles of different YF. A leaving-one-out cross validation procedure was carried out to assess the model performance.

3. Results and discussion

3.1. Anthocyanin profile characterization

Owing to the powerful colouring capacity of anthocyanins, these compounds were thoroughly characterized in the extracts obtained from the petals of each selected species. The extraction yields (mg of anthocyanin per 100 g of petals) obtained for each sample extract were: ~53% for dahlia; ~46% for rose; and ~23% for centaurea samples.

Nine anthocyanin compounds were detected in dahlia, two in rose and eight in centaurea extracts. Peak characteristics, tentative identification and compound quantification are presented in Table 1. Cyanidin (Cy; peaks 1, 2, 3, 4, 6, 10, 11, 13, 15, and 17), pelargonidin (Pg;

peaks 8, 9, 12, 16, and 18), and delphinidin (Dp; peaks 5 and 7) were identified as main aglycones, based on the observation of their characteristic fragments in MS² spectra. As reviewed by Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, and Galán-Vidal (2009), these non-methylated anthocyanidins are the most commonly found in flowers, being cyanidin derivatives the most abundant in the analysed samples.

The conjugated bonds of anthocyanins, the glycosylated form of anthocyanidins, result in red, blue, and purple-coloured plants (Khoo, Azlan, Tang, & Lim, 2017). Several foods, like yoghurt, are considered healthy, but they lack important components such as phenolic compounds. Therefore, the incorporation of plant extracts rich in anthocyanins in these fermented products might impart a desirable red colour, while enhancing their potential health effect (Mourtzinis et al., 2018).

Before incorporating the flower extracts in yogurts, their profiles in anthocyanins were thoroughly characterized. Peak 1, detected in rose and centaurea samples, was positively identified as cyanidin 3,5-di-O-glucoside based on the HPLC-DAD-MS results and comparison with our database library. This compound was already described as the main anthocyanin in petals of *R. damascena* (Velioglu & Mazza, 1991) and *R. hybrida* (Lee, Lee, & Choung, 2011) used with edible purposes, as well as in flowers from different *Centaurea* species (Mishio, Takeda, & Iwashina, 2015), highlighting its suitability to be incorporated in yogurt formulations. Peak 2, found in rose samples, was also positively identified as cyanidin-3-O-glucoside according with its retention time and mass spectral data by comparison with a standard. The presence of this reddish-purple anthocyanin was also reported in rose hips (*R. canina*) previously Hvattum (2002).

Peak 4 ([M]⁺ at m/z 711) was the majority anthocyanin in centaurea samples. Its MS² spectra yielded fragments at m/z 549 (–162 mu, loss of a hexose), 449 (–262 mu, loss of succinylhexose) and 287 (cyanidin), coherent with an identity as Cy-3-O-(6"-succinylglucoside)-5-O-glucoside, a compound consistently identified in centaurea flowers also referred to as centaurocyanin (Mishio et al., 2015; Kōsaku, Takeda & Tominaga, 1983), and whose combination with a flavone glycoside

Table 1

Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{\max}), mass spectral data, tentative identification, and quantification of anthocyanins in dahlia, rose, and centaurea extracts. Results are presented as mean \pm standard deviation.

Peak	Rt (min)	λ_{\max} (nm)	Molecular ion (m/z)	MS ² (m/z)	Tentative identification	Quantification ($\mu\text{g/g}$ extract)
Rose						
1	11.5	514	611	449(10),287(100)	Cyanidin 3,5-di-O-glucoside ^A	13.19 \pm 0.01
2	18.5	516	449	287 (100)	Cyanidin-3-O-glucoside ^A	0.131 \pm 0.004
Total Anthocyanins						13.326 \pm 0.002
Centaurea						
1	11.7	512	611	449(5),287(100)	Cyanidin 3,5-di-O-glucoside ^A	5.5 \pm 0.2
3	18.03	516	697	535(62),449(8),287(46)	Cyanidin 3-O-(6"-malonylglucoside)-5-O-glucoside ^A	6.2 \pm 0.3
4	20.38	516	711	549(3),449(48),287(100)	Cyanidin 3-O-(6"-succinylglucoside)-5-O-glucoside ^A	11.2 \pm 0.5
5	29.6	518	465	303 (100)	Delphinidin-hexoside ^C	1.5 \pm 0.2
6	31.5	518	463	287 (100)	Cyanidin-glucuronide ^A	0.85 \pm 0.06
7	32.6	518	561	303 (100)	Delphinidin-malonylhexoside ^C	tr
8	38.1	501	695	609(9),433(2),271(82)	Pelargonidin 3-O-(6"-succinylglucoside)-5-O-glucoside ^B	0.18 \pm 0.01
9	39.2	502	519	271 (100)	Pelargonidin-malonylhexoside ^B	0.17 \pm 0.01
Total Anthocyanins						26 \pm 1
Dahlia						
10	11.6	516	449	287 (100)	Cyanidin-hexoside ^A	2.98 \pm 0.01
11	13.4	504	449	287 (100)	Cyanidin-hexoside ^A	2.654 \pm 0.001
12	15.1	514	579	271 (100)	Pelargonidin-rutinoside ^B	1.4 \pm 0.1
13	17.2	514	491	287 (100)	Cyanidin-acetylhexoside ^A	5.36 \pm 0.01
14	19.4	501	431	269 (100)	Methylapigeninidin-hexoside ^A	4.1 \pm 0.1
15	20.8	518	595	287 (100)	Cyanidin-rutinoside ^A	0.8 \pm 0.1
16	28.5	504	595	271 (100)	Pelargonidin 3,5-di-O-glucoside ^B	0.8 \pm 0.1
17	31.5	518	491	287 (100)	Cyanidin-acetylhexoside ^A	0.33 \pm 0.02
18	32.7	516	433	271 (100)	Pelargonidin-hexoside ^B	0.450 \pm 0.001
Total Anthocyanins						18.8 \pm 0.2

tr-trace amounts; Standard calibration curves: A – cyanidin-3-O-glucoside ($y = 243287x - 1E + 06$; $R^2 = 0.995$); B – pelargonidin-3-O-glucoside ($y = 276117x - 480418$; $R^2 = 0.9979$); C– delphinidin-3-O-glucoside ($y = 557274x + 126.24$; $R^2 = 0.997$).

Table 2

Nutritional composition (g/100 g fresh weight) and energy values (kcal/100 g fresh weight) for different yogurt formulations (YF) and storage effect (SE). Results are presented as mean \pm standard deviation.^a

		Water	Fat	Protein	Ash	Carbohydrates	Galactose	Lactose	Energy
YF	BY	85.0 \pm 0.4	3.3 \pm 0.1	5.3 \pm 0.3	0.79 \pm 0.03	5.6 \pm 0.1	0.69 \pm 0.01 ^c	4.7 \pm 0.1	73 \pm 2
	RY	84.8 \pm 0.4	3.3 \pm 0.2	5.3 \pm 0.2	0.85 \pm 0.01	5.8 \pm 0.2	0.71 \pm 0.04 ^{bc}	4.7 \pm 0.2	74 \pm 3
	DY	85.0 \pm 0.1	3.4 \pm 0.1	5.4 \pm 0.1	0.86 \pm 0.02	5.4 \pm 0.1	0.71 \pm 0.01 ^b	4.8 \pm 0.1	73 \pm 1
	CY	84.8 \pm 0.1	3.2 \pm 0.1	5.4 \pm 0.1	0.86 \pm 0.02	5.7 \pm 0.1	0.76 \pm 0.02 ^a	4.8 \pm 0.1	74 \pm 1
	AY	84.9 \pm 0.2	3.4 \pm 0.1	5.3 \pm 0.1	0.82 \pm 0.02	5.5 \pm 0.1	0.72 \pm 0.02 ^b	4.9 \pm 0.1	74 \pm 1
	ANOVA <i>p</i> -value (n = 18) ^b	0.083	0.001	0.039	< 0.001	< 0.001	< 0.001	< 0.001	0.632
SE	0 days	85.0 \pm 0.2	3.3 \pm 0.1	5.3 \pm 0.2	0.84 \pm 0.04	5.6 \pm 0.2	0.73 \pm 0.03	4.8 \pm 0.1	73 \pm 1
	7 days	84.8 \pm 0.3	3.4 \pm 0.1	5.4 \pm 0.1	0.84 \pm 0.03	5.6 \pm 0.2	0.71 \pm 0.03	4.8 \pm 0.1	74 \pm 2
	ANOVA <i>p</i> -value (n = 45) ^c	0.056	0.061	0.119	0.763	0.258	0.100	0.408	0.081
YF \times SE	<i>p</i> -value (n = 90) ^d	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.272	< 0.001	< 0.001

^a Results are reported as mean values of each YF, aggregating results from 0 to 7 days, and mean values of SE, combining all YF.

^b If $p < 0.05$, the corresponding parameter presented a significantly different value for at least one YF.

^c If $p < 0.05$, the corresponding parameter presented a significant difference among stored and non-stored yogurts.

^d In this table, the interaction among factors was significant in all cases; thereby no multiple comparisons could be performed.

Table 3

Physicochemical parameters (CIE L^* , a^* and b^* and pH values) for different yogurt formulations (YF) and storage effect (SE). Results are presented as mean \pm standard deviation.^a

		L^*	a^*	b^*	pH
YF	BY	93 \pm 1	-3.5 \pm 0.1	9.8 \pm 0.4	4.3 \pm 0.1
	RY	88 \pm 1	2.2 \pm 0.1	9.0 \pm 0.3	4.3 \pm 0.1
	DY	84 \pm 1	2.1 \pm 0.3	17.7 \pm 0.4	4.4 \pm 0.1
	CY	90 \pm 1	-1.1 \pm 0.2	9.5 \pm 0.5	4.2 \pm 0.1
	AY	89 \pm 1	3.1 \pm 0.5	6.5 \pm 0.5	4.8 \pm 0.1
	ANOVA <i>p</i> -value (n = 18) ^b	< 0.001	< 0.001	< 0.001	< 0.001
SE	0 days	88 \pm 3	1 \pm 3	10 \pm 3	4.4 \pm 0.2
	7 days	89 \pm 3	0 \pm 2	11 \pm 3	4.4 \pm 0.2
	ANOVA <i>p</i> -value (n = 45) ^c	0.056	0.250	0.312	0.946
IF \times ST	<i>p</i> -value (n = 90) ^d	< 0.001	< 0.001	< 0.001	0.867

^a Results are reported as mean values of each YF, aggregating results from 0 to 7 days, and mean values of SE, combining all YF.

^b If $p < 0.05$, the corresponding parameter presented a significantly different value for at least one YF.

^c If $p < 0.05$, the corresponding parameter presented a significant difference among stored and non-stored yogurts.

^d In this table, the interaction among factors was significant in all cases; thereby no multiple comparisons could be performed.

and metal ions give rise to protocyanin, a stable complex pigment considered to be the main responsible for the blue colour of *Centaurea cyanus* flowers (Kosaku Takeda et al., 2005). This compound could have interesting colouring properties to be used as a natural additive in food products. Similarly, mass spectral characteristics of peak 3, with a molecular ion $[M]^+$ at m/z 697 and MS² fragments at m/z 535 (-162 mu, loss of a hexose), 449 (-248 mu, loss of malonylhexose) and 287 (cyanidin), allowed tentatively assigning it as Cy-3-O-(6''-malonylglucoside)-5-O-glucoside owing to its previous identification in flowers from different *Centaurea* species (Mishio et al., 2015). Peak 6 ($[M]^+$ at m/z 463) was another cyanidin derivative, tentatively identified as Cy-O-glucuronide based on the loss of 176 mu (a glucuronyl moiety) to yield the unique MS² product ion at m/z 287.

Peaks 8 and 9 in centaurea samples were associated to pelargonidin derivatives based on their characteristic absorption spectra showing λ_{max} at 501 nm and the fragment ion observed at m/z 271 (Pg). Peak 8 ($[M]^+$ at m/z 695), with similar fragmentation behaviour as peak 4, was identified as Pg-3-O-(6''-succinylglucoside)-5-O-glucoside, previously described in *Centaurea cyanus* flowers by Kosaku Takeda, Kumegawa, Harborne, and Self (1988). Peak 9 ($[M]^+$ at m/z 519) was tentatively assigned as a Pg-O-malonylhexoside based on the loss of 248

mu (malonylhexoside) to yield the aglycone ion at m/z 271. Pelargonidin differs from most anthocyanidins as it might provide an orange hue to flowers and red to some of the fruits and berries (Jaakola, 2013; Khoo et al., 2017), having also demonstrated a notable anti-inflammatory effect (Duarte et al., 2018). In a similar way, peak 7 ($[M]^+$ at m/z 561), yielding a unique MS² fragment at m/z 303 (-248 mu) was associated to delphinidin-O-malonylhexoside, whereas peak 5 ($[M]^+$ at m/z 465) was assigned as a Dp-O-hexoside; a possible identity as Dp-3-O-glucoside was excluded by comparison with peak characteristics with our database library. Delphinidin appears as a purple pigment in the nature, and the blue hue of flowers is often due this pigment, which was previously reported for its anti-inflammatory, anti-oxidant, and anti-tumorigenic activities (Ko et al., 2015), making it specially interesting as an ingredient of innovative food formulations (Khoo et al., 2017).

Similar reasoning was applied to identify anthocyanins in dahlia samples as cyanidin (peaks 10, 11, 13, 15 and 17) and pelargonidin derivatives (peaks 12, 16 and 18), which were previously reported in dahlia flowers (Deguchi et al., 2013; Kosaku, Takeda, Harborne, & Self, 1986; Yamaguchi et al., 1999). The presence of Pg-3,5-diglucoside in flowers of *Dahlia variabilis* was identified by Yamaguchi et al. (1999) and Deguchi et al. (2013), which could correspond to peak 16 ($[M]^+$ at m/z 595) in our samples. For the remaining compounds (peaks 10, 11, 12, 13, 15, 17, and 18), no conclusions about the precise identity of the anthocyanins could be obtained, and the glycoside moieties were assigned based on the mass losses observed in the MS² spectra, as hexosides (-162 mu), acetylhexosides (-204 mu) or deoxyhexosylhexosides (-308 mu). Curiously, none of the observed peak losses indicates the presence of malonylglucosides, a type of derivatives usually reported in dahlia flowers (Deguchi et al., 2013; Kosaku, Takeda et al., 1986; Yamaguchi et al., 1999). Cy-acetylhexoside (peak 13) was the most abundant compound in dahlia, representing the main responsible for the coloration of this edible flowers. In addition to improve the sensory characteristics of yogurt, that cyanidin might be a promising antiglycation agent for preventing or ameliorating AGEs-mediated diabetic complications (Suantawee, Cheng, & Adisakwattana, 2016). Finally, peak 14 presented a molecular ion $[M]^+$ at m/z 431 and a unique MS² fragment at m/z 269, which could match the mass of methylapigeninidin, so that it might be associated to a methylapigeninidin-hexoside, a pigment reported in red sorghum (Wu & Prior, 2005). Nevertheless, the absorption spectrum of peak 14 would not be coherent with such an identity, as the maximum absorption in the visible region of that compound should be expected around 470 nm (Awika, 2008; Yang, Dykes, & Awika, 2014). Thus, the identity of this peak as a 3-deoxyanthocyanin is uncertain, although in case it is confirmed it would be the first description of this type of pigments in dahlia flowers.

In general, these flowers have a great potential to be used as natural

Table 4
Fatty acids profile (relative percentage) of yogurt formulations (YF) and storage effect (SE). Results are presented as mean ± standard deviation.^a

	C4:0	C6:0	C8:0	C10:0	C12:0	C14:0	C15:0	C16:0	C16:1	C18:0	C18:1n9	C18:2n6	C18:3n3	SFA	MUFA	PUFA
YF																
BY	1.3 ± 0.2	1.7 ± 0.1	1.3 ± 0.1	2.9 ± 0.2	3.6 ± 0.1	11.9 ± 0.4	1.4 ± 0.1	35 ± 1	1.4 ± 0.1	11.0 ± 0.2	21 ± 1	2.3 ± 0.1	1.3 ± 0.1	71 ± 1	23 ± 2	5.0 ± 0.1
RY	0.8 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	2.8 ± 0.1	3.6 ± 0.1	12.2 ± 0.2	1.5 ± 0.1	36 ± 1	1.5 ± 0.1	11.5 ± 0.1	20 ± 1	2.2 ± 0.1	1.3 ± 0.1	72 ± 1	23 ± 1	4.6 ± 0.2
DY	1.0 ± 0.1	1.4 ± 0.1	1.1 ± 0.1	2.7 ± 0.1	3.5 ± 0.1	12.1 ± 0.1	1.5 ± 0.1	36 ± 1	1.5 ± 0.1	11.5 ± 0.2	21 ± 1	2.3 ± 0.1	1.5 ± 0.1	72 ± 1	23 ± 1	5.1 ± 0.1
CY	1.1 ± 0.1	1.5 ± 0.1	1.1 ± 0.1	2.7 ± 0.1	3.6 ± 0.1	12.0 ± 0.1	1.5 ± 0.1	36 ± 1	1.5 ± 0.1	11.5 ± 0.1	20 ± 1	2.5 ± 0.2	1.5 ± 0.1	72 ± 1	23 ± 1	5.3 ± 0.2
AY	1.2 ± 0.1	1.6 ± 0.1	1.2 ± 0.1	2.8 ± 0.1	3.7 ± 0.1	12.0 ± 0.3	1.5 ± 0.1	35 ± 1	1.4 ± 0.1	11.3 ± 0.1	21 ± 1	2.3 ± 0.1	1.4 ± 0.1	72 ± 1	23 ± 1	4.9 ± 0.2
ANOVA p-value (n = 18) ^b	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.002	< 0.001	< 0.001	< 0.001	< 0.001	0.133	< 0.001	< 0.001	< 0.001	0.125	< 0.001
SE																
0 days	1.1 ± 0.2	1.4 ± 0.1	1.1 ± 0.1	2.7 ± 0.1	3.5 ± 0.1	11.9 ± 0.3	1.5 ± 0.1	35 ± 1	1.5 ± 0.1	11.3 ± 0.3	21 ± 1	2.3 ± 0.1	1.5 ± 0.1	71 ± 1	23 ± 1	5.0 ± 0.2
7 days	1.0 ± 0.3	1.6 ± 0.1	1.2 ± 0.1	2.8 ± 0.1	3.6 ± 0.1	12.2 ± 0.2	1.5 ± 0.1	36 ± 2	1.5 ± 0.1	11.4 ± 0.1	20 ± 1	2.3 ± 0.1	1.4 ± 0.1	72 ± 1	22 ± 1	5.0 ± 0.1
t-student p-value (n = 45) ^c	0.018	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.737	0.001	0.984	0.213	< 0.001	0.063	< 0.001	< 0.001	< 0.001	0.532
IF × ST p-value (n = 90) ^d	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

^a Results are reported as mean values of each YF, aggregating results from 0 to 7 days, and mean values of SE, combining all YF.

^b If $p < 0.05$, the corresponding parameter presented a significantly different value for at least one YF.

^c If $p < 0.05$, the corresponding parameter presented a significant difference among stored and non-stored yogurts.

^d In this table, the interaction among factors was significant in all cases; thereby no multiple comparisons could be performed.

colorants, being an excellent source of anthocyanins to develop innovative products with new sensorial and bioactive characteristics.

3.2. Characterization of different fortified yogurts

Natural additives are generally considered as producing no harmful effects on consumers' health, contrarily to some artificial compounds (Carocho, Barreiro, Morales, & Ferreira, 2014). Nevertheless, the acceptability of these products is highly dependent on their appearance and rheological properties (Caleja et al., 2016; Santillán-Urquiza, Méndez-Rojas, & Vélez-Ruiz, 2017). In what concerns yogurt, similarly to several other food products, colour is a determining factor. Bearing this in mind, different plant species were selected as potential sources of colourants to be incorporated in yogurt. Yogurts prepared with different flower extracts were compared with each other and also with yogurts added with a commercial anthocyanin extract (E163, authorised by EFSA). In addition, a set of yogurts were used exactly as bought (free of any colouring agent), functioning as the “blank” yogurt control.

Besides comparing different yogurt formulations (YF), their stability throughout storage was also evaluated, specifically by performing the same evaluation assays on the preparation day and after 7 days of storage (SE).

Since the effect of each factor (YF or SE) might be affected by the second factor level (i.e., different storage effects according on each YF, or *vice versa*), the interaction (YF × SE) was also evaluated. In all cases where a significant interaction was found ($p < 0.050$), the multiple comparisons could not be performed. In those cases, the overall conclusions were obtained from the corresponding estimated marginal means (EMM) plots.

Starting by analysing the results for nutritional parameters (Table 2), a significant interaction among YF and SE (YF × SE) was found in all cases, thereby indicating that each YF reacted differently to storage. Considering each factor individually, YF-related differences were significant in most cases, except water and energy, while SE had no significant effect in any case. In either case, the nutritional profile is very similar among all tested samples, with water as the main component (≈ 85 g/100 g), followed by carbohydrates (slightly higher in RY and CY and lower in DY) and protein (a bit higher in CY and lower in RY), both corresponding to ≈ 5.5 g/100 g, fat (≈ 3.2 g/100 g in CY to 3.4 g/100 g DY and AY) and ash (< 0.9 g/100 g in all yogurts). This profile resulted in energy values around 74 kcal/100 g in all cases. Actually, owing to the low quantity of colorant added, it was not expectable to have differences of high magnitude among different YF, particularly in what concerns fat amounts (the flower extracts were prepared with water). Nevertheless, the plant species used in the extraction procedures had different nutritional composition (Pires et al., 2017), causing some minor changes in the corresponding yogurts. Even so, these results validate the maintenance of the nutritional quality of natural yogurt (herein identified as BY).

In what concerns individual sugars, lactose was the main compound (≈ 4.8 g/100 g, with slightly higher values in AY). Minor levels of galactose were also quantified, varying from the maximum values detected in CY (0.76 g/100 g) to the lowest in BY (0.69 g/100 g).

More significant differences were, as observable in Table 3, obtained in the case of colour parameters, which is in line with the main purpose of this work. Yogurts free of any additive (BY) showed the highest L^* values, followed by CY, AY, RY and DY. On the contrary, BY presented the lowest a^* values, while AY, RY and DY reached the highest (without significantly different values among them). On the other hand, the absence of significant differences for a^* values among AY, RY and DY, indicate that rose and dahlia extracts might be potential alternatives to E163.

Fatty acids profiles, especially for their potential usefulness as indicators of suitable conservation conditions, were also characterized (Barreira, Pereira, Oliveira, & Ferreira, 2010; Pereira et al., 2016). All fatty acids quantified in relative percentages above 1% are presented in

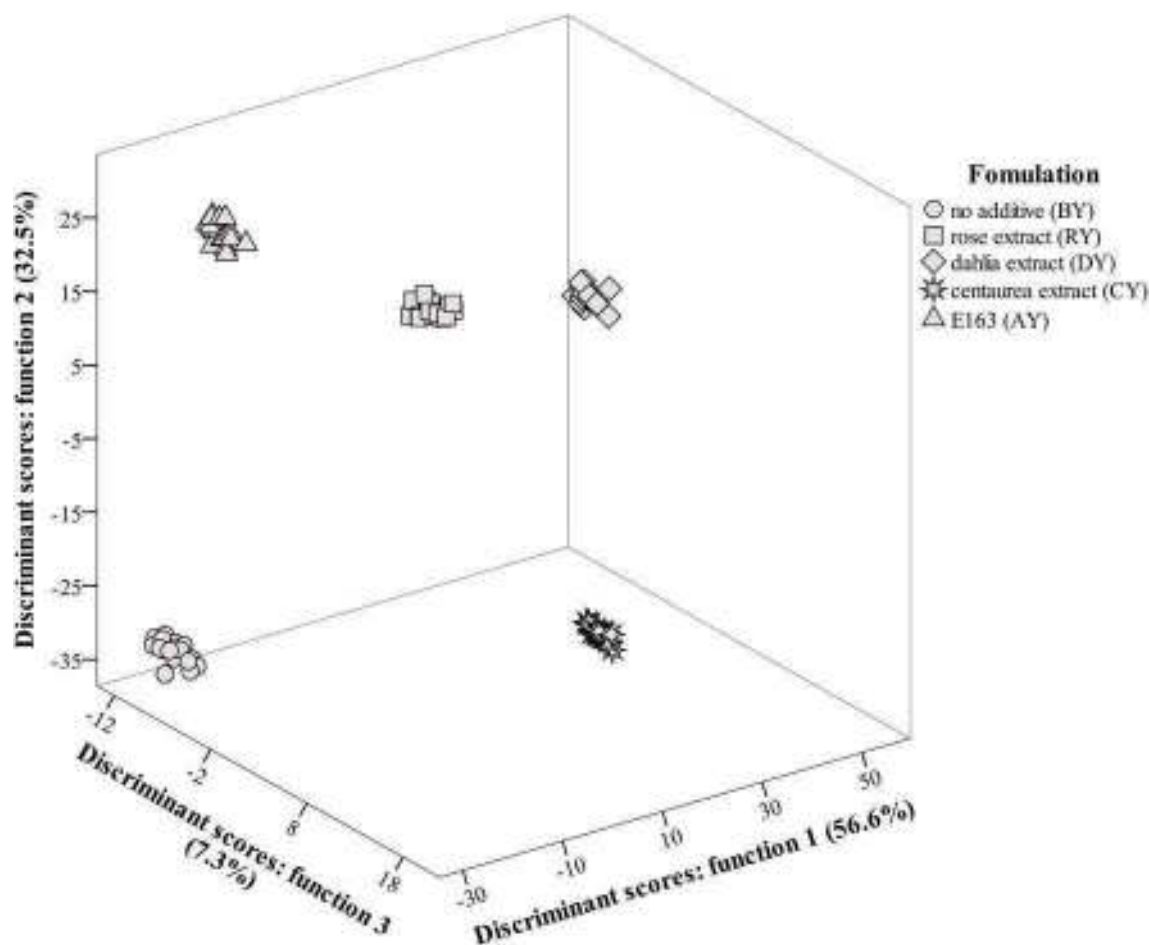


Fig. 1. Three-dimensional distribution of YF markers according to the canonical discriminant functions coefficients defined from different yogurt parameters.

Table 4, but the complete profiles included also C11:0, C13:0, C14:1, C17:0, C17:1, C18:3n6, C20:0, C20:1, C20:4n6, C20:5n3, C22:0, C23:0, C24:0 (however, all fatty acids were included in the Linear Discussion Analysis discussed in the next section).

Since milk was the main source of fatty acids in yogurt, and bearing in mind, once again, that the added extracts were aqueous, the high similarity among YF is coherent. Nevertheless, C18:1n9 ($p = 0.133$), SFA ($p = 0.180$) and MUFA ($p = 0.125$) were the only cases with no significant differences among tested YF, most likely because the added extracts might have different effectiveness in preventing the oxidation of specific fatty acids throughout time.

Since the interaction among factors (YF \times SE) the next conclusions were obtained from the EMM plots (data not shown): BY presented higher percentages of C4:0 (1.3%), C6:0 (1.7%), C8:0 (1.3%), and C10:0 (2.9%), while C15:0 (1.5%), C16:1 (1.5%), C18:0 (11.5%), C18:2n6 (2.5%) and PUFA (5.3%) showed the highest values in CY. Yogurts prepared with rose extract (RY), on the other hand, had the highest percentages of C14:0 (12.2%) and C16:0 (36%), whilst C12:0 was slightly higher in AY (3.7%).

In what concerns SE effect, almost all tabled fatty acids showed significant differences, except in the cases of C15:0 ($p = 0.737$), C16:1 ($p = 0.984$), C18:0 ($p = 0.213$), C18:2n6 ($p = 0.063$) and PUFA ($p = 0.532$). In stored samples, C6:0, C8:0, C10:0, C12:0, C14:0, C16:0 and SFA were quantified in higher percentages, while C4:0, C18:1n9, C19:3n3 and MUFA tended to present higher values in non-stored samples, thereby generally corroborating the higher resistance of saturated forms to storage.

3.3. Linear discriminant analysis

Despite the statistical significance of differences among different YF, we decided to verify if the magnitude of the detected differences was high enough to discriminate each YF. Accordingly, a linear discriminant analysis (LDA) was applied to find the variables with highest contribution to discriminate each YF.

The first three discriminant functions included 97.7% (first function: 61.4%; second function: 30.0%; third function: 6.3%) of the observed variance (Fig. 1). From the 41 variables under analysis, the discriminant model selected b^* , a^* , L^* , pH, C4:0, C8:0, C13:0, C16:1, C17:1, C18:3n3, C18:3n6, C20:1, C20:4n6, C20:5n3, C23:0, C24:0 and PUFA as those having discriminant ability, which clearly indicates that fatty acids and colour parameters were the variables with highest dissimilarity among the prepared YF.

In what concerns the correlations among functions and variables, function 1 was highly correlated with b^* and L^* , placing markers corresponding to DY and BY in the farthest positions due to their differences in both parameters (the highest b^* value was measured in DY, while the maximum L^* was measured in BY). Function 2, in turn, was mostly correlated with a^* , mostly separating markers corresponding to AY and RY (positive end of the axis) from BY (negative end of the axis). Function 3 also contributed to separate the markers of each YF, being especially effective in separating BY and CY. Owing to the higher proximity of their markers according to the three plotted discriminant functions RY and AY showed the highest similarity among the assayed parameters.

In the performed LDA, the classification performance was 100% accurate, either for original grouped cases, as well as for the cross-

validated grouped cases.

4. Conclusion

Overall, the natural extracts with highest potential as alternatives to E163 resulted to be RY, considering the main purpose of colouring yogurts in the yellow-orange series. In addition to the provided colour, these groups of yogurts (AY and RY) showed very similar nutritional value, free sugars and fatty acids composition.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Gomphrena globosa L. as a novel source of food-grade betacyanins: Incorporation in ice-cream and comparison with beet-root extracts and commercial betalains



Custódio Lobo Roriz^{a,b}, João C.M. Barreira^a, Patricia Morales^b, Lillian Barros^a, Isabel C.F.R. Ferreira^{a,*}

^a Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal

^b Dpto. Nutrición y Bromatología II, Facultad de Farmacia, Universidad Complutense de Madrid (UCM), Plaza Ramón y Cajal, s/n. E-28040, Madrid, Spain

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ABSTRACT

Currently, there are some examples of natural colourants with commercial use. However, these colourants are usually under-exploited, besides being obtained from a reduced number of plant or algal species. Accordingly, we propose using betalains obtained from an alternative plant species, *Gomphrena globosa*, which have a powerful colouring activity besides being strong antioxidants, as a novel ice-cream colourant. For comparison purposes, other ice-cream formulations were prepared, namely without colourants, added with commercial betalain or with *Beta vulgaris* extract. Besides evaluating the colour parameters L^* , a^* and b^* , the nutritional parameters, individual sugars and fatty acids profiles were also studied. These parameters were evaluated throughout time, up to a maximum of 60 days of freeze ($-22\text{ }^{\circ}\text{C}$) storage. Betacyanin quantification of each formulation was also performed to determine its maintenance along storage. In general, ice-creams prepared with *G. globosa* were similar (considering nutritional, colour, individual sugars and fatty acids profiles) to those including *B. vulgaris* extract, thereby validating the suitability of this alternative plant as a source of food colourants, particularly as ice-cream colourants. Furthermore, the positive effects induced by the addition of this natural colourant were maintained throughout storage time, as indicated by the markers distribution in the linear discriminant analysis.

1. Introduction

The importance of food appearance might be considered as being as important as its taste, being often considered as a top indicator of the overall quality of food (Bridle & Timberlake, 1997, pp. 103–109). Nowadays, the complex processing that most food products are submitted to might cause some unwanted changes to their visual appearance, especially by causing a fading effect in their colour (González, Gallego, & Valcárcel, 2002). In addition, there is growing concern about the health effects potentially induced by food, which is increasingly being considered as having the double purpose of satisfying nutritional needs, while exerting disease-preventing effects. This overall trend represents the main foundation of the functional foods market, in which novel food products, able to provide new consuming experiences, are continuously being developed (Edmonds, Wadhwa, & Wibisono, 2013). When these products are developed with the main purpose of achieving specific functional properties, foods poor in bioactive compounds present higher necessity of improvement. In this sense, ice-cream, a

product with high consumption level, would certainly benefit from the incorporation of bioactive natural substances, particularly if these substances could simultaneously improve its appearance (Edmonds et al., 2013). In general, frozen dairy desserts, such as ice-creams, are complex colloidal systems that consist in a combination of milk, sweeteners, emulsifiers, stabilizers, colouring and flavouring agents (Soukoulis, Lyroni, & Tzia, 2010). Despite the generalized use of artificial substances, most of these agents could be obtained from natural sources, making the resulting products very appealing to the consumers (Erkaya, Dağdemir, & Sengül, 2012).

In the case of colouring agents, and owing to the important role of colour when enjoying any food product or as a potential indicator of food quality (Bridle & Timberlake, 1997, pp. 103–109), it is mandatory to find natural-based colourants, particularly if the associated compounds might exert any kind of bioactivity. This becomes even more important if we remind that most artificial compounds are often regarded as causing several secondary and harmful effects, such as allergenic or intolerance reactions (Wissgott & Bortlik, 1996).

* Corresponding author.

E-mail address: iferreira@ipb.pt (I.C.F.R. Ferreira).

Despite the diversity of natural dyes (e.g., carotenoids, anthocyanins or betalains) in the market (Carocho, Morales, & Ferreira, 2015), most are far from being used according to their full potential, besides being obtained from a reduced number of natural sources. Betalains, which can be divided in red-violet betacyanins and yellow-orange betaxanthins, are a good example of under-exploited natural food colourants. Furthermore, betacyanins are almost exclusively obtained from beet-root, despite the availability of other alternative sources (Roriz, Barros, Prieto, Morales, & Ferreira, 2017). Besides their colouring capacity, these pigments have a high antioxidant activity, exerting also different chemopreventive effects (Spórna-kucab & Jagodzi, 2017). *Gomphrena globosa* L. presents a multitude of phytochemicals with interest, such as tocopherols, sugars and organic acids (Roriz, Barros, Carvalho, & Ferreira, 2014). In addition to these compounds, other substances with antioxidant properties have been identified in this plant, in particular bioactive substances such as phenolic compounds, but also molecules that can confer colour, such as betacyanins (Roriz, Barros, Carvalho, Santos-Buelga, & Ferreira, 2014). Herein, betacyanins extracted from the purple flowers of *Gomphrena globosa* L. (Amaranthaceae) were evaluated as colouring/functionalizing agents in ice-cream formulations, aiming to verify its potential improvement in colour parameters, as well as its effect on nutritional composition, individual sugars and fatty acids profiles. In order to acquire comprehensive conclusions, other ice-cream formulations were prepared namely, ice-cream without any colouring agent, ice-cream incorporated with commercial betalains and ice-cream added with beet-root extract. Betacyanin quantification of each formulation was also performed in order to determine its maintenance along storage. All parameters were analysed in five different moments: preparation day, and after 15, 30, 45 and 60 days (considered as the maximum desired storage limit, for this type of ice-cream) of storage (-22°C).

2. Material and methods

2.1. Standard and reagents

Acetonitrile (HPLC grade) was obtained from Lab-Scan (Lisbon, Portugal). The standard betalain and the fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Other reagents and solvents (analytical grade) were obtained from common sources. Milk (UHT entire milk Continente, composition per 100 mL: 273 KJ, or 65 Kcal energetic value; 3.6 g of lipids of which 2.3 are unsaturated; 4.9 g of carbohydrates, being the majority sugars; 3.3 g of protein; and 0.1 g of salt) and double cream (UHT cream Continente; composition per 100 mL: 1385 KJ, or 336 Kcal energetic value; 35 g of lipids of which 20 g are unsaturated; 3 g of carbohydrates; 2 g of protein; and 0.1 g of salt) were purchased at a local supermarket (Continente, Portugal). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.2. Sample collection

Gomphrena globosa L. samples were obtained from Ervital, a company established in a mountain region with high biodiversity, holding a vast collection of certified plant materials from different origins, obtained from sustainable harvesting of spontaneous local species and organic farming of exogenous ones. The botanical identification of the samples was confirmed by a botanical expert, responsible for the medicinal plant collection of *Escola Superior Agrária* herbarium (BRESA), Polytechnic Institute of Bragança (Trás-os-Montes, Portugal). After identification, samples were subjected to a mechanical treatment to separate the pigmented floral parts (bracts and bracteoles) from the inflorescences.

2.3. Sample preparation

2.3.1. Ultrasound assisted extraction (UAE)

The *G. globosa* extract enriched in betacyanins was obtained by UAE (QSonica sonicators, model CL-334, Newtown, CT, USA), working at 500 W, for 22 min, using water as the extraction solvent and a liquid-to-solid ratio of 5 g/L, as described by (Roriz, Barros, Prieto, Barreiro, et al., 2017).

2.3.2. Preparation of *B. vulgaris* extract

B. vulgaris extract obtained by UAE, was further filtrated and frozen prior to its lyophilization (FreeZone 4.5, Labconco, Kansas City, MO, USA), in order to obtain a dry extract.

2.4. Incorporation of the natural food colorant in the ice-cream

2.4.1. a) Ice-cream preparation

Ice-cream was prepared from a base recipe: 240 g of sugar were mixed with 500 mL of milk (UHT entire milk, Continente, Portugal), in order to dissolve the sugar. Meanwhile, 1000 mL of double cream (UHT cream, Continente, Portugal) were used to obtain whipped cream, which was previously added to the mixture. The final batter was left to stand for 12 h, further divided in four batches, each placed in an ice-cream machine equipment (Ice-cream Maker SECN 12 A1, SilverCrest, Hamburg, Germany).

2.4.2. b) Colouring agent addition

The ice-cream batches were identified as: i) control (ice-cream without colouring agents); ii) ice-cream added with betalain standard (200 mg, i.e. ≈ 46 mg/100 g ice-cream); iii) ice-cream with *G. globosa* extract (670 mg, i.e. ≈ 154 mg/100 g ice-cream); iv) ice-cream with *Beta vulgaris* extract (670 mg, i.e. ≈ 154 mg/100 g ice-cream).

The amount of each ingredient was added in order to obtain the desired colour. Moreover, the added quantities were different because commercial betalain (Sigma-Aldrich, St. Louis, MO, USA) is an isolated compound, obviously with a higher degree of purity in comparison with the extracts from *G. globosa* and *B. vulgaris*. *B. vulgaris* extract was obtained by grinding the sample with 10% of water, followed by a filtration step and freeze prior to its lyophilization (FreeZone 4.5, Labconco, Kansas City, MO, USA).

The samples were analysed immediately after preparation and after: 15, 30, 45 and 60 days (maximum desired storage limit for this type of ice-cream) of storage at -22°C .

2.5. Nutritional composition and physico-chemical analyses

2.5.1. Nutritional parameters

The samples were also analysed for proximate composition (moisture, protein, fat, ash and carbohydrates) using the AOAC procedures (AOAC, 2016). Crude protein content ($N \times 6.38$) was estimated by the Kjeldahl method (AOAC 978.04, AOAC, 2016); crude fat was determined by Rose-Gottlieb method (AOAC 905.02, AOAC, 2016); ash content was determined by incineration at $600 \pm 15^{\circ}\text{C}$ (AOAC 923.03, AOAC, 2016); total carbohydrates were calculated by difference, taking into account moisture content, and the results were expressed in fresh weight. Total energy was calculated according to the following equation: Energy (kcal) = $4 \times (\text{g proteins} + \text{g carbohydrates}) + 9 \times (\text{g lipids})$.

2.5.2. Individual sugars

Free sugars were detected by high performance liquid chromatography (HPLC) coupled to a refractive index detector. Sugars were identified by comparison with standards and further quantified (g/100 g of ice-cream) by the internal standard (melezitose) method (Barros et al., 2013).

2.5.3. Colour parameters

The CIE colour parameters were measured in three different points for each sample using a colorimeter (model CR-400, Konica Minolta Sensing Inc., Tokyo, Japan), and the average value of the different points was considered to determine this parameter. The illuminant C and a diaphragm aperture of 8 mm were selected. The Hunter colour L^* , a^* and b^* values were registered using the data software “Spectra Magic Nx” (version CM-S100W 2.03.0006, Konica Minolta Company, Japan). The instrument was calibrated to standard white tiles before analysis (Spectra Magic NX Instruction Manual, Konica Minolta Sensing, Inc. (ver. 2.0), 2009, Japan) (Fernandes et al., 2012).

2.5.4. Fatty acids

Fatty acids were analysed by gas chromatography (GC) coupled to a flame ionization detector (FID) at 260 °C, using a DANI model GC 1000 instrument equipped with a split/splitless injector and a Macherey-Nagel (Duren, Germany) column (50% cyanopropyl-methyl-50% phenylmethylpolysiloxane, 30 m × 0.32 mm i.d. × 0.25 µm df). The oven temperature programme was as follows: the initial temperature of the column was 50 °C, held for 2 min, then a 30 °C/min ramp to 125 °C, 5 °C/min ramp to 160 °C, 20 °C/min ramp to 180 °C, 3 °C/min ramp to 200 °C, 20 °C/min ramp to 220 °C and held for 15 min. The carrier gas (hydrogen) flow-rate was 4.0 mL/min (0.61 bar), measured at 50 °C. Split injection (1:40) was carried out at 250 °C. Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using the CSW 1.7 Software (DataApex 1.7) and expressed in relative percentage of each fatty acid (Barros et al., 2013).

2.5.5. Betacyanins

For betacyanins' determination an HPLC-DAD-ESI/MS analyses was performed with a Dionex Ultimate 3000 UPLC instrument (Thermo Scientific, San Jose, CA, USA) coupled with a diode-array detector and a mass spectrometer, as previously described by (Roriz, Barros, Prieto, Morales, et al., 2017). The separation was carried out in a Waters Spherisorb S3 ODS-2 C18, (3 µm, 4.6 mm × 150 mm, Waters, Milford, MA, USA) column operating at 35 °C and the solvents used were: (A) 0.1% trifluoroacetic acid (TFA) in water and (B) acetonitrile, using a gradient flow elution method. Betacyanins maximum absorbance is 530 nm, therefore this was the preference wavelength used to record all chromatograms. For quantitative analysis, a calibration curve was obtained based on gomphrenin III and betalain.

2.6. Evaluation of hepatotoxicity

The different extracts, the commercial standard and the final ice-cream formulations (maximal tested concentration: 400 µg/mL) were evaluated regarding their hepatotoxicity, following a methodology previously described (Abreu et al., 2011) and using porcine liver, which was acquired from certified abattoirs. A phase-contrast microscope was used to monitor the growth of the cell cultures. They were sub-cultured and plated in 96 well plates (density of 1.0×10^4 cells/well). Dulbecco's Modified Eagle Medium (DMEM) was used, with 10% of foetal bovine serum (FBS), 100 U/mL of penicillin and 100 µg/mL of streptomycin. Ellipticine was used as a positive control, and the results were expressed in GI_{50} values (growth inhibition values) in µg/mL (sample concentration that inhibited 50% of the net cell growth).

2.7. Statistical analysis

All statistical tests were performed with IBM SPSS Statistics for Windows, version 22.0 (IBM Corp., Armonk, NY, USA) considering a 5% significance level. Data were expressed as mean ± standard deviation (the number of significant numbers was maintained according to the standard deviation magnitude).

For each ice cream formulation, three different samples were used.

Each of these samples was further analysed in triplicate. A 2-way analysis of variance (ANOVA) with type III sums, using the general linear model (GLM) procedure was applied to compare all parameters among different ice-cream formulations (IF) or storage times (ST). Besides evaluating the effect of each factor, their interaction was also assessed. When no statistical significant interaction was found, the means were compared using Tukey's multiple comparison test, with a previous assessment of the equality of variances through a Levene's test. Otherwise, differences were analysed in the estimated marginal means plots obtained for all levels of each factor.

In addition, a linear discriminant analysis (LDA) was used to compare the effect of IF and ST over all parameters simultaneously. A stepwise technique was applied, based on the Wilks' λ test with the usual probabilities of F (3.84 to enter and 2.71 to be removed) for variable selection. Only variables with a statistically significant classification performance ($p < 0.050$) were maintained by the statistical model. This statistical classification tool was performed to estimate the relationship between single categorical dependent variables (ice-cream formulations) and the quantitative independent variables (results obtained in laboratorial assays). A leaving-one-out cross validation procedure was carried out to assess the model performance.

3. Results and discussion

Owing to the high consumers' acceptability, there are several studies reporting tentative improvements in ice-cream recipes, either by substituting its main ingredients, especially sugars (Moriano & Alamprese, 2017; Soukoulis & Tzia, 2017), or by adding compounds with different biological activity, e.g., probiotics (Chaikham & Rattanasena, 2017; dos Santos Cruxen et al., 2017) or antioxidants (Sanguigni, Manco, Sorge, Gnessi, & Francomano, 2016). There is an increasing tendency towards using natural extracts in recent studies (Carocho, Barreiro, Morales, & Ferreira, 2014; dos Santos Cruxen et al., 2017), for instance to replace the artificial dyes commonly utilized in ice-cream, as shown in a recent study reporting the presence of 18 artificial dyes in different ice-cream formulations (Machewad, Chatge, Chappalwar, Jadhav, & Chappalwar, 2012; Martin, Oberson, Meschiari, & Munari, 2016). The industrial use of natural extracts might represent an effective answer to the current consumers' concerns, who are gradually preferring food products prepared with natural additives instead of artificial compounds, often associated with unwanted effects.

Bearing this in mind, four different ice-cream formulations: i) control ice-cream (CI, ice-cream with no added colourants); ii) ice-cream added with betalain standard (BSI); iii) ice-cream with *G. globosa* extract (GGI); and iv) ice-cream with *B. vulgaris* extract (BVI), were characterized. In addition to the preparation day, this comparative study was extended to four storage periods, namely: 15, 30, 45 and 60 days at -22 °C.

As explained in the Materials and methods section, the effect of storage time (ST) was evaluated after a previous aggregation of results obtained for all formulations in a determined period; likewise, the effect of ice-cream formulation (IF) was evaluated after combining the results of all storage periods in each specific IF. This approach is expected to allow concluding which is best colouring agent, independently of ST, as well as the main changes along storage in differently formulated ice-creams. This is, in our opinion, the most useful information pertaining potential industrial applications. Since ST effect could depend on the ice-cream formulation (IF), and *vice-versa*, the interaction among both factors was also studied, in addition to evaluating the significance of each individual factor. In all cases where a significant ($p < 0.050$) interaction was found, not allowing the statistical classification of results, differences induced by each factor (if significant) were described according to the estimated marginal means (EMM) plots.

The nutritional composition and energy values for different ice-cream formulations (IF) and storage times (ST) are presented in Table 1. In terms of nutritional composition (Table 1), the interaction among IF

Table 1

Nutritional composition (g/100 g fresh weight) and energy values (kcal/100 g fresh weight) for different ice-cream formulations (IF) and storage times (ST). Results are presented as mean \pm standard deviation.¹

		Water	Fat	Protein	Ash	Carbohydrates	Sucrose	Lactose	Energy
IF	CI	59 \pm 1	14 \pm 2	5.0 \pm 0.2	0.35 \pm 0.05	22 \pm 2	19 \pm 1	2.5 \pm 0.3	234 \pm 13
	BSI	59 \pm 1	15 \pm 1	5.1 \pm 0.3	0.35 \pm 0.05	21 \pm 1	18 \pm 1	2.5 \pm 0.3	239 \pm 6
	GGI	59 \pm 1	15 \pm 1	5.1 \pm 0.4	0.37 \pm 0.05	21 \pm 1	18 \pm 1	2.5 \pm 0.3	239 \pm 7
	BVI	63 \pm 1	13 \pm 1	5.8 \pm 0.3	0.37 \pm 0.05	18 \pm 1	16 \pm 1	2.3 \pm 0.3	212 \pm 7
ANOVA <i>p</i> -value (n = 45) ²		< 0.001	< 0.001	< 0.001	0.243	< 0.001	< 0.001	0.036	< 0.001
ST	0 days	60 \pm 1	14 \pm 1	5.3 \pm 0.4	0.43 \pm 0.05	20 \pm 1	17 \pm 1	2.7 \pm 0.3	227 \pm 8
	15 days	60 \pm 1	15 \pm 1	5.3 \pm 0.5	0.32 \pm 0.04	20 \pm 2	18 \pm 1	2.3 \pm 0.2	236 \pm 8
	30 days	61 \pm 2	12 \pm 2	5.1 \pm 0.4	0.42 \pm 0.05	21 \pm 3	18 \pm 1	2.6 \pm 0.3	212 \pm 11
	45 days	60 \pm 2	15 \pm 1	5.3 \pm 0.5	0.28 \pm 0.04	20 \pm 2	18 \pm 1	2.4 \pm 0.2	236 \pm 11
	60 days	60 \pm 2	14 \pm 1	5.3 \pm 0.5	0.36 \pm 0.04	20 \pm 2	18 \pm 1	2.2 \pm 0.2	227 \pm 15
ANOVA <i>p</i> -value (n = 36) ³		0.026	< 0.001	0.219	< 0.001	< 0.001	0.002	< 0.001	< 0.001
IF \times ST	<i>p</i> -value (n = 180) ⁴	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

¹Results are reported as mean values of each ice-cream formulation (IF), including results from 0, 15, 30, 45 and 60 days, and mean values of each storage time (ST), considering all IF in each ST.

²If *p* < 0.05, the corresponding parameter presented a significantly different value for at least one IF.

³If *p* < 0.05, the corresponding parameter presented a significantly different value for at least one ST.

⁴In this table, the interaction among factors was significant in all cases; thereby no multiple comparisons could be performed.

Table 2

Colour parameters measured in different ice-cream formulations (IF) and storage times (ST). Results are presented as mean \pm standard deviation.¹

		Fresh			Lyophilized		
		<i>L</i> *	<i>a</i> *	<i>b</i> *	<i>L</i> *	<i>a</i> *	<i>b</i> *
IF	CI	94 \pm 1	-2.5 \pm 0.2	13 \pm 1	95 \pm 1	-2.3 \pm 0.2	11 \pm 1
	BSI	93 \pm 1	2.0 \pm 0.2	8.1 \pm 0.4	94 \pm 1	1.7 \pm 0.3	8 \pm 1
	GGI	86 \pm 3	8 \pm 1	2.4 \pm 0.3	87 \pm 2	8 \pm 1	2.5 \pm 0.3
	BVI	85 \pm 2	10 \pm 2	3.0 \pm 0.4	87 \pm 1	10 \pm 1	3.4 \pm 0.4
ANOVA <i>p</i> -value (n = 45) ²		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
ST	0 days	91 \pm 3	4 \pm 4	7 \pm 5	91 \pm 3	4 \pm 4	6 \pm 4
	15 days	90 \pm 4	4 \pm 6	7 \pm 5	90 \pm 5	5 \pm 5	6 \pm 3
	30 days	89 \pm 5	5 \pm 5	7 \pm 4	91 \pm 5	4 \pm 5	6 \pm 3
	45 days	88 \pm 5	4 \pm 5	6 \pm 4	91 \pm 3	4 \pm 5	6 \pm 4
	60 days	89 \pm 5	4 \pm 5	6 \pm 4	90 \pm 4	4 \pm 5	6 \pm 3
ANOVA <i>p</i> -value (n = 36) ³		0.030	0.824	0.879	0.854	0.912	0.985
IF \times ST	<i>p</i> -value (n = 180) ⁴	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

¹Results are reported as mean values of each ice-cream formulation (IF), including results from 0, 15, 30, 45 and 60 days, and mean values of each storage time (ST), considering all IF in each ST.

²If *p* < 0.05, the corresponding parameter presented a significantly different value for at least one IF.

³If *p* < 0.05, the corresponding parameter presented a significantly different value for at least one ST.

⁴In this table, the interaction among factors was significant in all cases; thereby, no multiple comparisons could be performed.

and ST had a significant influence in all evaluated parameters, thereby indicating that the effect of ST over water, fat, protein, ash, carbohydrates (which in this case corresponds to total soluble sugar content) and energy values varied according to each IF. Regarding the effect of each factor *per se*, it was also significant in most cases, except for protein (*p* = 0.219) along ST and ash (*p* = 0.243) among different IF. Despite not being possible to present the statistical classification, some general tendencies were obtained from the EMM plots. Among different IF, ice-creams incorporating the *B. vulgaris* extract showed the highest contents in water (63 \pm 1 g/100 g) and protein (5.8 \pm 0.3 g/100 g) and the lowest levels of carbohydrates (18 \pm 1 g/100 g), sucrose (16 \pm 1 g/100 g) and energy (212 \pm 7 kcal/100 g). These results were somehow surprising, since the same batter was used to prepare all ice-cream formulations, and the incorporation of different colouring additives, owing to the low added quantities, should not interfere with nutritional values. Nevertheless, it might be speculated that ice-cream

formulations prepared with *B. vulgaris* were not as stable as the remaining ones, since the presented values correspond to the average values obtained in each storage period. In what regards the effect of ST, 15 days and 45 days stored ice-creams presented the highest fat contents (15 \pm 1 g/100 g), whilst those stored during 30 days had the lowest energy levels (210 \pm 11 kcal/100 g).

In general, the nutritional profile is in agreement with the reported in typical ice-cream formulation (dos Santos Cruxen et al., 2017), except for protein contents that were detected herein, which were higher than commonly reported values (2.5–4.5 g/100 g) in ice-cream (Erkaya et al., 2012; Senaka Ranadheera, Evans, Adams, & Baines, 2013; Silva, Bezerra, Santos, & Correia, 2015). Protein content is important to the textural properties (emulsification, aeration, water retention, and viscosity) (Cheng, Ma, Li, Yan, & Cui, 2015), while carbohydrates, mainly sucrose and lactose, are essential to improve taste and retaining aromas (Karaman et al., 2014).

Table 3Major fatty acids (relative percentage > 0.5%) of different ice-cream formulations (IF) and storage times (ST). Results are presented as mean \pm standard deviation.¹

		C6:0	C8:0	C10:0	C12:0	C14:0	C14:1	C15:0	C16:0	C16:1	C17:0	C18:0	C18:1n9	C18:2n6	SFA	MUFA	PUFA
IF	CI	3.2 \pm 0.4	1.7 \pm 0.2	3.3 \pm 0.3	3.9 \pm 0.2	11.1 \pm 0.4	1.0 \pm 0.1	1.1 \pm 0.1	32 \pm 1	1.4 \pm 0.1	0.7 \pm 0.1	11.9 \pm 0.4	25 \pm 1	3.1 \pm 0.2	69 \pm 1	27 \pm 1	3.9 \pm 0.2
	BSI	3.2 \pm 0.3	1.7 \pm 0.1	3.4 \pm 0.2	4.1 \pm 0.2	11.3 \pm 0.3	1.0 \pm 0.1	1.1 \pm 0.1	32 \pm 1	1.5 \pm 0.1	0.7 \pm 0.1	11.8 \pm 0.4	24 \pm 1	3.1 \pm 0.2	70 \pm 1	27 \pm 1	3.9 \pm 0.3
	GGI	3.2 \pm 0.2	1.7 \pm 0.1	3.3 \pm 0.2	4.0 \pm 0.3	11.2 \pm 0.4	1.0 \pm 0.1	1.1 \pm 0.1	32 \pm 1	1.4 \pm 0.1	0.7 \pm 0.1	11.9 \pm 0.3	24 \pm 1	3.1 \pm 0.2	70 \pm 1	27 \pm 1	3.9 \pm 0.2
	BVI	3.3 \pm 0.4	1.7 \pm 0.2	3.3 \pm 0.2	4.0 \pm 0.2	11.1 \pm 0.3	1.0 \pm 0.1	1.0 \pm 0.1	32 \pm 1	1.5 \pm 0.1	0.7 \pm 0.1	11.8 \pm 0.3	24 \pm 1	3.1 \pm 0.1	69 \pm 1	27 \pm 1	3.9 \pm 0.2
	ANOVA <i>p</i> -value (n = 18) ²	0.360	0.849	0.217	0.051	0.039	0.084	0.833	0.402	0.003	0.141	0.193	0.002	0.615	0.072	0.073	0.494
ST	0 days	3.3 \pm 0.2	1.7 \pm 0.1	3.2 \pm 0.2	4.0 \pm 0.2	11.2 \pm 0.3	1.0 \pm 0.1	1.1 \pm 0.1	32 \pm 1	1.5 \pm 0.1	0.7 \pm 0.1	11.9 \pm 0.4	24 \pm 1	3.1 \pm 0.2	69 \pm 1	27 \pm 1	3.9 \pm 0.2
	15 days	2.9 \pm 0.3	1.6 \pm 0.1	3.2 \pm 0.2	4.0 \pm 0.2	11.3 \pm 0.4	1.0 \pm 0.1	1.0 \pm 0.1	33 \pm 1	1.4 \pm 0.1	0.7 \pm 0.1	12.0 \pm 0.3	24 \pm 1	3.0 \pm 0.2	70 \pm 1	27 \pm 1	3.8 \pm 0.2
	30 days	3.4 \pm 0.2	1.7 \pm 0.1	3.4 \pm 0.2	4.0 \pm 0.2	11.1 \pm 0.2	1.0 \pm 0.1	1.0 \pm 0.1	32 \pm 1	1.4 \pm 0.1	0.7 \pm 0.1	11.8 \pm 0.4	24 \pm 1	3.1 \pm 0.2	69 \pm 1	27 \pm 1	3.9 \pm 0.2
	45 days	3.3 \pm 0.3	1.8 \pm 0.2	3.5 \pm 0.3	4.2 \pm 0.2	11.3 \pm 0.4	1.0 \pm 0.1	1.1 \pm 0.1	32 \pm 1	1.4 \pm 0.1	0.7 \pm 0.1	11.7 \pm 0.3	24 \pm 1	3.1 \pm 0.2	70 \pm 1	27 \pm 1	3.9 \pm 0.3
	60 days	3.3 \pm 0.5	1.7 \pm 0.2	3.3 \pm 0.2	3.9 \pm 0.2	10.9 \pm 0.4	1.0 \pm 0.1	1.1 \pm 0.1	32 \pm 1	1.5 \pm 0.1	0.7 \pm 0.1	11.8 \pm 0.3	24 \pm 1	3.2 \pm 0.1	69 \pm 1	27 \pm 1	4.0 \pm 0.2
	ANOVA <i>p</i> -value (n = 36) ³	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.180	0.107	< 0.001	< 0.001	0.111	0.009	0.271	< 0.001	0.009	0.108	0.177
IF \times ST	<i>p</i> -value (n = 72) ⁴	< 0.001	< 0.001	< 0.001	< 0.001	0.001	0.307	0.258	0.001	< 0.001	0.135	0.002	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

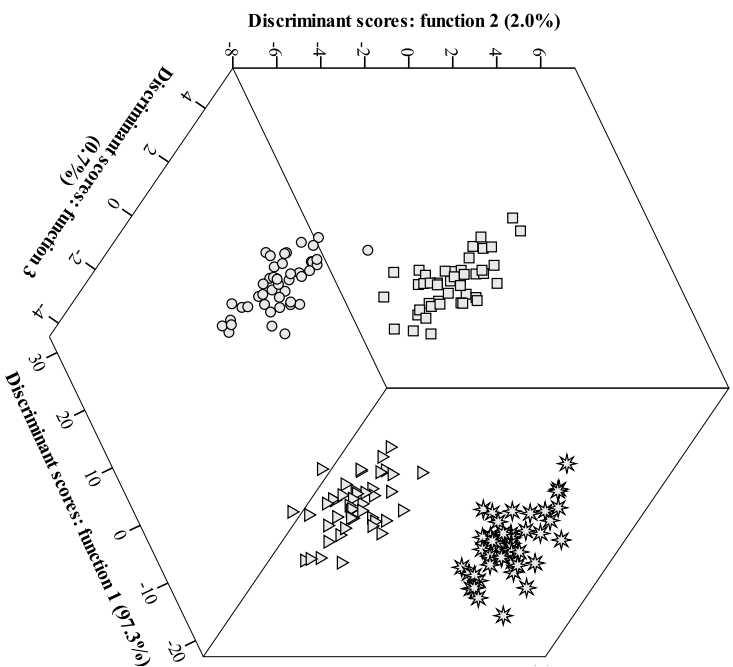
¹Results are reported as mean values of each ice-cream formulation (IF), including results from 0, 15, 30, 45 and 60 days, and mean values of each storage time (ST), considering all IF in each ST.²If *p* < 0.05, the corresponding parameter presented a significantly different value for at least one IF.³If *p* < 0.05, the corresponding parameter presented a significantly different value for at least one ST.⁴If *p* < 0.05, the interaction among factors is significant; in this case, no multiple comparisons can be performed.

Fig. 1. Spatial distribution of IF markers distributed according to the canonical discriminant functions coefficients defined from results obtained in the laboratorial assays. CI: control ice-cream (○); BSI: ice-cream added with beta-lain standard (□); GGI: ice-cream with *G. globosa* extract (△); BVI: ice-cream with *B. vulgaris* extract (*).

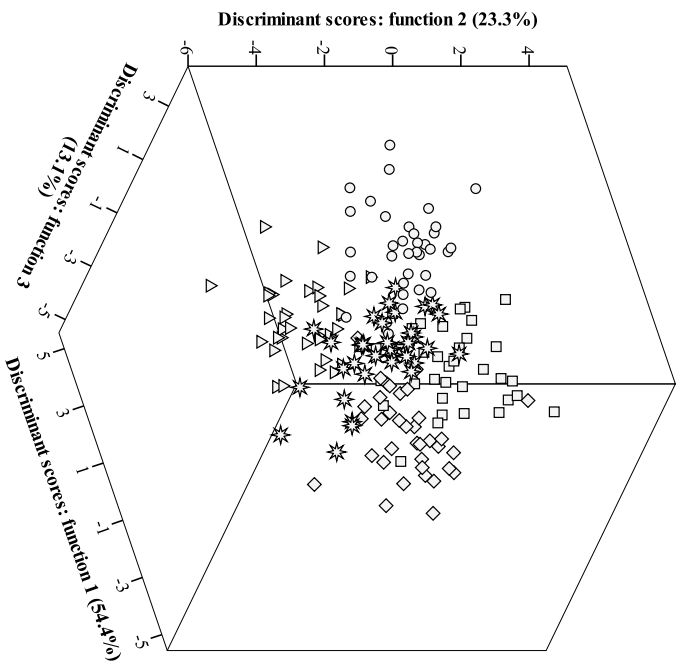


Fig. 2. Spatial distribution of ST markers distributed according to the canonical discriminant functions coefficients defined from results obtained in the laboratorial assays. 0 days: ○; 15 days: □; 30 days: △; 45 days: BVI; 60 days: *.

Table 2 presents the colour parameters measured for different IF and ST. Considering the main purpose of this work, finding an alternative natural colouring agent for ice-cream, the parameters lightness (L^*), redness (a^*) and blueness (b^*) were evaluated in fresh and lyophilized forms of all IF throughout ST (Table 2). In line with the

observed in nutritional composition, the studied factors, IF and ST, had a cooperative ($p < 0.001$) effect over colour parameters. However, the effect of ST was not significant in most cases, except L^* value in fresh samples ($p = 0.030$), while differences among different IF (as it might have been anticipated) were significant in all cases. In general, L^* and b^* presented the highest values in CI samples, fresh and lyophilized, which on the other hand showed the lowest a^* values, owing to the absence of any colouring agent. Concerning IF added with colourants, BSI presented higher L^* and b^* values, while GGI and BVI were characterized as having the highest a^* values. The lower values of L^* in GGI and BVI might be explained by the fact that both ingredients are natural extracts, therefore potentially presenting some opacity, as these were not purified compounds.

The major fatty acids of different IF and storage times are shown in Table 3. The profiles in fatty acids were influenced by ST, especially in the cases of short-chain fatty acids and unsaturated ones. Nevertheless, and despite their statistical significance, the observed differences correspond only to slight variations in the percentages of each fatty acid, which did not seem to be relevant enough to the overall quality of ice-creams. This hypothesis is corroborated by the effect of IF, which was only significant in the cases of C14:0, C16:1, C18:1 and MUFA. Likewise, the interaction among factors was not significant for C14:1, C15:0 and C17:0; however, since the individual effect of ST and IF was not significant in these cases, the statistical classification could not be presented. The analysis of the EMM plots of each fatty acid did not allow obtaining overall tendencies, except for the generally higher percentages of C12:0 in ice-creams stored for 45 days, C13:0 in non-stored ice-creams and C16:0 in ice-creams stored during 15 days.

In addition to the tabled fatty acids, C11:0, C13:0, C18:3n3, C20:0, C20:3n6 and C20:4n6 were also detected, but in percentages lower than 0.5%. Nevertheless, all quantified fatty acids were included in the linear discriminant analyses discussed in the next section.

Furthermore, the extracts, the commercial standard and the ice-cream samples did not show hepatotoxicity up to the maximal assayed concentration (400 $\mu\text{g}/\text{mL}$) as indicated by the results obtained with the PLP2 cell line (isolated from porcine liver). The quantities of betacyanins were also maintained along ST, as it was validated according to the chromatographic results from HPLC-DAD-ESI/MS, showing their suitability as potentially useful stable colouring agents.

3.1. Linear discriminant analysis

After comparing the evaluated parameters one by one, weighing the significance of changes caused by either IF or ST, a linear discriminant analysis was applied to find the parameters suffering the highest changes in each IF or ST. This was specifically achieved by assessing the linkage between IF or ST (categorical dependent variables) and the matrix of obtained results (quantitative independent variables).

Fig. 1 presents the spatial distribution of IF markers distributed according to the canonical discriminant functions coefficients defined from results obtained in the laboratorial assays. In what concerns the IF effect, the three defined discriminant functions included 100.0% (first function: 97.3%; second function: 2.0%; third function: 0.7%) of the observed variance (Fig. 1). From the 36 analysed variables, the model selected 17: water, protein, sucrose, energy, L^* (fresh and lyophilized), a^* (fresh and lyophilized), b^* (fresh and lyophilized), C6:0, C10:0, C11:0, C13:0, C16:1, C18:1 and C20:4 as those having discriminant effect. As it might be concluded, function 1 included a considerably high percentage of variance, thereby indicating that the variables more correlated with this function (specifically b^* in lyophilized samples, b^* in fresh samples and a^* in fresh samples) were the ones presenting the highest changes within different IF. Accordingly, function 1 separated mostly one group (high b^* values) formed by CI and BSI from a second group (high a^* values) formed by GGI and BVI. Function 2, on the other hand, was more highly correlated to water, sucrose and protein contents, being effective in separating markers corresponding to BVI. Finally,

function 3, despite the low percentage of explained variance, was useful to separate CI from BSI, mostly due to their differences in L^* (fresh and lyophilized), a^* (lyophilized) and energy values. The classification performance was 100% accurate, either for original grouped cases, as well as for the cross-validated grouped cases.

The spatial distribution of ST markers scattered according to the canonical discriminant functions coefficients defined from results obtained in the laboratorial assays is presented in Fig. 2. A completely different outcome was obtained when comparing the effect ST of all parameters simultaneously. In fact, the differences observed in each ST (0, 15, 30, 45 and 60 days) were not enough to discriminate their corresponding markers (Fig. 2). Actually, several results were misclassified (i.e., results from a determined storage time were classified as belonging to a different period) by the leave-one-out validation procedure, proving that ST, up to the assayed periods, did not act as a significant source of variability for nutritional and colour parameters, probably due to the low storage temperature (-22°C).

4. Conclusion

Overall, the extracts obtained from *G. globosa* might be a suitable alternative as natural colouring agents for ice-cream, as the obtained results in GGI and BVI were quite similar. Comparing both studied factors, ST and IF had significant effects in most nutritional parameters, while colour parameters were not affected by ST (except for L^* in lyophilized samples), contrarily to fatty acids, which were mainly altered by ST, showing only minor changes among different IF. Nevertheless, and as evidenced by LDA outputs, the effects of IF were more pronounced than those induced by ST, since markers were only clustered according to the levels of each factor in the case of IF.

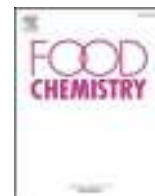
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Optimization of heat- and ultrasound-assisted extraction of anthocyanins from *Hibiscus sabdariffa* calyces for natural food colorants

José Pinela^a, M.A. Prieto^{a,b}, Eliana Pereira^a, Inès Jabeur^a, Maria Filomena Barreiro^{a,c}, Lillian Barros^a, Isabel C.F.R. Ferreira^{a,*}

^a Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal

^b Nutrition and Bromatology Group, Faculty of Food Science and Technology, University of Vigo, Ourense Campus, E32004 Ourense, Spain

^c Laboratory of Separation and Reaction Engineering – Laboratory of Catalysis and Materials (LSRE-LCM), Polytechnic Institute of Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal

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ABSTRACT

Heat- and ultrasound-assisted extraction methods were applied to recover anthocyanins from *Hibiscus sabdariffa* calyces. The extraction variables, time (t), ethanol proportion (S), and temperature (T) or ultrasonic power (P), were combined in a 5-level experimental design and analysed by response surface methodology for process optimization. The delphinidin-3-*O*-sambubioside (C1) and cyanidin-3-*O*-sambubioside (C2) levels were monitored by LC-DAD-ESI/MSⁿ and used as response criteria. The developed models were successfully fitted to the experimental data and used to determine optimal extraction conditions. UAE was the most efficient method yielding 51.76 mg C1 + C2/g R under optimal conditions ($t = 26.1$ min, $P = 296.6$ W and $S = 39.1\%$ ethanol, v/v). The dose-response effects of the solid/liquid ratio on the extraction rate were also determined. The anthocyanin levels herein reported are higher than those found in the literature, which support the potential use of *H. sabdariffa* as a sustainable source of natural colorants with application in different industrial sectors.

1. Introduction

The globalization of the industrial food sector, together with consumer's awareness about the existence of bio-based alternatives to the artificial additives, nowadays massively used, and with potential toxic effects in humans, has promoted the demand for food products formulated with natural ingredients recovered from plant materials (Carocho, Morales, & Ferreira, 2015; Martins, Roriz, Morales, Barros, & Ferreira, 2016). The scientific research in this area has gained international prominence (Almeida et al., 2018; Carocho et al., 2016; Pinela et al., 2017), as it is still necessary to expand the range of natural options and find new sources (e.g., plants, algae and insects), as well as to develop sustainable processes for an efficient recovery of the target compounds (e.g., anthocyanins, carotenoids, and beet derivatives).

The global food colouring market has grown rapidly in recent years and it is expected to continue growing by 10% to 15% annually (Carle & Schweiggert, 2016). The colour, in addition to be an important food sensory attribute, often related to flavour, safety and overall quality, also greatly influences product's marketing success. At the same time, there is a growing interest in replacing the artificially obtained colorants by natural counterparts, since the former have been associated

with adverse health effects, including hyperkinesia, skin rashes, tumours, kidney damage and migraine, among others (Ramesh & Muthuraman, 2018). Natural colorants can also provide an extensive range of colours, with the advantage of being innocuous and can provide beneficial health effects (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009). However, the high stability and low cost of the synthetic food colorants have limited the use of the natural counterparts by the industrial sector (Carocho et al., 2015). Moreover, there are only few natural alternatives approved by federal authorities (Martins et al., 2016).

Plants are an interesting source of natural pigments endowed with colouring potential and bioactivities (Jabeur et al., 2017). Among them, *Hibiscus sabdariffa* L. (Fam. Malvaceae), also known as roselle, is an annual medicinal shrub relatively easy to grow and used worldwide by the food and pharmaceutical industries (Da-Costa-Rocha, Bonnlaender, Sievers, Pischel, & Heinrich, 2014). It comprises two main varieties, the *altissima* Wester, cultivated for the jute-like fibre, and the *sabdariffa*, generally pigmented and cultivated for the edible calyces used in the preparation of herbal teas and beverages, and a number of pastry products (Sharma et al., 2016). In folk medicine, *H. sabdariffa* calyx infusions are used for their diuretic, febrifugal and hypotensive effects,

* Corresponding author.

E-mail address: iferreira@ipb.pt (I.C.F.R. Ferreira).

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and for helping to lower body temperature; while other preparations are used for treating sore throats and coughs, liver, cardiac and nerve diseases, and genital problems (Da-Costa-Rocha et al., 2014). Some of these traditional uses have been validated by scientific studies, which have shown that calyx extracts have strong antioxidant and antihypertensive capacities, together with antihypercholesterolaemic, antinociceptive, and antipyretic effects, among others (Ali, Al Wabel, & Blunden, 2005; Da-Costa-Rocha et al., 2014). Therefore, this plant has high potential to be used in the development of new functional and therapeutic products.

Most of the phytochemical studies on the *H. sabdariffa* constituents have been directed towards the characterization of pigments, namely anthocyanins (Ali et al., 2005; Beye, Hilgsmann, Tounkara, & Thonart, 2017; Jabeur et al., 2017). Delphinidin-3-glucoside, cyanidin-3-glucoside, and in particular delphinidin-3-sambubioside (hibiscin) and cyanidin-3-sambubioside (gossypicyanin) have been identified in calyx extracts (Alarcón-Alonso et al., 2012; Beye et al., 2017; Salazar-González, Vergara-Balderas, Ortega-Regules, & Guerrero-Beltrán, 2012). These anthocyanins are responsible for the characteristic red colour of the *H. sabdariffa* calyces and can be recovered for subsequent use as colorants in different industrial sectors.

In order to turn bio-based colorants into real and efficient alternatives to the widely used artificial analogues, it is necessary to find promising sources for their extraction and develop sustainable recovery processes. Today, several technologies are available to enhance extraction, including ultrasounds (López et al., 2018), microwaves (Liazi, Guerrero, Cantos, Palma, & Barroso, 2011), pulsed electric fields, and pressurized and supercritical fluids (Corrales, Toepfl, Butz, Knorr, & Tauscher, 2008; Garcia-Mendoza et al., 2017). Among them, ultrasound-assisted extraction (UAE) brings significant benefits over conventional heating methods in terms of time and solvent consumption and extraction yield (Chemat et al., 2017a; Marić et al., 2018). This “green” processing technique also reduces energy and water consumption, allows recycling of by-products through bio-refining, and ensures a safe and high quality product (Chemat et al., 2017a). In addition, UAE has been recognised as suitable for industrial applications (Vilkhu, Mawson, Simons, & Bates, 2008). However, the efficiency of these processes is affected by process variables (e.g., time, temperature, ultrasonic power and solvent). Therefore, it is necessary to use appropriate experimental designs and optimization tools to determine the optimal extraction conditions leading to the best responses in terms of recovering of target compounds.

This study was performed aiming at optimizing the recovery of the two major anthocyanins found in *H. sabdariffa* calyces by heat- and ultrasound-assisted extraction processes to serve as natural colorants (a workflow scheme is presented in Fig. A1). The three most relevant independent variables for each process were combined in a circumscribed central composite design, and response surface methodology (RSM) was used for process optimization. It is thus intended to identify which method and extraction conditions are the most suitable to extract these colouring compounds.

2. Material and methods

2.1. Plant material

Dried flowers of *H. sabdariffa* were supplied by a local company (Pragmático Aroma Lda, Alfândega da Fé, Bragança, Portugal) that produces medicinal and aromatic plants with organic certification. According to the producers, the plant material was dehydrated in a drying chamber with controlled conditions of temperature, relative humidity and air velocity, in order to ensure the quality of the final product. The red flower calyx consisting of 5 large sepals with a collar (epicalyx) of 8 to 12 slim, pointed bracts (or bracteoles) around the base was handpicked and reduced to a fine powder (~20 mesh). The powdered samples were kept at -80 °C until further use.

2.2. Extraction methods

2.2.1. Heat-assisted extraction

The heat-assisted extraction (HAE) was performed in a thermostated water bath using sealed vessels to avoid solvent evaporation. The dry powder samples (0.6 g) were mixed with 20 mL of solvent (ethanol:water mixtures) and processed under continuous electromagnetic stirring according to the experimental design presented in Table A1. The extraction time (t , 55–150 min), temperature (T , 20–90 °C) and ethanol proportion (S , 0–100%) were the considered independent variables. The solid/liquid ratio (S/L) was kept constant (30 g/L). After extraction, the mixture was centrifuged (6000 rpm for 10 min at room temperature) and the supernatant filtered through Whatman filter paper No. 4.

2.2.2. Ultrasound-assisted extraction

The ultrasound-assisted extraction (UAE) was performed using an ultrasonic equipment (QSonica sonicators, model CL-334, Newtown, CT, USA). The dry powder samples (1.5 g) were placed in a beaker with 50 mL of solvent (ethanol: water mixtures) and processed according to the experimental design presented in Table A1. The extraction time (t , 3–36.5 min), ultrasonic power (P , 100–500 W; at a frequency of 20 kHz) and ethanol proportion (S 0–100%) were the considered independent variables. The solid/liquid ratio (S/L , 30 g/L) and the temperature (30–35 °C) were kept constant during extraction. Then, the mixtures were centrifuged (6000 rpm for 10 min at room temperature) and the supernatant was filtered through Whatman filter paper No. 4.

A probe system was used for extraction because it delivers the ultrasonic intensity on a small surface compared to an ultrasonic bath, thus being more powerful and widely used for sonication of small volumes of sample (Chemat et al., 2017a; Sicaire et al., 2016). The probe was immersed directly into the reaction beaker (containing the solvent and the sample) so that less attenuation could happen. A special care was taken because of the fast rise of the temperature in the reaction system.

2.3. Determination of extraction yield

The residue or extract weight resulting from each extraction was determined gravimetrically using crucibles, subjected firstly to a partial evaporation of the water at 60 °C and then to heat treatment at 100 °C for 24 h. The results were expressed in percentage (% w/w).

2.4. Chromatographic analysis of anthocyanins

Each obtained solution was subjected to solvent evaporation at 35 °C and the obtained residue redissolved in water and filtered through a 0.22- μ m disposable LC filter disk. The chromatographic analysis was performed in a Dionex Ultimate 3000 UPLC system (Thermo Scientific, San Jose, CA, USA) equipped with a diode array detector coupled with an electrospray ionization mass detector (LC-DAD-ESI/MSⁿ), as previously described by Jabeur et al. (2017). Detection was carried out with a DAD (520 nm as the preferred wavelength) and a MS (Linear Ion Trap LTQ XL mass spectrometer, Thermo Finnigan, San Jose, CA, USA) equipped with an ESI source, working in positive mode. The anthocyanins were characterized according to their UV–Vis and mass spectra, and quantification was performed through a calibration curve performed using cyanidin-3-glucoside standard ($y = 243287x - 1E6$; $R^2 = 0.995$). The results were expressed as mg/g of plant material (P) or residue (R).

2.5. Extraction optimization by response surface methodology

2.5.1. Experimental design

A five-level circumscribed central composite design (CCCD) with three independent variables [X_1 (t , min), X_2 (T , °C or P , W) and X_3 (S ,

%) was applied to optimize the extraction of anthocyanins from *H. sabdariffa* calyces by HAE and UAE. The CCCD included 14 independent combinations and 6 replicates at the centre of the experimental design, chosen to maximize the predictive capacity of the models. In addition, the experimental points were generated on a sphere around the centre point to ensure that the variation of the model prediction is constant for all points equidistant from the centre. The experimental runs were randomized to minimize the effect of unexpected variability in the observed responses.

2.5.2. Response criteria used to understand the extraction behaviour

The extraction yield and levels of delphinidin-3-*O*-sambubioside (C1) and cyanidin-3-*O*-sambubioside (C2), as well as the total amount resulting from the sum of both compounds (CT), were the four response variables considered for the RSM optimization. In addition, the anthocyanin content was expressed using the Y_1 (mg/g P dw) and Y_2 (mg/g R) response formats in order to determine the concentration present in the dried plant material and in the obtained residue or extract, respectively.

2.5.3. Mathematical model

The response surface models were fitted by means of least squares calculation using the following second-order polynomial equation:

$$Y = b_0 + \sum_{i=1}^n b_i X_i + \sum_{i=1}^{n-1} \sum_{j=2}^n b_{ij} X_i X_j + \sum_{i=1}^n b_{ii} X_i^2 \quad (1)$$

where Y is the dependent (response) variable to be modelled, X_i and X_j define the independent variables, b_0 is the constant coefficient, b_i the coefficient of the linear effect, b_{ij} the coefficient of the interaction effect, b_{ii} the coefficient of the quadratic effect, and n is the number of variables.

2.5.4. Procedure to optimize the variables to a maximum response

The maximization of the model-produced responses was achieved using a simple method tool to solve non-linear problems (Heleno et al., 2016; Pinela et al., 2016). Limitations were made to the variable coded values to avoid unnatural conditions (i.e., time < 0).

2.6. Dose-response analysis of the solid/liquid ratio effect

After optimizing the experimental conditions for the variables X_1 , X_2 and X_3 , the solid/liquid ratio (S/L , g/L) was included as the fourth variable (X_4) to be optimized in order to design more productive and sustainable processes, as demanded by the industrial sector. The response effects as function of the S/L variation showed linear trends and were depicted using a general linear equation with intercept ($Y = b + mS/L$), where Y is the used response criteria (i.e. if Y_1 the units would be mg/g P dw) and b and m are the parameters (intercept and slope, respectively). The rate of the process parameter (m , if assessing the Y_1 response criterion the units would be mg/g P per g/L) provides information related to the extraction as function of S/L increase. Positive values will indicate an increase in the extraction responses, whereas negative values will designate a decrease in the extraction efficiency, as the S/L increase.

2.7. Fitting procedures and statistical analysis

The statistical analysis of the experimental results and models fitting was performed in three steps, using a Microsoft Excel spreadsheet, as follows:

(1) The measurement of the coefficients was achieved using the non-linear least-square (quasi-Newton) method provided by the macro “Solver” (Kemmer & Keller, 2010), by minimization of the sum of

the quadratic differences between the observed and model-predicted values.

- (2) The significance of the coefficients was obtained via “SolverAid” macro (de Levie, 2012) to determine the parametric confidence intervals. The terms that were not statistically significant (p -value > 0.05) were excluded to simplify the model.
- (3) The model reliability was confirmed by applying the following criteria: (a) the Fisher F -test ($\alpha = 0.05$) was used to determine the consistency of the constructed models to describe the obtained data (Shi & Tsai, 2002); (b) the “SolverStat” macro was used to make an assessment of the parameter and model prediction uncertainties (Comuzzi, Polese, Melchior, Portanova, & Tolazzi, 2003); (c) R^2 was determined to explain the variability proportion of the dependent variable obtained by the model.

3. Results and discussion

3.1. Experimental data for RSM optimization

Although some previous studies on the extraction of anthocyanins from *H. sabdariffa* calyces can be found in literature, no reports detailing the optimal conditions maximizing their extraction are presently available. In addition, the compositional diversity of anthocyanins' natural sources (e.g., fruits, flowers, leaves, stems and roots) does not allow to directly extrapolate the extraction conditions of these pigments from previously studied sources. Therefore, it is important to conduct independent studies to maximize the extraction of anthocyanins from *H. sabdariffa*, by selecting the relevant variables for each selected extraction method. Table 1 provides a bibliographical summary of the delphinidin-3-*O*-sambubioside, cyanidin-3-*O*-sambubioside and total anthocyanin levels in *H. sabdariffa* and other major plant sources, as well as the conditions used for their extraction. Although important conclusions can be derived from this summary, the results may be highly dependent on dissimilarities not foreseen in these studies, where certain variables remaining constant, together with raw-material's variability, can definitely influence the extraction process. Therefore, the first approach to optimize the efficiency of the HAE and UAE processes to recover anthocyanins from *H. sabdariffa* calyces consisted of the application of RSM coupled to a CCCD design with five levels of variation for the three independent variables as follows: t (30–150 min), T (30–90 °C) and S (0–100%) for HAE and t (3–45 min), P (100–500 W) and S (0–100%) for UAE. A detailed description of the coded and natural values of the selected variables for each extraction method in the CCCD design is presented in Table A1. The different steps carried out in this optimization study are illustrated in Fig. A1.

According to previous studies, high P can cause major alterations in plant materials by inducing greater shear forces, which result from the oscillation and collapse of cavitation bubble within the solvent. As a consequence, critical temperature and pressure are generated, inducing the formation of free radicals that can attack target metabolites and lead to their degradation (Chemat et al., 2017; Meullemiestre, Breil, Abert-Vian, & Chemat, 2016). However, this independent variable was optimized in order to apply the minimum power required to achieve the best results.

The experimental values obtained under the 20 runs of the five-level CCCD design applied to the HAE and UAE processes used in the recovery of anthocyanins from *H. sabdariffa* calyces are presented in Table 2. The different response criteria used (yield, Y_1 and Y_2) are of interest for industrial sectors dealing with the recovering of high added-value compounds from plant materials to be used as natural colorants, or other bio-based ingredients, providing information concerning the amount of plant material needed to obtain a certain quantity of the target compounds, and the concentration of these compounds in the produced extracts.

The extraction yield ranged from 34.45 to 62.80% and 14.38–56.21% for HAE and UAE, respectively (Table 2). In both cases,

Table 1
Bibliographical summary of the delphinidin-3-O-sambubioside, cyanidin-3-O-sambubioside and total anthocyanin levels in *H. sabdariffa* and other major plant species, and conditions used in their extraction.

Plant material Species and common name	Extraction conditions					Solvent		Content (mg/g)	Reference
	Used part	T (°C)	P (W)	t (min)	S/L (g/L)	Solvent type and proportion (v/v)	Acidification		
<i>Delphinidin-3-O-sambubioside</i>									
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	~25	–	120	100	Water	–	0.55 ± 0.07	R Salazar-González et al. (2012)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	100	–	5	5	Water	–	7.0 ± 0.2	R Jabeur et al. (2017)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	100	–	10	10	Water	–	4.11 ± 1.47	R Sindi et al. (2014)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	100	–	10	10	Water	1% formic acid	3.68 ± 0.34	R Sindi et al. (2014)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	~25	–	120	100	Ethanol/water (50:50)	–	1.33 ± 0.21	R Salazar-González et al. (2012)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	~25	–	120	100	Ethanol/water (70:30)	–	0.80 ± 0.14	R Salazar-González et al. (2012)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	25	–	60 × 2	33.3	Ethanol/water (80:20)	–	7.03 ± 0.04	R Jabeur et al. (2017)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	~25	–	120	100	Ethanol/water (96:4)	–	0.17 ± 0.02	R Salazar-González et al. (2012)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	~25	–	120	100	Ethanol/acidified water (85:15)	1.5 N HCl	0.50 ± 0.10	R Salazar-González et al. (2012)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	64,7	–	10	10	Methanol	–	2.26 ± 0.07	R Sindi et al. (2014)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	64,7	–	10	10	Methanol	1% formic acid	2.41 ± 0.09	R Sindi et al. (2014)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	50	–	30 × 3	20	Water	–	16.1–21.2	P dw Ifie et al. (2018)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	~25	–	60 + 60 × 5	83.3	Acetone + acetone/acidified water (70:30)	4% formic acid	3.54–9.19	P dw Beye et al. (2017)
<i>Vaccinium myrtillus</i> L. (bilberry)	Fruit	–	–	–	–	Methanol	0.5% HCl	0.26	R Du, Jerz, and Winterhalter (2004)
<i>Aristolelia chilensis</i> L. (maqui)	Fruit	~25	?	60 × 2	100	Methanol/water (70:30)	1% formic acid	0.19–0.73	P dw Gironés-Vilaplana et al. (2014)
<i>Cyanidin-3-O-sambubioside</i>									
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	~25	–	120	100	Water	–	0.31 ± 0.04	R Salazar-González et al. (2012)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	100	–	5	5	Water	–	4.08 ± 0.07	R Jabeur et al. (2017)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	100	–	10	10	Water	–	3.81 ± 1.21	R Sindi et al. (2014)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	100	–	10	10	Water	1% formic acid	2.98 ± 0.21	R Sindi et al. (2014)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	~25	–	120	100	Ethanol/water (50:50)	–	0.75 ± 0.09	R Salazar-González et al. (2012)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	~25	–	120	100	Ethanol/water (70:30)	–	0.45 ± 0.09	R Salazar-González et al. (2012)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	25	–	60 × 2	33.3	Ethanol/water (80:20)	–	4.40 ± 0.02	R Jabeur et al. (2017)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	~25	–	120	100	Ethanol/water (96:4)	–	0.11 ± 0.01	R Salazar-González et al. (2012)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	~25	–	120	100	Ethanol/acidified water (85:15)	1.5 N HCl	0.27 ± 0.06	R Salazar-González et al. (2012)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	64,7	–	10	10	Methanol	–	1.96 ± 0.03	R Sindi et al. (2014)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	64,7	–	10	10	Methanol	1% formic acid	2.10 ± 0.02	R Sindi et al. (2014)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	50	–	30 × 3	20	Water	–	3.06–5.17	P dw Ifie et al. (2018)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	~25	–	60 + 60 × 5	83.3	Acetone + acetone/acidified water (70:30)	4% formic acid	2.52–4.72	P dw Beye et al. (2017)
<i>Vaccinium myrtillus</i> L. (bilberry)	Fruit	–	–	–	–	Methanol	0.5% HCl	0.15	R Du et al. (2004)
<i>Aristolelia chilensis</i> L. (maqui)	Fruit	~25	*	60 × 2	100	Methanol/water (70:30)	1% formic acid	0.23–0.82	P dw Gironés-Vilaplana et al. (2014)
<i>Ribes rubrum</i> L. (red currant)	Fruit	–	–	? × 10	14	Methanol	1% HCl	0.43–0.60	P dw [†] Yang, Zheng, Laaksonen, Tahvonon, and Kallio (2013)
<i>Vigna unguiculata</i> ssp. <i>sesquipedalis</i> L. (yard-long bean)	Purple pod	4	–	2880	50	Methanol/water (40:60)	0.1% HCl	0.16	P dw Tae et al. (2010)
<i>Total anthocyanin content</i>									
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	100	–	5	5	Water	–	12.3 ± 0.3	R Jabeur et al. (2017)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	100	–	10	10	Water	–	8.53	R Sindi et al. (2014)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	100	–	10	10	Water	1% formic acid	7.36	R Sindi et al. (2014)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	25	–	60 × 2	33.3	Ethanol/water (80:20)	–	12.9 ± 0.1	R Jabeur et al. (2017)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	64,7	–	10	10	Methanol	–	4.88	R Sindi et al. (2014)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	64,7	–	10	10	Methanol	1% formic acid	5.14	R Sindi et al. (2014)
<i>Hibiscus sabdariffa</i> L. (roselle)	Petals	35	–	120 × 2	2.5	Methanol	0.1% HCl	33.92 ± 3.16	R Zhang et al. (2014)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	50	–	30 × 3	20	Water	–	19.57–27.10	P dw Ifie et al. (2018)
<i>Hibiscus sabdariffa</i> L. (roselle)	Calyx	~25	–	60 + 60 × 5	83.3	Acetone + acetone/acidified water (70:30)	4% formic acid	6.88–15.29	P dw Beye et al. (2017)
<i>Arbutus unedo</i> L. (strawberry tree)	Fruit	< 35	215.1	21.8	50	Ethanol/water (64.3:35.7)	0.05% HCl	0.49 ± 0.03	R López et al. (2018)
<i>Aristolelia chilensis</i> L. (maqui)	Fruit	~25	*	60 × 2	100	Methanol/water (70:30)	1% formic acid	6.14–9.84	P dw Gironés-Vilaplana et al. (2014)

(continued on next page)

Table 1 (continued)

Plant material	Extraction conditions				S/L (g/L)	Solvent	Acidification	Content (mg/g)	Reference
	Used part	T (°C)	P (W)	t (min)					
<i>Ipomoea batatas</i> L. (purple sweet potato)	Tuber	60	-	90	100	Ethanol/water (80:20)	0.1% HCl	2.18 ± 0.03	Cai et al. (2016)
<i>Ipomoea batatas</i> L. (purple sweet potato)	Tuber	50	200	45	100	Ethanol/water (90:10)	0.1% HCl	2.29 ± 0.05	Cai et al. (2016)
<i>Prunus avium</i> L. (sweet cherry)	Fruit	37	-	90	100	Methanol or ethanol	0.1% 12 N HCl	2.49 ± 0.04	Blackhall et al. (2018)
<i>Vigna unguiculata</i> ssp. <i>sesquipedalis</i> L. (yard-long bean)	Purple pod	4	-	2880	50	Methanol/water (40:60)	0.1% HCl	8.81	Tae et al. (2010)

P: Plant material; R: residue or extract; dw: dry weight; fw: fresh weight.

¹ Converted from a fresh weight value.

* Samples sonicated in an ultrasonic bath (power not indicated).

the higher values were achieved with the run 12, which combined the following conditions: $t = 90$ min, $T = 90$ °C and $S = 50\%$ for HAE, and $t = 24$ min, $T = 500$ W and $S = 50\%$ for UAE. In general, HAE originated the highest experimental yields, translated in higher amounts of residue or crude extract. A lower yield (28.3%) was obtained by Alarcón-Alonso et al. (2012) when using water at 55 °C with 2 h of extraction.

As described by other authors (Ifie, Ifie, Ibitoye, Marshall, & Williamson, 2018; Jabeur et al., 2017; Sindi, Marshall, & Morgan, 2014), C1 predominated over C2 in all cases (Table 2), with levels ranging from 1.02–6.88 mg/g P and 0.43–2.11 mg/g P for HAE and from 1.26 to 14.31 mg/g P and 0.92–7.29 mg/g P for UAE, respectively. The highest total content (CT) was achieved with the runs 13 (8.99 mg/g P) and 10 (21.48 mg/g P) of the HAE and UAE processes, respectively. Beye et al. (2017) recovered up to 9.19 mg C1/g P and 4.72 mg C2/g P from dried calyces of *H. sabdariffa* cultivars grown in Senegal, when subjected to a 60-min extraction with acetone followed by five more 60-min extraction cycles with acetone/acidified water (70:30, v/v; 4% formic acid). Higher levels of C1 (up to 21.2 mg/g P), but lower of C2 (up to 5.17 mg/g P) were obtained by Ifie et al. (2018) in dried calyces of a dark red variety of *H. sabdariffa* cultivated in Ibadan, South-Western Nigeria, in comparison with the quantities found in our samples harvested in north-eastern Portugal. These contents were obtained by three successive aqueous extractions of 30-min duration at 50 °C.

The CT content in the obtained residue ranged from 3.41 to 12.44 mg/g R and 14.39–47.93 mg/g R for HAE and UAE, respectively (Table 2). In general, these levels are higher than those found in the literature (Table 1). Salazar-González et al. (2012) obtained up to 2.08 mg CT/g P (1.33 mg C1/g and 0.75 mg C2/g) when performing a 120-min extraction with ethanol:water mixtures (50:50, v/v) at a S/L of 100 g/L and room temperature. Up to 7.92 mg CT/g R (4.11 mg C1/g and 3.81 mg C2/g) were achieved by Sindi et al. (2014) with aqueous extractions of 10 min at 100 °C. In another study, Jabeur et al. (2017) quantified 11.08 mg/g R (7.0 mg C1 + 4.08 mg C2) in extracts obtained by addition of boiling water (at 100 °C) to the samples and subsequent maceration for 5 min at room temperature (process known as infusion).

3.2. Theoretical response surface models

The parametric values obtained by fitting the response values (Table 2) to the second-order polynomial model of Eq. (1) using a nonlinear algorithm are presented in Table A2 (Eqs. (2)–(15)). These values translate the response patterns and are useful for developing mathematical models (Table A3), which indicate the complexity of the possible scenarios. However, not all Eq. (1) parameters were used in the development of the models since some coefficients were non-significant (ns); the significant ones were assessed at a 95% confidence level ($\alpha = 0.05$). The statistic lack-of-fit, used to test the adequacy of the obtained models, revealed that no considerable improvement was achieved by the inclusion of the statistically ns parametric values. The agreement between the experimental and predicted values provided an acceptable explanation of the obtained results (Table 2). Additionally, residues were randomly scattered around zero and no grouped data or auto-correlations were observed. The obtained coefficients of determination (R^2) were higher than 0.94 and 0.86 in the cases of HAE and UAE, respectively (Table A2), which indicates that the variability of each response can be explained by the independent variables involved in the process. Therefore, the models proved to be applicable and were used in the later prediction and optimization steps. Although the obtained model coefficients are empirical and cannot be associated with physical or chemical significance, they are useful to predict the outcome of untested experimental conditions (Ranic et al., 2014). Moreover, the sign of the parametric values determines part of the response; for positive effects, the response is higher at the high level, and when a factor has a negative effect, the response is lower at the high level. The higher of the parametric value, the more significant the weight of the

Table 2
Experimental CCCD design and results for the HAE and UAE processes used in the extraction of delphinidin-3-O-sambubioside (C_1), cyanidin-3-O-sambubioside (C_2), and total anthocyanins (C_T) from *H. sabdariffa* calyces. The extraction yield is given as percentage (%) and the anthocyanin content is expressed as mg/g of plant material (Y_1 , mg/g P dw) and mg/g of residue (Y_2 , mg/g R). The independent variables, natural values and ranges are presented in Table A1.

Experimental design			Heat-assisted extraction (HAE)									Ultrasound-assisted extraction (UAE)											
Coded values			HAE			UAE			Residue	Individual anthocyanins				Total anthocyanins		Residue	Individual anthocyanins				Total anthocyanins		
X_1	X_2	X_3	X_1 : t min	X_2 : T °C	X_3 : S %	X_1 : t min	X_2 : P W	X_3 : S %	Yield	Y_1C_1	Y_1C_2	Y_2C_1	Y_2C_2	Y_1C_T	Y_2C_T	Yield	Y_1C_1	Y_1C_2	Y_2C_1	Y_2C_2	Y_1C_T	Y_2C_T	
1	-1	-1	-1	54.3	42.2	20.3	11.5	181.1	20.3	51.85	5.01	1.67	9.67	3.22	6.68	12.89	18.78	5.16	2.59	27.45	15.54	7.75	42.99
2	1	-1	-1	125.7	42.2	20.3	36.5	181.1	20.3	55.67	4.72	1.86	8.47	3.34	6.58	11.82	35.24	9.14	3.08	25.94	9.84	12.22	35.78
3	-1	1	-1	54.3	77.9	20.3	11.5	418.9	20.3	53.80	4.28	1.86	7.96	3.46	6.14	11.42	27.16	3.18	3.90	11.71	16.17	7.08	27.88
4	1	1	-1	125.7	77.9	20.3	36.5	418.9	20.3	59.73	4.37	1.79	7.32	3.00	6.16	10.32	49.27	12.51	5.79	25.39	13.23	18.30	38.62
5	-1	-1	1	54.3	42.2	79.8	11.5	181.1	79.7	42.92	1.66	0.66	3.88	1.54	2.33	5.42	16.35	3.75	0.97	22.93	6.67	4.72	29.60
6	1	-1	1	125.7	42.2	79.8	36.5	181.1	79.7	43.44	1.70	0.61	3.92	1.39	2.31	5.32	39.00	5.26	3.00	13.49	8.67	8.26	22.16
7	-1	1	1	54.3	77.9	79.8	11.5	418.9	79.7	51.55	1.50	0.57	2.92	1.11	2.08	4.03	14.38	1.26	0.92	8.78	7.16	2.18	15.94
8	1	1	1	125.7	77.9	79.8	36.5	418.9	79.7	50.12	1.51	0.51	3.02	1.02	2.03	4.04	51.24	9.06	5.01	17.68	11.01	14.07	28.68
9	-1.68	0	0	30	60	50	3	300	50	46.29	1.10	0.84	2.37	1.82	1.94	4.19	23.28	6.62	2.04	28.45	9.88	8.67	38.33
10	1.68	0	0	150	60	50	45	300	50	49.00	1.10	0.78	2.26	1.59	1.88	3.84	55.38	14.31	7.17	25.84	14.58	21.48	40.41
11	0	-1.68	0	90	30	50	24	100	50	55.82	1.54	1.03	2.76	1.85	2.57	4.61	15.71	3.06	1.52	19.48	10.88	4.58	30.36
12	0	1.68	0	90	90	50	24	500	50	62.80	1.21	0.93	1.93	1.48	2.14	3.41	56.21	12.73	4.80	22.65	9.62	17.53	32.26
13	0	0	-1.68	90	60	0	24	300	0	55.30	6.88	2.11	12.44	3.82	8.99	16.26	30.03	7.26	2.61	24.17	9.77	9.86	33.94
14	0	0	1.68	90	60	100	24	300	100	34.45	1.31	0.43	3.82	1.26	1.75	5.07	32.69	3.21	1.33	9.82	4.57	4.54	14.39
15	0	0	0	90	60	50	24	300	50	55.22	1.06	0.91	1.92	1.64	1.97	3.56	37.23	10.06	6.68	27.02	20.19	16.74	47.21
16	0	0	0	90	60	50	24	300	50	55.37	1.02	0.96	1.84	1.73	1.98	3.57	37.36	10.28	6.54	27.51	19.71	16.82	47.22
17	0	0	0	90	60	50	24	300	50	57.32	1.34	0.96	2.33	1.68	2.30	4.01	37.49	10.14	6.76	27.06	20.30	16.90	47.35
18	0	0	0	90	60	50	24	300	50	56.72	1.23	0.89	2.16	1.58	2.12	3.74	38.51	10.25	7.29	26.61	21.32	17.54	47.93
19	0	0	0	90	60	50	24	300	50	51.10	1.21	0.91	2.36	1.79	2.12	4.15	38.08	10.35	6.92	27.19	20.44	17.27	47.63
20	0	0	0	90	60	50	24	300	50	56.12	1.25	0.92	2.23	1.63	2.17	3.86	37.90	10.49	6.68	27.66	19.83	17.16	47.49

governing variable is.

Certain features regarding the overall effects of the independent variables were inferred from the complexity of the parametric values, i.e. the variables were ordered in a decreasing form as a function of its significance in the extraction processes as follows: $S > > T > t$ for HAE; $S > P > t$ for the Y_2 response formats of UAE; and $t > P > S$ for the extraction yield of UAE. It was also possible to observe that all the evaluated responses were significantly affected by linear and quadratic effects, whose values were particularly higher for the variable S (with some exceptions, since, although the linear effects of the variable t were negligible in most cases in the HAE process, the corresponding values for the Y_1 response formats and extraction yield in the UAE process were quite high). The parametric values also revealed that strong interactions occurred in the UAE process, mainly between the variables $t \times P$. In turn, the interactions $t \times T$ and $P \times S$ were of minor relevance in the HAE and UAE processes, respectively. These results justify the use of RSM as an optimization tool, since one-variable-at-a-time approaches do not allow to assess the existence of interactive effects, which makes it difficult to determine optimum values.

To make all these combined effects more explicit and to visually describe the extraction trends, the results were presented in the response surface graphs discussed below.

3.3. Response surface analysis: Efficiency of the extraction conditions and methods

Fig. 1 shows the 3D response surface graphs of the extraction yield (residue) and total anthocyanin content (expressed in terms of mg/g of

plant material (Y_1) and mg/g of residue (Y_2)) obtained for both extraction methods (HAE and UAE). The net surfaces were predicted with the second-order polynomial model of Eq. (1), whose model equations are presented in Table A3. The binary actions between the variables are displayed when the excluded variable is positioned at the centre of the experimental domain (Table A1). Additionally, the goodness of fit of the model is illustrated by the ability to simulate response changes between the observed and predicted data, and the residual distribution as a function of each variable. In turn, Fig. 2 shows the 2D contour graphs resulting from the projections of the 3D response surfaces in the XY plane. These projections focused the optimal extraction conditions obtained for the residue (yield), delphinidin-3-O-sambubioside (C1) and cyanidin-3-O-sambubioside (C2), depending on the used extraction method. For each anthocyanin, the result is expressed in terms of mg/g of plant material (Y_1) and mg/g of residue (Y_2) to visually describe the extraction trends. The binary actions between variables are displayed when the excluded variable is positioned at the individual optimum (Table 3).

3.3.1. Extraction yield

After analysing the response surface and contour graphs shown in Figs. 1 and 2, it was possible to draw some conclusions regarding the effects of the independent variables on the extraction yield. For HAE, the variable S originated the most marked curvatures on the net surfaces, followed by the variable t . In the first case, the extraction was promoted with the increase in ethanol proportion up to 35% with subsequent decrease. The variable t had a comparable behaviour, since the medium-long extraction times were the most suitable ones. In turn,

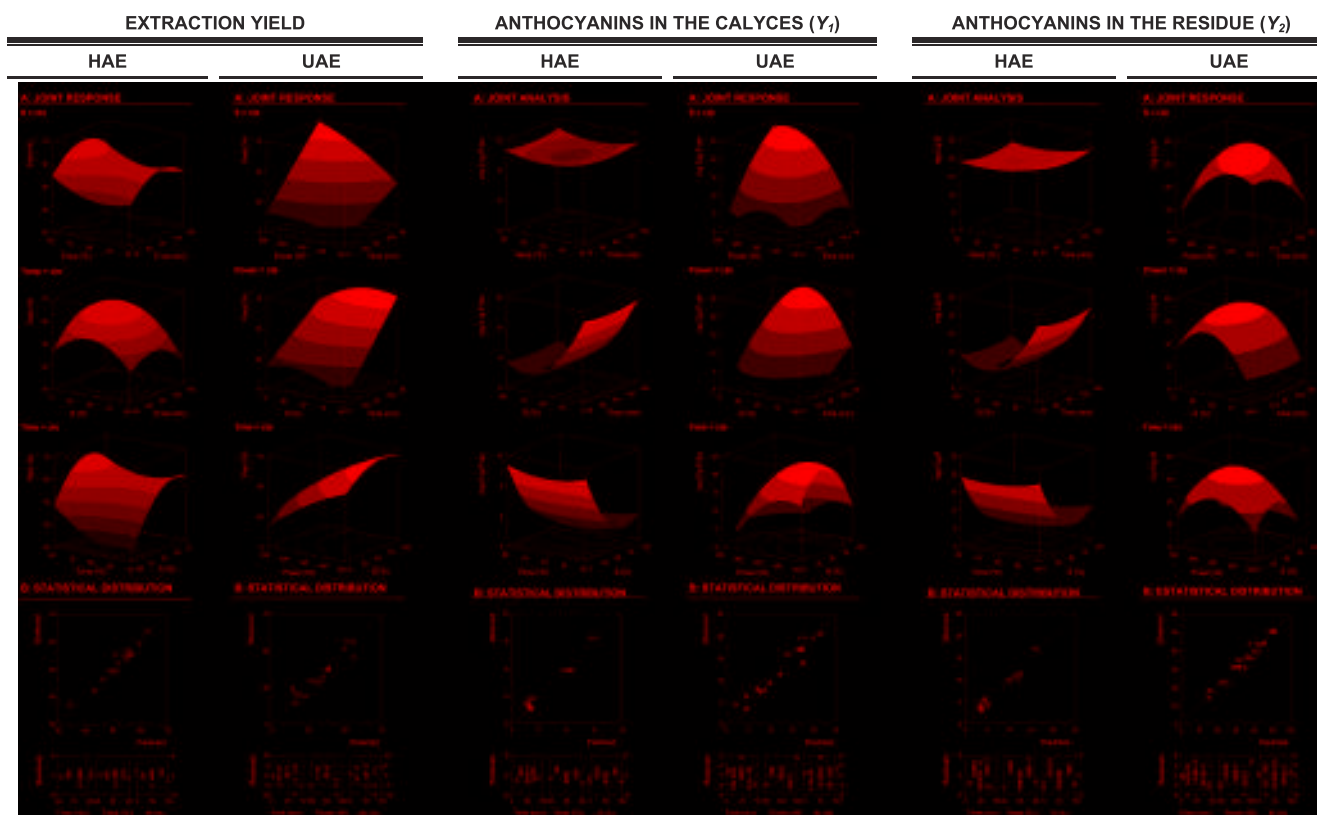


Fig. 1. Response surface graphs of the extraction yield (residue, %) and total anthocyanin content (expressed in terms of mg/g of plant material (Y_1) and mg/g of residue (Y_2)), obtained for both extraction methods (HAE and UAE). Part A: Joint graphical analysis as a function of the involved variables. Each net surface represents the 3D response surface predicted with the second-order polynomial model of Eq. (1) as a function of each variable and described by the equations given in Table A3. The binary actions between the variables are presented when the excluded variable is positioned at the centre of the experimental domain (Table A1). The experimental design and results are presented in Table 2 and the estimated parametric values are shown in Table A2. Part B: Illustration of the goodness of fit through two graphical statistic criteria, namely the ability to simulate response changes between the observed and predicted data, and the residual distribution as a function of each variable.

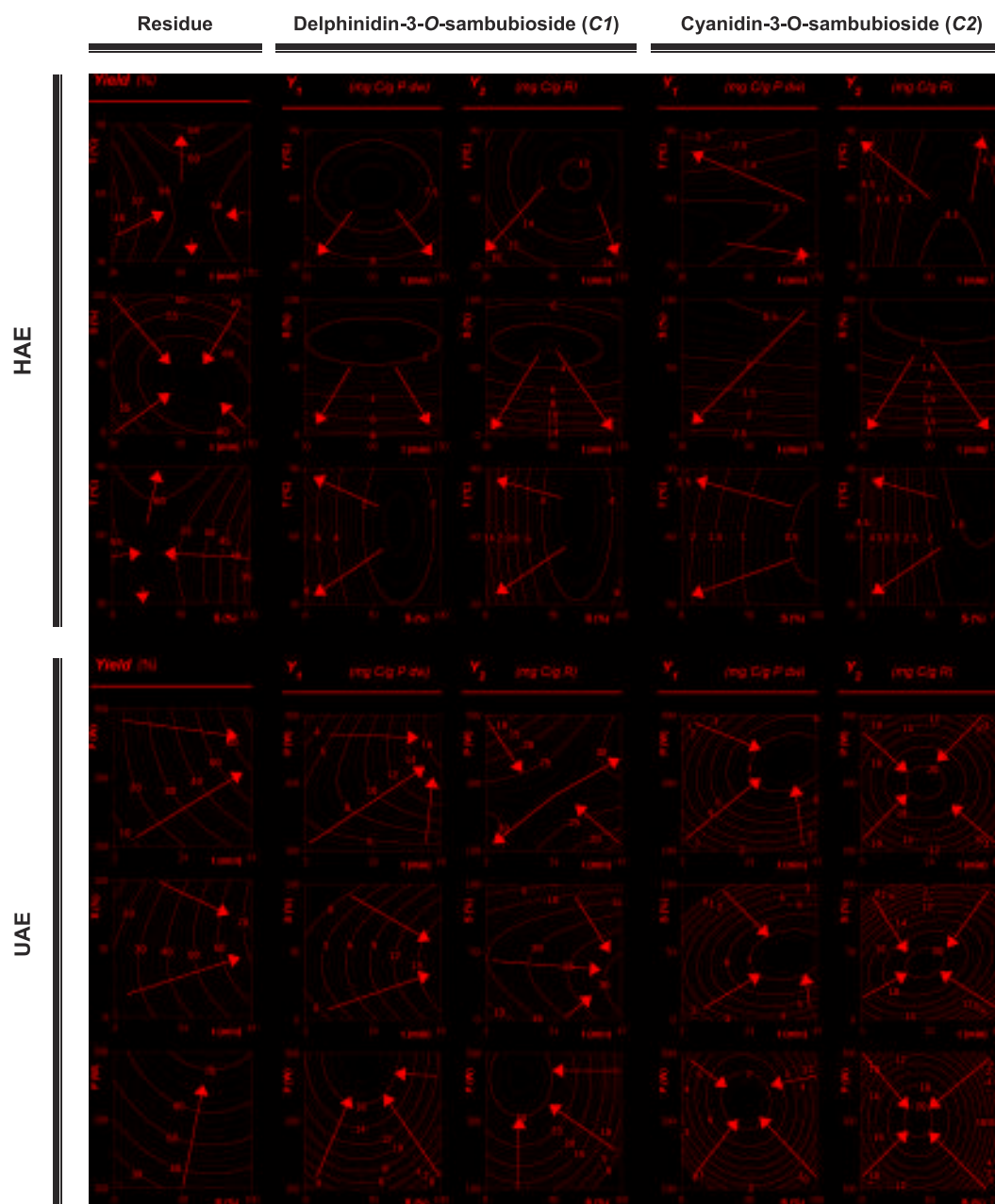


Fig. 2. 2D contour graphs focusing the optimal points for the extraction yield (residue) and levels of delphinidin-3-O-sambubioside (C1) and cyanidin-3-O-sambubioside (C2) obtained by HAE and UAE. For each anthocyanin, the result is expressed in terms of mg/g of plant material (Y_1) and mg/g of residue (Y_2) to visually describe the extraction trends. Each contour graph represents the projection of the theoretical 3D response surface predicted by the second-order polynomial model of Eq. (1) in the XY plane. The binary actions between variables are presented when the excluded variable is positioned at the individual optimum (Table 3). The experimental design and results are presented in Table 2 and the estimated parametric values are shown in Table A2.

higher T provided the highest extraction yields. The amount of residue was also affected by the interaction of the variable S with t (negative interaction) and T (positive interaction) (Table A2). In the UAE process, the most marked response surface curvatures were caused by the tested ranges of t and P , whose increase led to the higher extraction yields (Table A2). Additionally, the obtained yield was also promoted by the strong positive interaction between t and the other two variables (Table A2). Therefore, based on the optimal extraction conditions presented in Table 3, it was observed that HAE required a longer t and a lower S than UAE (while both methods required the highest tested T or P) to obtain optimum response values. The best yield ($73.30 \pm 4.51\%$) was

obtained with the UAE process under the following conditions: $t = 45.0$ min, $P = 500.0$ W, and $S = 71.4\%$ ethanol, v/v. Comparable results were obtained by López et al. (2018) when optimizing the HAE and UAE of anthocyanins from *Arbutus unedo* fruits, as they also associated the highest extraction yields with UAE process.

3.3.2. Anthocyanin contents

The variable S was the one that affected most the HAE of anthocyanins from *H. sabdariffa* calyces (Table A2), as verified from the obtained extraction yield with this method. Its significance is visually highlighted in the response surface graphs (Fig. 1), which show that the

Table 3

Operating conditions that maximize the amounts of residue (*yield*), delphinidin-3-*O*-sambubioside (C1), cyanidin-3-*O*-sambubioside (C₂), and total anthocyanins (C_T) extracted from *H. sabdariffa* calyces as a function of the extraction method (HAE and UAE) and response value format (Y₁, mg/g P, and Y₂, mg/g R).

Criteria		Optimal extraction conditions			Response optimum				
		X ₁ : t (min)	X ₂ : T (°C) or P(W)	X ₃ : S (%)					
<i>(A) Individual optimal extraction conditions</i>									
HAE	Yield		101.5	90.0	35.4	64.74	± 3.60	%	
		Y ₁	C1	30.0	30.0	0.0	8.57	± 2.07	mg C1/g P dw
			C2	30.0	90.0	0.0	2.66	± 1.15	mg C2/g P dw
	CT		30.0	30.0	0.0	10.61	± 3.26	mg CT/g P dw	
	Y ₂	C1	30.0	30.0	0.0	16.81	± 2.37	mg C1/g R	
		C2	30.0	90.0	0.0	4.59	± 1.24	mg C2/g R	
		CT	30.0	30.0	0.0	20.86	± 1.24	mg CT/g R	
	UAE	Yield		45.0	500.0	71.4	73.30	± 4.51	%
			Y ₁	C1	45.0	500.0	40.9	17.90	± 2.44
C2				36.0	360.0	47.9	7.64	± 1.13	mg C2/g P dw
CT		45.0		432.3	42.8	23.83	± 2.44	mg CT/g P dw	
Y ₂		C1	45.0	426.9	26.1	32.39	± 3.29	mg C1/g R	
		C2	22.7	300.0	41.7	20.55	± 3.21	mg C2/g R	
		CT	26.1	296.6	39.1	51.76	± 3.70	mg CT/g R	
<i>(B) Global optimal extraction conditions</i>									
HAE		Yield		30.0	30.0	0.0	44.85	± 8.12	%
	Y ₁		C1				8.55	± 1.73	mg C1/g P dw
			C2				2.26	± 0.29	mg C2/g P dw
		CT				10.60	± 1.11	mg CT/g P dw	
	Y ₂	C1				16.79	± 2.18	mg C1/g R	
		C2				4.47	± 0.86	mg C2/g R	
CT					20.83	± 2.76	mg CT/g R		
UAE	Yield		42.9	386.3	46.1	61.21	± 6.21	%	
		Y ₁	C1				16.17	± 1.22	mg C1/g P dw
			C2				7.38	± 1.91	mg C2/g P dw
	CT					23.08	± 2.96	mg CT/g P dw	
	Y ₂	C1				29.72	± 3.19	mg C1/g R	
		C2				12.76	± 1.91	mg C2/g R	
CT					47.57	± 4.37	mg CT/g R		

higher amounts were obtained when water ($S = 0\%$) was used as the extraction solvent. In turn, the effects of t and T were less marked, with the lowest tested ranges leading to the highest total anthocyanin values. For this method, the extraction conditions originating the higher response values were similar, regardless of the considered response format, except for the variable T . Table 3 shows that it was possible to obtain 8.57 ± 2.07 mg/g P and 2.66 ± 1.15 mg/g P of C1 and C2 from *H. sabdariffa* calyces, respectively, when applying the following HAE conditions: $t = 45.0$ min, $S = 0.0\%$ ethanol, v/v, and $T = 30$ or 90 °C for C1 and C2, respectively. The residue or extract obtained under the same extraction conditions contained approximately the double amount of C1 (16.81 ± 2.37 mg/g R) and C2 (4.59 ± 1.24 mg/g R).

The extraction of anthocyanins followed a different trend when using UAE. Fig. 1 shows that all variables caused accented response surface curvatures, as also indicated by the corresponding parametric values (Table A2). Moreover, contrary to the one verified for HAE, the extraction conditions differed according to the considered response format. However, in both cases, there was a strong $t \times P$ interaction with a positive impact on the obtained total anthocyanin contents, especially for Y₂. Interactions between t and S were also noted. Applying this method, it was possible to recover 17.90 ± 2.44 mg/g P and 7.64 ± 1.13 mg/g P of C1 and C2, respectively, from the dried red flower calyces (Table 3). In turn, 1 g of residue contained 32.39 ± 3.29 mg of C1 and 20.55 ± 3.21 mg of C2 when using the following conditions: $t = 45.0$ min, $P = 426.9$ W and $S = 26.1\%$ ethanol, v/v, and $t = 22.7$ min, $P = 300.0$ W and $S = 41.7\%$ ethanol, v/v, respectively. All these values were much higher than those obtained by HAE (approximately twice). Moreover, the effectiveness of the applied UAE process was highlighted since the obtained anthocyanin

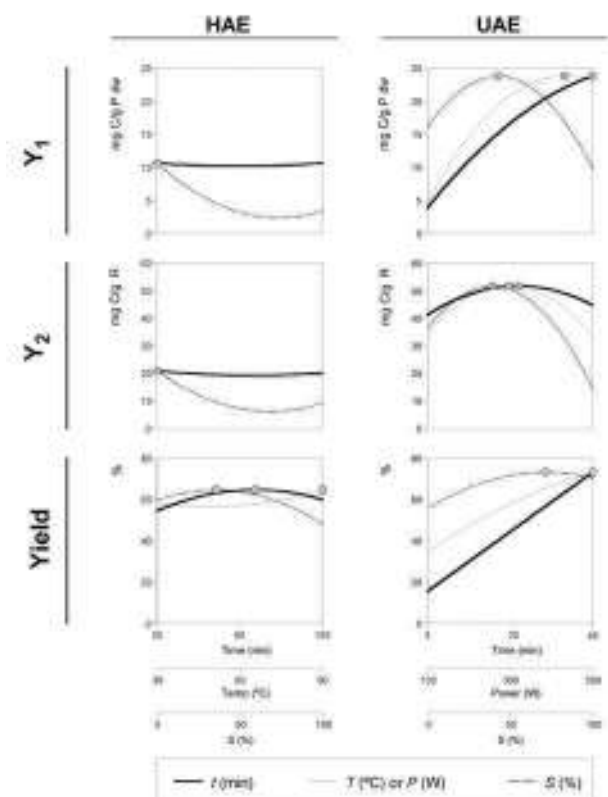
levels are much higher than those reported in other studies (Table 1).

The *H. sabdariffa* calyces are a promising source of anthocyanins for potential use as natural red colorants, since the amounts achieved by applying the optimal extraction conditions are superior to those already found in the fruits of *Prunus avium* L. (sweet cherry, 2.49 mg/g P fw) (Blackhall, Berry, Davies, & Walls, 2018) and *Aristotelia chilensis* L. (maqui, 9.84 mg/g P dw) (Gironés-Vilaplana et al., 2014), tubers of *Ipomoea batatas* L. (purple sweet potato, 2.29 mg/g P dw) (Cai et al., 2016), and purple pods of *Vigna unguiculata* ssp. *sesquipedalis* L. (yard-long bean, 8.81 mg/g P dw) (Tae et al., 2010). The anthocyanin-based colorants can replace the artificial counterparts and provide health-promoting effects (Castañeda-Ovando, de Pacheco-Hernández, & Páez-Hernández, Rodríguez, & Galán-Vidal, 2009; Martins et al., 2016).

3.3.3. Efficiency of HAE vs. UAE

As discussed above, the extraction of anthocyanins from the red flower calyces of *H. sabdariffa* was differently affected by the tested HAE and UAE methods. The highest yields were obtained using the non-conventional UAE method, which promotes the rupture of the plant tissue through cavitation forces and enhances the solvent entrance into the cells with consequent release of the intracellular compounds into the solvent, thus intensifying mass transfer phenomena (Antonio et al., 2016; Misra et al., 2017). This method yielded 51.76 ± 3.70 mg CT/g R when applying: $t = 26.1$ min, $P = 296.6$ W, and $S = 39.1\%$ ethanol, v/v, whereas HAE originated 20.86 ± 1.24 mg CT/g R when: $t = 30.0$ min, $T = 30.0$ °C, and $S = 0.0\%$ ethanol, v/v. These optimized methods shared some similarities in terms of T (~ 30 °C) and t (26–30 min), thus indicating that the lower HAE values were not caused by thermal degradation of these compounds. In contrast, the response

A: Optimized RSM variables



B: Solid-to-liquid ratio patterns

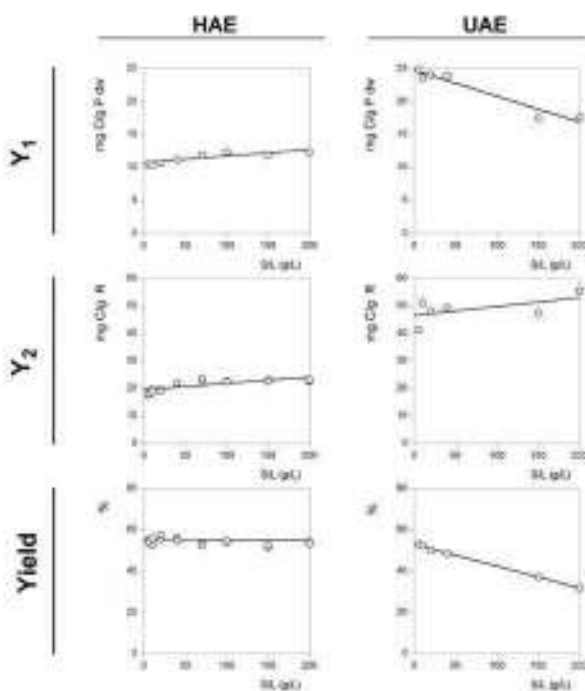


Fig. 3. Summary representation of the effects of the four-variable considered in the HAE and UAE processes. Part A: 2D individual responses and optimum values (○) of the three variables determined for the total anthocyanin content (CT) in mg/g P (Y_1) and mg/g R (Y_2) and the extraction yield (%). In each graph, each independent variable was positioned at the optimal value of the other two variables (Table 3). Lines and dots were generated by the second-order polynomial model of Eq. (1) (Table A3). Part B: Dose-response analysis of the solid/liquid ratio (S/L) at the optimal extraction conditions of the other three variables (Table 3). Dots (○) represent the experimental values and lines show the pattern predicted by linear equation (parametric values in Table A4).

optimum of UAE was achieved with the application of ultrasound and a higher ethanol proportion (meaning higher energy and solvent costs), while water was a suitable solvent for HAE. Nevertheless, the different maximum values achievable by each method are quite different and must be considered when selecting the most appropriate one.

In order to determine the most cost-effective option, it would be interesting to perform a life-cycle cost analysis (LCCA), not only of the sole extraction process as an individual unit operation, but considering the entire supply chain, including the production and harvesting of the plant material, equipment investment, natural resources and energy consumption, and environment hazardous emissions. A comparative LCCA between conventional solvent extraction and innovative methods using microwaves and ultrasounds was performed by Kyriakopoulou, Papadaki, and Krokida (2015), which found UAE the most viable and environmental friendly method to recover β -carotene from microalgae. In our study, the effects of the S/L variable were investigated to obtain more information about the efficiency of each extraction method.

3.3.4. Optimal extraction conditions for maximizing the responses criteria

Although the effects described above provided a guiding range of conditions maximizing the defined responses, optimal values can be determined using a simple method tool to solve nonlinear problems. The results of the application of this simple procedure are presented in Table 3 (part A), in which the extraction conditions that maximize each response in individual and global terms are provided. Additionally, Fig. 3 (part A) summarizes the information derived from the

mathematical equations, where 2D graphs are presented as a function of all assessed variables. These variables were positioned at the optimal global values of the other two variables (Table 3) (part B). For the three variables optimized for the recovery of total anthocyanins (CT) in mg/g P (Y_1) and mg/g R (Y_2) and extraction yield (%), the predicted individual responses are represented by lines and optimum values by points (○). The determined global optimal extraction conditions were experimentally tested to confirm the accuracy of the presented results and to assess the dose-response effect of the S/L variable.

3.4. Dose-response analysis of the solid/liquid ratio at the optimum conditions

Reduced extraction time and low solvent consumption are some of the desired requirements when designing novel extraction methods. The solvent volume should be sufficient only to dissolve the target compounds and promote mass transfer (Pinela et al., 2016). At industrial scale, higher solid/liquid ratios (S/L) are desirable to maximize the extraction yield with minimal solvent consumption, thus making the process more productive and sustainable. The extraction rate is also affected by the mass transfer resistance associated with the matrix structure (Marco, Agnese, & Giuseppe, 2012). The studies on S/L were performed at the global optimal conditions predicted by the polynomial models obtained for each extraction technique as previously described. Preliminary results indicated that the experimental limit value was proximal to 200 g/L. Therefore, the experiment was designed to assess

the dose-response effects of the S/L variable between 5 g/L and 200 g/L.

Fig. 3 (part B) shows the dose-responses analysis of S/L at the optimal global extraction conditions of the other three variables (Table 3 part B), where it is also possible to observe that the responses achieved by HAE and UAE are consistent with those previously obtained. In these 2D representations, dots (○) represent the experimental values and lines show the pattern predicted by a simple linear relation with intercept (parametric values in Table A4). Based on the parametric values presented in Table A4, a consistency was found between the CT values (for both Y_1 and Y_2 response formats) and those previously obtained for HAE and UAE (Table 3). Consequently, the dose response is explained by the slope (m) of the linear relation. Negative values of m describe decreasing extraction patterns as the S/L increases, and positive values describe increasing extraction patterns as the S/L increases, while a constant pattern is shown when the m value is *ns* (or zero). As can be observed, two cases showed negative m values (the extraction efficiency increases as the S/L rate decreases), and one case showed non-significant values, or a zero value of m (the efficiency doesn't change as the S/L increases). In all the other cases the m showed positive values (the efficiency increases as the S/L increases). The reliability of the obtained linear fittings is strongly consistent. In fact, high coefficients of determination were obtained ($R^2 \geq 0.94$) indicating a good agreement between predicted patterns and the obtained experimental data, validating the mathematical analysis selected to describe the reached solutions. The conclusions derived from this analysis are described below:

- For the Y_1 value format, the parametric values for HAE were $b = 10.76 \pm 1.31$ mg CT/g P dw and $m = 0.0097 \pm 0.001$, with $R^2 = 0.9510$; while for UAE, $b = 24.47 \pm 2.37$ mg CT/g P dw and $m = -0.0393 \pm 0.019$, with $R^2 = 0.9765$. For the HAE process, as the S/L increases, the extraction also slightly increases, leading to an increment of ~15% in the extracted compounds, when changing from 5 g/L (lower tested value) to 200 g/L (maximum tested experimental value). On the other hand, the observed decrease for UAE is relatively strong, which means that the increase of 1 g/L implies the loss of 0.0393 ± 0.019 mg CT/g P dw. Such values produce losses of ~25% when applying 200 g/L, comparatively with the tested lower value. Therefore, when working at the most economically attractive S/L value (200 g/L), both solutions will reach similar results.
- For the Y_2 value format, the parametric values for HAE were $b = 19.53 \pm 1.38$ mg CT/g R and $m = 0.0225 \pm 0.0091$, with $R^2 = 0.9434$; while for UAE, $b = 46.26 \pm 2.74$ mg CT/g R and $m = 0.0325 \pm 0.0098$, with $R^2 = 0.9674$. The positive m values show that the S/L increase leads to an increase in the extraction ability, conducting to a maximum extraction value when using 200 g/L. Nevertheless, the quantity extracted by UAE is nearly 3-fold superior to the quantity obtained by HAE.
- For the $Yield$ value format, the parametric values for HAE were $b = 55.14 \pm 3.56\%$ and a *ns* value of m (i.e., the obtained amount of residue does not vary as a function of the S/L increase), with $R^2 = 0.9794$; whereas for UAE, $b = 52.20 \pm 2.98\%$ and $m = -0.106 \pm 0.007$, with $R^2 = 0.9975$.

These results are in accordance with the ones reported in the literature, where UAE has been identified as a technique with potential to improve the extraction yield through the intensification of the mass transfer between the plant material and the solvent (Tomšik et al., 2016). Probably, the implosion of the cavitation bubbles generated during sonication led to an improved cell disruption and particle breakdown, which facilitated the release of the extractable compounds and allowed a greater penetration of the solvent into the sample matrix, thus increasing the contact surface area between the solid and liquid phases (Chemat et al., 2017b; Tomšik et al., 2016). However, in order to propose a more accurate sonication mechanism and to visualize the effects, it would be interesting to investigate different mechanisms

involved during UAE, such as fragmentation, erosion, capillarity, de-texturation and sonoporation, as well as the influencing parameters (Chemat et al., 2017b). Therefore, macroscopic and cyto-histochemical analyses, scanning electron microscopy (SEM), and environmental scanning electron microscopy (e-SEM), among other observations, should be performed (Khadhraoui et al., 2018).

The concept of “green extraction” is aligned with the societal challenges of the 21st century, to protect both the consumers and environment. This approach also promotes competition in the industrial sector, making it more innovative, efficient and sustainable (Chemat et al., 2017a; Khadhraoui et al., 2018; Sicaire et al., 2016). Therefore, the UAE process herein optimized can be adopted by industrials interested in replacing their traditional extraction process with this more ecological and competitive method. For this, experiments should be conducted from the laboratory to pilot-scale, to lead to the implementation of UAE at the industrial-scale (using countercurrent extractors) (Chemat et al., 2017a; Sicaire et al., 2016), which will be of great importance to recover pigments, aromas and antioxidants from plant materials (in this case, anthocyanins from *H. sabdariffa* calyces). However, UAE finds other relevant applications in the food, nutraceutical, pharmaceutical and bioenergy industries (Chemat et al., 2017a, 2017b; Meullemiestre et al., 2016; Sicaire et al., 2016).

4. Conclusions

Nowadays, consumers are increasingly choosing food products formulated with natural additives due to the understanding of the strong relation between health and diet. Therefore, it is important for the industrial food sector to find novel sources and efficient extraction methods to support the production of bio-based ingredients, including colorants. In this study, two extraction methods were applied, and optimized by combining the effects of three relevant independent variables, to maximize the recovery of anthocyanins from the red calyces of *H. sabdariffa*. The achieved experimental data were successfully fitted to the theoretical models used to determine the optimal extraction conditions. UAE was the most efficient method; it allowed to recover 23.83 ± 2.44 mg of the target anthocyanins per 1 g of dried plant material and obtain 51.76 mg of these pigments in 1 g of residue (or extract). For the S/L variable, whose effects were assessed at the optimum conditions, firstly determined for the defined three variables, the positive m values obtained for Y_2 showed that the S/L increase leads to an increase in the extraction ability, conducting to maximum values at 200 g/L. Furthermore, the amount of anthocyanins obtained by UAE was nearly 3-fold higher than the amount obtained by HAE. According to these results, it can be stated that *H. sabdariffa* calyces can be used as a viable source of anthocyanins to produce bio-based colouring agents, being one of the richest anthocyanin containing sources reported in the literature. In addition, this bench-scale application study can support the scale-up of natural colorants production, which is of interest to industrial suppliers of the food, pharmaceutical and cosmetic sectors, among others.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2018.09.118>.


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Article

Optimization of the Extraction Process to Obtain a Colorant Ingredient from Leaves of *Ocimum basilicum* var. *purpurascens*

Filipa Fernandes ¹, Eliana Pereira ¹, Miguel A. Prieto ^{1,2}, Ricardo C. Calhella ¹, Ana Ćirić ³, Marina Soković ³, Jesus Simal-Gandara ², Lillian Barros ^{1,*}
and Isabel C. F. R. Ferreira ^{1,*}

¹ Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal; filipafernandes1994@outlook.com (F.F.); eliana@ipb.pt (E.P.); mprieto@ipb.pt (M.A.P.); calhella@ipb.pt (R.C.C.)

² Nutrition and Bromatology Group, Department of Analytical and Food Chemistry, Faculty of Food Science and Technology, University of Vigo—Ourense Campus, E-32004 Ourense, Spain; jsimal@uvigo.es

³ University of Belgrade, Department of Plant Physiology, Institute for Biological Research “Siniša Stanković”, Bulevar Despota Stefana 142, 11000 Belgrade, Serbia; rancic@ibiss.bg.ac.rs (A.Ć.); mris@ibiss.bg.ac.rs (M.S.)

* Correspondence: lillian@ipb.pt (L.B.); iferreira@ipb.pt (I.C.F.R.F.);
Tel.: +351-273-303285 (L.B.); +351-273-303219 (I.C.F.R.F.)

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Abstract: Heat-Assisted Extraction (HAE) was used for the optimized production of an extract rich in anthocyanin compounds from *Ocimum basilicum* var. *purpurascens* leaves. The optimization was performed using the response surface methodology employing a central composite experimental design with five-levels for each of the assessed variables. The independent variables studied were the extraction time (t , 20–120 min), temperature (T , 25–85 °C), and solvent (S , 0–100% of ethanol, v/v). Anthocyanin compounds were analysed by HPLC-DAD-ESI/MS and the extraction yields were used as response variables. Theoretical models were developed for the obtained experimental data, then the models were validated by a selected number of statistical tests, and finally, those models were used in the prediction and optimization steps. The optimal HAE conditions for the extraction of anthocyanin compounds were: $t = 65.37 \pm 3.62$ min, $T = 85.00 \pm 1.17$ °C and $S = 62.50 \pm 4.24\%$, and originated 114.74 ± 0.58 TA mg/g of extract. This study highlighted the red rubin basil leaves as a promising natural matrix to extract pigmented compounds, using green solvents and reduced extraction times. The extract rich in anthocyanins also showed antimicrobial and anti-proliferative properties against four human tumor cell lines, without any toxicity on a primary porcine liver cell line.

Keywords: natural colorants; anthocyanins; *Ocimum basilicum* var. *purpurascens* leaves; red rubin basil; Heat-Assisted Extraction; extraction optimization

1. Introduction

Consumers' interest in food quality has been increasing, selecting foods with health benefits. Colour is the main organoleptic attribute in the selection and acceptance of foods [1,2]. Some vegetable matrices are composed by natural pigments, attracting much attention from the scientific community and leading to studies to characterize these compounds and explore their subsequent application, not only in the food industry as natural colorants, but also in the pharmaceutical sector, as antioxidants [3–5].

Anthocyanins are natural pigment studied worldwide; however, when these compounds are incorporated in food products, there are several intrinsic and extrinsic factors that affect and influence

their stability [6]. The pH is an important parameter, because it is crucial in the determination of the anthocyanin colour, which shows a significant pigmentation variability. In aqueous medium, they are red at pH = 1–3, colourless at pH = 4–5, purple at pH = 6–7, blue at pH = 7–8, and yellow at pH = 8–9 [3,7]. Other variables to be taken into account in anthocyanins' stability are the handling and storage temperature, the chemical composition of target products (presence of enzymes, proteins, metal ions and even other flavonoids), and exposure to light and oxygen [3].

The use of anthocyanins as food colorants is approved in several countries [8] and according to the Regulation (EU) nr 1129/2011 of the Commission of 11 November 2011, their application is authorised in numerous food products and processes, such as cured cheeses and cheese products of red marbled paste, vegetables in vinegar, oil or brine (except olives), jams, jellies and marmalades, fruit-flavored breakfast cereals, fish pastes and crustaceans, pre-cooked crustaceans, and smoked fish, among other products. The acceptable daily intake (ADI) is not regulated, which means that sufficient quantity can be added to food products to achieve the desired coloration effect [9].

Anthocyanins can be found in numerous natural sources, especially in fruits, cereals, leaves, flowers, and roots, such as in the leaves of *Ocimum basilicum* var. *purpurascens* (red rubin basil) [10]. Red rubin basil belongs to the *Lamiaceae* family, being a variety of *Ocimum basilicum*, and is used not only as an ornamental plant, but also in traditional medicine [10–12].

In order to apply sustainable extraction methodologies at an industrial level, mathematical studies are performed to maximize the extraction of compounds from natural matrices [13,14]. The patterns of the response variables of the extraction method, such as processing temperature, time and solvent [13,14] can be evaluated using the response surface methodology (RSM). This technique allows to save time, reagents and reduce the operational costs, meanwhile increases the efficiency of the optimization process. Aiming to promote the applicability of natural pigments present in the *Ocimum basilicum* var. *pupurascens* leaves at an industrial level, this work optimized the HAE extraction of anthocyanin compounds, particularly cyanidin and pelargonidin derivatives using Response Surface Methodology (RSM).

2. Results

2.1. Response Criteria for the RSM Analysis

The HPLC anthocyanin profile of the red rubin basil leaves extract from experimental run number 18 is shown in Figure 1. Up to 13 anthocyanin compounds were identified (Table 1) based their chromatographic characteristics (UV-Vis, mass spectral fragmentation patterns) and literature information [15,16].

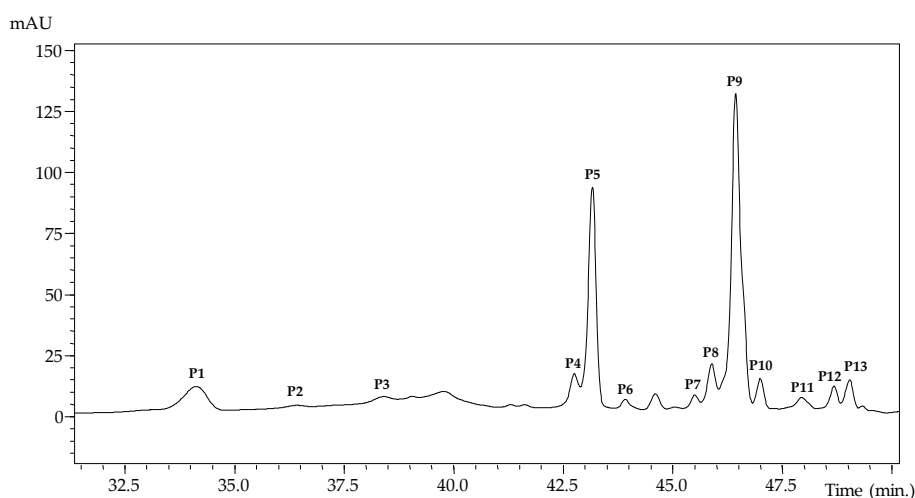


Figure 1. HPLC profile of anthocyanin molecules found in red rubin basil leaves extract obtained in the data set number 18 (as described in Table 2).

Table 1. Retention time (Rt), wavelengths of maximum absorption in the UV-Vis region (λ_{\max}), and tentative identification of anthocyanin compounds in *O. basilicum* var. *purpurascens* (mean \pm SD).

Peak	Rt (min)	λ_{\max} (nm)	[M + H] ⁺	Main Fragment ESI-MSn [Intensity (%)]	Tentative Identification
P1	34.1	520	919	757(49),449(6),287(13)	Cyanidin-3-(<i>p</i> -coumaroyl-6'-caffeoyl)sophoroside isomer 1 ^A
P2	36.4	520	919	757(49),449(6),287(13)	Cyanidin-3-(<i>p</i> -coumaroyl-6'-caffeoyl)sophoroside isomer 2 ^A
P3	38.4	522	1005	757(6),535(11),287(11)	Cyanidin-3-(6- <i>p</i> -coumaroyl)sophoroside-5-(6-malonyl)glucoside ^A
P4	42.8	522	757	595(100),449(11),287(61)	Cyanidin-3-(6- <i>p</i> -coumaroyl)glucoside-5-glucoside ^A
P5	43.2	530	1081	919(15),449(6),287(6)	Cyanidin-3-(6- <i>p</i> -coumaroyl-6'-caffeoyl)-5-glucoside isomer 1 ^A
P6	43.9	532	1167	919(44),757(5),287(20)	Cyanidin-3-(6- <i>p</i> -coumaroyl-6'-caffeoyl)sophoroside-5-(6-malonyl)glucoside isomer 1 ^A
P7	44.6	530	1167	919(27),757(5),287(6)	Cyanidin-3-(6- <i>p</i> -coumaroyl-6'-caffeoyl)sophoroside-5-(6-malonyl)glucoside isomer 2 ^A
P8	45.5	530	1081	919(100),449(11),287(20)	Cyanidin-3-(6- <i>p</i> -coumaroyl-6'-caffeoyl)sophoroside-5-glucoside isomer 2 ^A
P9	45.9	530	1065	903(20),449(5),287(3)	Cyanidin-3-(6,6'-di <i>p</i> -coumaroyl)sophoroside-5-glucoside ^A
P10	46.4	526	1151	989(10),903(5),287(5)	Cyanidin-3-(6,6'-di <i>p</i> -coumaroyl)sophoroside-5-(6-malonyl)glucoside ^A
P11	47.0	514	1049	887(33),433(9),271(5)	Pelargonidin-3-(6,6'-di <i>p</i> -coumaroyl)sophoroside-5-glucoside ^B
P12	48.0	526	1167	1005(63),919(23),449(8),287(13)	Cyanidin-3-(6- <i>p</i> -coumaroyl-X-malonyl-6'-caffeoyl)sophoroside-5-glucoside ^A
P13	48.7	530	1151	989(28),449(17),287(5)	Cyanidin-3-(6- <i>p</i> -coumaroyl-X-malonyl-6'- <i>p</i> -coumaroyl)sophoroside-5-glucoside ^A

Calibration curves used: ^A- cyanidin-3-*O*-glucoside ($y = 97,787x - 743,469$; $R^2 = 0.999$); ^B- pelargonidin-3-*O*-glucoside ($y = 43,781x - 275,315$; $R^2 = 0.999$).

Figure 2 shows a summary of the diverse stages used for optimization procedure, in order to recover the anthocyanin compounds from the red rubin basil leaves. The experimental values of the 28 experimental runs of the circumscribed central composite design (CCCD) design are presented in Table 2.

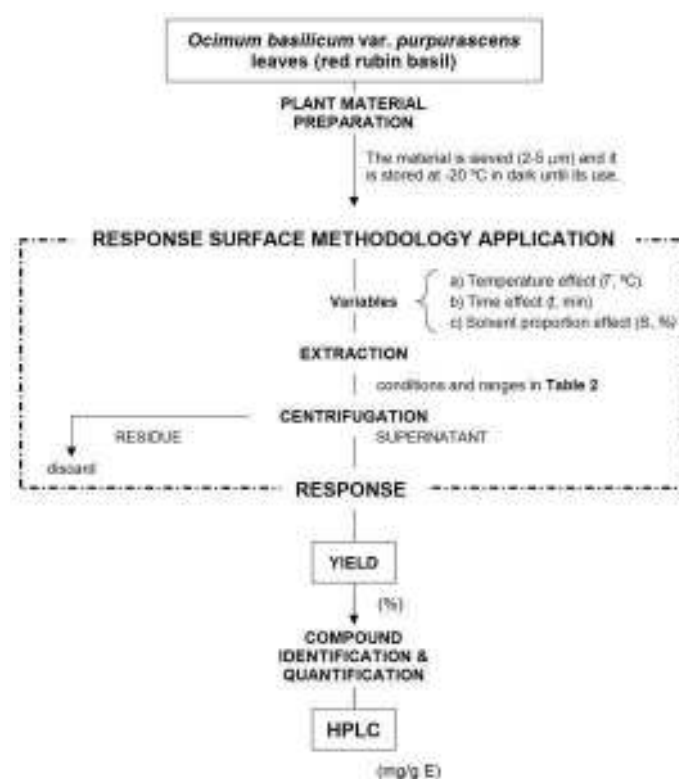
**Figure 2.** Diagram of the different steps carried out for optimizing the conditions that maximize the extraction responses of the anthocyanin compounds and the total extracted residue (Yield, %).

Table 2. The first part describes the experimental design that was applied in this work. The independent variables are presented in coded and natural values. The second part shows the response values for the detected anthocyanin compounds (mg/g E) and extraction yield (%) achieved for all the 28 experimental conditions performed for the HAE by the RSM design.

Five-Level CCD Experimental Design																													
Runs		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Coded values	X ₁ : Time (t)	−1	−1	−1	−1	1	1	1	1	1.68	−1.68	0	0	0	0	−1.68	−1.68	−1.68	−1.68	1.68	1.68	1.68	1.68	0	0	0	0	0	0
	X ₂ : Temp. (T)	−1	−1	1	1	−1	−1	1	1	0	0	−1.68	1.68	0	0	−1.68	−1.68	1.68	1.68	−1.68	−1.68	1.68	1.68	0	0	0	0	0	0
	X ₃ : Solvent (S)	−1	1	−1	1	−1	1	−1	1	0	0	0	0	−1.68	1.68	−1.68	1.68	−1.68	1.68	−1.68	1.68	−1.68	1.68	0	0	0	0	0	0
Natural values	X ₁ : t (min)	40.3	40.3	40.3	40.3	99.7	99.7	99.7	99.7	120.0	20.0	70.0	70.0	70.0	70.0	20.0	20.0	20.0	20.0	120.0	120.0	120.0	120.0	70.0	70.0	70.0	70.0	70.0	70.0
	X ₂ : T (°C)	37.2	37.2	72.8	72.8	37.2	37.2	72.8	72.8	55.0	55.0	25.0	85.0	55.0	55.0	25.0	25.0	85.0	85.0	25.0	25.0	85.0	85.0	55.0	55.0	55.0	55.0	55.0	55.0
	X ₃ : S (%)	20.3	79.7	20.3	79.7	20.3	79.7	20.3	79.7	50.0	50.0	50.0	50.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	50.0	50.0	50.0	50.0	50.0	50.0
Response Variables for RSM Application																													
P1	3.34	5.61	4.50	5.75	3.41	5.80	3.55	4.96	3.93	5.08	5.05	5.52	3.17	5.78	1.96	6.36	4.56	5.83	2.27	5.86	1.42	5.11	4.93	4.91	5.34	5.35	5.24	4.84	
P2	2.47	4.24	2.64	4.15	2.38	4.27	2.26	3.72	2.55	3.32	3.15	2.92	2.31	5.31	1.78	4.85	2.50	4.11	1.83	4.22	1.45	3.83	3.43	3.54	3.47	3.48	3.44	3.29	
P3	3.94	5.53	4.71	5.37	3.60	6.52	3.90	5.78	3.93	4.89	5.29	5.74	2.81	5.13	2.10	4.25	2.98	3.16	1.59	4.63	1.48	5.42	6.33	7.04	6.70	6.71	6.94	6.67	
P4	2.95	5.68	2.82	5.05	2.93	6.60	2.71	5.01	3.70	4.26	4.98	4.97	1.66	4.60	1.79	5.27	1.47	4.67	1.59	4.61	1.39	5.12	4.59	4.57	4.78	4.79	4.40	4.42	
P5	7.61	13.13	8.39	13.66	7.30	13.00	8.15	13.66	10.54	12.68	15.27	17.09	2.64	9.81	1.87	8.32	1.47	10.19	1.59	8.67	1.39	11.07	16.99	16.40	17.62	17.66	15.93	16.40	
P6	3.47	5.69	3.78	5.34	3.48	4.99	3.52	5.02	3.91	5.09	5.68	5.74	1.70	4.01	1.74	4.70	1.47	3.76	1.59	3.99	1.37	3.25	6.35	6.10	6.62	6.64	6.34	6.31	
P7	2.52	4.25	2.62	3.98	2.43	3.59	2.41	3.62	3.12	3.52	3.80	3.64	1.75	4.07	1.74	4.83	1.91	3.98	1.59	4.07	1.37	3.28	3.73	3.75	3.85	3.86	3.69	3.69	
P8	3.53	5.93	3.67	5.39	3.40	6.22	3.36	5.03	3.95	5.09	5.42	5.28	1.96	3.84	1.85	4.65	1.47	3.82	1.59	3.96	1.37	3.49	5.86	5.64	5.88	5.89	6.01	5.57	
P9	5.10	8.37	5.33	7.82	4.78	8.55	4.94	7.82	5.46	7.15	8.25	8.59	2.07	5.44	1.94	5.27	1.47	4.98	1.59	4.70	1.40	5.41	9.99	9.52	10.25	10.27	9.49	9.71	
P10	14.92	21.10	16.36	24.14	14.84	18.68	16.01	24.14	21.50	23.40	26.73	30.48	6.96	14.17	2.29	9.46	2.35	13.48	1.59	10.19	1.43	17.38	32.57	32.82	33.99	34.06	32.48	33.27	
P11	10.18	14.08	13.19	15.07	9.34	12.26	10.90	13.27	12.83	17.10	16.97	19.23	5.98	5.71	2.31	5.13	5.67	5.26	1.27	4.82	1.16	5.32	20.04	19.79	20.91	20.95	21.22	19.94	
P12	2.91	4.60	3.27	4.31	2.66	4.10	2.76	3.81	3.22	4.13	4.19	4.26	2.20	4.09	1.92	4.80	2.74	4.17	1.59	4.29	1.41	3.54	3.93	3.80	3.83	3.84	3.94	3.83	
P13	4.84	7.26	5.77	6.99	4.30	7.53	4.89	6.72	5.63	7.31	7.30	7.85	2.73	4.57	2.08	4.75	3.32	4.12	1.59	4.08	1.40	4.15	8.06	8.08	8.34	8.36	8.75	8.38	
TAC	67.78	105.46	77.06	107.01	64.86	102.12	69.37	102.56	84.25	103.00	112.07	121.30	37.96	76.52	25.38	72.65	33.37	71.53	21.22	68.10	18.04	76.37	116.79	115.97	111.59	111.85	117.87	116.32	
Yield	36.35	28.58	38.26	31.41	36.08	29.95	39.41	32.14	38.12	37.53	34.75	37.90	38.84	18.08	35.62	13.22	38.27	17.80	35.42	16.52	41.00	20.24	35.68	34.54	35.68	35.61	35.54	35.40	

P: anthocyanin compound; TAC: Total anthocyanin content.

The content in individual (P1 to P13) and grouped (TAC – total anthocyanin compounds) anthocyanin compounds were used as criteria to maximize their content and to optimize the extraction conditions of HAE from red rubin basil leaves under RSM assessment. The values of the extraction yield were also considered, and ranged from 13.22 to 41.00%, with the experimental runs no. 16 and 21, respectively (Table 2). In total, 15 response variables are taking into account for the optimization processes.

2.2. Theoretical Response Surface Models

Evaluating the precision of theoretical models to predict and comprehend the effects of independent variables in some response variable is necessary. This, as in many research fields, is achieved by fitting these models to the experimental values. In this study, a non-linear algorithm (least-squares estimates) has been used to adjust the response values (Table 2) to a second order polynomial model. The estimated coefficient values obtained from the polynomial model of Equation (1) and the coefficient of correlation (R^2) for each parametric response of the extraction process are shown in Table 3.

$$y = b_0 + \sum_{i=1}^n b_i X_i + \sum_{i=1}^{n-1} \sum_{\substack{j=2 \\ j > i}}^n b_{ij} X_i X_j + \sum_{i=1}^n b_{ii} X_i^2 \quad (1)$$

The parametric values obtained, not only it allows to translate response patterns, it also helps to understand the complexity of the possible interactions between variables. However, some of the parameters of Equation (1) whose coefficients were non-significant (*ns*) at a 95% confidence level ($\alpha = 0.05$) were not used for building the model. By means of the statistic lack of fit it is possible to prove the adequacy of the obtained models and in this way it was demonstrated that a considerable improvement was not achieved by means of the inclusion of the statistically *ns* parametric values. Each of the 15 assessed responses can be seen in models in Table 4 getting in all cases R^2 coefficients higher than 0.92 (Table 3). According to this value, it can be said that the percentage of variability of each response can be explained by the model. These workable models were applied in the subsequent prediction and optimization steps, with a good agreement between the experimental and predicted values, which indicates that the variation is explained by the independent variables.

Although the obtained model coefficients (Table 3) cannot be associated with physical or chemical significance and are empirical, they can however be used to predict the results of untested extraction conditions [17]. As the effect sign marks the performance of the response, if a factor has a positive effect, the response is higher at the high level. On the other hand, the response is lower at the high level when a factor has a negative effect. Therefore, the weight of the corresponding variable will be more important the higher the absolute value of a coefficient. Certain characteristics relating to the general effects of the variables based on mathematical expressions can be observed in Table 4. The relevance of the significant parametric values can be order as a function of the variables involved in a decreasing form as $S > t >> T$. Previous authors that work with similar matrices [14], have concluded that the most relevant variable on the HAE extraction of bioactive compounds is *S*. As for the study of the linear, quadratic, and interactive parametric effects of the developed equations, it allowed to conclude that all these parameters play an important and significant role in all evaluated responses. For the linear effect, the variables *S* and *t* had strong values, while the effect of *T* was less important in almost all cases. All independent variables had moderate quadratic or nonlinear effects. As for the interactions of the variable (*tT*, *TS* and *tS*), these were of minor importance. The results obtained were represented in the response surface plots that can be seen below so that in this way one can see in a more obvious way the combined effects as well as to be able to visually describe the tendencies of extraction. The optimal HAE conditions, that maximize their retrieval from red rubin basil leaves, are presented in Table 3.

Table 3. Estimated coefficients and R^2 determined for the models obtained for individual and grouped anthocyanin compounds and extraction yield (Table 3), and optimal HAE conditions and response values.

Response variables	Fitting Coefficients Obtained after Applying the Second-Order Polynomial Equation with Interactive Terms											R^2	t (min)	T (°C)	S (%)	Optimal Processing Conditions and Response Values
	Intercept	Linear Effect			Quadratic Effect			Interactive Effect								
	b_0	b_1 (t)	b_2 (T)	b_3 (S)	b_{11} (t ²)	b_{22} (T ²)	b_{33} (S ²)	b_{12} (tT)	b_{13} (tS)	b_{23} (TS)						
P1	5.06 ± 0.15	−0.28 ± 0.09	ns	0.92 ± 0.09	−0.16 ± 0.11	ns	−0.17 ± 0.11	−0.17 ± 0.06	0.07 ± 0.06	−0.15 ± 0.06	0.9441	81.06 ± 2.08	20.00 ± 1.73	100.00 ± 1.58	6.56 ± 0.31	
P2	3.37 ± 0.10	−0.15 ± 0.06	ns	0.76 ± 0.06	−0.20 ± 0.07	ns	0.11 ± 0.07	−0.04 ± 0.00	ns	−0.07 ± 0.04	0.9556	64.04 ± 5.07	20.00 ± 0.43	100.00 ± 9.11	5.15 ± 0.36	
P3	31.23 ± 1.68	ns	0.96 ± 0.95	ns	−2.14 ± 1.15	ns	−5.40 ± 1.15	ns	0.98 ± 0.68	ns	0.9359	70.00 ± 3.94	90.00 ± 6.07	50.00 ± 3.80	32.85 ± 2.47	
P4	4.65 ± 0.19	ns	−0.20 ± 0.11	1.06 ± 0.11	−0.11 ± 0.01	ns	−0.41 ± 0.13	ns	ns	ns	0.9225	70.00 ± 5.49	20.00 ± 1.94	88.44 ± 5.39	5.67 ± 1.05	
P5	15.59 ± 0.62	ns	0.33 ± 0.22	2.42 ± 0.35	−0.95 ± 0.42	ns	−2.85 ± 0.42	ns	ns	ns	0.9449	70.00 ± 1.26	90.00 ± 5.25	62.60 ± 5.86	16.66 ± 1.76	
P6	5.90 ± 0.27	−0.16 ± 0.15	−0.10 ± 0.01	0.74 ± 0.15	−0.31 ± 0.18	ns	−0.89 ± 0.18	ns	ns	ns	0.9336	62.47 ± 1.24	20.00 ± 0.31	62.27 ± 3.93	6.24 ± 0.56	
P7	3.63 ± 0.08	−0.16 ± 0.05	−0.09 ± 0.05	0.70 ± 0.05	−0.08 ± 0.06	ns	−0.23 ± 0.06	ns	ns	−0.07 ± 0.03	0.9701	40.93 ± 1.60	20.00 ± 1.49	100.00 ± 6.12	4.59 ± 0.34	
P8	5.59 ± 0.18	−0.13 ± 0.10	−0.14 ± 0.10	0.77 ± 0.10	−0.23 ± 0.12	ns	−0.80 ± 0.12	ns	ns	ns	0.9456	61.55 ± 4.64	20.00 ± 1.03	64.30 ± 1.89	6.03 ± 0.55	
P9	9.06 ± 0.43	ns	−0.12 ± 0.02	1.15 ± 0.24	−0.62 ± 0.30	ns	−1.52 ± 0.30	ns	ns	ns	0.9286	70.00 ± 1.16	20.00 ± 1.24	61.18 ± 2.85	9.47 ± 1.32	
P10	29.87 ± 1.32	ns	0.85 ± 0.74	2.89 ± 0.74	−2.07 ± 0.91	ns	−6.27 ± 0.91	ns	ns	ns	0.9377	70.00 ± 1.42	90.00 ± 2.46	56.85 ± 0.94	31.63 ± 2.42	
P11	8.93 ± 0.25	−0.32 ± 0.14	−0.12 ± 0.14	0.63 ± 0.14	−0.47 ± 0.17	ns	−1.64 ± 0.17	ns	ns	−0.10 ± 0.01	0.9359	59.99 ± 5.19	20.00 ± 0.59	57.18 ± 0.16	9.28 ± 0.38	
P12	3.91 ± 0.06	−0.22 ± 0.03	−0.03 ± 0.03	0.65 ± 0.03	−0.06 ± 0.04	ns	−0.25 ± 0.04	−0.05 ± 0.02	ns	−0.09 ± 0.02	0.9757	36.49 ± 0.30	20.00 ± 1.21	98.54 ± 9.29	4.71 ± 0.20	
P13	7.93 ± 0.20	−0.26 ± 0.11	−0.16 ± 0.11	0.73 ± 0.11	−0.37 ± 0.03	ns	−1.37 ± 0.13	ns	0.09 ± 0.08	−0.10 ± 0.01	0.9451	60.74 ± 0.00	20.00 ± 1.30	59.46 ± 0.04	8.38 ± 0.29	
TAC	109.78 ± 2.73	−1.93 ± 1.54	1.07 ± 0.32	14.30 ± 1.54	−6.20 ± 1.87	ns	−17.00 ± 1.87	ns	ns	ns	0.9577	65.37 ± 3.62	90.00 ± 1.17	62.50 ± 4.24	114.74 ± 0.58	
Yield	36.43 ± 1.46	0.49 ± 0.88	1.19 ± 0.87	−5.56 ± 0.87	ns	ns	−3.09 ± 0.84	ns	ns	ns	0.9592	120.00 ± 2.62	90.00 ± 7.72	23.23 ± 0.91	41.77 ± 1.59	

ns: non-significant coefficient; R^2 : Correlation coefficient; P: anthocyanin compound; TAC: total anthocyanin content.

Table 4. Mathematical models produced after fitting Equation (1) to the data set (individual and grouped values).

Anthocyanin Compounds	Equations	Equation Numbers
P1	$Y_{P1} = 5.06 - 0.28t + 0.92S - 0.16t^2 - 0.17S^2 - 0.17tT + 0.07tS - 0.15TS$	Equation (2)
P2	$Y_{P2} = 3.37 - 0.15t + 0.76S - 0.20t^2 - 0.11S^2 - 0.04tT - 0.07TS$	Equation (3)
P3	$Y_{P3} = 31.23 + 0.96T - 2.14t^2 - 5.40S^2 + 0.98tS$	Equation (4)
P4	$Y_{P4} = 4.65 - 0.20T + 1.06S - 0.11t^2 - 0.41S^2$	Equation (5)
P5	$Y_{P5} = 15.59 + 0.33T + 2.42S - 0.95t^2 - 2.85S^2$	Equation (6)
P6	$Y_{P6} = 5.90 - 0.16t - 0.10T + 0.74S - 0.31t^2 - 0.89S^2$	Equation (7)
P7	$Y_{P7} = 3.63 - 0.16t - 0.09T + 0.70S - 0.08t^2 - 0.23S^2 - 0.07TS$	Equation (8)
P8	$Y_{P8} = 5.59 - 0.13t - 0.14 + 0.77S - 0.23t^2 - 0.80S^2$	Equation (9)
P9	$Y_{P9} = 9.06 - 0.12T + 1.15S - 0.62t^2 - 1.52S^2$	Equation (10)
P10	$Y_{P10} = 29.87 + 0.85T + 2.89S - 2.07t^2 - 6.27S^2$	Equation (11)
P11	$Y_{P11} = 8.93 - 0.32t - 0.12 + 0.63S - 0.47t^2 - 1.64S^2 - 0.10TS$	Equation (12)
P12	$Y_{P12} = 3.91 - 0.22t - 0.03T + 0.65S - 0.06t^2 - 0.25S^2 - 0.05tT - 0.09TS$	Equation (13)
P13	$Y_{P13} = 7.93 - 0.26t - 0.16T + 0.73S - 0.37t^2 - 1.37S^2 + 0.09tS - 0.10TS$	Equation (14)
TAC	$Y_{TAC} = 109.78 - 1.93t + 1.07T + 14.30S - 6.20t^2 - 17.00S^2$	Equation (15)
Yield	$Y_{Yield} = 36.43 + 0.49t + 1.19T - 5.56S - 3.09S^2$	Equation (16)

2.3. Final Effects of the Studied Conditions of HAE on the Target Responses and Optimal Values that Maximize the Responses

Figure 3 shows the response surface plots of extraction yield, TAC and two other representative anthocyanins extracted (**P1** and **P10**), as well as their statistical analysis. Inspecting the given surface plots of the extraction yield (Figure 3), it is conceivable to confirm that the measure of removed material increments to an ideal point and afterward, by and large, it diminishes as a component of the included variables. Subsequently, the ideal values can be found similar to a solitary point, which permits figuring the extraction conditions that lead to the most extreme flat out. This behaviour is common to almost all responses, allowing us to determine the conditions that maximize the responses. In consequence, the ideal extraction values for the reactions shown in Figure 3 were determined for the HAE conditions (Table 3), as summarized below:

For yield, the optimal HAE conditions were: $t = 120.00 \pm 2.62$ min, $T = 85.00 \pm 7.72$ °C and $23.23 \pm 0.91\%$ of ethanol (v/v), and produced $41.77 \pm 1.59\%$.

For TAC, the optimal HAE conditions were: $t = 65.37 \pm 3.62$ min, $T = 85.00 \pm 1.17$ °C and $62.50 \pm 4.24\%$ of ethanol (v/v), and produced 114.74 ± 0.58 mg/g of E.

For **P1**, the optimal HAE conditions were: $t = 81.06 \pm 2.08$ min, $T = 25.00 \pm 1.73$ °C and $100.00 \pm 1.58\%$ of ethanol (v/v), and produced 6.56 ± 0.31 mg/g of E.

For **P10**, the optimal HAE conditions were: $t = 70.00 \pm 1.42$ min, $T = 85.00 \pm 2.46$ °C and $56.85 \pm 0.94\%$ of ethanol (v/v), and produced 31.63 ± 2.42 mg/g of E.

It is well-known that the utilization of high values of ethanol in the solvent, increases the extraction of bioactive compounds from plant materials [13]. The effects of the independent variables on the extraction of individual anthocyanin compounds from red rubin basil leaves are represented in 2D in Figure 4. The processing conditions that generated optimal response values (\odot) are numerically described in Table 3. The identified anthocyanin compounds were organized as a function of the maximum amount achieved (mg/g of extract) in a decreasing order as follows: **P3** (32.85) > **P10** (31.63) >> **P5** (16.66) >> **P9** (9.47) > **P11** (9.28) > **P13** (8.38) > **P1** (6.56) > **P6** (6.24) > **P8** (6.03) > **P4** (5.67) > **P2** (5.15) > **P7** (4.59) > **P2** (4.71).

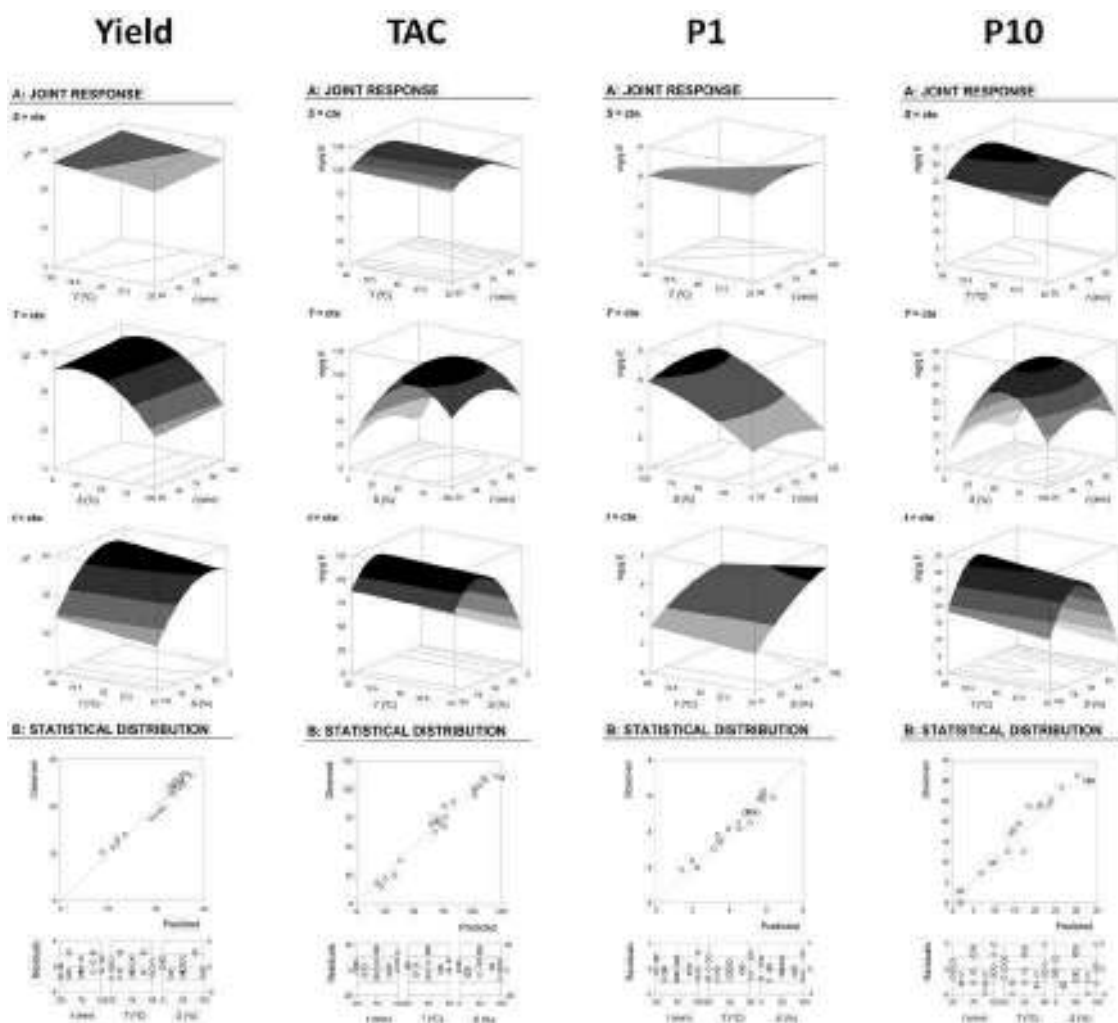


Figure 3. Illustrative representation of the extraction yield and grouped anthocyanin compounds (total anthocyanin acids, total flavonoids and total anthocyanin compounds) responses. The part A shows the 3D description as a function of each independent variable. The surfaces were constructed using the values presented in Table 3 and described by Equation (1). In each graph, the excluded variable was positioned at the optimum of their experimental domain (Table 3). Part B shows a summary of the goodness of fit using the observed/predicted and the residual distribution plots as a function of each variable.

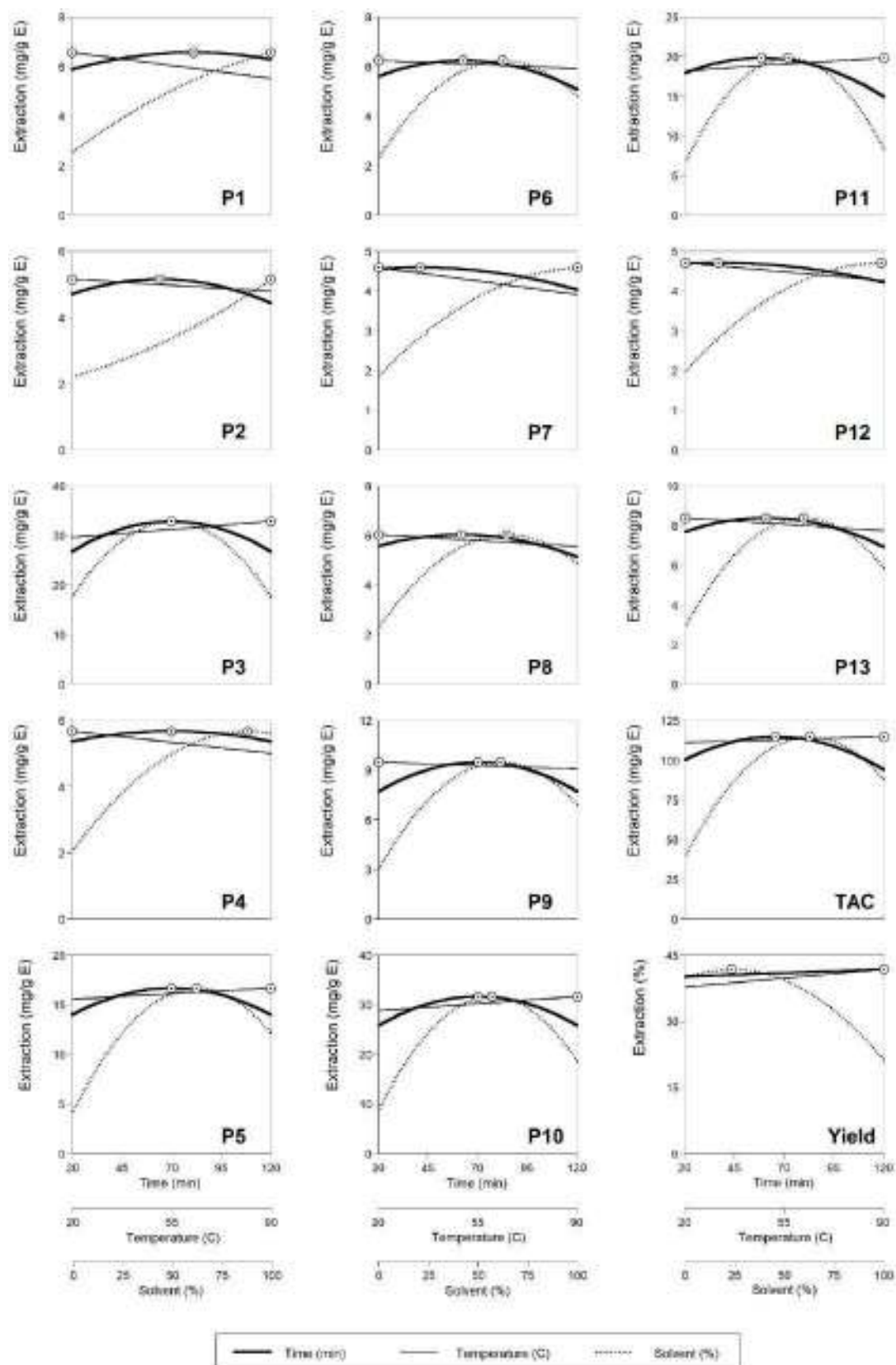


Figure 4. 2D graphical response of the effects of the independent variables on the extraction of anthocyanin compounds from red rubin basil leaves (see Figure 1 for peak identification). Dots (⊙) represent the optimal values. In each plot, each independent variable was positioned at the optimal value of the other two variables (Table 3).

The greater extraction values achieved under these optimized conditions highlight the suitability of HAE with RSM as an innovative process to recover a greater amount of anthocyanin compounds from red rubin basil leaves using shorter processing times and greener solvents.

2.4. Clustering of Anthocyanin Compounds According to the HAE Conditions that Maximize their Extraction

The maximum values for the response values of the different anthocyanin compounds and their concentrations if extracted under the optimal HAE conditions of the other compounds (Table 3) are presented in Table 5. The values of subparagraph (B) is the ratio of the optimum value of each compound between the maximum of the other compounds. When two compounds show values of 100%, i.e., the coefficient is 1, under the same conditions of HAE means that the optimal response value for both is in the same conditions. As example, the compounds P1, P2, P4, P7 and P12 were clustered in C1 under the same HAE conditions (Figure 5). By cons, if the coefficient is different from 1, it means that the conditions that are optimal for the extraction of a compound are not for the other (compounds 1 and 13).

Table 5. Maximum response values of each anthocyanin compound and their values at the optimal processing conditions of the other compounds presented in Table 3.

(A) Maximum Response Values (mg/g of Extract) of the Individual Anthocyanin Compounds													
Peak:	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13
Optimum:	6.56	5.15	32.85	5.67	16.66	6.24	4.59	6.03	9.47	31.63	9.28	4.71	8.38
(B) Values of each Anthocyanin Compound at the Optimal Conditions of the other Compounds													
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13
P1	1	0.99	0.77	0.96	0.81	0.84	0.95	0.85	0.83	0.79	0.81	0.94	0.82
P2	0.99	1	0.65	0.91	0.71	0.73	0.98	0.74	0.72	0.68	0.70	0.95	0.71
P3	0.45	0.42	1	0.63	0.97	0.87	0.33	0.85	0.88	0.99	0.88	0.33	0.88
P4	0.99	0.99	0.76	1	0.83	0.94	0.97	0.95	0.94	0.80	0.92	0.97	0.93
P5	0.65	0.66	0.97	0.80	1	0.93	0.61	0.93	0.93	0.99	0.92	0.61	0.93
P6	0.75	0.77	0.92	0.89	0.94	1	0.74	1.00	1.00	0.94	1.00	0.75	1.00
P7	0.97	0.99	0.76	0.97	0.80	0.90	1	0.91	0.88	0.79	0.87	1.00	0.88
P8	0.79	0.81	0.89	0.91	0.92	1.00	0.79	1	1.00	0.91	0.99	0.80	1.00
P9	0.72	0.72	0.93	0.86	0.96	1.00	0.66	0.99	1	0.95	0.99	0.66	0.99
P10	0.48	0.49	0.99	0.69	0.99	0.90	0.43	0.89	0.91	1	0.90	0.44	0.90
P11	0.61	0.63	0.94	0.80	0.93	0.99	0.61	0.99	0.99	0.94	1	0.63	1.00
P12	0.97	0.99	0.82	0.98	0.85	0.91	1.00	0.92	0.90	0.84	0.89	1	0.90
P13	0.69	0.70	0.91	0.84	0.91	1.00	0.67	1.00	1.00	0.92	1.00	0.68	1

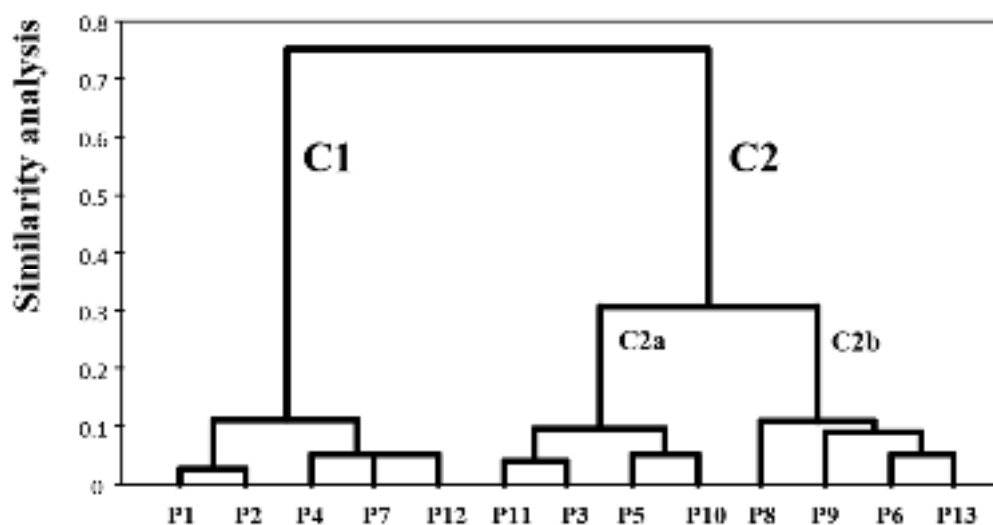


Figure 5. HCA dendrogram of anthocyanin compounds according to the HAE conditions that maximize their extraction from red rubin basil leaves.

In Table 5 it can be observed the formation of different groups of compounds of anthocyanin with maximum response values in conditions of HAE extraction similar. The division in these groups was made possible by the complete data set of Table 5 and by performing a multi objective optimization problem using an appropriate clustering algorithm. The results of Hierarchical Cluster Analysis (HCA) are presented in Figure 5. In the HCA dendrogram, the shorter distance between compounds, the higher similarity in terms of conditions that favour their extraction. Moreover, compounds belonging to the same group are better extracted under similar HAE conditions. Two significant clusters (C1 and C2), being the C2 divided in turn into 2 subgroups (a and b). Other less important subgroups were created, but they can be considered as a residual noise produced by the algorithm.

Cluster C1 included the compounds **P1**, **P2**, **P4**, **P7** and **P12**. The extraction of these compounds for maximize by medium t , high S and low/high T (Table 3 and Figure 3). The subgroups were mainly differentiated by the T values.

Cluster C2 included all other compounds **P11**, **P3**, **P5**, **P10**, **P8**, **P9**, **P6** and **P13**, which were subdivided in C2a and C2b. For maximizing the extraction of the compounds in C2a low T and medium S was used. On the other hand, the compounds in C2b was maximized when using high T and medium S .

Although it was expected that if the compounds have similar chemical characteristics also would have similar HAE conditions, the HCA analysis was an interesting and innovative approach in the field of extraction of high added-value compound from natural sources since this analysis highlighted suitable HAE conditions for maximize the simultaneous recovery of specific groups of compounds from red rubin basil leaves.

2.5. Dose-Response Analysis of the Solid-to-Liquid Effect at the Optimum Conditions

Thanks to the precise results obtained by HPLC, the S/L effect was tested under the optimal conditions provided for each extractive technique by the polynomial models, using the amount of anthocyanin as response. As confirmed by the preliminary results (data not shown), the maximum experimental value is close to 30 g/L, since at higher values of S/L it is observed experimental stirring, so an experiment was designed for each extractive process in which to check the S/L behaviour at values between 1 and 30 g/L. The obtained results are consistent with previous responses. It was observed that the effect caused by the S/L ratio follows a simple linear model with an intercept, and that this model follows a slightly decreasing pattern proportional to the increase of S/L in all the assays. However, that pattern, explained by the parametric coefficient of the slope, was non-significant with a confidence interval level of 95 % ($\alpha = 0.05$) and the decreasing effect was not taken into account for further analysis. In conclusion, it can be affirmed that the increase in the S/L ratio has very little effect on the TAC extraction, besides that saturation effects were not observed at any value below 30 g/L.

2.6. Evaluation of the Colorant Potential of the Extract Rich in Anthocyanin Compounds Obtained under Optimum Conditions from Leaves of *O. basilicum* var. *purpurascens*

The results of the chromatic analysis in the CIE $L^*a^*b^*$ colour space of the extract rich in anthocyanins present in the leaves of *O. basilicum* var. *purpurascens* are shown in Table 6. The colour of the pigmented extract showed an L^* value, lightness (0 to 100), of 20.5 ± 0.5 ; and in parameters a^* (colour intensity from green to red (−120 to 120)) and b^* (colour is evaluated at the intensity level from blue to yellow (−120 to 120)), the values were 33.0 ± 0.1 and 8.2 ± 0.4 , respectively.

For a better understanding of the colour values, these were converted to RGB values and the colour obtained from the extract, red-berry, can be visualized. These results can be justified by the presence of anthocyanin compounds in the extract, which, in addition to having darker shades, are also characterized by blue, red and purple tones. The concentration of total anthocyanin compounds, obtained in the optimized extract, was similar to that predicted by the model.

Table 6. Amount of anthocyanins (cyanidin and pelargonidin derivatives) and color parameters under optimal conditions (mean \pm SD).

Quantification (mg/g E)	L*	a*	b*	Conversion Color to RGB Values
115.4 \pm 0.4	20.5 \pm 0.5	33.0 \pm 0.1	8.2 \pm 0.4	

L* lightness; a* chromatic axis from green (−) to red (+); b* chromatic axis from blue (−) to yellow (+).

2.7. Evaluation of the Bioactive Properties of the Extract Rich in Anthocyanin Compounds Obtained under Optimal Conditions from Leaves of *O. basilicum* var. *purpurascens*

2.7.1. Antimicrobial Activity

Table 7 shows the results of the antimicrobial activity obtained from the extract rich in anthocyanins present in the leaves of *O. basilicum* var. *purpurascens*. The results demonstrate antibacterial activity of the pigmented extract for all microorganisms' strains. In this way, the best results are obtained against *Bacillus cereus* (*B.c.*) (MIC = 0.037 mg/mL; MBC = 0.075 mg/mL) and *Escherichia coli* (*E.c.*) (MIC = 0.037 mg/mL; MBC = 0.075 mg/mL) strains. However, the pigmented extract also showed a high activity against *Listeria monocytogenes* (*L.m.*) (MIC = 0.05 mg/mL; MBC = 0.075 mg/mL), *Staphylococcus aureus* (*S.a.*), *Enterobacter cloacae* (*En.cl.*) (MIC = 0.075 mg/mL, MBC = 0.15 mg/mL), and *Salmonella typhimurium* (*S.t.*) (MIC = 0.15 mg/mL; MBC = 0.30 mg/mL).

Table 7. Antibacterial activity (MIC and MBC, mg/mL) and antifungal activity (MIC and MFC, mg/mL) of the anthocyanins rich extract obtained under optimal extraction conditions.

		Antibacterial Activity					
		<i>B.c.</i>	<i>S.a.</i>	<i>L.m.</i>	<i>E.c.</i>	<i>En.cl.</i>	<i>S.t.</i>
Anthocyanins rich extract	MIC	0.037	0.075	0.05	0.037	0.075	0.15
	MBC	0.075	0.15	0.075	0.075	0.15	0.30
Streptomycin ⁽¹⁾	MIC	0.10	0.04	0.20	0.20	0.20	0.20
	MBC	0.20	0.10	0.30	0.30	0.30	0.30
Ampicillin ⁽¹⁾	MIC	0.25	0.25	0.40	0.40	0.25	0.75
	MBC	0.40	0.45	0.50	0.50	0.50	1.20
		Antifungal Activity					
		<i>A.fun.</i>	<i>A.o.</i>	<i>A.n.</i>	<i>Pf.</i>	<i>P.o.</i>	<i>P.v.c.</i>
Anthocyanins rich extract	MIC	0.037	0.002	0.075	0.075	0.30	0.30
	MFC	0.075	0.075	0.15	0.15	0.45	0.45
Ketoconazole ⁽¹⁾	MIC	0.25	0.20	0.20	0.20	2.50	0.20
	MFC	0.50	0.50	0.50	0.50	3.50	0.30
Bifonazole ⁽¹⁾	MIC	0.15	0.10	0.15	0.20	0.20	0.10
	MFC	0.20	0.20	0.20	0.25	0.25	0.20

⁽¹⁾ Positive controls. *B.c.*: *Bacillus cereus*; *S.a.*: *Staphylococcus aureus*; *L.m.*: *Listeria monocytogenes*; *E.c.*: *Escherichia coli*; *En.cl.*: *Enterobacter cloacae*; *S.t.*: *Salmonella typhimurium*; *A.fun.*: *Aspergillus fumigatus*; *A.o.*: *Aspergillus ochraceus*; *A.n.*: *Aspergillus niger*; *Pf.*: *Penicillium funiculosum*; *P.o.*: *Penicillium ochrochloron*; *P.v.c.*: *Penicillium verrucosum* var. *cyclopium*. MIC—minimum inhibitory concentration; MBC—minimum bactericidal concentration; MFC—minimum fungicidal concentration.

Regarding antifungal activity, the extract showed a high potential against most of the tested fungi. *Aspergillus ochraceus* (*A.o.*) was the most susceptible species to the extract (MIC = 0.002 mg/mL; MFC = 0.075 mg/mL); however, no antifungal activity was observed against *Penicillium verrucosum* var. *cyclopium* (*P.v.c.*) (MIC = 0.30 mg/mL; MFC = 0.45 mg/mL). These results indicated a promising antimicrobial activity, and this can be explained due to the high concentration of anthocyanin compounds that have a high antimicrobial potential [18].

2.7.2. Cytotoxic Activity

Table 8 shows the results obtained in the cytotoxicity evaluation assays in extracts rich in anthocyanin compounds, obtained through optimal extraction conditions. The extract exhibited anti-proliferative capacity in HeLa ($GI_{50} = 213 \pm 9 \mu\text{g/mL}$) and HepG2 ($GI_{50} = 198 \pm 9 \mu\text{g/mL}$) tumour cell lines.

Table 8. Cytotoxic activity of the anthocyanins rich extract obtained under optimal extraction conditions (mean \pm SD).

Tumor Cell Lines	Concentrations (GI_{50} Values, $\mu\text{g/mL}$)
MCF-7 (breast carcinoma)	>400
NCI-H460 (lung carcinoma)	>400
HeLa (cervical carcinoma)	213 ± 9
HepG2 (hepatocellular carcinoma)	198 ± 9
Non-Tumour Cells	
PLP2 (non-tumor porcine liver primary cells)	>400

GI_{50} values - concentration that inhibited 50% of cell growth. Ellipticin GI_{50} (positive control): 1.21 $\mu\text{g/mL}$ (MCF-7), 1.03 $\mu\text{g/mL}$ (NCI-H460), 0.91 $\mu\text{g/mL}$ (HeLa), 1.10 $\mu\text{g/mL}$ HepG2) and 2.29 $\mu\text{g/mL}$ (PLP2).

These results may also be explained by the high levels of anthocyanin compounds present in the extract, since these molecules have been described, by several authors, as a potential anti-proliferative agent in tumor cell lines [19]. Regarding the assay performed on primary non-tumor cell culture (PLP2), the extract evidenced the absence of toxicity up to the maximal tested concentration ($GI_{50} > 400 \mu\text{g/mL}$).

3. Materials and Methods

3.1. Samples

Ocimum basilicum var. *purpurascens* (Lamiaceae) variety was obtained in Cantinho das Aromáticas, Vila Nova de Gaia, Portugal. The samples acquired were planted to grow in greenhouse at the Polytechnic Institute of Bragança and then collected (September 2017). The fresh leaves were separated through a mechanical procedure, posteriorly lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA), reduced to a fine and homogeneous dried powder (~20 mesh) and stored protected from light and heat.

3.2. Heat-Assisted Extraction

Heat-Assisted Extraction (HAE) was performed in a water reactor agitated internally with a Cimarec™ Magnetic Stirrer at a constant speed (~500 rpm, Thermo Scientific, San Jose, CA, USA), following a procedure previously performed by Roriz et al. [20]. The powdered samples (300 mg) were extracted with solvent (20 mL of ethanol/water) under diverse conditions, as previously defined by the established RSM plan (Table 2). The ranges of the experimental design were: time (t or X_1 , 20 to 120 min), temperature (T or X_2 , 25 to 85 °C) and ethanol content (S or X_3 , 0 to 100%). The solid-to-liquid ratio (S/L) was kept at 15 g/L for all conditions.

When all the individual extraction conditions were carried out, the samples were immediately centrifuged ($4750 \times g$ during 20 min at 10 °C) and filtered (paper filter Whatman n° 4) to eliminate the non-dissolved material. The supernatant was collected and divided in two portions for HPLC and extraction yield analysis. The portion separated for HPLC analysis (2 mL) was filtered through a LC filter disk (0.22 μm), whereas the portion for the extraction yield determination (5 mL) was dried at 105 °C during 48 h and thereafter weighted.

3.3. Calculation of the Extraction Yield

The extraction yields (%) were calculated based on the dry weight (crude extract) obtained after evaporation of the solvent. In all cases, the filtrates were concentrated at 35 °C in a rotary evaporator (Büchi R-210, Flawil, Switzerland) under reduced pressure and the aqueous phase was then lyophilised to obtain a dried extract.

3.4. Chromatographic Analysis of Anthocyanin Compounds

The samples were analysed using Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA) coupled to a diode array detector (chromatograms recorded at 520 nm) and to a Linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an ESI source working in positive mode, following a procedure previously reported [21]. Quantitative analysis was performed using a calibration curve obtained using cyanidin-3-glucoside ($y = 97,787x - 743,469$; $R^2 = 0.9993$) and pelargonidin-3-glucoside ($y = 43,781x - 275,315$; $R^2 = 0.9989$) and results were expressed in mg per g of extract (mg/g E).

3.5. Experimental Design, Modelling and Optimization

3.5.1. Experimental Design

A RSM of five-level CCCD of 28 runs with 6 replicated values at centre points was applied to optimize the HAE conditions for the extraction of anthocyanin compounds. Coded and natural values of the independent variables X_1 (processing time (t), min), X_2 (temperature (T), °C) and X_3 (solvent (S), % of ethanol, v/v) are presented in Table 1.

3.5.2. Mathematical Modelling

The response surface models were fitted by means of least-squares calculation using the following second-order polynomial equation with interactive terms (Equation (1)). In this equation, Y represents the dependent variable (response variable) to be modelled, X_i and X_j are the independent variables, b_0 is the constant coefficient, b_i is the coefficient of linear effect, b_{ij} is the coefficient of interaction effect, b_{ii} is the coefficient of quadratic effect, and n is the number of variables. The extraction yield and the individual and grouped anthocyanin compounds, 13 individual compounds plus the total anthocyanin content (TAC), were used as dependent variables.

3.5.3. Maximization of the Responses

For the extraction yield and the recovery of phenolic compounds responses, a *simplex* method was used for maximize the models developed of Equation (1) [22]. In all cases, restrictions were added to limit the values of the conditions assessed.

3.6. Grouping the Responses by Cluster Analyses

A cluster analysis was performed to group the anthocyanin compounds according to the extraction conditions that maximize their response values using the Excel add-in "XLSTAT 2016" (Addinsoft, Barcelona, Spain). A comparative agglomerative hierarchical clustering analysis (HCA) with automatic truncation based on entropy and Pearson correlation coefficient were used for clustering (similarity analysis).

3.7. Fitting Procedures and Statistical Analysis

Fitting procedures, coefficient estimates and statistical calculations were performed as previously described by Prieto and Vázquez [23]. In brief: (a) fitting procedure by nonlinear least-square (quasi-Newton) as provided by the Excel add-in "Solver"; (b) coefficient intervals determination by the Excel add-in "SolverAid"; and (c) the model consistency by common statistical tests for each

model developed: (i) the Fisher F-test ($\alpha = 0.05$); (ii) parametric assessment by the Excel add-in “SolverStat”; (iii) the determination of R^2 .

3.8. Preparation of the Extract Rich in Anthocyanin Compounds Obtained under Optimum Conditions from the Leaves of *O. basilicum* var. *purpurascens*

For the preparation of an extract rich in anthocyanin compounds, extraction from the leaves of *O. basilicum* var. *purpurascens* was performed, following the previously optimized procedure (Table 1). The samples (300 mg) were placed together ethanol/water (20 mL, 55:45, *v/v*) acidified with 0.25% citric acid (pH = 3) in a glass vial with a stopper. The extraction followed established conditions of temperature ($T = 72$ °C) and time (60 min). After the procedure described, the sample was centrifuged (Centurion K24OR, West Sussex, UK) at 5000 rpm for 5 min at 10 °C. They were then filtered through filter paper (Whatman n° 4) to remove suspended solids. The ethanol fraction was removed at a temperature of 35 °C and the aqueous fraction obtained was frozen and lyophilized (FreeZone 4.5), affording an extract rich in anthocyanin compounds. The lyophilized extract was stored away from the light for further analysis.

3.9. Evaluation of the Colorant Potential of the Extract Rich in Anthocyanin Compounds Obtained under Optimum Conditions from the Leaves of *O. basilicum* var. *purpurascens*

The evaluation of the colorant potential of the extract was carried out by measuring the colour and the measurement of the colouring compounds by chromatography, in order to corroborate the data provided by the MRS. The colour was measured using a colorimeter (model CR-400, Konica Minolta Sensing, Inc., Osaka, Japan) with an adapter for granular materials (model CR-A50), according to a procedure described by Pereira et al. [24]. The measurements were made in the CIE $L^*a^*b^*$ colour space, using the illuminant C and a diaphragm aperture of 8 mm. Data were processed with the “Spectra Magic Nx” (version CM-S100W 2.03.0006 software, Konica Minolta). Quantitation of anthocyanin compounds was accomplished by chromatography using an HPLC-DAD-ESI/MS system as described in Section 3.4.

3.10. Evaluation of the Bioactive Properties of the Extract Rich in Anthocyanin Compounds Obtained under Optimal Conditions from the Leaves of *O. basilicum* var. *purpurascens*.

3.10.1. Antimicrobial Activity

The antimicrobial activity was evaluated using the methodology described by Carocho et al. [25]. Gram-negative (*Enterobacter cloacae* (American Type Culture Collection (ATCC) 35030), *Escherichia coli* (ATCC 35210) and *Salmonella typhimurium* (ATCC 13311)) and Gram-positive (*Bacillus cereus* (clinical isolate), *Listeria monocytogenes* (NCTC (National collection of type cultures) 7973) and *Staphylococcus aureus* (ATCC 6538)) bacteria strains were used. For the calculation of the minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations, the microdilution method was applied and the results were expressed in mg/mL.

For the antifungal activity, a procedure previously described by Carocho et al. [25] was followed. *Aspergillus fumigatus* (ATCC 1022), *Aspergillus niger* (ATCC 6275), *Aspergillus ochraceus* (ATCC 12066), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112) and *Penicillium verrucosum* var. *cyclopium* (food isolate) were used. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were also determined by using the microdilution method and the results were also expressed in mg/mL.

3.10.2. Cytotoxic Activity

The evaluation of the cytotoxic potential of the extract rich in anthocyanin compounds was performed by the Sulfarodamine B (SRB) assay previously described by Barros et al. [26] MCF-7 (breast carcinoma), NCI-H460 (lung carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular

carcinoma) were used as human tumor cell lines. For the hepatotoxicity assay, the extract rich in anthocyanin compounds was tested in a primary non-tumor cell culture obtained from porcine liver (PLP2).

Ellipticine (Sigma-Aldrich, St. Louis, MO, USA) was used as the positive control and the results were expressed as GI_{50} values (sample concentration that inhibits the growth of cells by 50%), and expressed in $\mu\text{g}/\text{mL}$.

4. Conclusions

Colorants are one of the most important additives in terms of marketing, because their presence in food products is considered the principal factor influencing customer choice. To the authors' best knowledge, the potential industrial use of the anthocyanin compounds from red rubin basil leaves have not been explored previously. In such a context, the present work presents a new rapid method to extract anthocyanin compounds from red rubin basil leaves. RSM and other mathematical strategies were successfully employed to optimize extraction conditions that maximize the anthocyanin recovery to produce a rich extract with potential for industrial application as a natural colouring additive.

The scientific literature shows clear evidence that extraction procedures of target compounds from plant-based products, must be assessed individually. Therefore, a nonstop effort needs to be performed, because agro-industrial and food sectors are looking for byproduct valorisation into added-value products. However, in order to take full advantage of the technological advances, the extraction conditions need to be optimized. Mathematical solutions, such as RSM tools, could increase the efficiency and profitability of the process and help to change conventional extraction approaches.

In this study, the suitability of HAE for extracting anthocyanin compounds from red rubin basil leaves was demonstrated and the variables of t , T and S were combined in a five-level *CCCD* design coupled to RSM for optimization. According to the results, a good agreement between experimental and theoretical results was observed. In general, the recovery of anthocyanin compounds was maximized when high temperatures, high ethanol concentrations and medium extraction times were applied, validating this Heat-Assisted Extraction.

The colour analysis in the pigmented extract revealed interesting values, showing dark tones, more directed to a red tonality. It was also evident the antimicrobial and anti-proliferative potential against several strains and tumour cell lines, respectively, without presenting toxicity for non-tumor cells.

These results should promote interest in conducting further studies on *O. basilicum* varieties, highlighting the potential of ruby red basil as a potential source of natural and bioactive ingredients with application in several industrial factors, namely in the food and pharmaceutical areas.

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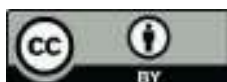
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Sample Availability: Samples are available from the authors.



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Recovery of bioactive anthocyanin pigments from *Ficus carica* L. peel by heat, microwave, and ultrasound based extraction techniques

Emanuéli Backes^{a,b}, Carla Pereira^a, Lillian Barros^a, M.A. Prieto^{a,c}, Aziza Kamal Genena^d, Maria Filomena Barreiro^{a,b}, Isabel C.F.R. Ferreira^{a,*}

^a Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal

^b Laboratory of Separation and Reaction Engineering, Laboratory of Catalysis and Materials (LSRE-LCM), Polytechnic Institute of Bragança, Campus Santa Apolónia, 5300-253 Bragança, Portugal

^c Nutrition and Bromatology Group, Faculty of Food Science and Technology, University of Vigo, Ourense Campus, E32004 Ourense, Spain.

^d Departamento Acadêmico de Alimentos (DAALM), Universidade Tecnológica Federal do Paraná, Campus Medianeira, 85884-000 Paraná, Brazil

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ABSTRACT

Due to its coloration, the fig (*Ficus carica* L.) peel, a by-product of fruit processing and/or consumption, is a potential source of anthocyanin compounds. In the present study different extraction techniques (heat, ultrasound, and microwave) were compared aiming to recover the anthocyanin pigments and optimize its extraction conditions. A response surface methodology tool with three factors and five levels for each factor was used according to a circumscribed central composite design. The variables tested for the heat and microwave extraction methods were time, temperature, and solvent proportion (ethanol/water ratio), meanwhile, for the ultrasound method, the variables tested were the ultrasonic power, time, and solvent proportion. The anthocyanin composition of the extract was determined by HPLC-DAD-ESI/MS, and the used criteria responses were: i) quantification of cyanidin 3-rutinoside (C) in the extracted residue (mg C/g R) and in the dried peel (mg C/g P dw), and the extraction yield of the obtained residue (g R/g P dw). Ultrasound extraction was the most effective method, yielding 3.82 mg C/g R at the optimal global extraction conditions (21 min, 310 W, and 100% of ethanol). Additionally, the solid-to-liquid ratio effect was studied at the optimal conditions, using a dose-response format, in view of its plausible transference to industrial level. For the ultrasound method, an increased non-linear relationship was observed for concentrations in the range 5 to 200 g/L, being the optimal solution close to 150 g/L. In brief, the obtained results show the potential of fig peels as a source of anthocyanin pigments, with potential uses in various industrial fields, such as food, pharmaceutical, and cosmetic.

1. Introduction

Figs are the infructescences of a tree belonging to the family of Moraceae, called *Ficus carica* L., with extensive production in the Mediterranean region (Palassarou et al., 2017). When mature, the fig can acquire different colouring hues depending on its cultivar, which range from green to black-violet as a function of the anthocyanin concentration in the infructescence peel (Wang, Cui, Vainstein, Chen, & Ma, 2017). Recent studies have shown that the fig peel contains anthocyanins at levels higher than the ones present in most of their typical natural sources (Harzallah, Bhourri, Amri, Soltana, & Hammami, 2016; Vallejo, Marín, & Tomás-Barberán, 2012). Figs consumption varies with the region, but for industrial purposes it is mostly used peeled (Harzallah et al., 2016), which makes the fig peel a potential by-

product that can be valorised in the recovering of anthocyanin pigments.

In the last decade, researchers have proved that the regular consumption of artificial colouring agents can cause several adverse toxicological side effects on humans, such as allergic reactions, minor health disorders, and behavioural changes (Fattore et al., 2016; Montesano et al., 2008; Salem et al., 2014). Faced with this problem, and driven by consumer's needs, the food industry is decreasing the amount of used artificial colorants and, whenever possible, replacing them by more innocuous natural counterparts. These natural solutions are, not only harmless to humans when regularly consumed, but also exhibit a complementary range of important bioactivities, with beneficial health effects. These include antioxidant activity, protection against cellular oxidation, anti-inflammatory capacity, and prevention

* Corresponding author.

E-mail address: iferreira@ipb.pt (I.C.F.R. Ferreira).

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of chronic non-transmissible diseases, among others (Gowd, Jia, & Chen, 2017; Rodriguez-Amaya, 2016). Therefore, the attention given by the industrial sector to these natural colorant alternatives is increasing over time, due to the urge need to find reliable solutions to replace the prevalent artificial ones. Other important features, such as the possible variation of anthocyanin chemical structure (Ongkowijoyo, Luna-Vital, & Gonzalez de Mejia, 2018) that allow the existence of a high variety of colorations, together with their high solubility in water (Rustioni, Di Meo, Guillaume, Failla, & Trouillas, 2013), rises the interest to screen different raw-material sources, able to reach industrial scale and contribute to make these natural solutions viable (Salem et al., 2014).

A huge array of solid-liquid extraction procedures are available to recover compounds of interest from natural matrices (Chemat et al., 2017; Montesano et al., 2008; Zhu et al., 2017). Briefly, the solid-liquid extraction consists in keeping the solid sample (usually in powder form) in direct contact with a solvent for a specific time, and by applying a certain level of energy (conventional heat, ultrasound or microwave radiation, pressure, etc.) (Fattore et al., 2016; García-Moreno et al., 2014; Zhu et al., 2016). The common solid-liquid procedures comprise the conventional methods, such as Soxhlet and heat assisted extraction (HAE, also known as maceration). These methods are easy to apply and relatively inexpensive; nevertheless several authors have pointed out some disadvantages, which are mainly associated with their application at industrial level, i.e. the use of large amounts of solvent and long extraction times (Azmir et al., 2013).

Nowadays, modern solid-liquid extraction technologies are available, such as microwave and ultrasound assisted extraction (MAE and UAE), which are perceived as more sustainable, green techniques and efficient solutions for industrial application, in particular UAE (Chemat et al., 2017; Dai & Mumper, 2010). However, conventional methods are still important at industrial level, mainly due to the lack of comparative results showing the advantages of the alternative modern techniques. For instance, the UAE can improve the recovery of bioactive components, mainly the ones that are sensitive to heat at prolonged extraction times, by keeping these variables at low levels. UAE is an effective extraction technique, in comparison to conventional methods, because the ultrasound radiation is able to disrupt cellular walls allowing a better penetration of solvents in the matrix material, thus improving mass transfer and increasing cell's content release (Bonfigli, Godoy, Reinheimer, & Scenna, 2017; Chemat, Rombaut, Sicaire, et al., 2017). On the other hand, MAE is a process in which the applied energy accelerates the extraction (Tsatsop, Djiobie, Kenmogne, Regonne, & Ngassoum, 2016). This method has a good performance in terms of extraction yield, solvent consumption, and extraction time, being considered a potential substitute for conventional methods (Chan, Yusoff, & Ngoh, 2013; Meullemiestre, Breil, Abert-Vian, & Chemat, 2016).

The recovery of natural components from vegetable matrices for implementation as food ingredients must be made under the best extraction conditions to promote its application at industrial scale and compete against the low economic cost of producing artificial dyes. The particularities of the aforementioned extraction methods, and their effectiveness, cannot be applied in a generalized way to all matrices, demanding specific optimization for each case (Jacotet-Navarro et al., 2016). Moreover, several factors may affect anthocyanin stability and, consequently, their deterioration rate, reinforcing the importance of determining the conditions that maximize the extraction yield of these compounds (Estupiñan, Schwartz, & Garzón, 2011). In this context, the response surface methodology (RSM) arises as an important statistical method to optimize the extraction conditions and maximize responses (Sang, Sang, Ma, Hou, & Li, 2017). The RSM consists in an assembly of mathematical and statistical techniques, helping to describe process patterns (e.g. extraction patterns) of any data set and perform predictions. The RSM procedure is essential when responses are interactively influenced by different factors, its application simplifies the system performance and optimizes the extraction conditions while maximizing the responses assessed (Bezerra et al., 2008).

Thus, the present study aims to optimize and compare anthocyanin extraction, from the peel of *F. carica* infructescences, through different extraction methodologies (HAE, UAE, and MAE). For that purpose, the joint effect of the identified relevant variables for each technique will be described through RSM. This study will allow to achieve the optimal conditions to recover anthocyanins, important pigments with a large range of colours and various industrial applications, from this natural source.

2. Material and methods

2.1. Samples, standards and reagents

The infructescences of *F. carica* were obtained in a local production at the municipality of Bragança, in Trás-os-Montes, Northeast of Portugal. They were peeled, and the peels lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA), powdered to 20 mesh size, and stored in the freezer at -20 °C for subsequent extractions assays.

PA grade ethanol, hydrogen chloride, formic acid, citric acid, and HPLC grade acetonitrile were acquired from Fisher Scientific (Lisbon, Portugal). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.2. Description of the extraction techniques and associated relevant variables

The relevant variables to be considered in the optimization study by RSM were time (t , min), temperature (T , °C), and solvent proportion (S , % v/v of ethanol in hydroalcoholic mixtures) for HAE and MAE extraction methods. For the UAE method, ultrasonic power (P , W), together with t and S were used. For all the extraction techniques, the solid-to-liquid ratio (S/L) was kept constant (50 g/L). Next, the technical requirements for each technique are briefly described.

2.2.1. Heat-assisted extraction (HAE)

The lyophilized powdered peel samples (1 g) were placed in a beaker with 20 mL of solvent acidified with citric acid (pH = 3). The beaker was placed in a thermostatic water bath (Bath Shaker, OVAN, Barcelona, Spain) and the mixture was kept under continuous electromagnetic stirring (Cimarec™ Magnetic, Thermo Scientific, San Jose, CA, USA) for the required t . The variables and tested ranges were: t (X_1 , 5–68.8 min), T (X_2 , 20–90 °C), and S (X_3 , 0–100%).

2.2.2. Microwave-assisted extraction (MAE)

The MAE process was performed in a Biotage Initiator Microwave (Biotage® Initiator+, Uppsala, Sweden) using closed vessels. The lyophilized powdered peel samples (0.5 g) were introduced in a closed reaction vessel with 10 mL of acidified solvent (pH 3, using citric acid). The variables of pressure and T are correlated in the microwave extraction and only one can be used to maximize the responses. The effect of T was used letting the other one reach the corresponding values. The microwave power was set in all cases at a constant value of 400 W. Another important issue in the microwave extraction systems is the time interval needed to reach the selected T (value that increases as the T increases). For the conditions used, the time interval was always < 20 s, therefore, under this quick heating process, time interval was neglected considering only the studied extraction t range. In consequence, the tested variables and ranges were t (X_1 , 5–35 min), T (X_2 , 40–115 °C) and S (X_3 , 0–100%).

2.2.3. Ultrasound-assisted extraction (UAE)

The UAE was studied in a QSonica sonicators equipment (CL-334, Newtown, CT, USA) using a reaction vessel of 50 mL of the acidified solvent (pH 3, using citric acid) and 2.5 g of lyophilized powdered. The tested variables and ranges were at t (X_1 , 5–55 min), P (X_2 , 100–400 W) and S (X_3 , 0–100%). The T was monitored to ensure that the reaction

was always below 30–35 °C.

2.3. Anthocyanin identification and quantification

The extraction solutions were centrifuged (600 rpm for 20 min) and filtered through a paper filter n° 4 to remove the suspended solids. The solvent was then evaporated at 35 °C (rotary evaporator Büchi R-210, Flawil, Switzerland) and the extracted residue gravimetrically quantified. Afterwards, it was re-dissolved in acidified water (citric acid solution with pH 3) and filtered through a LC filter disk (0.22 µm) to a 1.5 mL amber vial. This solution was analysed by high-performance liquid chromatography (Dionex UltiMate 3000 UPLC, Thermo Scientific, San Jose, CA, USA), coupled to a DAD (using 520 nm as the preferred wavelength), and to a mass spectrometer working in positive mode using a Linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an ESI source (Gonçalves et al., 2017). Data acquisition was performed using the Xcalibur® data system (Thermo Finnigan, San Jose, CA, USA) and quantitative analysis was performed from a 5-level calibration curve obtained from the injection of known concentrations of cyanidin 3-rutinoside ($Y = 146,924 \times - 671,583; R^2 = 0.9989$).

2.4. Experimental design, model analysis, and statistical evaluation

2.4.1. RSM experimental design

To centre correctly the experimental design preliminary trials were conducted based in one-at-the-time analysis of the studied variables for each of the selected techniques. After those preliminary analysis (data not presented), the relevant ranges were selected for each one of the studied techniques and presented in Table A1 (supplemental material). The experimental design used was the *circumscribed central composite design (CCCD)* with 28 response combinations using five levels for each variable.

2.4.2. Responses applied to analyse the results

The extraction results were expressed in three response formats (Y): Y_1 , mg of cyanidin 3-rutinoside (C) obtained in the extracted dried weight residue (R; mg C/g R), which was specifically used to evaluate the purity of the target compound in the extract; Y_2 , mg of C per g of peel (P) dry matter (mg C/g P dw), specifically used to analyse the extraction yield in C; and Y_2/Y_1 , obtained by dividing the responses Y_2 and Y_1 , which provides information regarding the extraction yield in R (g of R/g P dw).

2.4.3. Mathematical model to describe the responses

The RSM data was fitted by means of least-squares calculation using the following second-order polynomial equation:

$$Y = b_0 + \sum_{i=1}^n b_i X_i + \sum_{i=1}^{n-1} \sum_{j=2}^n b_{ij} X_i X_j + \sum_{i=1}^n b_{ii} X_i^2 \quad (1)$$

where Y is the dependent variable (response) to be modelled, X_i and X_j define the independent variables, b_0 is the constant coefficient, b_i is the coefficient of linear effect, b_{ij} is the coefficient of interaction effect, b_{ii} is the coefficient of quadratic effect, and n is the number of variables. As responses, the three response formats were used: Y_1 (mg C/g R), Y_2 (mg C/g P dw), and Y_2/Y_1 (g R/g P dw).

2.4.4. Procedure to optimize the variables to a maximum response

To optimize the extraction conditions and maximize the responses, a simplex method was used. The predictive model obtained by RSM was employed under non-linear system with restrictions to avoid variables with unnatural physical conditions (i.e., $t \geq 0$) and a maximization was obtained (Vieira et al., 2017).

2.5. Dose-response description of the solid-to-liquid ratio effect

The analysis of the solid-to-liquid ratio (S/L or X_4 , expressed in g/L) was performed by a dose-response at the optimal conditions of the variables found by the RSM (X_1 , X_2 , and X_3). The aim was to achieve the S/L conditions that leads to a more productive processes for industrial applications. To depict the response effect as function of the S/L , the Weibull (W) equation (Prieto, Curran, Gowen, & Vázquez, 2015) for increasing (↑) and decreasing (↓) responses was used (with some parametric modifications to fit the searched purposes):

$$\left\{ \begin{array}{l} W(X_4) = K \exp \left[\ln \left(1 - \frac{n}{100} \right) \left(\frac{X_4}{m_n} \right)^a \right] \text{ or } \\ W(X_4) = K - K \exp \left[\ln \left(1 - \frac{n}{100} \right) \left(\frac{X_4}{m_n} \right)^a \right] \end{array} \right. \quad (2)$$

where K is the maximum extraction value (i.e., if Y_2 the units would be in mg C/g P dw), a is a shape parameter related to the maximum slope of the response, n is any desired level between 0 and 100% of the response (Y_1 , Y_2 , and Y_2/Y_1) that would be achieved and m_n would be the S/L value (X_4) for the selected n response level (m_{10} , m_{25} , m_{75} , m_{95} , etc.). For example, if the n value is selected as 99%, the m_n parameter will display the S/L needed to achieve the 99% of the assessed response ($m_{99\%}$). When the response shows increasing patterns (↑), the Weibull equation that is used to describe the response will present a m_n parameter of $n = 99\%$. When the response shows decreasing patterns (↓), a m_n parameter with $n = 50\%$ will be used. These different levels of the responses as a function of their increasing or decreasing patterns are logical relations of the intrinsic solutions for industrial purposes. When the response increases, it is logical to know the maximum S/L leading to a 99% level of the assessed response ($m_{99\%}$). However, when the response decreases, the value of $m_{99\%}$ will tend to zero, therefore, it seems to be logical to search for values not decreasing our response more than the half of the maximum (such as $m_{50\%}$). If other m_n is required, Eq. (2) can be modified to produce any other desirable result. However, the selected values for the parameters K and m_n will provide key information related to the pattern of the response to assess the effect of the S/L .

2.6. Numerical methods, statistical analysis, and graphical illustrations

Fitting procedures, coefficient estimates, and statistical calculations were achieved as previously described by other authors (Prieto & Vázquez, 2014). In brief, a) the parameter's determination was accomplished using the quasi-Newton algorithm (least-square) by running the integrated macro 'Solver' in Microsoft Excel by minimizing the differences between observed and predicted values; b) the coefficient significance was evaluated using the 'SolverAid' macro to determine their intervals ($\alpha = 0.05$); and c) the model consistency was checked by means of several statistical criteria: ci) the Fisher F -test ($\alpha = 0.05$) was used to assess the adequacy of the models to describe the observed data; cii) the 'SolverStat' macro was used to assess parameter and model prediction uncertainties (Murado & Prieto, 2013); and ciii) the R^2 value was interpreted as the amount of variability of the dependent variable that is explained by the model.

3. Results and discussion

3.1. Optimization of the RSM analysis using the 3 relevant variables for each extraction technique

3.1.1. Preliminary experiments to select the relevant variables and ranges for designing an appropriate RSM

The extraction of target compounds from natural matrices requires specific considerations due to the intrinsic features and stability of these compounds, and such analysis cannot be extrapolated from similar sources. An in-depth extraction study is required to determine the best

solid-to-liquid extraction method, and operating conditions, for the extraction of target compounds from a certain natural matrix, otherwise, results may lead to erroneous conclusions.

The anthocyanin profile of fig peel extracts was obtained by HPLC-DAD-ESI/MS is presented in Fig. A1. Among another minor component, cyanidin 3-rutinoside ($[M-H]^-$ at m/z 595), which is the molecule responsible for the colorant capacity, was identified. To maximize the extraction of cyanidin 3-rutinoside (C), it is indispensable to identify the effects of variables on responses. A minimum time, energy, and solvent consumption, in order to achieve the most cost-effective and profitable extraction system, is intended (Dai & Mumper, 2010). The RSM design allows optimizing all the variables simultaneously considering interactive effects and predicting the most efficient conditions. Based on the tested experimental range and by using second order polynomial models with interactions, the RSM technique provides for the selected responses used as criteria, a truthful description and the optimal conditions that maximize/minimize them (Bezerra et al., 2008; Ferreira et al., 2007; Kalil & Maugeri, 2000). When applying RSM, the initial difficulties are to select the important involved factors and their relevant experimental range. To overcome those issues, preliminary laboratory tests using the one-factor-at-the-time method (keeping the other variables constant) were performed. Once these preliminary analyses were completed (data not shown), the factors selected for the RSM application were t , T , and S for the HAE and MAE systems, and t , P , and S for the UAE system. Similar findings were reported by other authors when performing optimization studies with other natural matrices (Albuquerque et al., 2016; Caleja et al., 2017). A detailed description for all tested values for each technique can be found in Table A1 (supplemental material section). Concerning the type of employed solvent, and since anthocyanins are polar pigments, they were extracted with hydroalcoholic solutions (ethanol/water mixtures). In all cases, ethanol content was tested in the range 0 to 100%, and confirmed as impacting significantly the achieved anthocyanin extraction yield. In order to maintain anthocyanin stability, citric acid was added to the extraction solvent in order to obtain a pH value around 3. For the RSM study, the S/L variable was kept at 50 g/L (constant value).

In conclusion, the efficiency of the HAE, UAE, and MAE processes for extracting cyanidin 3-rutinoside from fig peel was performed by applying a RSM using three variables in a CCCD (five values for each factor). The coded values, and respective natural values, are presented in Table A1. Once the optimal conditions (t , T , and S for HAE and MAE, and t , P , and S for UAE) were optimized, the study was further advanced towards the study of the S/L effects.

Fig. A2 (presented in the supplemental material section) shows a diagram summary of the work achieved and the steps carried out to optimize the conditions that maximize the extraction of the detected anthocyanin compound in the fig peel (cyanidin 3-rutinoside).

3.1.2. Developed mathematical models after the RSM application

Table 1 shows the results of the responses considered. The Eq. (1) was employed to fit the responses in Table 1 using nonlinear least-squares estimations. The parametric values with higher confidence interval values than the parameter value, were considered as non-significant (ns), and were not used for the model development (Ranic et al., 2014). Table 2 part A shows the significant parametric values of Eq. (1) obtained and the confidence interval values ($\alpha=0.05$). The ns parameters of RSM approaches (Table 2A) do not improve the reached solutions, but rise the uncertainties of all significant coefficients, and in addition, the ns parameters will alter the solutions in untested conditions. Based on the results of Table 2A, the final significant models for each assessed extraction technique are described below:

For the response format Y_1 (mg C/g R):

for HAE:

$$\text{for HAE: } Y_{HAE}^Y = 3.71 - 0.22t + 0.22T + 1.30S - 1.17t^2 - 0.05T^2 + 0.17tT - 0.08TS \quad (3)$$

for UAE

$$Y_{UAE}^Y = 5.51 + 0.21t + 0.61P + 2.05S - 0.63P^2 + 0.27S^2 + 0.19tS \quad (4)$$

for MAE

$$Y_{MAE}^Y = 6.17 - 0.35t - 0.52T + 1.58S - 0.49T^2 - 0.35tT - 0.31tS - 0.36TS \quad (5)$$

For the response format Y_2 (mg C/g P dw):

for HAE

$$Y_{HAE}^{Y_2} = 14.74 - 0.86t - 1.36T + 3.89S - 0.98T^2 - 0.91S^2 - 0.74tT - 0.9TS \quad (6)$$

for UAE

$$Y_{UAE}^{Y_2} = 4.24 + 0.11t + 0.42P + 0.88S - 0.067t^2 - 0.39P^2 - 0.26S^2 + 0.09tS - 0.07PS \quad (7)$$

for MAE

$$Y_{MAE}^{Y_2} = 4.46 - 0.28t - 0.36T + 1.09S - 0.39T^2 - 0.23S^2 - 0.24tT - 0.24TS \quad (8)$$

For the response format Y_2/Y_1 (g R/g P dw):

for HAE

$$Y_{HAE}^{Y_2/Y_1} = 0.69 + 0.02t + 0.02T - 0.01tT + 0.01tS + 0.01TS \quad (9)$$

for UAE

$$Y_{UAE}^{Y_2/Y_1} = 0.78 + 0.01t + 0.01P - 0.09S - 0.01t^2 - 0.05S^2 \quad (10)$$

for MAE

$$Y_{MAE}^{Y_2/Y_1} = 0.72 - 0.01S - 0.01T^2 - 0.01S^2 - 0.01tS \quad (11)$$

The variables of models (3) to (11) derived from Eq. (1) where X_1 (t , min), X_2 (T , °C or P , W), and X_3 (S , %), whereas Y is the response, sub-indices indicate the applied technique, and the super-indices the three used response criteria (Y_1 in mg C/g R, Y_2 in mg C/g P dw, and Y_2/Y_1 in g R/g P dw). Therefore, eqs. (3) to (11) translate the response patterns, showing a relatively high complexity (higher than 6 parameters) of the possible sceneries for Y_1 and Y_2 value formats and relatively simple solutions for the Y_2/Y_1 response value format (lesser than 6 parameters).

Because the experimental plan is based on coded values of the variables, the obtained model coefficients are empirical and cannot be associated with physical or chemical significance. However, their numerical values can be used for direct comparisons. In fact, the higher is the absolute value of the coefficient, the more important will be the weight of the corresponding variable. Correspondingly, when a factor has a positive effect, the response increases as the values of the involved variable rises, and when the factor has a negative effect, the response decreases.

Eqs. (3) to (11) provide a comprehensive summary of the effects produced by each of the variables defined for the assessed extraction techniques. Several statistical tests were employed to evaluate the ability of the obtained Eqs. (3) to (11) and the results are presented in Table 2B. Overall, the statistical tests are conclusive providing the same findings: Eqs. (3) to (11) are efficient in the subsequent prediction stages. In this regard, and comparatively to the most common statistical criteria presented in Table 2B, the coefficients R^2 and R_{adj}^2 , almost in all cases, displayed results higher than 0.9, which indicates a good agreement between the experimental and predicted values. This implies that the variation of the experimental results can be explained by the independent processing variables by using the specific parametric values presented in Table 2A, which validates the models of Eqs. (3) to

Table 1

Experimental RSM results of the CCD for the optimization of the three main variables involved (X_1 , X_2 , and X_3) in the HAE, UAE, and MAE for the three response value formats assessed (Y_1 , mg C/g R; Y_2 , C/g P dw; and Y_2/Y_1 g R/g P dw). Variables, natural values and ranges in Table A1. Three replicates were performed for each condition for each technique.

Variable coded values			Experimental responses			UAE			MAE		
X_1	X_2	X_3	HAE			Y_1	Y_2	Y_2/Y_1	Y_1	Y_2	Y_2/Y_1
			Y_1	Y_2	Y_2/Y_1						
-1	-1	-1	1.031	0.709	0.688	1.783	1.304	0.731	2.997	2.376	0.793
-1	-1	1	6.731	5.137	0.763	8.272	5.820	0.704	7.993	5.623	0.704
-1	1	-1	1.753	1.072	0.612	3.537	2.591	0.732	3.738	2.908	0.778
-1	1	1	5.944	4.660	0.784	6.932	5.002	0.722	7.602	5.413	0.712
1	-1	-1	1.016	0.682	0.671	2.265	1.635	0.722	3.213	2.384	0.742
1	-1	1	5.042	3.792	0.752	9.915	7.333	0.740	7.702	5.357	0.695
1	1	-1	2.622	1.869	0.713	2.091	1.606	0.768	3.159	2.477	0.784
1	1	1	6.163	4.738	0.769	8.585	6.112	0.712	6.267	4.393	0.701
-1.68	0	0	3.702	1.782	0.661	4.865	3.604	0.741	5.994	4.501	0.751
1.68	0	0	2.326	1.570	0.675	5.574	3.880	0.696	6.324	4.522	0.715
0	-1.68	0	3.489	2.325	0.666	2.647	1.885	0.712	6.351	4.576	0.720
0	1.68	0	3.206	2.133	0.665	5.096	3.767	0.739	2.498	1.706	0.683
0	0	-1.68	0.999	0.688	0.689	2.299	1.749	0.760	2.531	1.982	0.761
0	0	1.68	6.051	4.345	0.718	10.307	4.576	0.444	7.209	5.324	0.739
-1.68	-1.68	-1.68	1.098	0.765	0.696	0.576	0.406	0.705	1.449	0.948	0.655
-1.68	-1.68	1.68	6.777	2.405	0.355	4.875	1.936	0.397	7.834	5.546	0.708
-1.68	1.68	-1.68	2.665	1.864	0.699	3.140	2.417	0.770	2.775	1.930	0.696
-1.68	1.68	1.68	4.975	3.716	0.747	7.648	3.273	0.428	6.757	5.142	0.761
1.68	-1.68	-1.68	0.773	0.518	0.670	0.421	0.348	0.827	1.066	0.841	0.788
1.68	-1.68	1.68	3.553	2.636	0.742	6.213	2.443	0.393	8.641	5.958	0.689
1.68	1.68	-1.68	2.128	1.551	0.729	2.623	2.302	0.878	0.488	0.360	0.739
1.68	1.68	1.68	5.747	4.234	0.737	9.145	4.324	0.473	1.973	1.505	0.762
0	0	0	2.428	1.639	0.675	4.734	3.496	0.738	6.720	4.882	0.726
0	0	0	2.558	1.669	0.652	5.751	4.654	0.809	6.251	4.591	0.734
0	0	0	2.510	1.657	0.660	5.256	4.165	0.792	6.065	4.212	0.695
0	0	0	3.049	2.090	0.686	5.340	4.449	0.833	6.063	4.244	0.700
0	0	0	2.558	1.733	0.678	5.177	4.020	0.776	5.863	4.233	0.722
0	0	0	2.555	1.725	0.675	5.907	4.652	0.788	6.747	2.376	0.684

(11) and allows to move to the interpretation of the independent variable effects and the determination of the optimal conditions that maximize the responses.

3.1.3. Effect of the independent variables on the target responses and optimal extraction conditions

The second-order polynomial model of Eq. (1), which has proved its suitability in the previous section, describe two types of effects (linear and non-linear) for each variable and one interactive effect between two pairs of variables. The patterns of the extraction can be explained by means of the parametric values of the second-order polynomial models shown in Table 2A or Eqs. (3) to (11), as described above, or can be illustrated by graphical representations. In this regard, Fig. 1, Fig. A3, and Fig. A4 (supplementary material) show the extraction results for the three response criteria formats for each assessed technique, respectively. The figures are divided in three columns, each one showing the response results (Y_1 , Y_2 , and Y_2/Y_1) for each of the tested techniques. Additionally, each column is divided into two sections (A and B):

- Section A shows the three-dimensional surface figures for the three possible variable combinations produced by the developed Eqs. (3) to (11). The studied responses (dependent variables) are visualized as a function of the corresponding independent variables by drawing the responses generated by the models. The plots were built by fluctuating two variables within the experimental range and holding the excluded variable constant at the centre of their experimental domain (Table A1). It can be observed that the obtained amount of cyanidin 3-rutinoside is highly dependent on the S variable, for all the tested extraction methods. For the UAE, the effect of the P variable is also considerable, although not so significant as the effect of S . The analysis of the 3D graphs presented in Fig. 1 shows the low influence of t and T on the response of the HAE and MAE methods.

The quadratic effect of the variables observed in HAE was found important, whereas for UAE and MAE the detected relevant effects were the interactive ones.

- Section B illustrates the capability to predict the obtained results and the residual distribution as a function of each one of the considered variables. The statistical information is displayed using two basic graphical criteria, depicting the capacity to predict the obtained results. As previously described in Table 2B, in all cases, the coefficient R^2 displays values higher than 0.9 and the graphical distribution shows the experimental data versus the ones predicted by the model with a linear arrangement, proving the consistency of the RSM models. In addition, the distribution of the residuals as function of each variable are graphically represented and no grouped data or autocorrelations were observed.

3.1.4. Optimal conditions that favours the extraction of anthocyanins and its experimental verification

Once models are statistically and graphically validated, the optimization of the variable conditions to maximize the response values (or minimize, depending on the requirements) was performed by numerical analysis using the simplex procedure. Table 3A shows the variable conditions that optimize the three assessed responses (Y_1 , Y_2 , and Y_2/Y_1), individually, while Table 3B presents the results of an overall optimization of these responses.

Once the numerical values of the optimal conditions and the maximum responses are achieved, the tendencies of each response were represented in Fig. 2. The graphical illustration allows the visual selection of the most favourable conditions, taking into account simultaneously all responses when the excluded variable is positioned at the individual optimal values of the others (Table 3A). Fig. 2 shows the isolines projections for the combination of the three main involved variables (\times_1 , \times_2 , and \times_3) in the HAE, MAE, and UAE, for the three

Table 2
Parametric values, symmetric confidence intervals and statistical results of the second-order polynomial model of Eq. (1) after fitting the three response format values (Y_1 , mg C/g R; Y_2 , C/g P dw; and Y_2/Y_1 g R/g P dw) for the HAE, UAE, and MAE extracting techniques. The parametric subscript 1, 2, and 3 stands for the variables involved $t(X_1)$, T or $P(X_2)$, and $S(X_3)$, respectively. The parameters are presented in coded values according to the CCD with 5 range levels (Table A1).

Coefficients	Parametric responses to the central composite designs for each technique																		
	HAE						UAE						MAE						
	Y_1	Y_2	Y_2/Y_1	Y_1	Y_2	Y_2/Y_1	Y_1	Y_2	Y_2/Y_1	Y_1	Y_2	Y_2/Y_1	Y_1	Y_2	Y_2/Y_1	Y_1	Y_2	Y_2/Y_1	
Fitting coefficients obtained																			
Intercept	b_0	3.706	± 0.333	14.744	± 0.837	0.687	± 0.009	5.509	± 0.442	4.244	± 0.478	0.775	± 0.029	6.173	± 0.322	4.461	± 0.222	0.724	± 0.005
Linear effect	b_1	-0.224	± 0.188	-0.859	± 0.471	0.020	± 0.006	0.206	± 0.162	0.114	± 0.262	0.012	± 0.010	-0.355	± 0.181	-0.278	± 0.125	ns	
	b_2	0.216	± 0.188	-1.361	± 0.471	0.021	± 0.006	0.613	± 0.162	0.421	± 0.262	0.013	± 0.010	-0.518	± 0.181	-0.361	± 0.125	ns	
	b_3	1.297	± 0.187	3.894	± 0.470	ns		2.047	± 0.162	0.884	± 0.262	-0.086	± 0.010	1.580	± 0.181	1.094	± 0.125	-0.007	± 0.005
Quadratic effect	b_{11}	-0.171	± 0.102	ns		ns		ns		-0.067	± 0.345	-0.009	± 0.007	ns		ns		ns	
	b_{22}	-0.057	± 0.030	-0.980	± 0.573	ns		-0.626	± 0.162	-0.393	± 0.345	ns		-0.485	± 0.220	-0.386	± 0.152	-0.008	± 0.005
	b_{33}	ns		-0.908	± 0.573	ns		0.274	± 0.162	-0.261	± 0.345	-0.050	± 0.010	-0.348	± 0.220	-0.226	± 0.152	0.010	± 0.005
Interactive effect	b_{12}	0.171	± 0.135	-0.738	± 0.339	-0.012	± 0.002	ns		ns		ns		-0.310	± 0.130	-0.241	± 0.090	ns	
	b_{13}	ns		ns		0.013	± 0.002	0.189	± 0.162	0.092	± 0.189	ns		ns		ns		-0.007	± 0.005
	b_{23}	-0.084	± 0.049	-0.904	± 0.338	0.014	± 0.001	ns		-0.066	± 0.188	ns		-0.360	± 0.130	-0.235	± 0.090	ns	
Statistical information of the fitting analysis																			
Obs		28		28		28		28		28		28		28		28		28	
R^2		0.9478		0.9129		0.8155		0.9359		0.9206		0.9194		0.9263		0.9352		0.9135	
R^2_{adj}		0.9056		0.8933		0.8022		0.9005		0.8954		0.8924		0.9016		0.8974		0.9036	

ns: non-significant coefficient; Obs: number of observations; R^2 : coefficient of determination; R^2_{adj} : adjusted determination coefficient for the model.

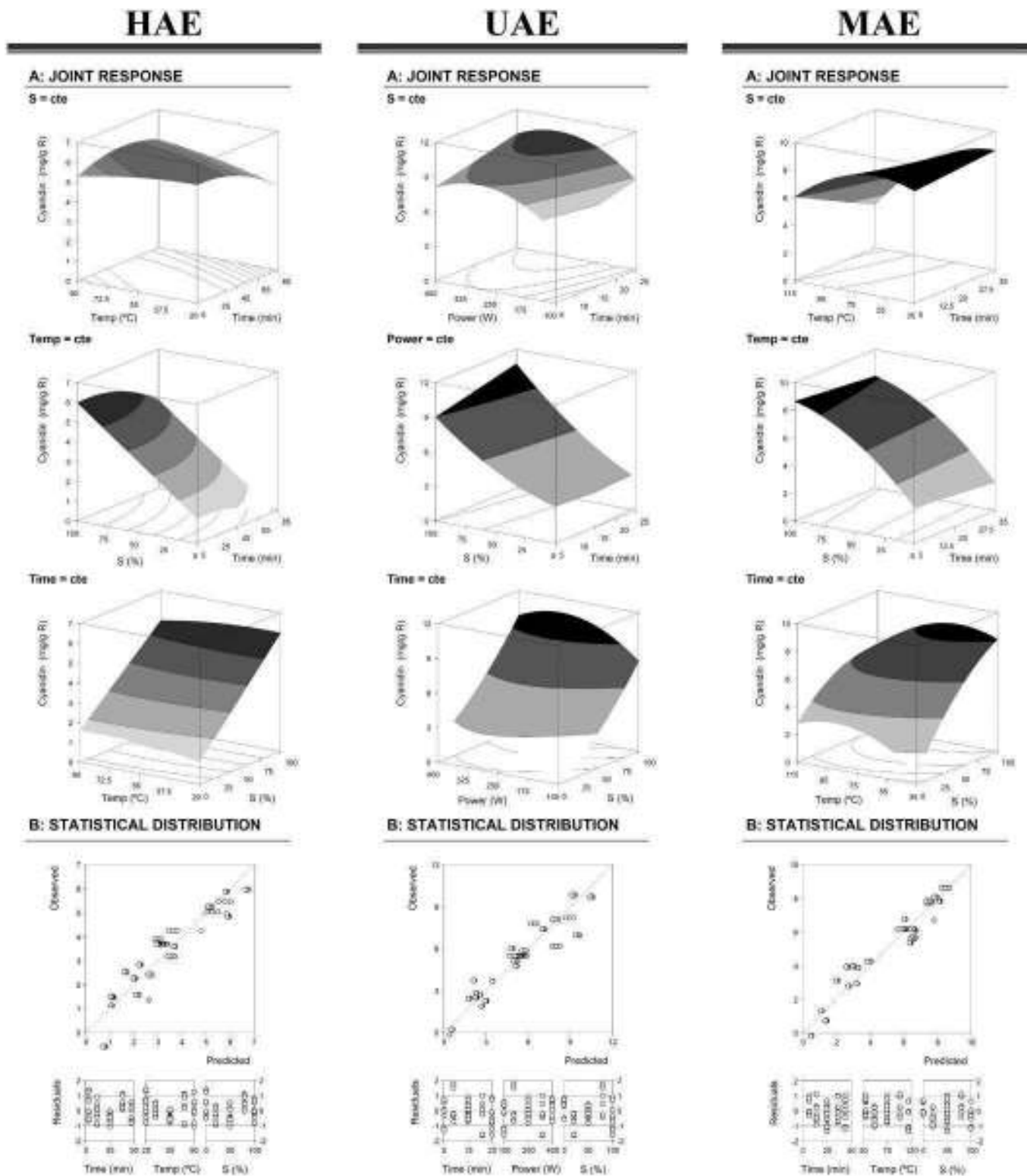


Fig. 1. Part A: Illustrates the three-dimensional analysis in terms of the extraction behaviour for the Y_1 (mg C/g R) responses for the optimization of the three main variables involved (x_1 , x_2 , and x_3) in the HAE, UAE, and MAE. Each net surface shows the predicted response performed by the second order polynomial of Eq. (1). The interactions between each pair of variables in each graph are built when the factor excluded is constantly positioned at the centre of the experimental domain (Table A1, supplementary material). Experimental results are displayed in Table 1 and parametric values used in Eq. (1) are shown in Table 2. Part B: Shows a statistical illustration of the goodness of fit by presenting the differences between predicted and observed results and the residual distribution as a function of each of the variables.

response value formats (Y_1 , mg C/g R; Y_2 , mg C/g P dw; and Y_2/Y_1 g R/g P dw). The isolines presented in the XY plane are derived from the three-dimensional responses obtained from the second-order

polynomial equation of Eqs. (3) to (11). Their analysis is important for decision making when choosing the best conditions to perform the extractions.

Table 3

Optimal individual and global variable conditions (in natural values) that lead to maximal response values for each of the response value formats (Y_1 , mg C/g R; Y_2 , C/g P dw; and Y_2/Y_1 g R/g P dw) for each extracting technique assessed (HAE, UAE, and MAE).

Criteria	Optimal Variable Conditions			Optimum Response	
	X_1 : t (min)	X_2 : T (°C) or P (W)	X_3 : S (%)		
A) Individual optimal variable conditions:					
HAE	Y_1	14.24 ± 0.16	28.26 ± 2.83	100.00 ± 2.12	5.99 ± 0.60 mg C/g R
	Y_2	49.25 ± 2.48	90.00 ± 8.10	100.00 ± 2.83	4.50 ± 0.27 mg C/g P dw
	Y_2/Y_1	85.00 ± 8.50	90.00 ± 3.60	100.00 ± 1.43	0.80 ± 0.08 g R/g P dw
UAE	Y_1	21.00 ± 1.89	309.53 ± 3.10	100.00 ± 1.45	9.56 ± 0.48 mg C/g R
	Y_2	25.00 ± 1.75	285.07 ± 19.95	100.00 ± 2.04	5.32 ± 0.37 mg C/g P dw
	Y_2/Y_1	23.61 ± 1.18	394.76 ± 3.95	19.96 ± 1.42	0.84 ± 0.08 g R/g P dw
MAE	Y_1	5.00 ± 0.10	60.27 ± 4.22	100.00 ± 3.19	8.63 ± 0.69 mg C/g R
	Y_2	5.00 ± 0.05	64.21 ± 1.28	100.00 ± 2.48	6.21 ± 0.56 mg C/g P dw
	Y_2/Y_1	35.00 ± 1.05	75.00 ± 6.75	0.00 ± 1.63	0.78 ± 0.02 g R/g P dw
B) Global optimal variable conditions:					
HAE	Y_1	13.74 ± 1.91	35.64 ± 7.98	100.00 ± 1.36	5.78 ± 0.12 mg C/g R
	Y_2				4.03 ± 0.22 mg C/g P dw
	Y_2/Y_1				0.56 ± 0.02 g R/g P dw
UAE	Y_1	21.34 ± 0.55	310.58 ± 25.89	100.00 ± 1.23	9.01 ± 0.76 mg C/g R
	Y_2				4.32 ± 0.14 mg C/g P dw
	Y_2/Y_1				0.51 ± 0.21 g R/g P dw
MAE	Y_1	5.00 ± 0.30	62.41 ± 0.57	100.00 ± 3.73	7.43 ± 0.78 mg C/g R
	Y_2				4.11 ± 0.37 mg C/g P dw
	Y_2/Y_1				0.76 ± 0.04 g R/g P dw

Finally, Fig. 3A shows a 2D graphical representation, in which the lines show the response predicted by the models described in Eqs. (3) to (11), when the other studied variables are fixed at their optimal values. The response axes (Y) were maintained on the same scale to facilitate the comparison among the used extraction techniques. The dots (⊙) presented alongside the line highlight the location of the optimal value (Table 3A).

When combining the information produced by the three response criteria (Y_1 , Y_2 , and Y_2/Y_1), the complete behaviour of each relevant variable influencing the responses is defined in global terms. The global optimizing results are presented in Table 3B and summarized below:

- For the HAE system: the optimal global conditions were at 13.74 ± 1.91 min, 35.64 ± 7.98 °C, and 100.00 ± 1.36% of ethanol, producing 5.78 ± 0.12 mg C/g R (Y_1), 4.03 ± 0.22 mg C/g P dw (Y_2), and 0.56 ± 0.02 g R/g P dw (Y_2/Y_1).

- For the UAE system: the optimal global conditions were at 21.34 ± 0.55 min, 310.58 ± 25.89 W, and 100.00 ± 1.36% of ethanol, producing 9.01 ± 0.76 mg C/g R (Y_1), 4.32 ± 0.14 mg C/g P dw (Y_2), and 0.51 ± 0.21 g R/g P dw (Y_2/Y_1).

- For the MAE system: the optimal global conditions were at 5.00 ± 0.30 min, 62.41 ± 0.57 °C, and 100.00 ± 9.00% of ethanol, producing 7.43 ± 0.78 mg C/g R (Y_1), 4.11 ± 0.37 mg C/g P dw (Y_2), and 0.76 ± 0.04 g R/g P dw (Y_2/Y_1).

For all techniques, the conditions that led to the optimal values were experimentally tested in order to ensure the accuracy of the presented results. Consequently, for the response Y_1 , the UAE technique produced the best results, as the increase of t and S led to a residue with greater purity in cyanidin 3-rutinoside. Regarding the response Y_2 , the MAE produced the higher values, but the increase of the t and T variables led to the degradation of the anthocyanin compound. The UAE gives rise to

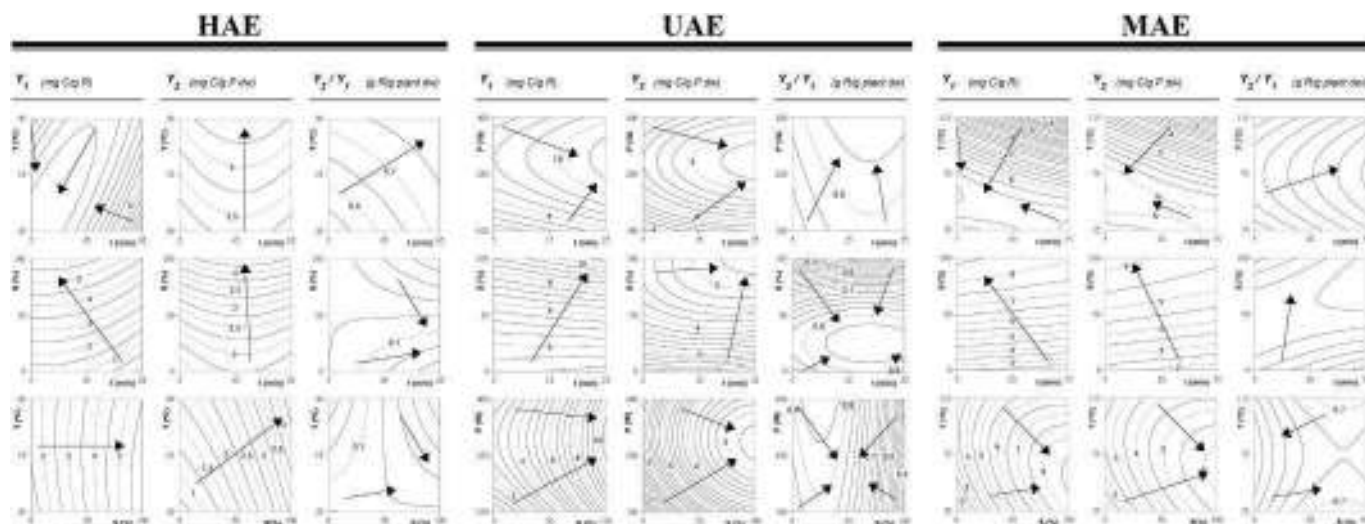


Fig. 2. Shows the contour graphs in terms of the three response value formats assessed (Y_1 , mg C/g R; Y_2 , mg C/g P dw; and Y_2/Y_1 g R/g P dw) as a function of two pair of variables of the three variables involved (X_1 , X_2 , and X_3) in the HAE, MAE and UAE. This analysis allows to describe visually the tendencies of each response and guide the selection of the most favourable conditions. Each of the contour graphs represents the isoline projection predicted with the second order polynomial of Eq. (1). The interactions between each pair of variables in each contour graphs are built when the factor excluded is constantly positioned at the individual optimal values of the others (Table 3A). The statistical design and experimental results are described in Table 1. Estimated parametric values are shown in Table 2.

A: Illustration of the interaction between variables

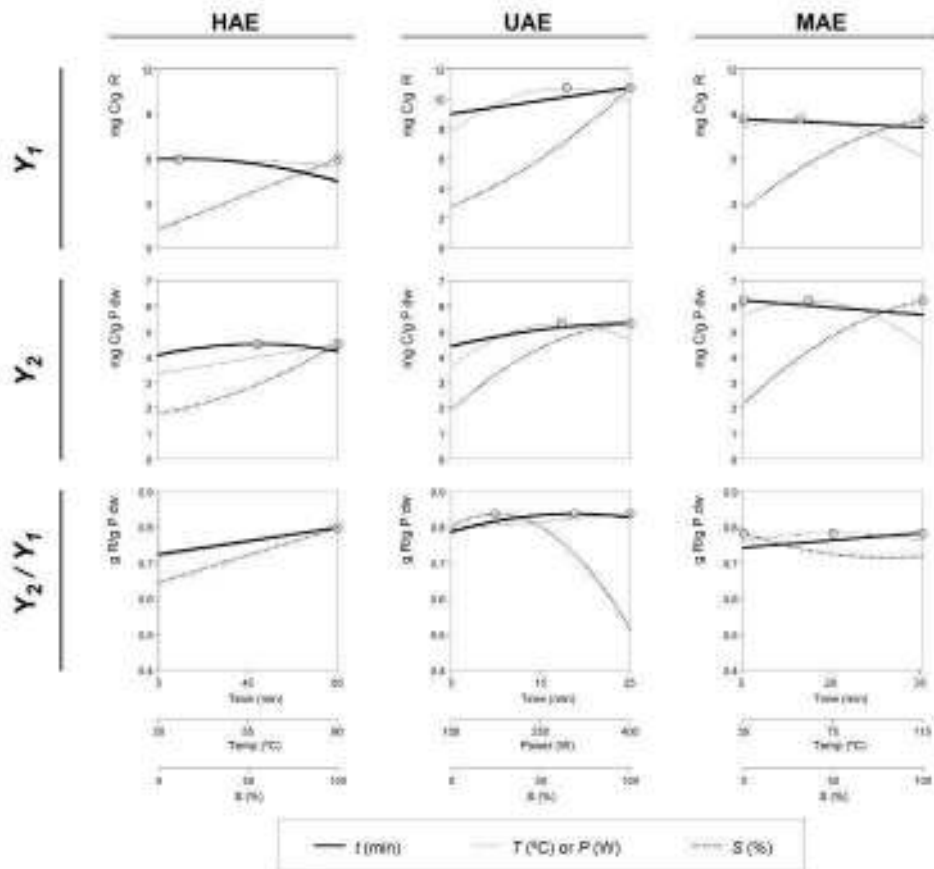
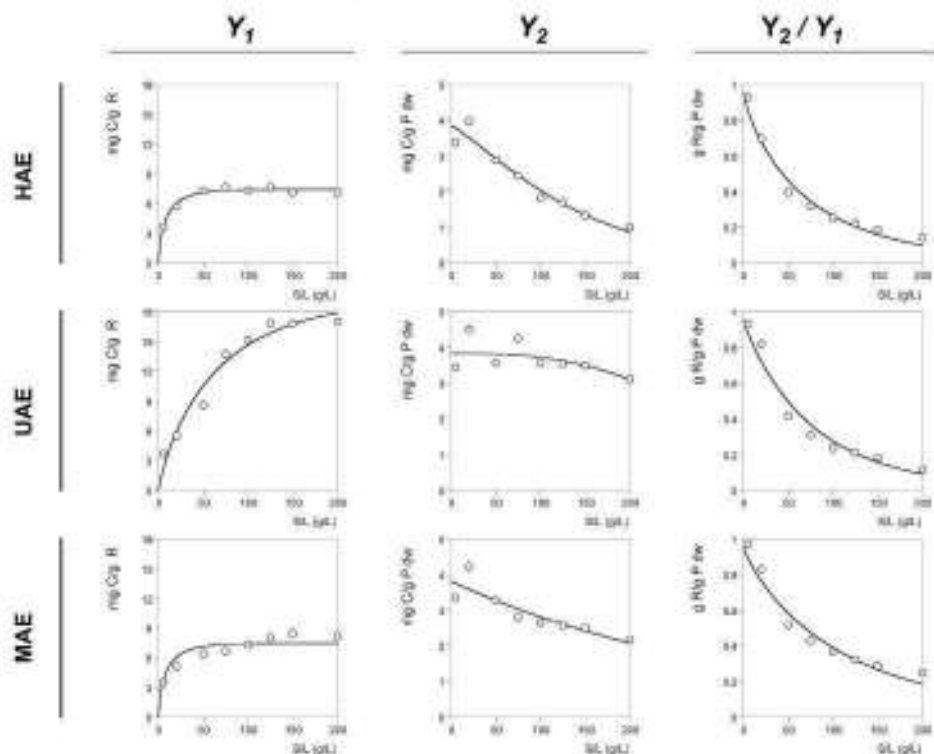


Fig. 3. Summary of the effects of each of the variables assessed for HAE, UAE and MAE systems. Part A: Shows the individual responses as a function of all the variables assessed. The variables in each graph were positioned at the individual optimal values of the others (Table 3). The points (⊙) illustrates the position of the optimum value. Lines and dots are generated by the theoretical second order polynomial models derived from Eq. (1). Part B: Shows the dose response of S/L at the global optimal values of the other three variables (Table 3B). Experimental results are represented by dots (⊙), meanwhile lines are the predicted results described by Eq. (2).

B: Solid-to-liquid ratio patterns



lower Y_2 values, but the extraction was favoured if longer t and S were used. The HAE extraction system ensures a significant yield value as the variables S and T increases, but when the variable t increases the anthocyanin content decrease. However, the conditions optimized in global terms, the value of the variable S that maximized the cyanidin 3-rutinoside extraction is always 100%.

In conclusion, UAE is the best extraction technique, closely followed by MAE, and HAE, which showed to be less effective solutions. These results prove that alternative solid-liquid extraction methods (UAE and MAE) are more suitable than the conventional HAE for the extraction of cyanidin 3-rutinoside from fig peel.

3.2. Dose-response analysis of the solid-to-liquid effect at the optimum conditions

As already described in the bibliography, the ideal solid-to-liquid ratio should be the one that allows the solvent to properly penetrate into the structure of the solid matrix (Pinela et al., 2017), but also the one that allows the solvent to dissolve the target compounds (Albuquerque et al., 2016). Therefore, a study aiming to evaluate the S/L effect was conducted at the global optimal conditions predicted by the polynomial models obtained for HAE, UAE, and MAE techniques.

Primary tests were performed to find the limit value of S/L . The results showed that over 200 g/L the reaction system could not be homogenized properly, thus the dose-response analysis process was designed to analyse the S/L from 5 to 200 g/L.

The dose-response results to S/L effects of the three response value formats (Y_1 , Y_2 , and Y_2/Y_1) and for the three assessed extraction techniques were evaluated by fitting the Eq. (2) (increasing or decreasing form) to the experimental responses. The obtained parametric values are presented in Table A2 (supplementary material). The effects caused by the S/L on the response value formats are graphically shown in Fig. 3B for the three studied techniques. Fig. 3B shows the experimental results (points) and their respective predictions made by the mathematical model of Eq. (2) (lines). In general, a non-linear effect can be observed for all responses as the S/L dose-response increases, causing a saturation-increasing effect (\uparrow) for the Y_1 (mg C/g R) value format and saturation-decreasing effects (\downarrow) for Y_2 (mg C/g P dw) and Y_2/Y_1 (g R/g P dw) value formats. The analysis of the results can be interpreted by means of the two main parameters K and m_n (at 50% or 99% of the response). The parameter K shows the maximum extraction value that can be obtained as a function of the S/L dose-response. Thus, the lower the m_n values are, the higher are the reached extraction levels at a shorter dose-response value, which would limit the possibility of reducing the amount of needed solvent, for industrial purposes. Given these considerations, both values are important to understand the trends of the S/L dose-response effect.

In a more detailed analysis, the following aspects can be observed:

- For Y_1 values, response that gives the purity in C in the extracted residue: a saturation-increasing dose-response pattern was observed, which means that Y_1 initially increases as the S/L increases, but when a certain S/L level is reached, the anthocyanin purity remains constant. For HAE, the response rises until S/L reaches values close to 43.58 ± 0.11 g/L (parametric value $m_{99\%}$ from Eq. (2), Table A2), allowing a concentration of 7.41 ± 0.59 mg C/g R (parametric value K from Eq. (2), Table A2) that remains constant at high S/L values. For UAE, the $m_{99\%}$ value was 302.48 ± 3.48 g/L, with a K value of 19.40 ± 0.78 mg C/g R, and for MAE, 53.26 ± 8.91 g/L were obtained for $m_{99\%}$, with a K of 9.71 ± 1.38 mg C/g R. In the performed HAE and MAE, the increased levels of anthocyanin purity achieved by increasing the S/L were not as pronounced as those observed with UAE, fact that may be related to instrumental limitations.
- For Y_2 values, the response that reflects the C content in the peels: a saturation-decreasing dose-response pattern was observed, which

means that Y_2 initially decreases to zero as S/L increase. For HAE, UAE, and MAE the obtained $m_{50\%}$ values were 107.39 ± 9.67 , 302.46 ± 27.22 , and 225.07 ± 2.25 g/L, respectively, presenting K values of ~ 3.8 mg C/g P dw for the three tested techniques. These results may probably reflect the total available anthocyanin content in the fig peels, once a maximum concentration was achieved for all the tested extraction methods. Besides, when the S/L increased, a saturation of the solvent was observed, with a decrease of cyanidin 3-rutinoside levels. However, for the modern extraction techniques (UAE and MAE) this decreasing effect is less noted comparatively with the conventional extraction method (HAE). In fact, for UAE and MAE the S/L effect remains almost constant until ~ 120 g/L.

- For Y_2/Y_1 values, response that represents the extraction yield of the residue: a saturation-decreasing dose-response pattern was found, which means that Y_2 initially decreases as S/L increases. For HAE, UAE, and MAE the obtained $m_{50\%}$ values were 47.32 ± 1.89 , 53.32 ± 0.53 , and 74.41 ± 1.49 g/L, respectively, with a maximum residue levels of ~ 0.95 g R/g P dw (K), for all the assessed techniques.

Given the widespread interest for anthocyanins, there has been an effort to modernize the extraction protocols, reducing the amount of organic solvents (ecological point of view) and improving the extraction yield (economic point of view) (Jiménez et al., 2018). The lack of optimization approaches, specifically in what concerns anthocyanins extraction contributed to detract the use of these natural solutions in food industry. The study concludes that UAE and MAE, reduce both economic and ecological impacts in comparison with the typically HAE process, in the extraction of cyanidin 3-rutinoside from fig peel in an industrial level (Sicaire et al., 2016).

3.3. Comparison with other studies involving the extraction of anthocyanins

To recover anthocyanins from *Ficus carica* L. infructescences peel to be used as natural food colorant, the optimization of the extraction process is one of the crucial stages towards the industrial implementation. Nevertheless, along with the scarcity of studies on the evaluation of anthocyanin content in *F. carica* (entire infructescence, pulp, or peel), to the best of our knowledge none of these studies has previously optimized their extraction from these matrices, which hinders results comparison for this particular matrix. Consequently, the reported total anthocyanin values in the fig peel will be compared with bibliographic results in which the entire infructescence was used, as well as with those that have been reported as the major natural sources of anthocyanins (Khadhraoui et al., 2018).

Previous studies have shown that figs contain greater anthocyanin levels than other atypical natural sources (Harzallah et al., 2016; Vallejo et al., 2012). In a recent study conducted by Wojdyło, Nowicka, Carbonell-Barrachina, and Hernández (2016), dealing with the assess of different fig varieties, it was possible to recover 0.01 to 1.2 mg of anthocyanins per g of entire infructescence dw, using UAE with methanol as the extraction solvent. These values are lower than those obtained in this study for fig peel at the optimal conditions (~ 3.8 mg/g P dw). The higher concentration obtained with the fig peel could be partly attributed to the performed optimization procedure, which led to an increased extraction efficiency, and thus anthocyanin yield, when compared with results reported for the entire infructescence (Wojdyło et al., 2016). Moreover, further differences could be explained by the use of different extraction solvents (water-ethanol mixtures vs. methanol). It is a fact that methanol presents a high extraction power, especially for polar molecules such as phenolic compounds. Nevertheless, its use for the extraction of anthocyanin compounds seems to be unrealistic. On the other hand, the hydroalcoholic mixture water-ethanol can be considered a bio-solvent alternative for food industry, if properly removed, leading to higher anthocyanin recovery (Bosiljkov et al., 2017).

However, the differences found between anthocyanin levels in the entire infructescence and in the peel could be mainly explained by the fact that these compounds tend to be accumulated in the outer parts of the infructescence, such as peels, in the maturity stages chosen for harvest. This fact is well known and has been investigated with other bio-residues rich in anthocyanins, such as mango and sugarcane peels, which present ~0.1 mg/g P dw (Lopes et al., 2016; Zhao et al., 2018). Even so, the anthocyanin concentration detected in these matrices are far below those found in the fig peels assessed in the present study. Given the fact that in almost all industrial processes involving fig infructescence's they are used in very mature stages, the discarded parts of the fig, including the peels, can become relevant by-products if recovered for extraction of anthocyanins, compounds with natural colouring properties that could be applied as natural additives.

Generally, the most common rich sources of anthocyanins are those derived from *Oryza sativa* L. (var. Glutinosa), presenting 42 mg/g dw (Chen, McClung, & Bergman, 2017), *Phaseolus vulgaris* L. (common beans), with 32 mg/g dw (Mojica, Berhow, & Gonzalez de Mejia, 2017), and *Rubus fruticosus* L. fruits (blackberries), with 17.10 mg/g dw (Elisia, Hu, Popovich, & Kitts, 2006). Although these values are considerably higher than those presented by fig peels, these sources have an already recognized commercial value for other purposes, contrarily to fig peels. Considering that other matrices, such as eggplant peel (0.6 mg/g dw) (Todaro et al., 2009) or grape bark (1 mg/g dw) (Chen et al., 2015), are currently used as common sources of anthocyanins, even presenting a lower concentration than the one found in fig peels, this matrix arises as a promising by-product to be explored for colorant industrial application purposes.

4. Conclusions

Due to the great interest of using natural pigments, such as anthocyanins, in food, pharmaceutical, and cosmetic industries, in the present work different solid-liquid extraction methods were studied and optimized by RSM. The selected techniques were HAE, UAE, and MAE, using three independent variables (t , T , and S for HAE and MAE, and t , P , and S for UAE) to maximize the defined responses: mg C/g R (Y_1); mg C/g P dw (Y_2); and g R/g P dw (Y_2/Y_1). The UAE was the most effective method of this study, capable of producing 3.8 mg C/g P dw and 0.95 g R/g P dw, with an anthocyanin content of 19.4 mg C/g R. These values were obtained at the optimum conditions (21.34 ± 0.55 min, 310.58 ± 25.89 W, 100% ethanol, and 183.01 ± 22.82 g/L). The HAE and MAE techniques revealed similar performances, but slightly lower than the one obtained for UAE. When S/L variable was tested, a significant effect was noticed with UAE, resulting in an extract with an increased purity as the S/L increased.

The analysis presented provides important data that allows the comparison between different extraction methods, in terms of efficiency, and consequent related decision making. In an industrial level, these methodologies reduce costs related to energy, solvent consumption, equipment investment, etc. Achieving the optimal conditions and maximum the responses is an important step to guide the choice of a suitable and sustainable process.

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Abbreviations

General abbreviations

HPLC-DAD-ESI/MS High-performance liquid chromatography coupled to photodiode array detector and mass spectrometer
 Eq. or Eqs. Equation or equations
 C Cyanidin 3-rutinoside
 P Fig peel
 R Extracted dried weight residue
 dw Dry weight
 RSM Response surface methodology
 CCCD *Circumscribed central composite design*
 MAE Microwave assisted extraction
 UAE Ultrasound assisted extraction
 HAE Heat assisted extraction
 Response surface methodology experimental design and model equations:

Variables

t Time or X_1 (min)
 T Temperature or X_2 (°C)
 S Solvent proportion or X_2 (% v/v of ethanol in hydroalcoholic mixtures)
 P Ultrasonic power or X_2 (W)
 S/L Solid-to-liquid ratio or X_2 (g/L)
 Responses
 Y_1 mg of cyanidin 3-rutinoside obtained in the extracted dried weight residue (R; mg C/g R)
 Y_2 mg of cyanidin 3-rutinoside per g of peel dry matter (mg C/g P dw)
 Y_2/Y_1 obtained by dividing the responses Y_2 and Y_1 , which provides information regarding the extraction yield of the dried weight residue (g of R/g P dw)Equation
 Eq. (1) Second-order polynomial equation
 Y Dependent variable to be modelled
 X_i and X_j Independent variables to be assessed
 b_0 Constant coefficient.
 b_i Coefficients of linear effect.
 b_{ij} Coefficients of interaction effect
 b_{ii} Coefficient of quadratic effect
 n Number of variables
 Eqs. (3) to (11) Second-order polynomial equation derived from Eq. (1) that describe all response formats for all techniques testedDose-response description of the solid-to-liquid ratio effect
 W Weibull equation
 $W (\uparrow)$ Increasing Weibull equation
 $W (\downarrow)$ Decreasing Weibull equation
 Eq. (2) Number of the Weibull equation in its increasing and decreasing form
 K Maximum extraction value. Units would depend in the response format (Y_1 , Y_2 , and Y_2/Y_1) assessed (i.e., if Y_2 the units would be in mg C/g P dw)
 a Shape parameter related to the maximum slope of the response
 n Any desired level between 0 and 100% of the response (Y_1 , Y_2 , and Y_2/Y_1)

m_n	The S/L parametric value for the selected n response level (m_{10} , m_{25} , m_{75} , m_{95} , etc.)
ns	Statistical evaluation
ns	Non-significant coefficient
Obs	Number of observations
R^2	Coefficient of determination
R^2_{adj}	Adjusted determination coefficient

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodres.2018.07.016>.

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Article

Ultrasound as a Rapid and Low-Cost Extraction Procedure to Obtain Anthocyanin-Based Colorants from *Prunus spinosa* L. Fruit Epicarp: Comparative Study with Conventional Heat-Based Extraction

Maria G. Leichtweis ¹, Carla Pereira ¹, M.A. Prieto ^{1,2}, Maria Filomena Barreiro ^{1,3},
Ilton José Baraldi ⁴, Lillian Barros ^{1,*}, and Isabel C.F.R. Ferreira ^{1,*}

¹ Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal; mg.leichtweis@hotmail.com (M.G.L.); carlap@ipb.pt (C.P.); michaelumangelum@gmail.com (M.A.P.); barreiro@ipb.pt (M.F.B.)

² Nutrition and Food Science Group, Dept. of Analytical and Food Chemistry, CITACA, CACTI, University of Vigo-Vigo Campus, Vigo, Spain

³ Laboratory of Separation and Reaction Engineering – Laboratory of Catalysis and Materials (LSRE-LCM), Polytechnic Institute of Bragança, Campus Santa Apolónia, 5300-253 Bragança, Portugal

⁴ Departamento Acadêmico de Alimentos (DAALM), Universidade Tecnológica Federal do Paraná, Campus Medianeira, 85884-000, Paraná, Brasil; ijbaraldi@gmail.com

* Correspondence: lillian@ipb.pt (L.B.); iferreira@ipb.pt (I.C.F.R.F); Tel.: +351-273-303285 (L.B.); +351-273-303219 (I.C.F.R.F); Fax: +351-273-325405 (L.B.); +351-273-325405 (I.C.F.R.F)

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Abstract: An ultrasound rapid and low-cost procedure for anthocyanin-based colorants from *Prunus spinosa* L. fruit epicarp was developed, and the advantages were compared with conventional heat-based extraction. To obtain the conditions that maximize anthocyanins' extraction, a response surface methodology was applied using the variables of time, temperature, and ethanol content, in the case of heat extraction, whereas for ultrasound assisted extraction, temperature was replaced by ultrasound power. Two anthocyanin compounds were identified by HPLC-DAD-ESI/MS—namely, cyanidin 3-rutinoside and peonidin 3-rutinoside. The responses used were the extraction yield and the content of the identified anthocyanins. Ultrasound extraction was the most effective method at 5.00 ± 0.15 min, 400.00 ± 32.00 W, and $47.98\% \pm 2.88\%$ of ethanol obtaining $68.60\% \pm 2.06\%$ of extracted residue, with an anthocyanin content of 18.17 mg/g (extract-basis) and 11.76 mg/g (epicarp-basis). Overall, a viable green process was achieved that could be used to support pilot-scale studies for industrial production of anthocyanin-based colorants from *P. spinosa* fruit epicarp.

Keywords: *Prunus spinosa* L. fruit epicarp; wild fruit valorization; cyanidin 3-rutinoside; peonidin 3-rutinoside; heat and ultrasound assisted extraction; response surface methodology

1. Introduction

Prunus spinosa L. (blackthorn) is a spontaneous wild shrub found in Portugal, Spain, and other European countries. Its fruits are commonly used for liqueur and jam preparations, as well as for medicinal purposes [1]. Nevertheless, no reports were found regarding the industrial, or large scale, use of these fruits, probably because of their bitter and astringent taste.

The valorization of agricultural products has gained much attention in the late years as a mean for a sustainable management, which can concomitantly increase the profit of local economies. In this regard, *P. spinosa* constitutes an underexploited source and can serve as a raw material for the

recovery/production of compounds for food applications [2]. As with other *Prunus* species, anthocyanin compounds can be found in blackthorn fruits at high levels, being responsible for their typical coloration [3,4]. In fact, a complex profile of anthocyanins was previously identified in *P. spinosa* fruits, among which cyanidin 3-rutinoside and peonidin 3-rutinoside were found to be predominant [5,6].

Anthocyanins are natural pigments belonging to the phenolic compounds group and, within that, to the flavonoids class, presenting a range of colors between red, blue, and violet that are characteristic of various fruits and vegetables [7]. Beyond their various physiological benefits, which include effects against cardiovascular diseases, atherosclerosis, and cancer, recently, an increasing interest in these compounds began to arise because of their colorant properties [8,9].

The industrial production of natural-based colorants has been established for years and consists mainly of obtaining colorant-rich extracts through conventional heat assisted extraction (HAE, or maceration) using water as a solvent followed by several isolation/drying steps. This type of conventional process, although used at large-scale, is known for requiring high-energy consumption and long extraction times [10–12]. Alternative extraction processes, able to replace traditional ones, have been established to shorten the needed time, decrease energy requirements, and reduce solvent consumption. Among the non-conventional procedures applied to anthocyanins' extraction, ultrasound, microwave, and supercritical fluid assisted extraction techniques have attracted, in the recent years, the attention of industrials and researchers [10,13]. Regarding ultrasound assisted extraction (UAE), it is considered an inexpensive, simple, and efficient alternative to conventional techniques [14]. The extraction capability of UAE is attributed to mechanical and cavitation phenomena, which lead to cells' disruption, particle size reduction, and enhanced mass transfer across the cell membrane [11,13].

To obtain anthocyanin-rich extracts, it is crucial to consider the factors affecting the stability of these compounds, including structure and concentration, pH, temperature, light exposure, oxygen levels, and used extraction solvents [15]. Thus, the choice of the extraction method, along with the optimization of relevant extraction variables, are essential to guarantee a maximum recovery efficiency [16]. Additionally, the efficiency is also strongly affected by the variability observed among different matrices [17]. Through response surface methodology (RSM), it is possible to optimize the relevant variables simultaneously, obtaining polynomial models capable of describing, within the tested experimental interval, the ideal conditions that maximize the used response criteria [13].

In the present study, the goal was to explore blackthorn anthocyanin composition and promote a higher commercial value of these wild fruits through the development of an anthocyanin-based coloring extract. For that purpose, the fruit epicarp was used because it has a much more intense color than the pulp, and thus a higher concentration of anthocyanins and less interfering compounds in the extraction process (e.g., sugars). To the best of our knowledge, and according to a thorough literature survey, no reference or report on the optimization of anthocyanin compounds extraction from fruit epicarps of *P. spinosa* was found. Therefore, the present study aimed to optimize the extraction of these compounds from *P. spinosa* fruit epicarps through HAE and UAE techniques, evaluating the following variables: i) type of solvent (water and green organic solvents); ii) extraction time; iii) solid-to-liquid ratio; and iv) temperature (for HAE) or pressure (for UAE). The most efficient parameters were obtained by response surface methodology (RSM). The identification and quantification of the anthocyanin compounds present in the extracts was assessed by HPLC-DAD-ESI/MS.

2. Results

2.1. Development of RSM Models to Optimize Responses and Conditions

The RSM is a valuable instrument to assess the impact of the main extraction factors and their interactions on one or more responses. The technique uses fixed experimental designs with the major goals of minimizing the experimental labor and finding optimal solutions. In this regard, the work presented here uses the *circumscribed central composite design (CCCD)* design plan, which is a popular

design among researchers when trying to optimize food processing methods [18], such as the extraction of anthocyanin compounds.

In a previous study [5], authors identified, using HPLC-DAD-ESI/MS, the anthocyanin compounds of cyanidin 3-rutinoside ($[M + H]^+$ at m/z 595) and peonidin 3-rutinoside ($[M + H]^+$ at m/z 609) in *P. spinosa* fruits, and highlighted that the colorant capacity of these fruits is mainly attributed to these compounds. Although authors quantify the content of those anthocyanin compounds in *P. spinosa* fruits, the conditions of extraction were not optimized. Therefore, based on those preliminary findings, it seems logical to continue to explore the potential of *P. spinosa* fruits as a source of anthocyanin compounds. In this regard, the study applies a RSM technique under a CCCD to optimize the operating conditions of the extraction of two common techniques in the industrial environment (HAE and UAE) with the intention of maximizing their extraction. However, because the major quantity of anthocyanin compounds in *P. spinosa* fruits is located in the fruit epicarp, in this study, we ignored the inside parts of the fruit and focused the attention on the fruit epicarps. Additionally, by focusing on the epicarps, we are avoiding a high content of interfering compounds in the extraction process (e.g., sugars) that would require further purification steps. Figure A1 (supplemental material) shows a complete summary of all the steps used for the optimization procedure in order to recover the anthocyanin compounds from the epicarps of *P. spinosa* fruits. Figure A2 (supplementary material) shows a chromatographic example of HPLC-DAD-ESI/MS results for the quantification of anthocyanin compounds in the epicarps of *P. spinosa* fruits.

Table 1 shows the experimental results derived from the CCCD used to optimize the extraction of anthocyanins from the fruits epicarps (Y_1 , mg C/g R; Y_2 , mg C/g E dw; and *Yield*, %) for each one of the computed extraction techniques (HAE and UAE). As described, the CCCD experimental results are subjected to the mathematical analysis of Equation (1), by applying a fitting procedure coupled with non-linear least-squares estimations. The parametric values of Equation (1) derived from this analytical procedure, the corresponding confidence interval of the parameters ($\alpha = 0.05$) found after modelling the extraction response values, and basic statistical information of the mathematical procedure are presented in Table 2. The parametric values considered non-significant (*ns*) values were excluded from model construction and the final equations for describing the responses assessed using significant terms are presented in Table A1 (supplementary material).

The significant parametric values in Table 2 are presented as a function of the codification criteria of the CCCD. Although they could be presented as the real numerical ranges of the variables assessed (X_1 to X_3), such information would not provide any additional insights of the regression analysis performed or the possible effects that may occur. The key information is the weight of the numerical values of the significant parameters; therefore, it seems logical to present them under a codification mode that allows us to compare the values between them effortlessly. Therefore, based on the numerical values derived, some global conclusions can be deduced as follows:

- For the HAE technique: In global terms, the significant parametric values within the linear effect (LE) group have a far more relevant contribution to the description of the responses than the interactive effect (IE), with the quadratic effect (QE) group being the less representative one ($LE > IE \gg QE$). In the extraction *Yield* response, the three variables assessed (t , T , and S) showed similar contributions to its description. Regarding the response values of Y_1 (mg CT/g R) and Y_2 (mg CT/g E dw), the contribution of the variables is $S \gg T > t$.
- For the UAE technique: The contribution to the description of effects of the responses by the significant parametric values is distributed as $LE > QE \gg IE$. In all the responses assessed (extraction *yield*, Y_1 and Y_2 values), the contribution of the variables is $S \gg P > t$.

Table 1. Experimental results of the *CCCD* used for the response surface methodology (RSM) optimization of the three main variables involved (X_1 , X_2 , and X_3) in the heat assisted extraction (HAE) and ultrasound assisted extraction (UAE). Responses comprised three format values assessed (Y_1 , mg C/g R; Y_2 , mg C/g E dw; and *Yield*, %).

Experimental Design										HAE						UAE							
Coded Values			HAE			UAE			Residue	Individual Content			Total Content			Residue	Individual Content			Total Content			
X_1	X_2	X_3	$X_1: t$ min	$X_2: T$ °C	$X_3: S$ %	$X_1: t$ min	$X_2: P$ W	$X_3: S$ %	<i>Yield</i> %	Y_1C1 mg/g R	Y_1C2 mg/g R	Y_2C1 mg/g E	Y_2C2 mg/g E	Y_1CT mg/g R	Y_2CT mg/g E	<i>Yield</i> %	Y_1C1 mg/g R	Y_1C2 mg/g R	Y_2C1 mg/g E	Y_2C2 mg/g E	Y_1CT mg/g R	Y_2CT mg/g E	
1	-1	-1	-1	21.2	34.2	20.3	9.1	160.8	20.3	53.75	6.65	3.04	3.58	1.64	9.73	5.21	61.24	9.26	3.65	5.67	2.24	12.91	7.91
2	-1	-1	1	21.2	34.2	79.7	9.1	160.8	79.7	45.43	7.54	4.65	3.42	2.11	12.22	5.54	52.87	9.45	4.44	5.00	2.35	13.89	7.34
3	-1	1	-1	21.2	75.8	20.3	9.1	339.2	20.3	57.26	6.08	2.81	3.48	1.61	8.78	5.09	70.17	8.96	3.44	6.29	2.41	12.40	8.70
4	-1	1	1	21.2	75.8	79.7	9.1	339.2	79.7	48.85	8.35	4.44	4.08	2.17	12.74	6.25	58.80	9.36	4.51	5.50	2.65	13.87	8.15
5	1	-1	-1	68.8	34.2	20.3	20.9	160.8	20.3	55.45	6.88	2.90	3.82	1.61	9.66	5.42	59.12	10.66	3.69	6.30	2.18	14.35	8.48
6	1	-1	1	68.8	34.2	79.7	20.9	160.8	79.7	50.00	10.05	4.05	5.03	2.02	14.03	7.05	55.22	8.99	4.14	4.97	2.29	13.14	7.25
7	1	1	-1	68.8	75.8	20.3	20.9	339.2	20.3	60.18	6.72	2.81	4.04	1.69	9.37	5.73	67.05	8.79	2.86	5.90	1.92	11.65	7.82
8	1	1	1	68.8	75.8	79.7	20.9	339.2	79.7	53.09	11.01	4.39	5.84	2.33	15.26	8.17	57.19	8.69	4.24	4.97	2.42	12.92	7.39
9	-1.68	0	0	5	55	50	5	250	50	53.88	8.15	3.80	4.39	2.05	12.01	6.44	68.58	10.53	4.77	7.22	3.27	15.30	10.49
10	1.68	0	0	85	55	50	25	250	50	56.51	9.09	3.70	5.14	2.09	12.74	7.23	56.13	10.14	4.56	5.69	2.56	14.71	8.25
11	0	-1.68	0	45	20	50	15	100	50	49.49	11.09	4.70	5.49	2.33	15.59	7.82	55.99	12.41	5.45	6.95	3.05	17.86	10.00
12	0	1.68	0	45	90	50	15	400	50	60.78	8.68	3.42	5.27	2.08	11.96	7.36	76.95	10.60	4.40	8.16	3.38	15.00	11.54
13	0	0	-1.68	45	55	0	15	250	0	54.73	3.81	1.63	2.09	0.89	5.46	2.98	50.18	8.22	1.92	4.12	1.19	10.14	5.31
14	0	0	1.68	45	55	100	15	250	100	47.62	5.68	2.69	2.70	1.28	8.23	3.99	34.40	11.06	1.15	3.81	0.39	12.21	4.20
15	-1.68	-1.68	-1.68	5	20	0	5	100	0	54.39	4.24	1.85	2.30	1.01	6.01	3.31	47.94	8.15	3.73	3.91	1.79	11.88	5.69
16	-1.68	-1.68	1.68	5	20	100	5	100	100	36.34	2.45	1.65	0.89	0.60	4.12	1.49	33.12	6.25	1.50	2.07	0.50	7.75	2.57
17	-1.68	1.68	-1.68	5	90	0	5	400	0	56.79	3.53	1.59	2.00	0.90	5.13	2.90	61.16	10.89	4.46	6.66	2.73	15.36	9.39
18	-1.68	1.68	1.68	5	90	100	5	400	100	47.24	4.51	2.39	2.13	1.13	6.88	3.26	28.92	11.57	4.64	3.34	1.34	16.21	4.69
19	1.68	-1.68	-1.68	85	20	0	25	100	0	51.72	4.45	2.00	2.30	1.03	6.40	3.33	45.30	9.99	4.55	4.53	2.06	14.54	6.59
20	1.68	-1.68	1.68	85	20	100	25	100	100	39.88	4.46	1.75	1.78	0.70	6.21	2.48	28.91	9.60	1.71	2.78	0.50	11.31	3.27
21	1.68	1.68	-1.68	85	90	0	25	400	0	64.04	2.14	0.78	1.37	0.50	2.95	1.87	51.96	9.85	3.88	5.12	2.02	13.73	7.13
22	1.68	1.68	1.68	85	90	100	25	400	100	61.84	6.75	2.82	4.17	1.74	9.54	5.91	23.34	10.08	3.72	2.35	0.87	13.80	3.22
23	0	0	0	45	55	50	15	250	50	56.07	8.65	3.78	4.85	2.12	12.53	6.97	65.72	10.85	4.50	7.13	2.96	15.35	10.08
24	0	0	0	45	55	50	15	250	50	56.55	8.79	4.13	4.97	2.34	13.00	7.31	65.90	10.93	4.34	7.20	2.86	15.27	10.06
25	0	0	0	45	55	50	15	250	50	54.57	8.99	4.09	4.91	2.23	13.22	7.14	66.06	10.44	4.04	6.89	2.67	14.47	9.56
26	0	0	0	45	55	50	15	250	50	54.35	8.65	3.78	4.70	2.05	12.36	6.76	67.94	11.08	4.35	7.53	2.96	15.43	10.48
27	0	0	0	45	55	50	15	250	50	54.57	9.26	4.20	5.02	2.27	13.33	7.14	67.80	10.27	4.09	6.96	2.77	14.36	9.74
28	0	0	0	45	55	50	15	250	50	54.35	9.12	4.18	5.02	2.30	13.37	6.76	68.10	10.26	3.92	6.99	2.67	14.18	9.66

In general, positive and highly significant effects of LE, QE, and IE are found to moderately affect the studied responses. In both techniques assessed (HAE and UAE), the variable *S* is the most relevant one. Initial increases of *S* cause an increase of the extraction efficiency until it reaches a maximum, in which case the increase will cause a decrease in the extraction, but its interactive effect with the variable *t* and *T* or *P* causes a more favorable influence.

Additionally, using the significant parametric values of Table 2 coupled with a simplex methodology, it is possible to determine the absolute/relative optimal values of conditions to maximize the responses individually or globally, in order to obtain the most efficient extraction process. Table 3 shows the individual and global optimal response values and the corresponding conditions that produced them. In consequence, the extracting techniques (HAE and UAE) according to the three response value formats (Y_1 , mg C/g R; Y_2 , mg C/g E dw; and *Yield*, %) for each assessed anthocyanin (C1 and C2), as well as for the total anthocyanin content (CT = C1 + C2), are depicted.

Table 2. Parametric results of the second-order polynomial equation of Equation (1) for the HAE and UAE techniques assessed and for the three response value formats (Y_1 , mg C/g R; Y_2 , mg C/g E dw; and *Yield*, %). The parametric subscript 1, 2, and 3 stands for the variables involving *t* (X_1), *T* or *P* (X_2), and *S* (X_3), respectively. Analyses of significance of the parameters ($\alpha = 0.05$) are presented in coded values. Additionally, the statistical information of the fitting procedure to the model is presented.

PARAMETERS	RESIDUE	INDIVIDUAL CONTENT				TOTAL CONTENT		
		<i>Yield</i>	Y_1C1	Y_1C2	Y_2C1	Y_2C2	Y_1CT	Y_2CT
HAE								
Intercept	b_0	54.86±0.72	9.35±0.38	4.15±0.19	5.07±0.17	2.26±0.08	13.48±0.55	7.29±0.23
Linear effect	b_{11}	1.54±0.43	0.33±0.21	ns	0.25±0.09	ns	0.28±0.21	0.26±0.13
	b_{12}	3.12±0.43	ns	ns	0.15±0.09	0.05±0.02	ns	0.19±0.13
	b_{13}	-3.07±0.43	0.56±0.21	0.33±0.11	0.17±0.09	0.11±0.05	0.88±0.31	0.27±0.13
Quadratic effect	b_{111}	ns	-0.26±0.21	-0.09±0.07	-0.10±0.05	-0.05±0.03	-0.35±0.26	-0.16±0.15
	b_{222}	ns	ns	ns	ns	ns	ns	ns
	b_{333}	-1.24±0.42	-1.60±0.26	-0.69±0.13	-0.94±0.11	-0.40±0.06	-2.26±0.37	-1.33±0.15
Interactive effect	b_{112}	0.78±0.31	0.00±0.00	ns	ns	ns	ns	ns
	b_{113}	0.54±0.31	0.26±0.15	0.04±0.02	0.18±0.07	0.04±0.03	0.31±0.22	0.22±0.09
	b_{233}	0.69±0.31	0.31±0.15	0.14±0.08	0.21±0.07	0.09±0.03	0.45±0.22	0.29±0.09
Statistics (R^2)		0.9375	0.9100	0.8755	0.9443	0.9272	0.9046	0.9489
UAE								
Intercept	b_0	68.11±1.70	10.42±0.47	4.10±0.28	6.98±0.22	2.83±0.13	14.46±0.62	9.75±0.31
Linear effect	b_{11}	-1.70±0.96	ns	ns	-0.13±0.12	-0.10±0.07	ns	-0.22±0.17
	b_{12}	2.12±0.96	0.23±0.21	0.18±0.15	0.25±0.12	0.14±0.07	0.37±0.35	0.36±0.17
	b_{13}	-6.46±0.96	ns	-0.16±0.15	-0.55±0.12	-0.26±0.07	ns	-0.82±0.17
Quadratic effect	b_{111}	-2.29±1.16	ns	0.26±0.20	-0.13±0.11	ns	ns	ns
	b_{222}	ns	ns	0.35±0.20	ns	0.19±0.09	0.66±0.42	0.27±0.21
	b_{333}	-7.33±1.16	-0.36±0.27	-0.84±0.20	-1.02±0.15	-0.68±0.09	-1.22±0.42	-1.84±0.21
Interactive effect	b_{112}	ns	-0.34±0.20	-0.11±0.11	-0.18±0.09	-0.07±0.05	-0.46±0.25	-0.23±0.12
	b_{113}	ns	ns	ns	ns	ns	ns	ns
	b_{233}	-1.29±0.69	0.17±0.10	0.22±0.11	-0.08±0.05	ns	0.41±0.25	ns
Statistics (R^2)		0.9431	0.7825	0.9032	0.9316	0.9035	0.8986	0.9380

Table 3. Variable conditions in natural values that lead to optimal global and individual response values for RSM for each of the extracting techniques assessed (HAE and UAE), for the three response value formats (Y_1 , mg C/g R; Y_2 , mg C/g E dw; and *Yield*, %), for each compound assessed (C1 and C2), and for the total compounds (CT=C1+C2).

CRITERIA	OPTIMAL VARIABLE CONDITIONS			OPTIMUM RESPONSE		
	X ₁ : t (min)	X ₂ : T (°C) or P(W)	X ₃ : S (%)			
A) Individual optimal variable conditions						
HAE	<i>Yield</i>	85.00±8.50	90.00±4.50	38.01±3.04	65.10±3.91	%
	C1	64.89±5.19	90.00±8.10	62.01±3.10	9.71±0.49	mg C1/g R
	Y_1 C2	47.18±3.30	90.00±7.20	62.22±6.22	4.27±0.34	mg C2/g R
	CT	58.85±5.89	90.00±9.00	61.97±6.20	13.89±0.14	mg CT/g R
	C1	84.27±0.84	90.00±3.60	63.11±3.79	5.64±0.51	mg C1/g E dw
	Y_2 C2	48.06±1.92	90.00±0.90	59.98±3.60	2.38±0.21	mg C2/g E dw
	CT	70.76±5.66	90.00±4.50	60.82±3.04	7.89±0.55	mg CT/g E dw
	<i>Yield</i>	12.79±0.51	400.00±32.00	32.51±1.95	74.53±2.24	%
	C1	5.00±0.10	400.00±28.00	61.80±1.85	11.82±0.71	mg C1/g R
UAE	Y_1 C2	5.00±0.10	400.00±28.00	53.58±1.07	6.45±0.45	mg C2/g R
	CT	5.00±0.10	400.00±20.00	58.39±4.09	18.32±1.47	mg CT/g R
	C1	5.00±0.50	400.00±4.00	40.11±2.81	7.88±0.16	mg C1/g E dw
	Y_2 C2	5.00±0.40	400.00±28.00	44.35±4.43	3.96±0.28	mg C2/g E dw
	CT	5.00±0.35	400.00±20.00	43.37±4.34	12.23±0.86	mg CT/g E dw
	B) Global optimal variable conditions					
HAE	<i>Yield</i>				50.89±3.05	%
	C1				9.71±0.29	mg C1/g R
	Y_1 C2				4.22±0.13	mg C2/g R
	CT	49.02±2.94	90.00±7.20	50.00±0.50	13.93±0.42	mg CT/g R
	C1				5.57±0.11	mg C1/g E dw
	Y_2 C2				2.36±0.05	mg C2/g E dw
	CT				7.93±0.08	mg CT/g E dw
	<i>Yield</i>				68.60±2.06	%
	C1				11.74±0.23	mg C1/g R
UAE	Y_1 C2				6.43±0.32	mg C2/g R
	CT	5.00±0.15	400.00±32.00	47.98±2.88	18.17±1.82	mg CT/g R
	C1				7.81±0.47	mg C1/g E dw
	Y_2 C2				3.95±0.24	mg C2/g E dw
	CT				11.76±0.82	mg CT/g E dw

2.2. Alternative Visual Illustration of the Effects of the Extraction Variables on the Target Responses Used

Although the parametric values show the behavior of the responses, and could be used to understand their patterns, a more visual way to express the effects of variables on the extraction of any type of response is to generate 3D surface and/or contour plots, by varying two variables in the experimental range under investigation and holding the other one at a fixed level. In this regard, Figure 1 and Figure 2 show the 3D surface and 2D contour plots, respectively, representing the influence of the investigated effects of HAE and UAE parameters on the extraction behavior. The plots enable one to visualize the influence and interaction between the variables. Visual analyses of 3D surface and 2D contour plots are in accordance with parametric values derived from the multiple regression analysis, as described in Table 2 (parametric values) and Table A1 (full mathematical models, supplementary material).

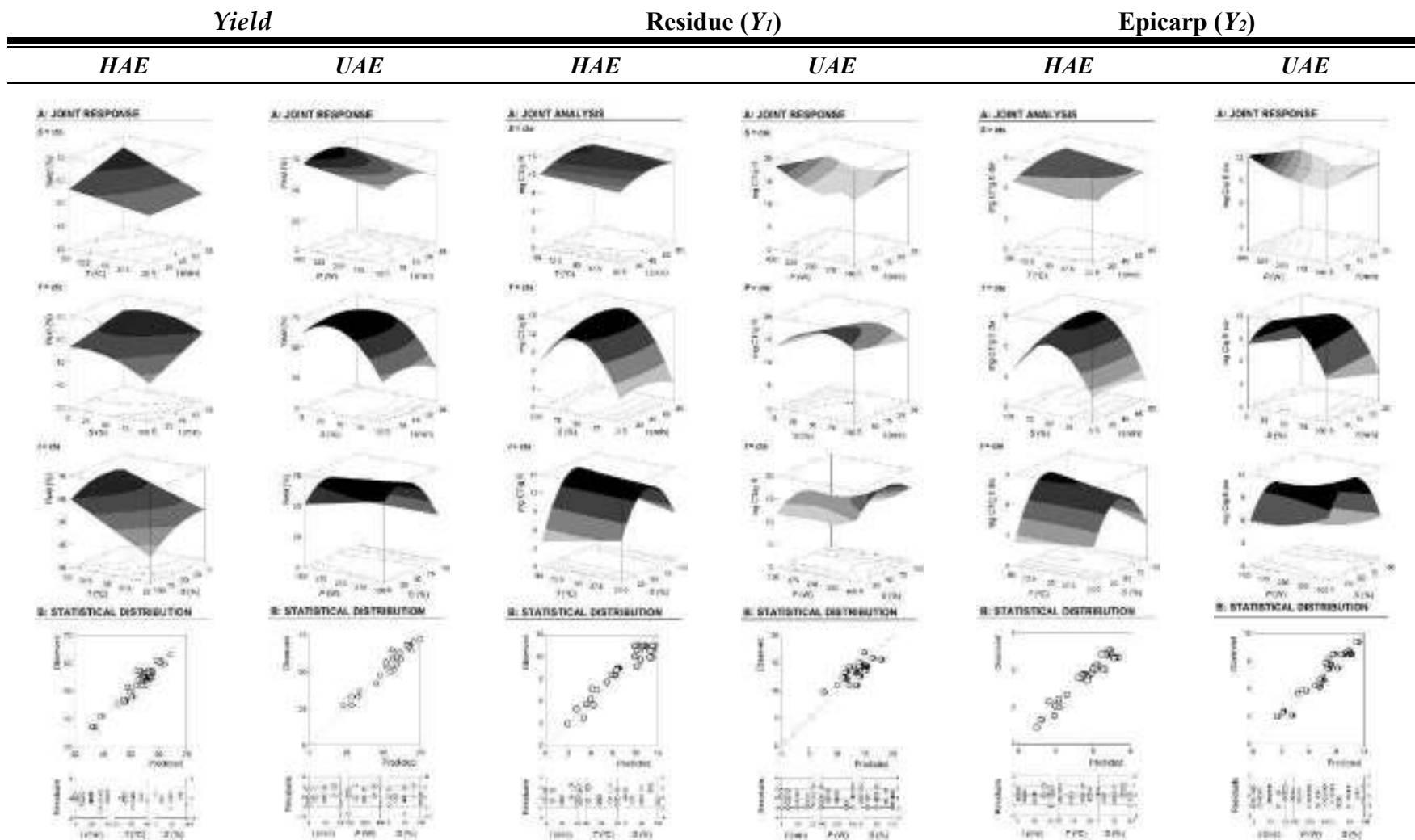


Figure 1. Illustration of the graphical results obtained by heat assisted extraction (HAE) and ultrasound assisted extraction (UAE) for the extraction *yield* of the residual content material (R) and the total detected anthocyanin compounds (cyanidin 3-rutinoside and peonidin 3-rutinoside, $CT = C1 + C2$) in terms of two response formats (Y_1 , mg C/g R and Y_2 , mg C/g E dw). Full results are described in Table 1. Every figure is presented in two parts. Part A shows the 3D net surfaces predicted by Equation (1) when the excluded variable is positioned at the individual optimum (Table 3). Part B describes the statistical analysis in a graphical form to show the goodness of fit of the models applied.

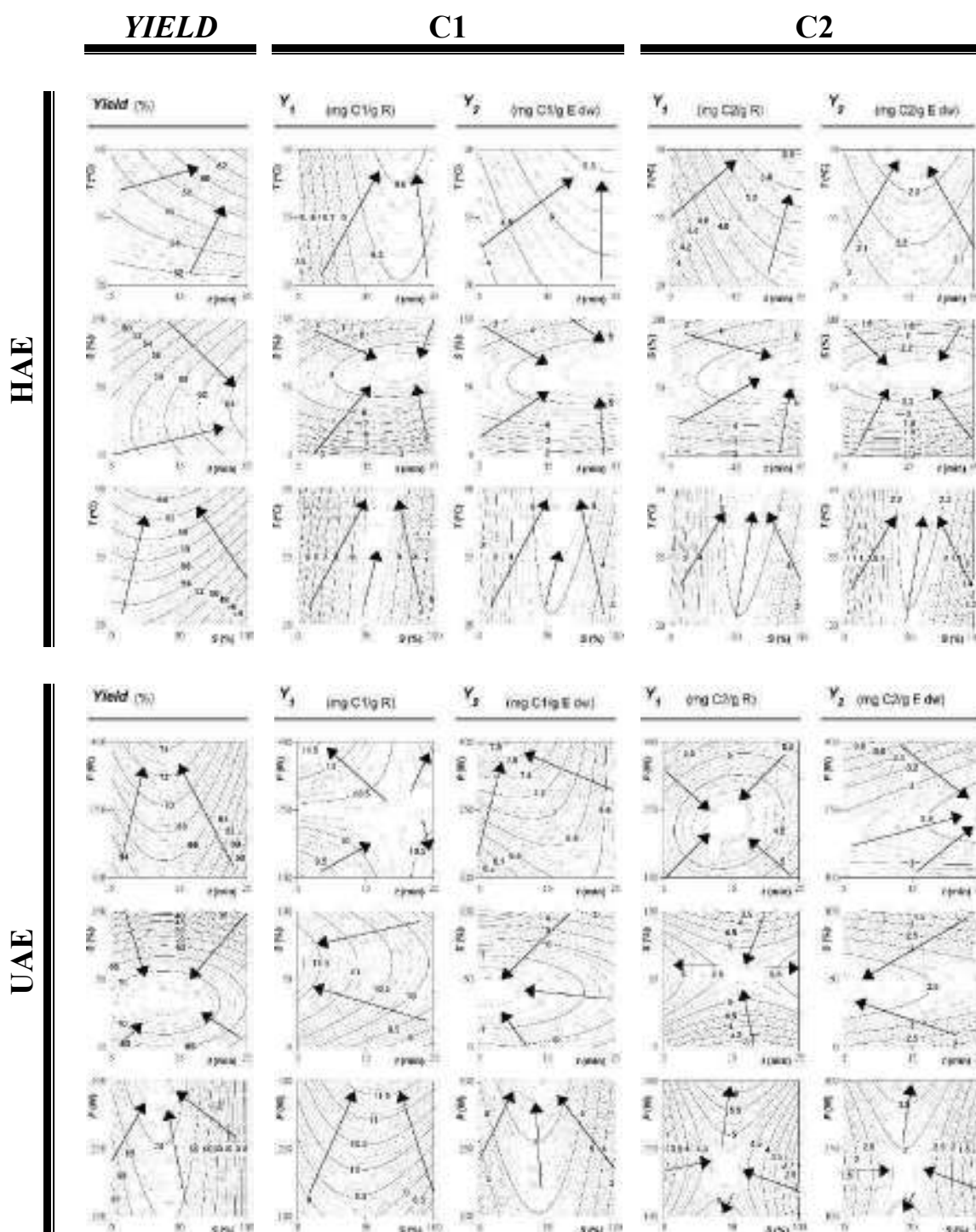


Figure 2. The optimized isolines projections for the extraction of C1 (cyanidin 3-rutinoside) and C2 (peonidin 3-rutinoside) as a function of the combination of the three main variables involved (X_1 , X_2 , and X_3) in the HAE and UAE. For each compound, the two response value formats (Y_1 , mg C/g R and Y_2 , mg C/g E dw) are presented to describe the most favorable conditions. Furthermore, the response projections of the *yield* of the extracted residual material are presented. All the contour graphs were built by the second order polynomial models generated by Equation (1) (Table A1) when the excluded variable is positioned at the individual optimum (Table 3).

The extraction results for HAE and UAE, as function of the combination of the three main involved variables (X_{1-3} : t , T or P , and S), can be observed in Figure 1 and Figure 2. In this regard, Figure 1 shows the 3D surface plots of the extracted R (*Yield*, %), and CT, in two response formats (Y_1 , mg CT/g R and Y_2 , mg CT/g E dw). On the other hand, Figure 2 shows the optimized isolines projections for C1 (cyanidin 3-rutinoside) and C2 (peonidin 3-rutinoside) extraction, in the two response value formats (Y_1 , mg C/g R and Y_2 , mg C/g E dw). These figures show, respectively, optimized 3D graphical and 2D isolines projection results for the extracted anthocyanins (C1 or C2) as function of the three combined variables (t , T or P , and S) in HAE and UAE. The total anthocyanins (C1+C2) are accounted together (CT) in Figure 1, and individually in Figure 2. They are helpful to visualize the tendencies of each response and lead to define of the maximum favorable conditions, considering all together all responses.

Additionally, Figure 1B exemplifies the competence to predict the obtained results. In statistical terms, the distribution of residues (Figure 1) presents, for the majority of the cases, more than 90% of reliability, showing a good agreement between experimental and predicted values. This is also verified by the achieved high R^2 values (Table 2), which indicates the percentage of variability explained by the model.

In HAE, small differences between the extraction behavior of the two considered anthocyanins (when comparing C1 and C2, or Y_1 and Y_2) were clearly distinguished. The opposite occurred in UAE, the effects were distinct for each one of the detected anthocyanins, as well as according to the response format. However, for both extraction techniques, the S variable was the most significant one, producing a relevant impact on the level of extraction of all anthocyanins assessed. As described above, the LE and the QE of the significant parametric values of the variable S can be perceived in all figures. In almost all cases, the variable S indicates a maximum level at ~50% of hydroalcoholic mixture (water/ethanol, v/v). The negative impact of quadratic term of the variable S can be explained through the increase of water in the process, which expands the yield of extraction. Other negative effects such as those between T or P and S may suggest that the further use of lower P , in combination with higher S , will avoid the anthocyanin degradation. The results are in accordance with others recently reported by other authors [19–21], in which inclined surfaces to the side of T or P may increase the solubility of target compounds by using stronger energies, and consequently improve their release from the sample matrix, while destroying the integrity of connective and structural tissues.

2.3. Conditions That Maximize the Anthocyanins Extraction and Experimental Verification

The aim of this study was to maximize the extraction yield of targeted anthocyanin compounds from epicarps of *P. spinosa* fruits, in the applied HAE and UAE techniques, within pre-set variable conditions and ranges. The values of the variable conditions that lead to optimal response values for RSM using a *CCCD*, obtained with the aid of *simplex* procedure, for each of the assessed extracting techniques are shown in Table 3. Figure 3 part A shows the individual summary of the effects of all variables assessed for HAE and UAE systems in 2D illustrations, where the variables are positioned at the individual optimal values of the others (Table 3). The dots (⊙) presented alongside each line highlight the location of the optimum value, meanwhile lines are the predicted behavior found by the mathematical analysis of Equation (1) generated by the theoretical second-order polynomial models described in Table A1. Next, some relevant details of the results produced are highlighted:

- For the HAE: the global optimal variable conditions were found at 49.02 ± 2.94 min, 90.00 ± 7.20 °C, and $50.00\% \pm 0.50\%$ of ethanol, producing maximum response values of 13.93 ± 0.42 mg CT/g R (Y_1), 7.93 ± 0.08 mg CT/g E dw (Y_2), and $50.89\% \pm 3.05\%$ (*yield* of the extracted residue).
- For the UAE: the global optimal variable conditions were found at 5.00 ± 0.15 min, 400.00 ± 32.00 W, and $47.98\% \pm 2.88\%$ of ethanol, producing maximum response values of 18.17 ± 1.82 mg CT/g R (Y_1), 11.76 ± 0.82 mg CT/g E dw (Y_2), and $68.60\% \pm 2.06\%$ (*yield* of the extracted residue).

Considering both the individual and global values, the higher amount of extracted compounds was obtained for the UAE technique. The ideal solvent composition was almost the same, and the two techniques required high energy values, where the highest values of T and P proposed by the

experimental design were the optimal, but the UAE needed less t than HAE (~90% less). The obtained results are in accordance with similar conclusions found previously [10,17,22], in which UAE proved to consume less energy because of the lower t needed, and provide higher extraction values while increasing the purity and, additionally, aiding to meet the requirements of a green extraction concept.

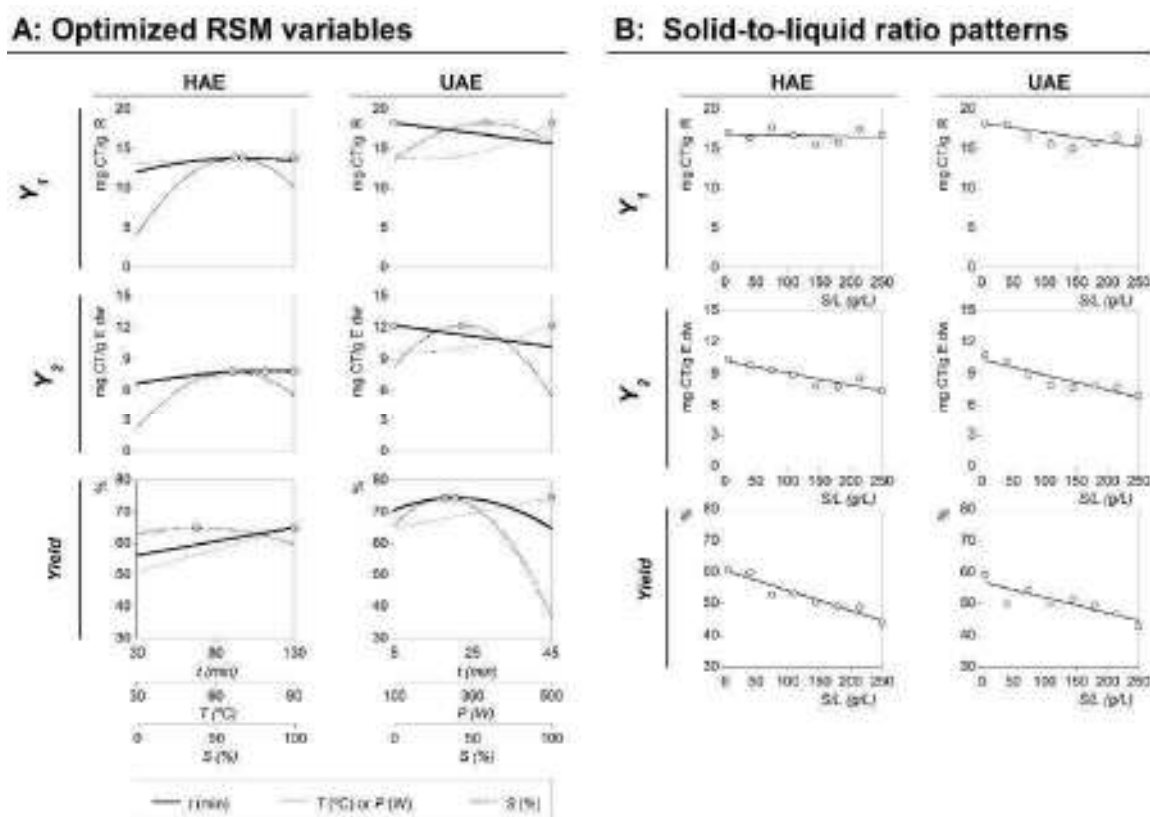


Figure 3. Final graphical effects of all variables assessed for HAE and UAE systems. Part A shows the individual 2D responses as a function of all the variables assessed that were positioned at the individual optimal values of the others (Table 2). The points (⊙) presented alongside each line highlight the location of the optimum value. Lines and dots are generated by the theoretical second order polynomial models generated by Equation (1) (Table A1). Part B shows the dose response of S/L at the global optimal values of the other three variables (Table 3). The limit value (~150 g/L) shows the maximum achievable experimental concentration until the sample cannot be physically stirred at laboratory scale. RSM—response surface methodology.

2.4. Dose-Response Analysis of the Solid-to-Liquid Ratio Effect at the Optimal Conditions

The study of S/L effect was performed at the optimal conditions (Table 3) predicted by the polynomial models obtained for each extraction technique (HAE and UAE) using the total anthocyanin content (CT), as quantified by HPLC analysis, as the response factor. The individual S/L study for each individual anthocyanin (C1 or C2) was not presented because the behavior was similar to the pattern of the total amount. In both processes, the S/L was designed to verify the behavior between 5 and 250 g/L. The maximum value of 250 g/L was used as a limit condition because of the impossibility of producing a homogenized extraction when higher values were used.

The obtained dose responses of the S/L were consistent for both HAE and UAE systems, and could be described by a simple linear relationship (shown in Part B of Figure 3). All experimental points are distributed around the linear equation; consequently, the dose response is explained by the slope (m) of the linear relationship. None of the cases showed positive m values (the extraction efficiency increases as the S/L rate increases), and two cases showed non-significant values or a zero value of m (the efficiency doesn't change as the S/L increases). In all the other cases, the m showed negative values (the efficiency decreases as the S/L increases). The responses from the Y_1 value format, for HAE and UAE, were the ones that showed non-significant m values, whereas all other responses

showed significant negative values of m (Y_1 and *Yield* for HAE and UAE). The conclusions derived from this analysis are described below:

- For the Y_1 value format, the response of the parametric m value in HAE and UAE presents a non-significant interval of confidence, which means that the changes in the response are not statistically supported and, therefore, the parameter must be considered equal to zero. In other words, the amount of anthocyanins in the extracted residue does not vary as a function of the S/L increase. The extraction values were defined numerically by the intercept parametric value (b) of the linear equation as 14.85 ± 2.29 and 18.25 ± 3.95 mg CT/g R for HAE ($R^2 = 0.9920$) and UAE ($R^2 = 0.9817$), respectively.
- For the Y_2 value format, the parametric values for HAE were $b = 9.21 \pm 1.37$ mg CT/g E dw and $m = -0.0113 \pm 0.0051$, with $R^2 = 0.9566$; while for UAE, $b = 10.32 \pm 1.48$ mg CT/g E dw and $m = -0.0143 \pm 0.0038$, with $R^2 = 0.9244$. Negative m values show that the S/L increase leads to a decrease in the extraction ability, obtaining a maximum value of extraction at 5 g/L and a minimum at 250 g/L. However, the observed decrease is slight (less than -0.02), which means that the increase of 1 g/L implies the loss of 0.0113 ± 0.0051 mg CT/g E dw for the HAE process and 0.0143 ± 0.0038 mg CT/g E dw for UAE. Such values produce losses at the maximum tested experimental value (250 g/L) of $\sim 15\%$, comparative with the one extracted at 5 g/L. Nevertheless, the economic advantages of working at 250 g/L are far more superior than the possible benefits of extracting at the optimal S/L value.
- For the *Yield* value format, the parametric values for HAE were $b = 54.62\% \pm 4.87\%$ and $m = -0.0636 \pm 0.0123$, with $R^2 = 0.9516$; whereas for UAE, $b = 58.90\% \pm 7.77\%$ and $m = -0.0491 \pm 0.0116$, with $R^2 = 0.9618$. Although, at the initial S/L values, the results obtained for HAE and UAE conducted to similar extraction yields, these values decreased as the S/L increased. The m parametric value is significantly lower for the UAE process, resulting in higher extraction yield values at 250 g/L. These results are in accordance with the conclusions highlighted in the literature, where UAE is reported as enhancing the extraction process by increasing the mass transfer between the plant material and the solvent [23]. The UAE leads to better cell disruption, facilitating the release of the extractable compounds by increasing the contact surface area between the solid and liquid phases [22,23].

2.5. Comparison with Other Studies Involving the Extraction of Anthocyanins

There are few works in the literature dealing with anthocyanins in *P. spinosa* fruits. In one of these studies with *P. spinosa* fruits, Guimarães et al. [5] performed the extraction using methanol with 0.5% TFA added as solvent, and identified eight different anthocyanins, predominantly peonidin 3-rutinoside and cyanidin 3-rutinoside, with 34.47 ± 0.03 $\mu\text{g}/100$ g fruits dw and 31.12 ± 0.11 $\mu\text{g}/100$ g fruits dw, respectively. Other authors [6], found 3.5 ± 0.5 mg of anthocyanins/100 g dw of *P. spinosa* fruits. Both authors used the whole fruit, while in this study, only the epicarp was used as the extraction material, a fact that may justify the significant differences between the encountered results, when compared with the present study. Compared with the pulp, the fruit epicarp presents a greater intensity of color and, therefore, a higher concentration of anthocyanins, in addition to less interfering compounds, is obtained. Moreover, another fact that aided the production of large amounts of anthocyanins from the extracted material was the optimization of the extraction process, which led to increased extraction efficiency and yield. In another study that used *P. spinosa* fruits as a source of anthocyanins [6], the total content was quantified by spectrophotometric methods, presenting values that cannot be compared with those found in the present study.

Some examples of other plant-based sources of anthocyanins are *Oryza sativa* L. (var. Glutinosa) bran, which shows 42.00 mg/g [24]; *Phaseolus vulgaris* L. (common beans) fruit coat, presenting 32.00 mg/g [25]; and *Rubus fruticosus* L. (blackberries) fruit, which possess 17.10 mg/g [26]. Although these values are slightly higher than those presented by *P. spinosa* fruits, in general, the referred fruits and vegetables already have a high commercial value and other industrial purposes, unlike *P. spinosa* fruits. On the other hand, residues such as grape vine (*Vitis vinifera* L.) pomace, and mango (*Mangifera indica*) skin presented lower anthocyanin amounts, that is, 6.33 mg/g [27] and 2.03 to 3.60 mg/g [28],

respectively. Thus, these wild fruits revealed to be an excellent source of anthocyanins, serving as a base raw-material for the production of natural colorant additives for commercial purposes.

3. Discussion

The minimalism of using conventional methods (HAE or maceration) versus the compensations of new non-conventional technologies (microwave, ultrasound, cold pressing, squeezing, etc.) to recover compounds from plant materials, as well as by-products, is a principal topic in the list of many industries in order to increase profitability by decreasing energy costs and reducing greenhouse gas emissions to meet legal requirements. Additionally, non-conventional technologies favor the safety of processes and the quality of products, as well as the functionality and product standardization.

Scientific literature shows clear evidence that extraction procedures of target compounds from plant-based products must be assessed individually. Therefore, a nonstop effort needs to be performed, as agro-industrial and food sectors are looking for by-products' valorization into added-value products. However, in order to take full advantage of the technological advances, the extraction conditions need to be optimized. Mathematical solutions, such as RSM tools, could increase the efficiency and profitability of the process and help to change conventional extraction approaches.

Colorants are one of the most important additives in terms of marketing because their presence in food products is considered to influence customers' perceptions, choices, and preferences. *P. spinosa* fruit epicarps have been scarcely explored and, to the best of the authors knowledge, the potential industrial use of their anthocyanin compounds has not been previously investigated. In such a context, the present work presents a new rapid method to extract anthocyanin compounds from *P. spinosa* fruit epicarps. RSM and other mathematical strategies were successfully employed to optimize the extraction conditions that maximize the anthocyanin compounds' recovery to produce a rich extract with potential industrial application as a natural coloring additive.

4. Materials and Methods

4.1. Plant Material

Ripe *P. spinosa* fruits were harvested in Bragança (Trás-os-Montes, Northeast Portugal) in September 2017, the epicarp was separated from the rest of the fruit body, frozen, and lyophilized. They were then triturated, to be reduced to a fine powder (~20 mesh), and stored under refrigeration, protected from light until further use.

4.2. Extraction Procedures for *P. Spinosa* Fruit Epicarps

4.2.1. Heat Assisted Extraction (HAE)

HAE was performed in a water reactor agitated internally with a Cimarec™ Magnetic Stirrer at a constant speed (~500 rpm, Thermo Scientific, San Jose, CA, USA), following a procedure previously performed [13]. The powdered epicarp samples of *P. spinosa* (1.0 g) were extracted with 20 mL of solvent (ethanol/water) acidified with citric acid (pH = 3), under diverse conditions, as previously defined by the established RSM plan (Table 1). The ranges of the experimental design were as follows: time (t or X_1 , 5 to 85 min), temperature (T or X_2 , 20 to 90 °C), and ethanol content (S or X_3 , 0% to 100%). The solid-to-liquid ratio (S/L or X_4) was kept at 50 g/L for all conditions.

4.2.2. Ultrasound-Assisted Extraction (UAE)

An ultrasonic device (QSonica sonicators, model CL-334, Newtown, CT, USA) equipped with a water reactor (EUP540A, Euinstruments, France) at a fixed frequency (40 kHz) was used for UAE procedure. The variables considered were as follows: ultrasonic power (P , in watts), S , and t , which were programmed according to the defined RSM plan (Table 1), following a procedure previously performed [29]. The powdered epicarp samples (2.5 g) were placed in a reactor with 50 mL of solvent (ethanol/water) acidified with citric acid (pH = 3), and extracted under diverse conditions,

maintaining the *S/L* constant at 50 g/L. The ranges of the experimental design were as follows: *t* (X_1 , 5 to 25 min), *P* (or X_2 , 100 to 400 W), and *S* (or X_3 , 0% to 100%).

4.2.3. Post-Extraction Sample Processing

When all the individual extraction conditions were carried out (for HAE and UAE), the samples were immediately centrifuged (6000 rpm during 20 min at 10 °C) and filtered (paper filter Whatman n° 4) to eliminate the non-dissolved material. The supernatant was collected and divided into two portions for HPLC and extraction yield analysis. The portion separated for HPLC analysis (3 mL) was dried at 35 °C, re-dissolved in acidified water (citric acid solution with pH 3), and filtered through an LC filter disk (0.22 µm), whereas the portion for the extraction yield determination (5 mL) was dried at 105 °C during 48 h and thereafter weighted.

4.3. Identification and Quantification of Anthocyanins by HPLC

The extract was analyzed using an HPLC-DAD-ESI/MSn (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA) system, previously described [30]. The detection was carried out using a DAD (520 nm as the preferred wavelength) and mass spectrometer (Linear Ion Trap LTQ XL mass spectrometer, Thermo Finnigan, San Jose, CA, USA) equipped with an ESI source. The anthocyanins present in the samples were characterized according to their UV and mass spectra. The anthocyanins cyanidin 3-rutinoside (C1) and peonidin 3-rutinoside (C2) were the most relevant compounds found, and were, therefore, quantified using a five-level calibration curve of known concentrations (200–20 µg/mL) of cyanidin 3-glucoside ($y = 243287 x - 1000000$; $R^2 = 0.9953$, Polyphenols, Sandnes, Norway) and peonidin 3-glucoside ($y = 122417 x - 447974$; $R^2 = 0.9965$, Polyphenols, Sandnes, Norway).

4.4. Response Value Formats for Results Presentation

The two anthocyanin compounds (C, either C1 or C2) and their sum (C total, CT) were used as responses in each applied technique. The results were presented according to two response formats (*Y*): Y_1 , in mg of C per gram of extracted residue (mg C/g R), which was specifically used to evaluate the C purity in the extracts; and Y_2 , in mg of C per g of fruit epicarp dry weight (mg C/g E dw), which was specifically used to analyses the C extraction yield. Both responses were equally analyzed, but additional considerations regarding the last one (Y_2 , mg C/g E dw) were taken in the results presentation, because it is considered as an important guiding response when dealing in terms of optimization for industrial transference. Note that by dividing those responses, Y_2/Y_1 , the extracted residue quantity (g R/g E dw) is obtained, which provides information regarding the third response criterion expressed (*Yield*, %).

4.5. Experimental Design, Model Analysis, and Statistical Evaluation

4.5.1. RSM Experimental Design

Trials based on one-at-the-time analysis (analysis of each of the variables for each one of the selected techniques) were conducted, the ranges originating significant changes were selected (Table 1). The joint effects of the three defined variables were studied using a *circumscribed central composite design* (CCCD), using five levels for each one with twenty eight response combinations, as described previously [31].

4.5.2. Mathematical Model

The experimental data produced by the RSM design were analyzed mathematically by means of least-squares calculation, using the following second-order polynomial equation with interactive terms [32]:

$$Y = b_0 + \sum_{i=1}^n b_i X_i + \sum_{i=1}^{n-1} \sum_{\substack{j=2 \\ j>i}}^n b_{ij} X_i X_j + \sum_{i=1}^n b_{ii} X_i^2 \quad (1)$$

where Y is the dependent variable (response variable) modelled, X_i and X_j define the independent variables, b_0 is the constant coefficient, b_i is the coefficient of linear effect, b_{ij} is the coefficient of interaction effect, b_{ii} the coefficient of quadratic effect, and n is the number of variables. As responses, the following three value formats were used: Y_1 (mg C/g R), Y_2 (C/g E dw), and *Yield* (%).

4.5.3. Procedure to Optimize the Variables to a Maximum Response

A simplex algorithm method was used to find the optimum values by solving nonlinear problems in order to maximize the extraction yield and the recovery of anthocyanin compounds, as explained previously [33]. Certain limitations were imposed (i.e., times cannot be lower than 0) to avoid variables with unnatural and unrealistic physical conditions.

4.5.4. Dose-Response Analysis of the Solid-to-Liquid Ratio

Once the optimal conditions (X_1 , X_2 , and X_3) were found, the following natural optimization step was used to describe the pattern of the S/L (or X_4 , expressed in g/L). The objective was to achieve more productive conditions as required by industrial applications. In all cases, experimental points are distributed following linear patterns as the S/L increases, consequently, linear models with intercepts were used to evaluate the responses. The parametric value of the slope (m) was used to assess the dose response. Positive values will indicate an increase in the extraction responses, whereas negative values will designate a decrease in the extraction efficiency, as the S/L increases.

4.6. Mathematical Procedures

The analytical procedures to model the data, to determine the parametric values, confidence intervals, and statistical calculations, were obtained following the descriptions of other authors [34]. In brief, (a) the parametric values were obtained using the quasi-Newton algorithm (least-square) by running the integrated macro 'Solver' in Microsoft Excel; (b) the coefficient significance of the parameters produced ($\alpha = 0.05$) was assessed using the 'SolverAid' macro to conclude their confidence intervals; and c) the model consistency was proven by means of several statistical criteria, such as (i) the Fisher F -test ($\alpha = 0.05$); (ii) the 'SolverStat' macro; and (iii) the R^2 coefficient.

5. Conclusions

The efficiency of the UAE was higher than that obtained with HAE. The main anthocyanins identified were cyanidin 3-rutinoside and peonidin 3-rutinoside, being the ones quantified. Through the optimization of the extraction process, it was possible to reach by UAE 18.17 ± 1.82 mg CT/g R (Y_1), 11.76 ± 0.82 mg CT/g E dw (Y_2), and $68.60\% \pm 2.06\%$ (yield of the extracted residue), with the optimal parameters of extraction being 5.00 ± 0.15 min, 400.00 ± 32.00 W, and $47.98\% \pm 2.88\%$ of ethanol. The used mathematical models (RSM and dose-response models) were statistically significant and allowed the optimization of the anthocyanins extraction. For the S/L effects, inspected at the optimum conditions, the responses for all assessed criteria followed a decreasing linear relation until 250 g/L.

In conclusion, the present study contributed to the valorization of the wild fruits of *P. spinosa* by exploring anthocyanin-rich extracts that can find potential application as natural colorants in different industrial fields. For that purpose, an optimized extraction method was obtained using advanced and efficient extraction systems.

Author Contributions: Formal analysis, M.A.P.; Investigation, M.G.L.; Methodology, C.P. and L.B.; Resources, M.F.B.; Supervision, I.J.B. and I.C.F.R.F.

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Sample Availability: Samples of the plant and extracts are available from the authors.



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ValorNatural – Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar.

Entregável nº 3.2.1.

Versão do Documento: 1

Data de Submissão: 31/05/2019

Responsável: IPB-CIMO

Nome do Documento: Folheto descritivo das condições que garantem a maior estabilidade dos ingredientes corantes

Histórico de Revisão

Revisão	Data	Parceiros Envolvidos	Descrição



Lista de Autores

Maria Inês Dias



Sumário

Neste folheto estão descritas as condições (técnicas de encapsulação e polímeros naturais usados como encapsulantes) que garantem a maior estabilidade dos ingredientes corantes extraídos das matrizes vegetais previamente estudadas e de extratos corantes.



Índice

1. Identificação	5
2. Informação	6



1. Identificação

<i>Deliverable</i>	E.3.2.1. Folheto descritivo das condições que garantem a maior estabilidade dos ingredientes corantes
<i>Tipo de deliverable</i>	Folheto descritivo
Nível de disseminação	Público
PPS	PPS3. Corantes Naturais

2. Informação

MATRIZES VEGETAIS

Beta vulgaris L.



Nome Comum: Beterraba

Origem geográfica: Bragança, Portugal

pH do extrato corante: 6

Técnicas de encapsulação: *Spray-drying*

Polímeros naturais usados como encapsulantes:
Maltodextrina (20%).

Gomphrena globosa L.



Nome Comum: Perpétua-roxa

Origem geográfica: Mezio, Portugal

pH do extrato corante: > 3 e < 7

Técnicas de encapsulação: *Spray-drying*

Polímeros naturais usados como encapsulantes:
Maltodextrina (20%).

Sambucus nigra L.



Nome Comum: Sabugueiro

Origem geográfica: Bragança, Portugal

pH do extrato corante: 4,6

Técnicas de encapsulação: *Spray-drying*

Polímeros naturais usados como encapsulantes:
Maltodextrina (20%).

EXTRATOS CORANTES

Curcumina



Origem: Extrato comercial de curcumina, extraído de *Curcuma longa* L. com 80% de pureza.

Técnicas de encapsulação: *Spray-congealing*

Polímeros naturais usados como encapsulantes: Cera de abelha (98,5%).



ValorNatural – Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar.

Entregável nº 4.1.1

Versão do Documento: 1

Data de Submissão: 28/02/2019

Responsável: UP (FEUP-LSRE)

Nome do Documento: Relatório com as especificações de extração dos aromas por extração supercrítica.

Histórico de Revisão

Revisão	Data	Parceiros Envolvidos	Descrição



Lista de Autores

José Carlos Lopes

Madalena Dias

Isabel Martins

Sumário

Este entregável apresenta a seleção das plantas aromáticas e frutos secos que serão usados para a obtenção de aromas naturais por extração supercrítica com CO₂, assim como as especificações a serem definidas para o processo de extração.

Índice

1. Identificação	5
2. Informação	6

1. Identificação

<i>Deliverable</i>	E 4.1.1 Relatório com as especificações de extração dos aromas por extração supercrítica.
<i>Tipo de deliverable</i>	Relatório
<i>Nível de disseminação</i>	Confidencial
<i>PPS</i>	4. Aromas e modelos de aromas

2. Informação

O Projecto “Valor Natural” tem como objetivo a valorização de recursos naturais através da extração de ingredientes de elevado valor acrescentado obtidos por extração supercrítica ou na forma de hidrolatos. Para o estudo da extração dos aromas foram selecionadas como plantas aromáticas o alecrim, o tomilho e os orégãos. Relativamente aos frutos secos foram selecionados as nozes, amêndoas e avelãs. A caracterização destas espécies será efetuada tanto no fruto como na pele.

A extração dos óleos será realizada através de uma unidade de extração de CO₂ supercrítico [1]. Esta instalação experimental encontra-se dividida em 4 zonas de operação (ver Figura 1). Uma zona de média pressão, cerca de 60 bar, onde o CO₂ 99.9% (grau indústria alimentar) se encontra armazenado numa garrafa de gás e será arrefecido e comprimido para uma gama de pressão de extração entre 80-200 bar usando uma bomba de alta-pressão. Posteriormente, o CO₂ comprimido será aquecido até 40°C através de uma bomba de aço inoxidável e conduzido até à célula de extração (1 L) que contém as amostras para extração dos aromas (zona de alta pressão). Os ensaios serão realizados em modo estático, deixando o CO₂ supercrítico em contato com o material vegetal ou os frutos secos a pressão e temperatura constantes, durante 2 horas. A recuperação dos extratos supercríticos será realizada em dois passos de despressurização, o primeiro a uma gama de pressão de 20-50 bar, zona de média pressão, e o segundo a uma gama de pressão de 1-6 bar, zona de baixa pressão. Os extratos supercríticos isolados serão armazenados a 4 °C antes de serem caracterizados [2].

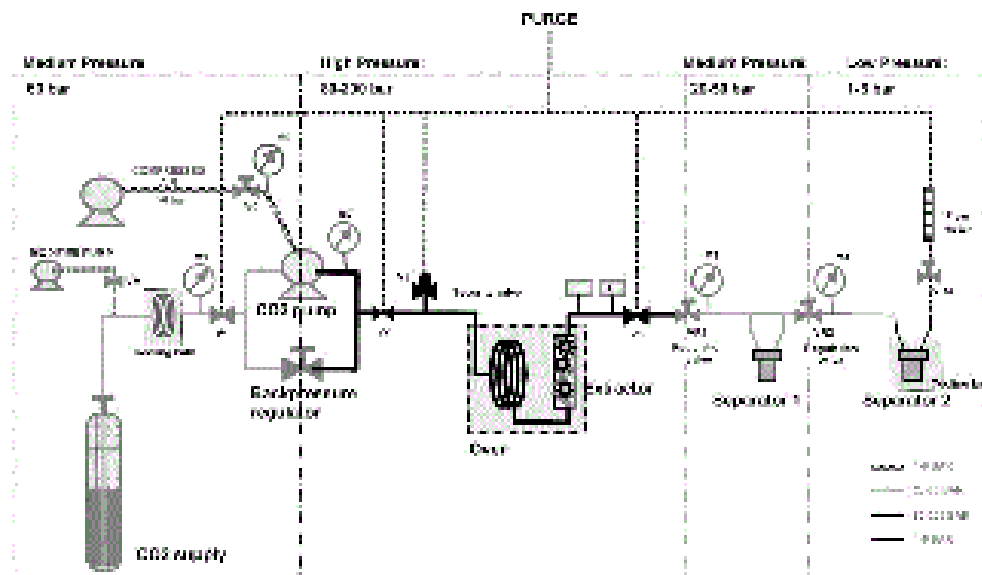


Figura 1. Esquema da instalação experimental do processo de extração de aromas usando CO₂ supercrítico [1].

Referências:

- [1] – Gomes, P., Mata, V., Rodrigues, A. *Production of rose geranium oil using supercritical fluid extraction*. J. of Supercritical Fluids 41 (2007) 50-60.
- [2] – Costa P., Velasco, C., Rodrigues, A. *Effect of cosmetic matrices on the release and odour profiles of the supercritical CO₂ extract of *Origanum majorana* L.* International Journal of Cosmetic Science 38 (2016) 364-374.



ValorNatural – Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar.

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Versão do Documento: 1

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Responsável: IPB-CIMO

Nome do Documento: Folheto com procedimentos de recolha dos bio-resíduos de cogumelos

Histórico de Revisão

Revisão	Data	Parceiros Envolvidos	Descrição



Lista de Autores

Ângela Fernandes

Sandrina Heleno

Sumário

Este entregável resume os procedimentos necessários para a recolha dos bio-resíduos de cogumelos.

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1. Identificação

<i>Deliverable</i>	5.1.1. Folheto com procedimentos de recolha dos bio-resíduos de cogumelos
<i>Tipo de deliverable</i>	Folheto
<i>Nível de disseminação</i>	Público
<i>PPS</i>	5. Bioativos naturais

2. Informação

Agaricus bisporus



Os bio-resíduos da Indústria produtora de *Agaricus bisporus*, correspondendo a cerca de 15% da produção, são fornecidos pela empresa “Mogaricus”, Mogadouro, Portugal. Estes bio-resíduos são todas as partes dos cogumelos que apresentam irregularidades a nível fisiológico. Alguns exemplos são a parte de baixo e volva devido à sua textura dura, ou a matéria orgânica presente no efluente gerado nos processos de lavagem e branqueamento. Além disso, durante o cultivo e a colheita dos cogumelos, os espécimes com dimensões e forma irregulares são descartados. Aquando da colheita dos cogumelos na sua fase de maturação apropriada para consumo, todos estes bio-resíduos são colocados num recipiente refrigerado e transportados até ao laboratório onde são imediatamente congelados, liofilizados e armazenados a 4 °C ao abrigo da luz.



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Entregável nº 5.1.2.

Versão do Documento: 1

Data de Submissão: 30/11/2018

Responsável: IPB-CIMO

Nome do Documento: Relatório do procedimento de conversão do ergosterol em vitamina D2

Histórico de Revisão

Revisão	Data	Parceiros Envolvidos	Descrição



Lista de Autores

Ângela Fernandes

Sumário

Este entregável resume o procedimento de conversão do ergosterol em vitamina D2.

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1. Identificação

<i>Deliverable</i>	5.1.2. Relatório do procedimento de conversão do ergosterol em vitamina D2
Tipo de <i>deliverable</i>	Relatório
Nível de disseminação	Confidencial
PPS	5. Bioativos naturais

2. Informação

A vitamina D2 (ergocalciferol) tem como precursor o ergosterol que é amplamente encontrado em cogumelos. O ergosterol é fotossensível e sob influência da irradiação ultravioleta (UV) ocorre a clivagem fotoquímica do anel B e em seguida, a pré-vitamina D2, sofre um rearranjo térmico levando à formação da vitamina D2. As doses de irradiação, duração da exposição e distância da fonte de luz UV serão otimizadas.



ValorNatural – Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar.

Entregável nº 5.1.3.

Versão do Documento: 1

Data de Submissão: 30/11/2018

Responsável: IPB-CIMO

Nome do Documento: Relatório com as especificações técnicas dos bioativos a desenvolver

Histórico de Revisão

Revisão	Data	Parceiros Envolvidos	Descrição



Lista de Autores

Ângela Fernandes

Sandrina Heleno



Sumário

Este entregável resume as especificações técnicas dos bioativos a desenvolver: ergosterol e vitamina D2.

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1. Identificação.....	5
2. Informação.....	6

1. Identificação

<i>Deliverable</i>	5.1.3. Relatório com as especificações técnicas dos bioativos a desenvolver
Tipo de <i>deliverable</i>	Relatório
Nível de disseminação	Confidencial
PPS	5 Bioativos naturais

2. Informação

A vitamina D2 é uma vitamina lipossolúvel, é relativamente estável após incorporação nos alimentos que têm caráter lipofílico. A fermentação, a cozedura e o armazenamento têm pouco efeito na sua atividade. É considerada uma vitamina relativamente robusta, sendo estável durante a cozedura até 200 °C.

O ergosterol apresenta uma estrutura química anfipática, pelo que a sua solubilidade quer em meio polar, quer em meio apolar é reduzida. No entanto apresenta maior solubilidade em meio lipofílico, é resistente à fermentação, a alterações de pH, a altas temperaturas (podendo suportar até 250 °C sem degradação) e à refrigeração; pelo que a sua utilização como agente bioativo na indústria alimentar não apresenta limitações técnicas do ponto de vista da sua estabilidade após a incorporação como agente funcionalizante em alimentos lipofílicos. Para a sua utilização em meios aquosos é necessária a sua estabilização através de técnicas de encapsulação de forma a aumentar a sua solubilidade. Estas técnicas serão otimizadas.



ValorNatural – Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar.

Entregável nº 5.1.4.

Versão do Documento: 1

Data de Submissão: 28/02/2019

Responsável: FEUP

Nome do Documento: Relatório das condições de extração ótimas para a obtenção das moléculas bioativas

Histórico de Revisão

Revisão	Data	Parceiros Envolvidos	Descrição



Lista de Autores

Ângela Fernandes

Sandrina Heleno

Sumário

Este entregável resume os procedimentos necessários para a extração das moléculas bioativas: ergosterol e vitamina D2.

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1. Identificação

<i>Deliverable</i>	5.1.4. Relatório das condições de extração ótimas para a obtenção das moléculas bioativas
Tipo de <i>deliverable</i>	Relatório
Nível de disseminação	Confidencial
PPS	5. Bioativos naturais

2. Informação

Agaricus bisporus L.



Extração de ergosterol

Os bio-resíduos da indústria produtora de *Agaricus bisporus*, previamente liofilizados e triturados são sujeitos a uma extração assistida por ultrassons tendo-se otimizado as condições de extração (temperatura, tempo, potência do equipamento, razão sólido/líquido e diferentes solventes), que permitem a obtenção da maior quantidade de ergosterol. O solvente etanol 100% foi o solvente mais eficaz para a extração deste bioativo. As condições de operação do equipamento que permitem a maior quantidade de ergosterol extraída são: temperatura ambiente, tempo de 15 min e potência de 375 W. Após a extração, segue-se uma filtração e posterior evaporação do solvente. O resíduo é de seguida redissolvido numa concentração conhecida e o ergosterol é quantificado através da técnica de cromatografia líquida de alta eficiência acoplada a um detetor de díodos a 280 nm. Estas condições ótimas de extração permitem a obtenção de 671.5 ± 0.5 mg de ergosterol/100 g de cogumelo seco.

Extração da Vitamina D2

Os bio-resíduos da indústria produtora de *Agaricus bisporus*, previamente liofilizados e triturados são sujeitos a uma extração assistida por ultrassons tendo-se otimizado as condições de extração (temperatura, tempo e diferentes solventes), que permitem a obtenção da maior quantidade de vitamina D2. O solvente hexano foi o solvente mais eficaz para a extração deste bioativo. As condições de operação do equipamento que permitem a maior quantidade de vitamina D2 extraída são: temperatura de 45

°C e tempo de 30 min. Após a extração, segue-se uma filtração e posterior evaporação do solvente. O resíduo é de seguida redissolvido numa concentração conhecida e a vitamina D2 é quantificada através da técnica de cromatografia líquida de alta eficiência acoplada a um detetor de díodos a 280 nm.



ValorNatural – Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar.

Entregável nº 5.1.5.

Versão do Documento: 1

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Histórico de Revisão

Revisão	Data	Parceiros Envolvidos	Descrição



Lista de Autores

Sumário

Este entregável resume os procedimentos necessários para a refinação das moléculas bioativas: ergosterol e vitamina D2.

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1. Identificação

<i>Deliverable</i>	5.1.5. Relatório dos procedimentos de refinação dos ingredientes bioativos
Tipo de <i>deliverable</i>	Relatório
Nível de disseminação	Confidencial
PPS	5. Bioativos naturais

2. Informação

Agaricus bisporus L.



Refinação dos bioativos

Não foi necessária refinação.



ValorNatural – Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar.

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Lista de Autores

Isabel Ferreira

Márcio Carcho

Sumário

Publicações relativas aos ingredientes com maior capacidade hipocolesterémica e que não apresentam toxicidade.

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1. Identificação

<i>Deliverable</i>	5.1.6
<i>Tipo de deliverable</i>	Publicação
Nível de disseminação	Público
PPS	5

2. Informação

As publicação relativas aos ingredientes com maior capacidade hipocolesterémica e sem toxicidade são:

Enhanced extraction of ergosterol from *Pleurotus ostreatus* using response surface methodology (RSM)

Taofiq O., Heleno S. A., Carocho M., Costa C., Prieto M. A., Barros J., Ferreira I., Nunes J., Barros L., Ferreira I. C. F. R.

XX EuroFoodChem Conference

Ayadi R., Functionalation of cheese with mycosterol extracts, 2019, Tese de Mestrado apresentada ao Instituto Politécnico de Bragança



3. Anexos

Enhanced extraction of ergosterol from *Pleurotus ostreatus* using response surface methodology (RSM)

Oludemi Taofiq¹, Sandrina A. Heleno¹, Márcio Carochó¹, Cristina Costa², Prieto M.A.^{1,3}, Joana Barros², Inês Ferreira², João Nunes², Lillian Barros¹, Isabel C.F.R. Ferreira^{1,*}

¹Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal;

²Centre Bio R&D Unit, Association BLC3 – Technology and Innovation Campus, Rua Nossa Senhora da Conceição, n2, 3405-155 Oliveira do Hospital, Portugal

³Nutrition and Bromatology Group, Faculty of Food Science and Technology, University of Vigo, Ourense Campus, E32004 Ourense, Spain

* iferreira@ipb.pt

Pleurotus ostreatus (Jacq. ex Fr.) P. Kumm., is one of the most widely consumed mushrooms in the world with interesting health-promoting benefits, mainly due to its richness in several bioactive compounds [1]. Mushrooms produce ergosterol as one of their main sterols, which has been considered a contributor to their anti-inflammatory, antioxidant, and antitumor properties [2]. Obtaining an ergosterol enriched extract depends on different variables, such as the extraction method, solvent type, temperature, extraction time, and the solid-liquid ratio [2]. Therefore, it is essential to define the main variables and relevant response criteria to maximize the extraction yield and purity, combining the economic competitiveness.

In the present work, response surface methodology (RSM) was applied to optimize a heat assisted extraction system (HAE), combining time (t) and temperature (T) effects, and using a circumscribed central composite design (CCCD) for the recovery of ergosterol from the fruiting bodies of *P. ostreatus* produced with lignocellulose substrate. The obtained responses were the quantification of ergosterol by HPLC-UV (Y_1 : mg of ergosterol per g of extract residue and Y_2 : mg of ergosterol per 100 g of dry weight mushroom), and the extraction yield (Y_3 : %). The CCCD consist of 16 response combinations and 4 centre points. Response surface models were fitted by using the following second order polynomial equation:

$$Y = b_0 + \sum_{i=1}^n b_i X_i + \sum_{i=1}^{n-1} \sum_{j=2}^n b_{ij} X_i X_j + \sum_{i=1}^n b_{ii} X_i^2$$

The results obtained showed a significant interaction between the variables. For all the three responses (Y_1 , Y_2 , and Y_3), the model successfully explained more than 80% variation in the experimental data (i.e. $R^2 > 0.8$). The individual optimum conditions and responses were as follows; Y_1 (10 min, 30°C, 57.6 mg/g), Y_2 (150 min, 61°C, 246.3 mg/100 g dw), and Y_3 (10 min, 80.9°C, 9.3%). The global optimum conditions predicted by the model were: 150 min and 54.3 °C, capable of yielding 7.3 %, 33.3 mg/g and 244.3 mg/100 g dw. The values predicted by the model are in close agreement with the experimental observations with very low residual distribution, proving the validity of the applied model. The results also showed the usefulness of the predictions for future scale up based on the desired responses. The obtained ergosterol enriched extract can be considered as a bioactive ingredient for pharmaceutical, cosmeceutical and nutraceutical purposes.

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Functionalization of cheese with mycosterol extracts

Rihab Ayadi

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Supervisors

Sandrina Heleno
Isabel C.F.R. Ferreira
Ichrak Charfi

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Abstract

Due to the structural similarity with phytosterols, it is believed that ergosterol acts identically in the lowering of blood cholesterol levels. Due to the easier solubility of ergosterol in lipophilic media, an interesting approach is the incorporation of pure ergosterol and mycoesterol extracts into lipid matrices such as cheese, facilitating its solubility and consequent availability to exert the biological activity. In this perspective, the objective of this dissertation was the incorporation of mycoesterol extracts (obtained from *A. bisporus*) and pure ergosterol in traditional sheep cheese. The incorporation was based on the data regarding phytosterols and their ability to reduce cholesterol absorption. The developed cheeses were fully characterized before and after 2 months of maturation in terms of nutritional value, physicochemical parameters, profiles in fatty acids, lactose, lactic acid, and ergosterol/cholesterol. The cytotoxicity of pure ergosterol, cholesterol and *A. bisporus* extract was also evaluated in PLP2 and in CaCo2 cells to find the subtoxic concentration for application in the cell transport assay. Ergosterol, cholesterol, *A. bisporus* extract and the final cheese formulations were tested in Caco2 cells to infer the reduction in the cholesterol absorption. From the obtained results, it can be observed that the nutritional value did not vary between the control cheese and the cheeses with the incorporations. In terms of physicochemical parameters, for the color analysis, overtime, the cheese with the extract increased in yellowness and reduced lightness, showing a browner tone. Among the fatty acids, no variations were detected between samples, being palmitic acid the most abundant one, followed by oleic and myristic acids. The saturated fatty acids predominated in all samples, followed by monounsaturated and polyunsaturated fatty acids. Lactose was, as expected, identified in the cheeses and was greatly reduced overtime, while lactic acid increased during the two months, confirming the decrease of lactose, which is converted during the maturation. A subtoxic concentration of 50 µg/mL was selected to perform the absorption tests. It was possible to determine that Caco2 cells absorbed 30% of cholesterol from the control cheese. On the other hand, cheese with pure ergosterol reduced the cholesterol absorption in ~12%, while the cheese with mushroom extract reduced the absorption in ~15%. These results highlight the ability of ergosterol in reducing the cholesterol absorption, being an interesting candidate for the development of functional foods for hypocholesterolemic effects.

Keywords: Mycoesterol; sheep cheese; hypocholesterolemic agents; functional foods

Resumo

Devido à semelhança estrutural com os fitoesteróis, é possível que o ergosterol atue de forma similar na redução dos níveis de colesterol. Graças à sua solubilidade em meio lipofílico, uma abordagem interessante é a incorporação de extratos enriquecidos em ergosterol e de ergosterol puro em matrizes lipídicas como o queijo, facilitando a sua solubilidade e consequente disponibilidade. Assim, o objetivo desta dissertação foi a incorporação de extratos de micosteróis obtidos a partir de *A. bisporus* e ergosterol puro, em queijo de ovelha tradicional. A incorporação foi baseada nos dados referentes aos fitoesteróis e na sua capacidade de reduzir a absorção de colesterol. Os queijos desenvolvidos foram totalmente caracterizados antes e após 2 meses de maturação em termos de valor nutricional, parâmetros físico-químicos, perfis em ácidos gordos, lactose, ácido láctico e ergosterol/colesterol. A citotoxicidade do ergosterol, colesterol e ainda do extrato de *A. bisporus* foi avaliada em células PLP2 e CaCo2 de forma a encontrar a concentração subtóxica destas substâncias para posterior aplicação no ensaio de transporte celular. Assim, uma concentração subtóxica de ergosterol, colesterol, extrato de *A. bisporus* e das formulações finais de queijo foram testadas em células CaCo2 para verificar a sua capacidade de redução da absorção de colesterol. A partir dos resultados obtidos, foi possível observar que o valor nutricional não variou entre o queijo controle e os queijos incorporados com os agentes bioativos. Relativamente aos parâmetros físico-químicos, para a análise de cor, ao longo do tempo, o queijo com o extrato aumentou a cor amarela e reduziu a luminosidade, apresentando um tom mais acastanhado. Relativamente aos ácidos gordos, não foram detetadas variações entre as amostras, sendo o ácido palmítico o mais abundante, seguido dos ácidos oléico e mirístico. Os ácidos gordos saturados predominaram em todas as amostras, seguidos dos ácidos gordos monoinsaturados e poliinsaturados. A lactose presente nos queijos foi bastante reduzida após o processo de maturação, tendo originado o aumento do ácido láctico devido ao consumo de lactose pelas bactérias lácticas. Após os ensaios de transporte celular, foi possível determinar que as células CaCo2 absorveram 30% do colesterol do queijo controle, enquanto que o queijo com ergosterol puro reduziu a absorção de colesterol em ~12%, e o queijo com extrato de reduziu a absorção em ~15%. Esses resultados destacam a capacidade do ergosterol em reduzir a absorção de colesterol, sendo um candidato interessante para o desenvolvimento de alimentos funcionais para efeitos hipocolesterolémicos.

Palavras-Chave: Micosteróis; Queijo de ovelha; agentes hipocolesterolémicos; alimentos funcionais

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List of abbreviations

ABC	ATP-Binding Cassette
ABCG	ATP-Binding Cassette G
ABCGaqbcg	ATP-Binding Cassette G Gene
ALP	Alkaline Phosphatase Activity
AOAC	Association of Official Analytical Chemists
ATP	Adenosine Tri-Phosphate
A_w	Water Activity
CACO2	Human Colorectal Adenocarcinoma Cell Line
CC	Control Cheese
CCM	Maturated Control Control
CEXT	Cheese with <i>A. bisporus</i> Extract
CEXTM	Maturated Cheese with <i>A. bisporus</i> Extract
CPE	Cheese with Pure Ergosterol
CRP	C-reactive protein
CVD	Cardiovascular disease
CPEM	Maturated Cheese with Pure Ergosterol
DMEM	Dulbecco's Modified Eagle's Medium
DRBC	Agar Dicloran Rosa Bengala Cloranfenicol Base
ECACC	European Collection of Cell Cultures
EMM	Estimated Marginal Means
FAME	Fatty Acid Methyl Esters
FBS	Fetal Bovine Serum
FID	Flame Ionization Detector
FW	Fresh Weight
GC	Gas Chromatography
GI₅₀	Concentration that inhibits 50% of cell growth
HBSS	Hank's Balanced Salt Solution
HDL	High Density Lipoprotein
HPLC	High Performance Liquid Chromatography
HEPES	Hydroxyl-Ethyl-Piperazine-Ethane-Sulfonic-Acid Solution

Functionalization of cheese with mycoesterol extracts

ISO	International Standards Organization
IT	Tukey's multiple comparison test
LDL	Low Density Lipoprotein
LXR	Liver X Receptor
<i>mf</i>	Final Mass
<i>mi</i>	Initial Mass
MRS	Manrogosa And Sharpe Agar
MT	Maturation time
MUFA	Monounsaturated Fatty Acids
NPC1L1	Niemann-Pick C1-Like 1 transporter
PCA	Plate Count Agar
PDA	Photodiode Array Detector
PLP2	Porcine Liver Primary Culture
PUFA	Polyunsaturated Fatty Acids
PW	Peptone Water
RH	Relative Humidity
RI	Refraction Index
SFA	Saturated Fatty Acids
SRB	Sulforhodamine
ST	Student's T Test
TPA	Texture Profile Analyzer
TEER	Transepithelial Electrical Resistance
UFC	Colony Forming Unit
UAE	Ultrasound-assisted extraction
UV	Ultraviolet radiation
VLDL	Very Low Density Lipoprotein
VRBG	Violet Red Bile Glucose Agar
VRLA	Violet Red Bile Lactose Agar

1. Introduction

1.1. Bioavailability of phytosterols in the human body

1.1.1. Absorption and metabolism of sterols

Sterols or steroid alcohols are important molecules, involved in the metabolic pathway and in the structure of the eukaryotic cells. Different sterols can be found in the serum plasma of the body, some of them are derived from endogenous biosynthesis of cholesterol, while others are ingested through the diet. Sterols derived from plant sources are called phytosterols. The enzyme acetyl-CoA is used for the synthesis of sterols via several reaction steps with a remarkable level of conservativeness between species (Vecka et al., 2009).

The most common sterol in the human body is cholesterol. It contributes to the structure of membranes, and is one of the most abundant lipids in the human brain, representing the key molecule of the steroid hormone biosynthesis and bile acids (Ogbe et al. 2015). There are two sources of cholesterol: dietary cholesterol (from food) and endogenous cholesterol synthesis by the liver. The intestine usually absorbs cholesterol, but only half of the cholesterol absorbed will enter the cells, the rest will be eliminated in the feces (Danacol Monograph, 2009). Being a hydrophobic molecule, cholesterol is transported through the body bonded to lipoproteins. There are three types of lipoproteins, being the differences among them related to their density: low density lipoprotein (LDL), high density lipoprotein (HDL), very low density lipoprotein (VLDL) (Danacol Monograph, 2009). The consumption of dietary food with high levels of cholesterol can cause heart diseases and even strokes, and according to the World Health Organization, raised cholesterol levels in the human body are estimated to cause 2.6 million deaths (4.5% of total) and 29.7 million disability adjusted life years. Moreover, a 10% reduction in cholesterol serum levels in men aged 40 has been reported to result in a 50% reduction in heart diseases within 5 years; the same reduction for men aged 70 can result in an average of 20% reduction of heart diseases occurring in the next 5 years”.

Phytosterols can be ingested and consequently absorbed by the human body and appear in the serum plasma, leading to a direct absorption competition with the internally produced cholesterol, thus reducing the absorption of cholesterol into the cells, thus decreasing its potential accumulation. (Vecka et al., 2009)

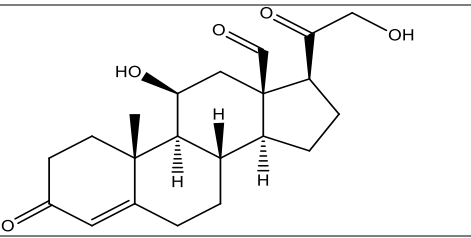
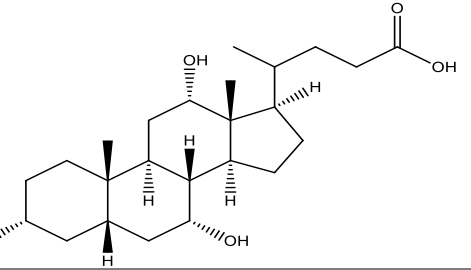
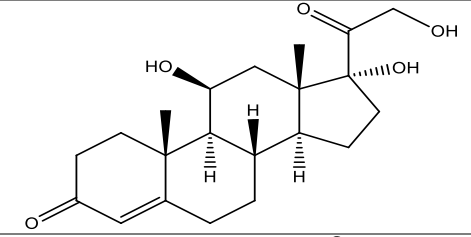
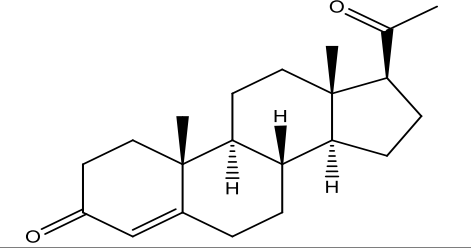
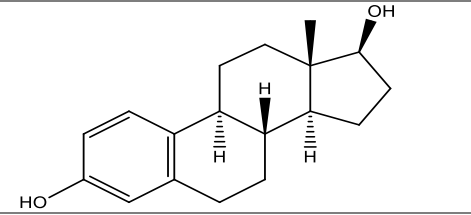
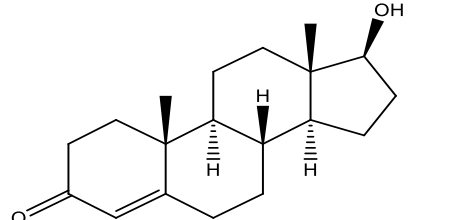
On **Table 1** it is possible to observe the types of sterols found in different organisms, and in **Table 2** the different derivatives of sterols are shown.

Functionalization of cheese with mycoesterol extracts

Table 1. Sterol structures

Name	Chemical structure
Cholesterol	
Latosterol	
Demosterol	
Campesterol	
Ergosterol	
□-sitosterol	

Table 2. Sterol derivates.

Name	Structure
Aldosterone	
Cholic acid	
Cortisol	
Progesterone	
Estradiol	
Testosterone	

The dietary lipids are first digested in the oral cavity through exposure to the lingual lipases, secreted by glands in the tongue. The digestion is continued in the stomach, where gastric enzymes further breakdown these nutrients and emulsify them to then enter the duodenum, as fine lipid droplets which react with pancreatic and bile enzymes to change some of the physical and chemical characters (Iqbal and Hussain 2009; Ogbe et al. 2015).

Functionalization of cheese with mycoesterol extracts

Once the digestion is finished the emulsion droplets will turn into monoglycerides, cholesterol, soluble vitamins, and fatty acids. These structures will bind to bile salts and phospholipids forming micelles (Shoshana and Garti 2006) which facilitate the transport of the fat that will be absorbed by the microvilli. Only free fatty acids and monoglycerides can be absorbed by the enterocytes to re-synthesis the triglycerides (Ogbe et al. 2015). The triglycerides will further aggregate with cholesterol, fat-soluble vitamins and proteins forming chylomicron. These newly formed lipoproteins move into the lymph capillaries, transporting the lipids to the rest of the body (Hui and Howles, 2005). Unabsorbed chylomicrons turn back to the liver where they release triglycerides and a portion of cholesterol that is converted to Low Density Lipoprotein (LDL). LDL's carry triglycerides and cholesterol esters and deposit these molecules in cells of different peripheral tissues. Since LDL and Very-Low Density Lipoprotein (VLDL) have the ability to transport lipids to the arteries, they are the main contributors to the formation of atheromatous plaques. Contrarily, high density lipoprotein (HDL) carries cholesterol to the liver for excretion because it is the primary organ that can remove the excess cholesterol from the bile in its native form or in the form of bile acids (Danacol Monograph, 2009) (**Figure 1**).

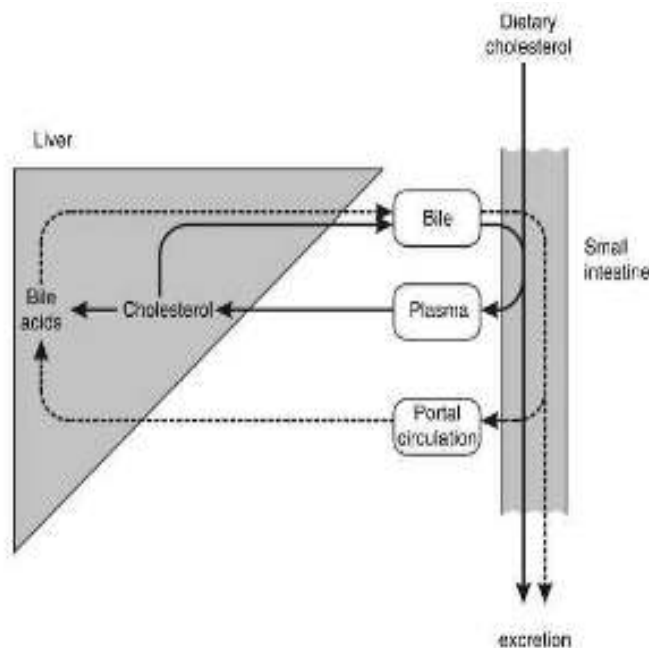


Figure 1. Cholesterol pathway of the cholesterol inside the human body (Agellon, 2006).

The absorption of cholesterol by the intestine is controlled by ATP (adenosine triphosphate) binding cassettes (ABC) which are membrane protein transporters located on the brush membranes of the intestine that send cholesterol back to the intestine lumen using ATP. Once

cholesterol is inside the enterocytes, its systemic absorption is inhibited by the activity of efflux transporters, consisting of a pair of ATP-binding cassette proteins known as ABCG5 and ABCG8. Each of these ABC's forms one half of a transporter that turns back sterols and non-esterified cholesterol from the enterocyte into the intestinal lumen (Ogbe et al., 2015).

1.1.2. Effects of phytosterols on the intestinal cholesterol absorption

Phytosterols, as mentioned above, are plant sterols with a similar structure to cholesterol, having an additional substitution of an extra hydrophobic carbon chain at the C24 position (**Figure 2**).

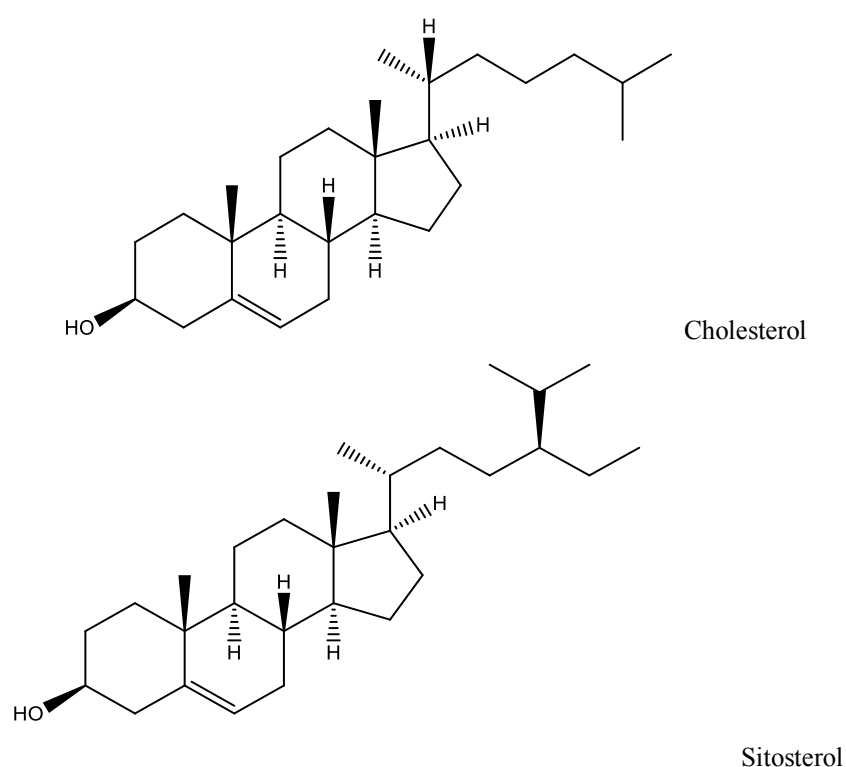


Figure 2. The difference between cholesterol and phytosterol (e.g., sitosterol) structures

There are two types of phytosterols (**Figure 3, 4**) (Ogbe et al., 2015) :

- Unsaturated molecules, the sterols

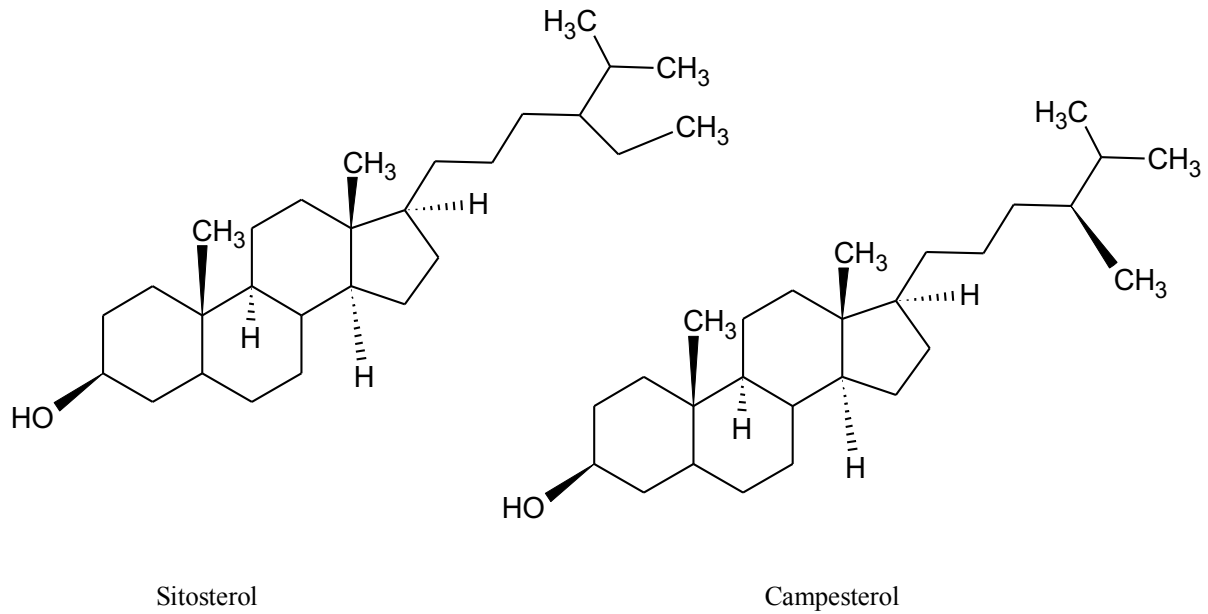


Figure 3. Chemical structure of sterols

- Saturated molecules, the stanols:

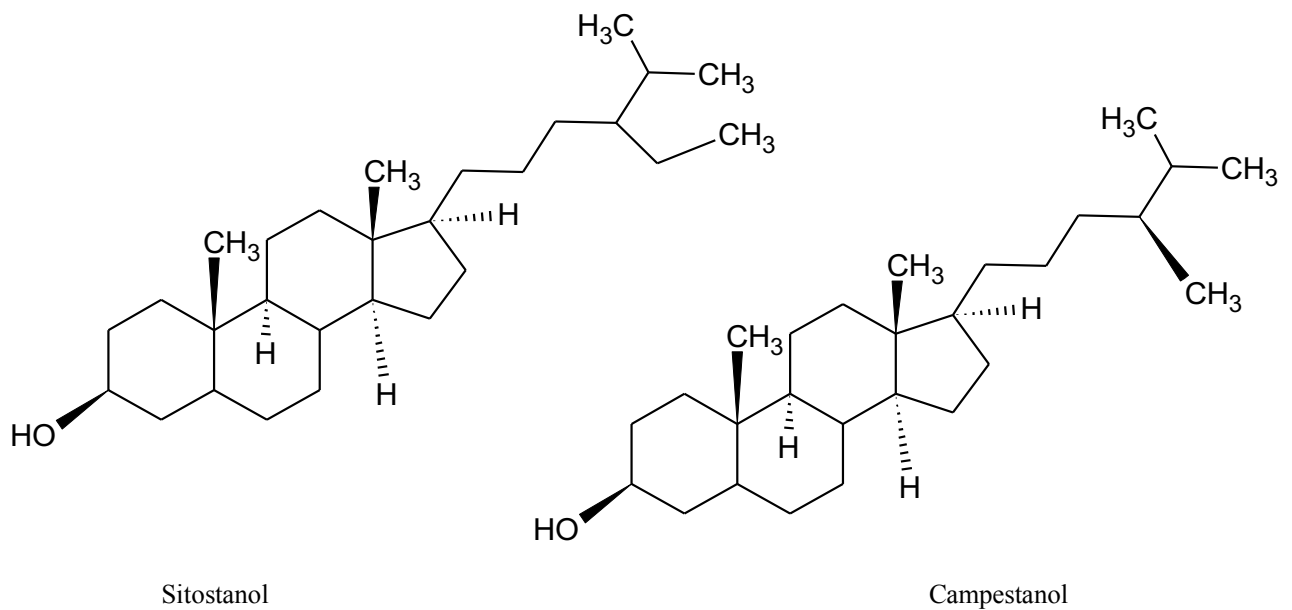


Figure 4. Chemical structure of stanols

1.1.2.1. Physicochemical effects

Reduction of cholesterol absorption is based on the co-crystallization of the cholesterol and phytosterols, leading to the formation of a mixture of insoluble crystals during food lipolysis in the gastro-intestinal tract (Christiansen et al., 2003). Cholesterol and phytosterols are present in a water and oil emulsion, because of the low degree of enzymatic lipid hydrolysis. In the small intestine, the enzymatic conversion of the gastric emulsion intensifies, with the degradation of triacylglycerol, free fatty acids and phospholipids, as well as the transformation of sterol esters into free sterols. Thus, the rapid reduction of the lipid phase volume in the small intestine is believed to be a prerequisite for the precipitation of sterols. In addition, increased intake of sterols, particularly phytosterols, leads to the increase in the competition with cholesterol molecules by the lipid phase which facilitates intestinal precipitation of cholesterol and other sterols. This is also influenced by the ratio between cholesterol and phytosterol and their amounts in the aqueous phase (Trautwein et al., 2003).

1.1.2.2. Competition between cholesterol and phytosterol for micellar solubilization

When phytosterols and cholesterol are present in the chylomicrons; 70% of cholesterol will be esterified and absorbed although for phytosterols, the absorption only amounts to 12% (Shoshana and Garti 2006; Trautwein et al. 2003). Micelles which are lipid molecules such as fatty acids, mono-glycerides and sterols that arrange themselves in a spherical form in aqueous solutions formed during digestion, play an important role in the whole absorption mechanism (Shoshana and Garti, 2006).

These structures act as a vehicle that transports both non-polar and polar molecules towards the intestinal wall (enterocytes). Enterocytes can only absorb them after hydrolyzation of the free cholesterol with the help of the esterase pancreatic cholesterol enzyme. Some theories suggest that the decrease of cholesterol absorption is linked to the low capacity of micelles to solubilize sterols. Contrarily, it has also been proven by *in vivo* and *in vitro* experiments, that the solubility of cholesterol is lower than phytosterol due to the higher affinity of phytosterols to the dietary mixed micelles. Thus, as a result, the phytosterols limit the micellar solubilization of cholesterol (Ikeda et al., 1988).

1.1.2.3. Effect on the absorption site

Before the absorption of cholesterol, cholesterol esters must be split by the pancreatic enzyme cholesterol-esterase. Interestingly, phytosterol esters can also be a substrate for this enzyme, which can result in two different effects:

- Phytosterols can bind to cholesterol-esterase to reduce or block the splitting of all the dietary cholesterol esters. This will reduce the amount of cholesterol in the absorbable form. Therefore the dietary amount of cholesterol esters will decrease, but this is not the only cholesterol-lowering effect of phytosterols esters (Ikeda et al., 2002).
- The pancreatic enzyme cholesterol-esterase is able to split all the phytosterols esters that will be absorbed. When the enzyme does not have the ability to split phytosterol esters at enough speed, thus, the phytosterol esters will stay in the intestinal lumen acting as a solvent for other lipophilic compounds. As a consequence, a part of the total cholesterol and bile acids absorption in the intestinal lumen may take place in distal parts of the intestine, where there is less absorption efficiency (Lu et al., 2001).

The presence of phytosterols and phytosterol esters in the mixed micelles at the distal part of the gastrointestinal tract can influence the mixed micellar structure. This could result in a drain of the cholesterol pool, as the body will compensate for the bile salt loss. (Trautwein et al., 2003). Micelles are absorbed into enterocytes by Niemann-Pick C1-Like transporter (NPC1L1) (Brown et al., 2010), which is found in the intestinal epithelium and the hepatobiliary interface, being responsible for facilitating the uptake of sterols by enterocytes as shown in **Figure 5**. In the other hand, phytosterols can act as active intestinal liver x receptor (LXR) which is oxysterol-activated nuclear receptors that regulate the expression of genes which are involved in intestinal cholesterol absorption.

Initially, the active intestinal liver x receptor and phytosterols can reduce the intestinal cholesterol absorption and promote transintestinal cholesterol excretion. In addition it can also stimulate biliary cholesterol secretion (Cedó et al., 2017).

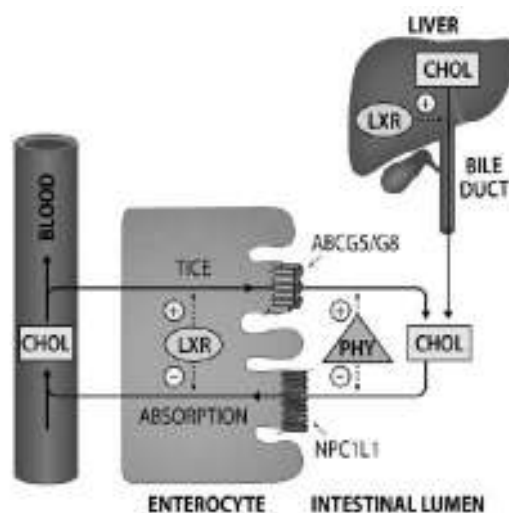


Figure 5. The effect of phytosterols in the cholesterol absorption(Cedó et al., 2017).

1.2. Mycoesterols as hypocholesterolemic agents: alternative to phytosterols

1.2.1. Mushroom as sources of mycoesterols

Mushrooms are consumed all over the world due to their texture, aroma, flavor and health properties (Taofiq et al., 2016). In addition to the different sensorial properties, they present an interesting nutritional value since they are constituted by proteins containing all the essential amino acids and present low lipid contents (Cardoso et al., 2017). Furthermore, they have high amounts of carbohydrates, fibers and significant contents of minerals and vitamins, such as thiamine and riboflavin, ascorbic acid and vitamin D (Raquel et al., 2013).

Mushrooms are described as having several bioactive components, such as phenolic compounds, terpenes, mycoesterols and polysaccharides (Guillamón et al., 2010), that according to several studies are responsible for the mushroom's antioxidant, anti-inflammatory, antitumor, anti-hyperlipidemic, antihyperglycaemic (Oludemi et al., 2016), antimicrobial and antiviral properties (Erreira, 2007). Their consumption is important in helping the body in the prevention of cardiovascular illnesses. Moreover, similarly to phytosterols, the mycoesterols are described as showing a strong capacity to act as cholesterol lowering or hypocholesterolemic agents.

Among the mushrooms species, *Agaricus bisporus* L., commonly known as white button mushroom, is the one presenting the highest amount in mycoesterols, being a strong candidate for mycoesterols obtention (Caz et al., 2016). These mushrooms contain high amounts of ergosterol (ergosta-5,7,22-trien-3 β -ol), the main mycoesterol, which can also be a direct competitor to cholesterol for intestinal absorption (Guillamón et al., 2010).

1.2.2 Chemical structures and biosynthetic pathway of mycoesterol

The first steps of sterol synthesis are common for cholesterol, phytosterol, and ergosterol; initiating with the transformation of acetyl-coA into squalene; for ergosterol the squalene will be then transformed by sequentially enzymatic reactions into lanosterol (Dupont et al., 2012). The only difference in the chemical structure is that fungi produce ergosterol which differs from phytosterols by having a double bond on its B ring (Barreira et al., 2013) (**Figure 6**).

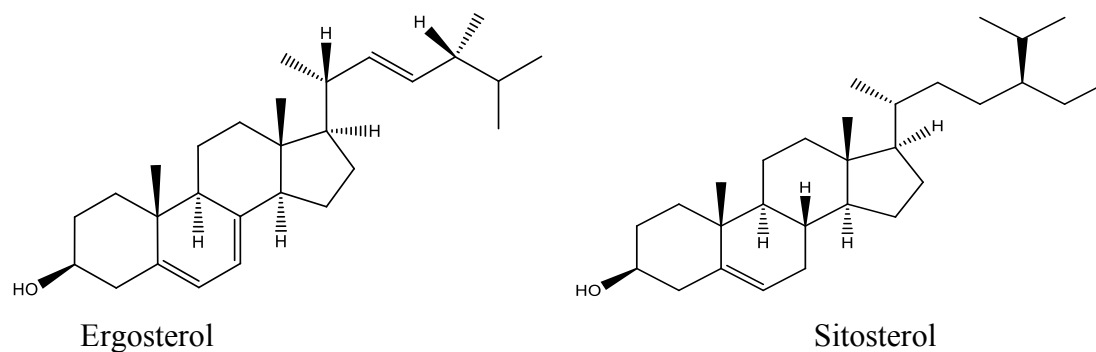


Figure 6. The difference in the chemical structures between phytosterol (sitosterol) and ergosterol

In terms of the biosynthetic pathway for ergosterol, the reaction starts with lanosterol which is the first sterol formed after squalene oxide cyclization and represents a branching point of the pathway. One branch leads towards zymosterol by sequential demethylation at C-14 and C-4. The other branch starts with methylation at C-24 producing eburicol and continues with a sequence of demethylations at both C-14 and C-4 (two methyl groups). Finally, the pathways converge at 24 methylcholesta-8, 24(28)-dien-3-ol (fecosterol) level. The second part of ergosterol biosynthesis pathway consists on the double-bond rearrangements in the steroid, nucleus and in the side chain, isomerization of the double connection in the C-8 to the C-7 followed by the desaturation of the C-5 and C-22, and the reduction of the C-24. The C-24 alkylation is catalyzed by S-adenosylmethionine-Sterol-C-methyl transferases. Fecosterol is converted to episterol by the C-8 sterol isomerase. Once episterol is synthesized, the transformation from episterol to ergosterol may occur through diverse alternative routes and some of them may even coexist in the same organism. **Figure 7** details the biosynthesis of ergosterol (Alcazar-fuoli et al., 2008)

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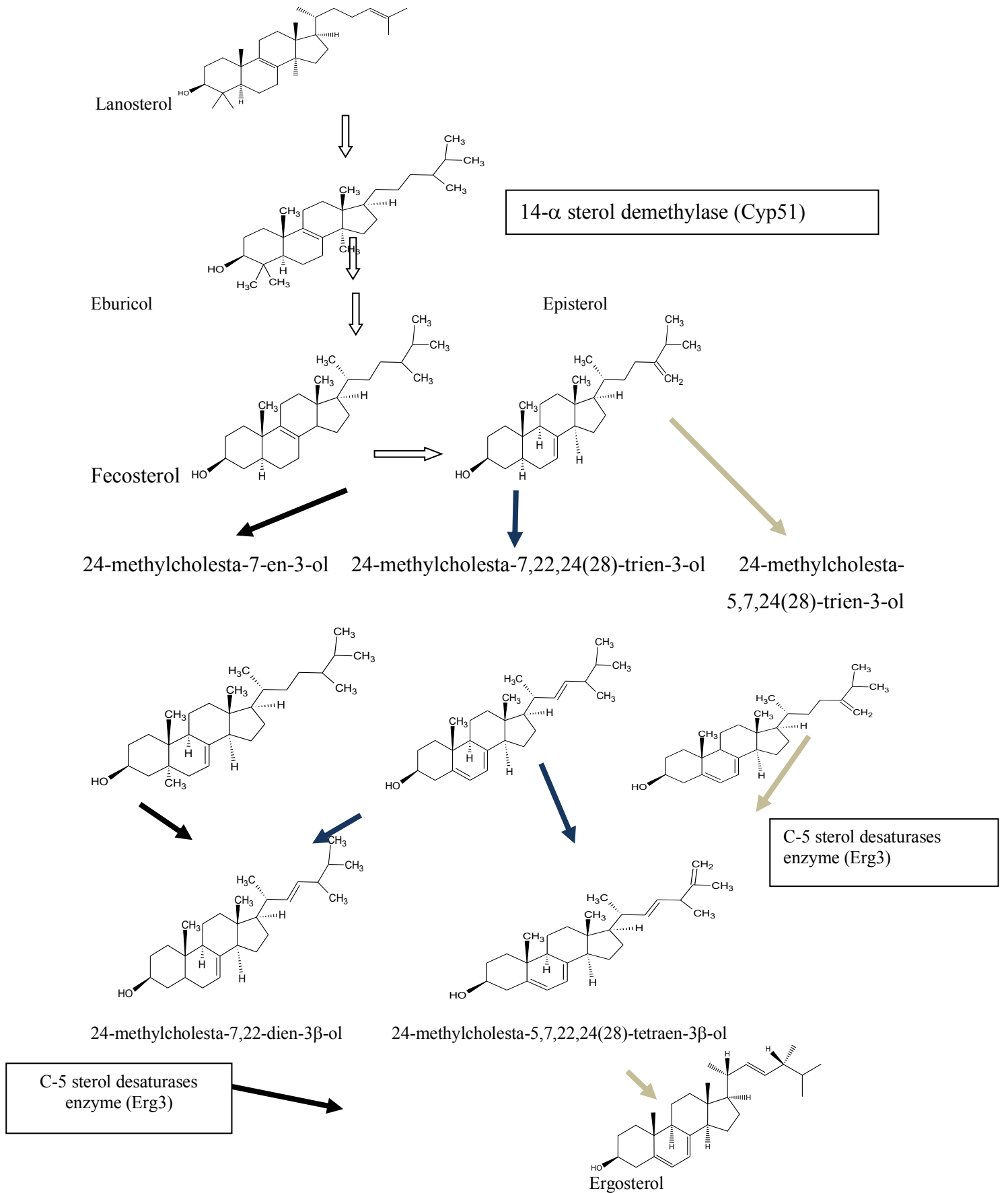


Figure 7. Biosynthetic pathway of ergosterol

1.2.3. Hypocholesterolemic potential of mycoesterols

Many studies prove that ergosterol has the same hypocholesterolemic effect as phytosterols, by decreasing the intestinal cholesterol absorption through the action of Niemen–Pick C1-like 1 (NPC1L1) protein, the excretion of cholesterol into the bile and liver through complex protein transporters ABCG5 and ABCG8. Furthermore, as phytosterols, there is a competition between cholesterol and ergosterol for the incorporation of dietary mixed micelles which will reduce the amount of the cholesterol absorbed by the enterocytes (Caz et al., 2016).

The (22E)-ergost-22-ene-1,3-diol can act as an agonist to the liver X receptor and inhibit the cholesterol absorption by up-regulating ABCG5 and ABCG8 genes in the small intestine (Makishima, 2003). The ergosterol-enriched diet causes overexpression of the cholesterol regulators protein ABCG5, ABCG8, and NPC1L1 (Caz et al., 2016).

Ergosterol can also combine with niacin to inhibit the activity of the enzyme hydroxymethylglutaryl-CoA reductase; which controls the synthesis of cholesterol by the liver. As a result, the amount of endogenous cholesterol will decrease (Moselhy et al., 2016).

1.3. Functional foods with hypocholesterolemic effects

1.3.1. New trends on functional foods

Human diets have contributed to the growth of a number of diseases: deficits in micronutrients and minerals, cancer, obesity, and coronary heart disease. Therefore, consumers are encouraged to adopt behaviors that promote their health and well-being. From this point of view, a new concept is developed to meet consumer demand for functional foods whose role is more preventive than curative.

Functional foods generated a revolution in the food industry, which took place in two phases; the first, from the beginning to the end of the 1990s, that started in Japan with the development of the first functional foods. This “revolution” was composed of three main strategies: the “recycling” of certain products, creation of new product categories and new markets, and a special emphasis on some of the nutritional properties of functional foods.

The second phase began in the early 2000’s with the consolidation of the functional food industries. This is the case, for example, of Unilever, that achieved a drop in bad cholesterol after introducing their hypocholesterolemic spreads, or even Danone with their probiotics. The consolidation phase is still ongoing and is expected to last a few more years. The functional food market is thus progressively evolving from a small market to a mass market as evidenced by the numerous annual attempts to introduce new functional foods.

In addition, the concerted action of the European Union supports the development of two types of assertions of health concerning the functional foods:

- Improving the desired function, referring to specific physiological, psychological and biological functions going beyond their role established in the growth, the development and the other normal functions of the body.
- Reducing the risk of diseases related to the consumption of a food or a food component that may help reduce the risk of a particular disease or condition due to the presence of specific nutrients or non-nutrients.

1.3.2. Dairy products with sterols for hypocholesterolemic effects

As fatty acid esterification improves lipid solubility of phytosterols, these are often incorporated into high-fat foods including margarine, yogurts, salad dressings, cheese, mayonnaise, milk, chocolate bars, baked goods, meat products, cereal bars, and fruit juices (Jones and Ph, 2014)

In a recent meta-analysis, the consumption of more than 2 daily grams of phytosterols -enriched fat food products reduced LDL-cholesterol concentrations by 0.33-0.54 mmol/L. However, some studies have examined the hypocholesterolemic effects of phytosterols supplementation in low-fat dairy products such as milk, yogurt, and other beverages. Therefore an absolute decrease in plasma cholesterol concentration has been observed (Hansel et al., 2007). The use of sterols represents a simple and safe tool to reduce plasma cholesterol in patients with mild cholesterol elevations, thus enhancing the attainment of LDL cholesterol in hypercholesterolemic patients (Mannarino et al., 2008). This evidence can be used to manufacture more effective dairy products in lowering blood cholesterol (Kwak et al., 2001). Some studies have shown that a daily intake of phytosterols, by people with moderate hypercholesterolemia decreases blood cholesterol levels from the 3rd week and remain present after 6 weeks of active consumption of phytosterols (Mannarino et al., 2009).

Also, in a recent study, it has been proven that yogurts incorporated with ergosterol show higher antioxidant, antitumor and anti-inflammatory activities than the commercial samples incorporated with phytosterols; being the ergosterol content in the yogurts much lower than the ones with phytosterols, highlighting the stronger activity obtained with lower amounts of ergosterol (Heleno et al., 2017).

In the European Union, the cheese consumption is around 10 million tons per year, averaging a consumption of about 14.2 kg in 2017 per capita. The top cheese consumer is Denmark, with an average consumption of 28.1 kg of cheese, followed by Iceland with 27.7 kg, Finland at 27.3

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kg and France with 27.2 kg. The high-fat dairy products are known to increase high density lipoprotein (HDL) and low-density lipoprotein (LDL)-cholesterol concentrations which predicts risk of cardiovascular diseases. Analyses conducted in Iran on 1.752 participants (782 men and 970 women) demonstrated that cheese eaters had higher levels of C-reactive protein, apolipoprotein A, HDL cholesterol, while fasting blood pressure, total cholesterol, LDL cholesterol, Apo B and triglyceride were not any higher (Nilsen et al., 2015). Considering all this evidence, cheese can be an outstanding candidate for incorporation of mycosterol or extracts containing this molecule.

1.4. Objectives

Hypercholesterolemia is a major risk factor for premature development of coronary heart disease. Dietary modification is the first step to reduce total cholesterol and LDL cholesterol. Thus, the new trend is to combine phytosterols with other ingredients that promote targeted health benefits accurate for health.

Based on scientific reports, it can be concluded that mycoesterols are bioactive ingredients, being its incorporation in food formulations to develop new functional foods, a topic of high interest. In this work, the intention was to incorporate *Agaricus bisporus* L. extracts containing mycoesterols, and pure ergosterol in traditional sheep cheese, in order to develop a functional food with hypocholesterolemic effects.

1.4.1. Specific objectives:

- Obtention of mycoesterol extracts from *A. bisporus*;
- Incorporation of *A. bisporus* powder, extracts and pure ergosterol in the sheep cheese;
- Evaluation of the nutritional value, individual composition in fatty acids, physicochemical parameters (color, pH, texture and water activity) and microbial load in the final formulations;
- Evaluation of the effects of the incorporation in the maturation process;
- Evaluation of hypocholesterolemic effects of the extract/ergosterol and final formulations;
- Evaluation of the stability of the ergosterol molecule over the maturation process and shelf life of the cheese.

2. Material and methods

2.1. Mushroom samples

2.1.1. *Agaricus bisporus* L.

The samples of *A. bisporus* L. were provided by the local mushroom producer "Mogarius mushrooms - Sociedade Unipessoal Lda" and consisted of biowaste. The mushrooms were frozen at -30 °C and further lyophilized (FreeZone 4.5 model 7750031, Labconco, KS, USA). The dried mushrooms were reduced to a fine dried powder (20 mesh) (**Figure 8**).



Figure 8. Mushrooms preparation: A-Lyophilized mushroom; B- Mushrooms grinding; C- mushroom powder

2.2. Extraction procedures

2.2.1. Mycoesterols extraction from *A. bisporus*

The mycoesterol extracts were obtained by ultrasound-assisted extraction (UAE) as previously optimized by Heleno et al. (2016). Briefly, 3 g of lyophilized mushroom powder were extracted with 150 mL of ethanol into the UAE device (Hielscher UIP1500hdT Ultrasonic Homogenizer) during 15 min and a potency of 375 w. The extract solution was filtered through a Whatman paper No. 4 and the solvent was further evaporated under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) (**Figure 9**).

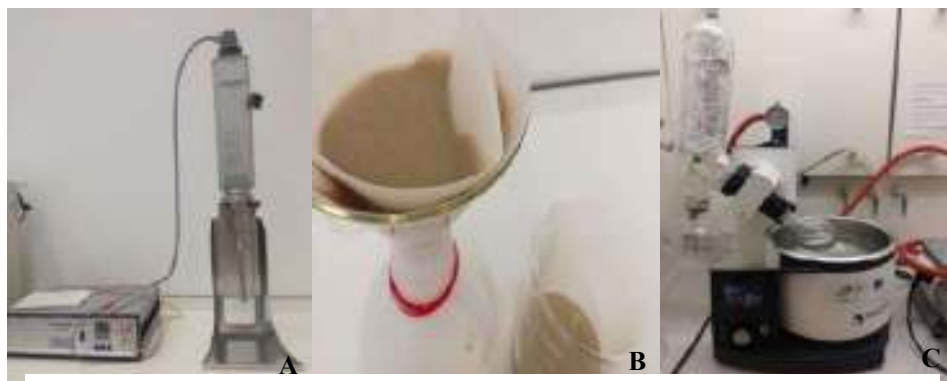


Figure 9. Obtention of mycoesterol extracts: A- UAE extraction; B- Filtration; C- Solvent evaporation

2.2.2. Obtention of the cheese extracts

Extraction of cheese samples (for ergosterol and cholesterol quantification) by UAE did not render as much yield as Soxhlet extraction, therefore, this technique was used to extract higher amounts of compounds for the analysis of the cheese samples (Heleno et al., 2016). Briefly, 3 g of cheese powder were extracted with 150 mL of ethanol during 5h (14 cycles), refluxing in a Soxhlet apparatus. Then the solvent was evaporated under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) (**Figure 10**).

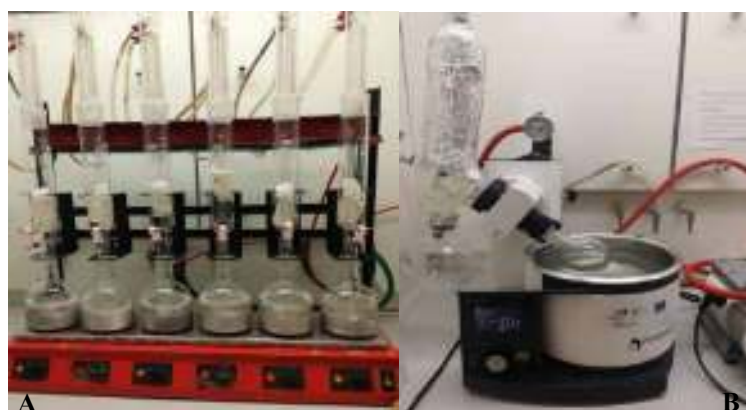


Figure 10. Cheese extraction by Soxhlet: A- Soxhlet extraction; B- Solvent evaporation

2.3. Incorporation of *A. bisporus* extract and pure ergosterol in sheep cheese

2.3.1. Traditional cheese making process

The sheep cheese was made in the artisanal manufacturing plant of the enterprise “Queijaria Vaz” located in Mirandela, Portugal. For the elaboration of sheep cheese, after milk collection, it was warmed up in water bath to 30°C and the rennet was added for coagulation (4-5 h). After the milk was clotted, a lateral tap was opened for the removal of the whey and the crude was cut in small portions. Afterwards, with the help of a cloth, the crude portions were manually pressed to filtrate and remove the remaining whey; and further inserted in perforated cylindrical recipients to maintain the traditional shape. The pressing and molding process were continued by an automatic system linked to a pump to remove the maximum of whey for up to 6 hours. Finally, the cheese’s were removed from the recipients, salt was added in the top, and was placed in maturation chambers, with a mean temperature between 15°C and 19°C and mean relative humidity (RH) of 90% for 2 months (**Figure 11**).



Figure 11. Traditional cheese making process: A- Sheep milk with added rennet during coagulation; B- Curdled milk; C- sliced curdle; D, E- Manual pressing for removing the remaining whey; F- Cheese in the cylindrical recipient; G- Automatic pressing; H- Final cheese with salt for maturation process; I- Matured cheese

2.3.2. Incorporation process

The incorporated amounts were based on data regarding the phytosterol incorporations in other lipid food matrices which added 2g of phytosterols per 25g of margarine (Law, 2000). Thus, sheep cheeses weighting 500g after maturation were incorporated with three different formulations to analyze which one was more economically viable for the enterprise, had higher ergosterol stability and least processing time. The first formulation was the incorporation of 40 g of *A. bisporus* extract in 500 g of cheese, but this amount of extract was not viable since this content affected the rheologic properties of the cheese, causing it to lose integrity and fragment into small pieces, demanding for a reduction to 20 g per cheese. The second formulation was the incorporation of 1.5 g of pure ergosterol in the cheeses, which represents the same amount of ergosterol as 40 grams of extract. Finally, in an attempt to reduce the costs and the time-consuming process related to the extraction process, a third formulation was tried, consisting of the incorporation of dried mushroom powder in the cheese. The amount of mushroom powder was established based on the maximum content that did not affect the rheological characteristics and set at 35g of mushroom powder per 500g of cheese. For this last formulation, the mushroom powder was decontaminated by ultraviolet germicidal irradiation.

The different formulations were incorporated during the manual pressing procedure of the cheese preparation (**Figure 12**). This step was chosen due to previous steps having high contents of whey, which could cause significant losses of the extract/ergosterol in posterior whey removal procedures.

The *A. bisporus* extract, the pure ergosterol and the mushroom powder were directly added to the curdle and well homogenized by hand. Then, the cheese was placed in a cylindrical recipient, subjected to an automatized pressure treatment. Finally, before placing the samples in maturation rooms, salt was added to the upper and lower faces of the cheese, to help improve maturation and reduce microbial contamination.

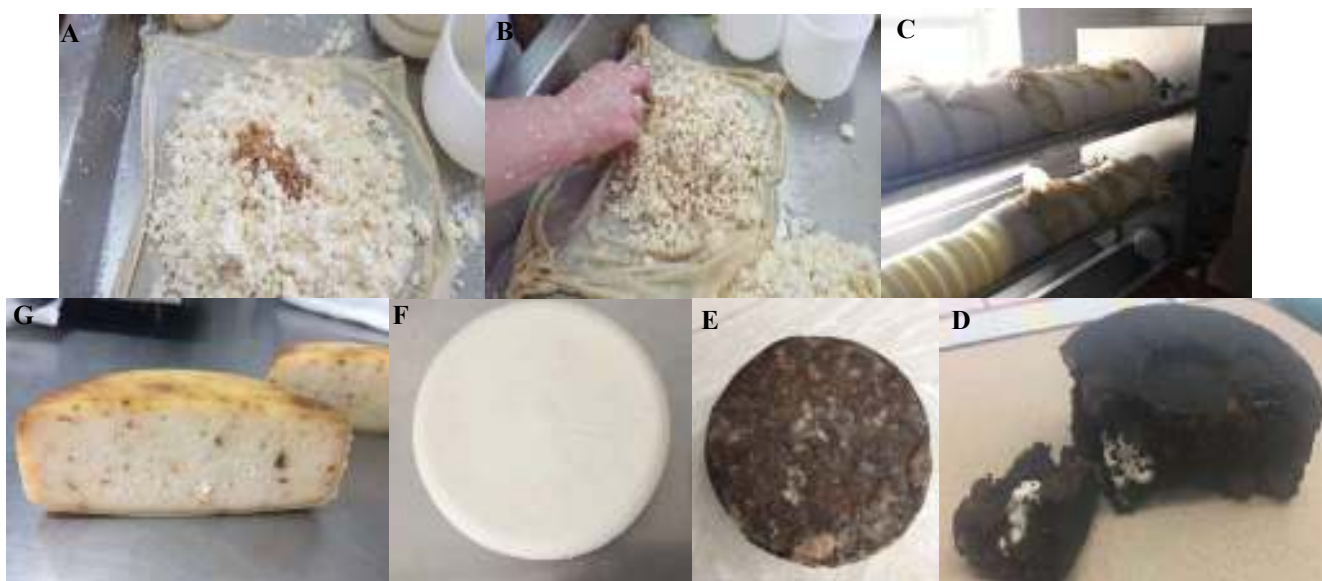


Figure 12. Incorporation process: A- Addition of *A. bisporus* extract/pure ergosterol/mushroom powder; B- Homogenization; C- Automatic pressing; D, E- Matured cheese with mushroom powder; F- Matured cheese with pure ergosterol; G- Matured cheese with mushroom extract

All the cheeses were analyzed before and after the maturation process (2 months) in order to evaluate the effect of the incorporation in the maturation process, except for the cheese incorporated with mushroom powder that drastically changed its appearance, rendering it impossible to be analyzed. Furthermore, its appearance was not appealing and thus was discarded (**Figure 12 D-E**). The remaining 5 cheese formulations were selected for further analysis: Cheese with *A. bisporus* extract at time 0 and after maturation (CEXT and CEXTM, respectively); Cheese with pure ergosterol at time 0 and after maturation (CPE and CPEM, respectively) and a control cheese at time 0 and also after maturation (CC and CCM, respectively).

2.4. Chemical composition

2.4.1. Nutritional value

The nutritional value (moisture, proteins, fat, carbohydrates and ash) was performed following the standard AOAC procedures (AOAC, 2016). The crude protein content ($N \times 6.38$) of the samples was estimated by the macro-Kjeldahl method; the crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600 ± 15 °C. Total carbohydrates were calculated by difference. Energy was calculated according to the following equation: Energy

(kcal)= $4 \times (\text{g protein} + \text{g carbohydrate}) + 9 \times (\text{g fat})$; KJ= Kcal*4.1868. Results were expressed as g/100 of fresh weight.

2.4.1.1. Moisture

The cheeses samples (2g) were put in the metal plate and placed in the balance moisture analyzer (Adam Equipment, PMB 163) as showed in **Figure 13**. This equipment increases the temperature gradually to 105°C to force moisture to evaporate from the food. When the weight is constant, with no evaporation being recorded, the sample is weighed again. The results were obtained using the following function: $\% \text{ Moisture} = (m_i - m_f) / m_i \times 100$. Where m_i is the initial weight and m_f is the weight after reaching a constant weight.



Figure 13. Moisture analyzer

2.4.1.2. Proteins

The protein analysis was carried out using the M. Kjeldahl method, which quantifies the crude protein content as a function of the nitrogen content of the sample. Birefly, 500 mg of the cheese samples was weighed and introduced into the digestion tube with 15 mL of concentrated sulfuric acid and two selenium pellets as digestion catalysts.

The tubes were placed in the digester (Foss™ Digester) for about 70 min at 400°C. After cooling, the digestion tubes were placed in the Kjeldahl apparatus, which automatically performs distillation and titration. The protein content was then calculated by multiplying the value obtained for nitrogen by a conversion factor selected on the apparatus (**Figure 14**).

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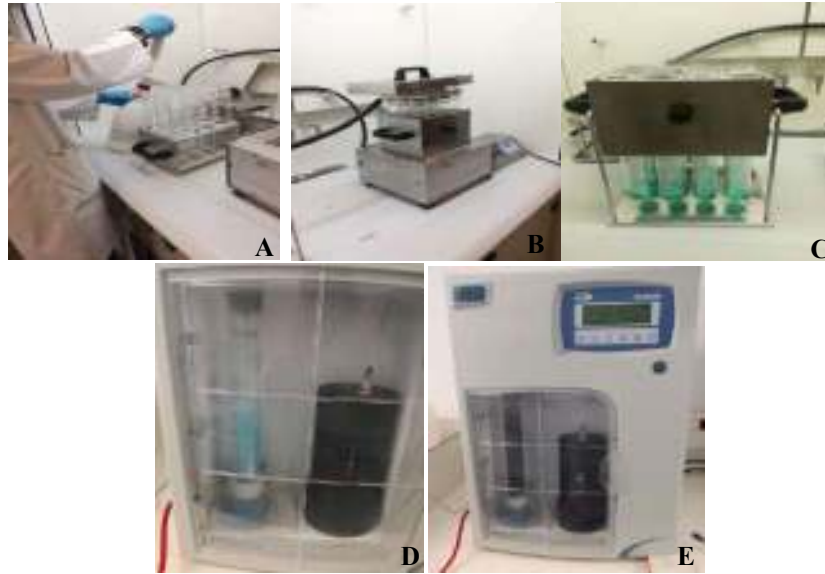


Figure 14. Proteins analysis: A- addition of sulfuric acid; B- Digestion; C- Digested sample; D- Destillation; E- Titration

2.4.1.3. Fat

The fat content was obtained by extracting 3 g of each sample with petroleum ether in a Soxhlet apparatus. The obtained solution was then placed in an oven to evaporate the petroleum ether and leaving the crude fat behind, which was then weighed (**Figure 15**).



Figure 15. Fat extraction

2.4.1.4. Ash

From each sample, 250 mg were weighted in a crucible and placed in the muffle furnace at 600 ± 15 °C for incineration overnight. The results were expressed as a percentage of total ash, according to following expression: $Ash \% = \left(\frac{mf}{mi}\right) * 100$. Where the initial mass represented by *mi* and the final mass by *mf*, Corresponding to the residues after incineration in the muffle (Figure 16).



Figure 16. Muffle for incineration

2.4.2. Salt determination

The salt determination was achieved using the Mohr's method as described by (Osaili et al., 2014). Initially, 1 g of the sample was homogenized in 20 mL of distilled water and filtered through a Whatman No.4 paper. The powder was then homogenized with an additional portion of 20 mL and this procedure was repeated a total of 5 times. Then the pH of the aqueous solution was adjusted to 8.5 with sodium hydroxide and 1 mL of potassium chromate solution (5%) was added. The mixture was titrated against $AgNO_3$ (0.05 mol/L until the appearance of the first reddish color (Ag_2CrO_4 precipitate). The salt concentration was calculated using the following equation: $Salt\ content\ \% = \frac{[V_{titrated\ of\ AgNO_3} \times 0.00292]}{[m\ sample]} \times 100$ (where 1 mL of $AgNO_3$ corresponds to 0.00292 g of NaCl). The results were expressed in g/100 g of fresh weight (Figure 17).

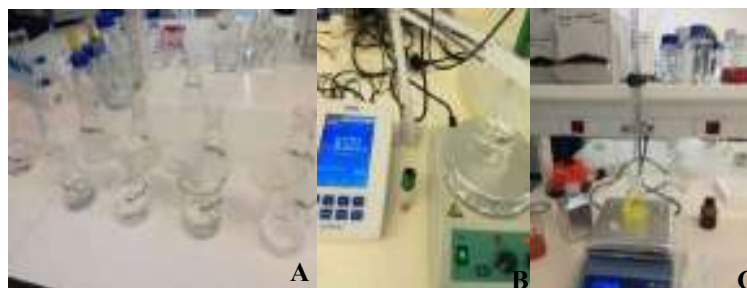


Figure 17. Salt determination: A- Samples washing; B- pH adjusting; C- Titration

2.4.3. Fatty acids

After obtaining the lipid fraction by Soxhlet extraction, the fatty acids were determined through a transesterification process employing a 5 mL methanol:sulphuric acid:toluene 2:1:1 (v: v: v) solution, for at least 12 h in a water bath at 50 °C and 160 rpm. After this process, 3 mL of distilled water was added to obtain two separate phases. The fatty acid methyl esters (FAME) were recovered with 3 ml of diethyl ether, vortexed and finally the hydrophobic phase was collected into vials with anhydrous sodium sulphate in order to eliminate traces of water. Afterwards, the samples were filtered with a 0.2 µm nylon filter (Whatman) and analyzed by gas chromatography (DANI 1000, Contone, Switzerland) coupled with a flame ionization detection (GC-FID)/capillary column. The analysis was carried out with a split/splitless injector, an FID at 260 °C, and a Zebron-Kame column (30 m × 0.25 mm i.d. × 0.20 µm film thicknesses, Phenomenex, Torrance, CA, USA). The carrier gas (hydrogen) flow rate was 1.1 mL/min, measured at 100 °C. Split injection (1:50) was carried out at 250 °C. Fatty acids identification and quantification were achieved by comparing the relative retention times of the fatty acids methyl ester peaks with standards. The results were recorded and processed using CSW 1.7 software (DataApex 1.7) and expressed in the relative percentage for each fatty acid as described by Pinela et al., 2011 (**Figure 18**).



Figure 18. GC-FID

2.4.4. Lactose

For the lactose determination, 1 g of each sample was mixed with raffinose (internal standard, IS, 25 mg/mL) and extracted with 40 mL of 80% aqueous ethanol at 80 °C for 1.30 min. The resulting suspension was centrifuged (Centorion K24OR-2003 refrigerated centrifuge) at 15000 g for 10 min. The supernatant was filtered, and subsequent solvent evaporation and fat excess removal was carried out by washing the suspension 3 x 10 ml of ethyl ether.

The lactose content was determined by HPLC, which consisted of an integrated system with a pump (Knauer, Smartline system 1000), degasser system (Smartline manager 5000) and auto-sampler (AS-2057 Jasco), coupled to a refraction index detector (RI detector Knauer Smartline

2300) showed in **Figure 19** as previously described by the authors Pinela et al., 2011. The chromatographic separation was achieved with a Eurospher 100-5 NH₂column (4.6 × 250 mm, 5 μm, Knauer) operating at 30 °C (7971 R Graceoven). The mobile phase was acetonitrile/deionized water (70:30, v/v) at a flow rate of 1 mL/min.

Lactose was identified by comparing the relative retention time of sample peaks with a standard. Data were analyzed using Clarity 2.4 Software (DataApex). Quantification was based on the RI signal response of the standard, using the internal standard method and by using calibration curves obtained from a commercial standard. The results were expressed in g per 100 g of fresh weight.



Figure 19. HPLC-RI

2.4.5. Lactic acid

The analysis of lactic acid was performed according to Silva et al., 2019. Briefly, 1 g of each sample was mixed with 25 mL of metaphosphoric acid (4.5%). The mixture was protected from light and extracted by maceration at room temperature for 20 min. After this process, the sample was filtered through a 0.2 μm nylon filter (Whatman). The lactic acid was determined by ultra-fast liquid chromatography UFLC Shimadzu 20A series (Shimadzu Corporation, Kyoto, Japan) coupled to a photodiode array detector (PDA). A SphereClone (Phenomenex, Torrance, CA, USA) reverse phase C18 column (5 μm, 250 mm × 4.6 mm i.d.—internal diameter.) thermostatted at 35 °C was used. The elution was performed with sulphuric acid (3.6 mM) using a flow rate of 0.8 mL/min. Detection was carried out using 215 and 245 nm (for ascorbic acid) as preferred wavelengths. For the quantitative analysis, a calibration curve with

known concentrations of a commercial standard was constructed, and the lactic acid present in the two samples was determined by peak area comparison at 215 (**Figure 20**).



Figure 20. UPLC-DAD

2.4.6. Ergosterol and cholesterol quantification

The obtained mushroom extracts by UAE and the cheese extracts obtained by Soxhlet extraction were dissolved in methanol in order to obtain a concentration of 20 mg/mL, and filtered through a 0.2 μm nylon filter for ergosterol and cholesterol quantification by HPLC-UV according to Heleno et al., 2016. The equipment consisted of the same Knauer system described above. Chromatographic separation was achieved with an Inertsil 100A ODS-3 reversed-phase column (4.6×150 mm, 5 μm , BGB Analytik AG, Boeckten, Switzerland) operating at 35 °C (7971R Grace oven). The mobile phase was acetonitrile/methanol (70:30, v/v) at a flow rate of 1 mL min⁻¹, the injection volume was 20 μL and the detection was performed at 280 nm for ergosterol and 200 nm for cholesterol. The quantification was achieved based on calibration curves obtained from commercial standards using the internal standard method with cholecalciferol as the internal standard. Data were analyzed using Clarity 2.4 Software (DataApex) (**Figure 21**).



Figure 21. HPLC-RI

2.5. Physical parameters

2.5.1. Water activity

For each cheese sample the water activity “ a_w ” was determined at 20 °C for 5 min at the surface of each sample slice by using an activity meter instrument (AQUALAB 4TE) based on the dew-point method and with an absolute error of 0.003. The average a_w value of each slice was calculated from the a_w values estimated at its surface and at the adjacent slice surface (**Figure 22**).



Figure 22. Water activity determination

2.5.2. Colour

From each cheese sample, the external color was analyzed. This was performed with a portable CR400 colorimeter from Konica Minolta (Chiyoda, Tokyo, Japan) with the D₆₅ illuminant, a standard illuminant defined by the International Commission on Illumination (CIE) which represents the midday light in Europe (daylight illuminant). The CIE L* a* b* color space of 1976 was used, with L* representing lightness, a* representing redness (red-green), and b* representing yellowness (yellow-blue), with a 10° observer angle and 8 mm aperture (**Figure 23**).



Figure 23. Colorimeter

2.5.3. Texture

Texture analysis was carried out on a Stable Micro Systems (Vienna Court, Godalming 191 UK) TA.XT Plus Texture Analyzer with a 30 Kg load cell, using the P/45 45mm aluminium cylinder probe (**Figure 24**) according to Carocho et al., 2019. The texture profile analysis (TPA) was determined on the samples with a pre-test speed of 5 mm/s, with a post-test speed of 5 mm/s and a test speed of 3 mm /s. The target mode was set to strain 5% of the cheese samples that were compressed twice for 5 seconds with timeout of another 5 seconds. The trigger force for starting the measurement was set at 50 g. The cheese texture properties such as hardness, adhesiveness, springiness, cohesiveness, chewiness and resilience were calculated using a macro using the Exponent 199 program.



Figure 24. Texture analysis

2.6. Bioactivity

2.6.1. Cytotoxicity

In order to evaluate the effects of the bioactive samples in non-tumor cells and to establish a subtoxic concentration of these samples in the tumor cell line of Human adenocarcinoma cell line (CaCo2) obtained from ECACC (European Collection of Cell Cultures) CaCo2 cells.

The normal cell line was a porcine liver primary culture (PLP2) which is established at the Mountain Research Centre. The cytotoxicity was carried out using the sulforhodamine B (SRB) assay according to the procedure previously described by Abreu et al., 2011. Briefly, both cell lines were routinely maintained as adherent cell cultures in RPMI-1640 containing heat-inactivated FBS (10%), glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 µg/mL) and were further incubated at 37 °C with humidified air and 5% CO₂.

To evaluate the cytotoxicity, the cell lines were plated in 96-well microplates, along with the different dilutions of the sample under analysis (6.25-500 µg/mL), and incubated for 48 hours

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at 37°C with 5% CO₂. After the incubation period, the adherent cells were fixed by the addition of 10% trichloroacetic acid previously refrigerated (100 µL) and incubated for 60 minutes at 4°C. Afterwards, the microplates were washed with deionized water and dried. After this process, SRB (0.1% in 1% acetic acid, 100µL) was then added to the wells of the microplate and incubated for 30 minutes at room temperature. Subsequently, non-adhered SRB was removed by washing with 1% acetic acid solution and the plate was allowed to dry. The adhered SRB was solubilized with 10 mM Tris (200 µL) and the absorbance was read at a wavelength of 540 nm in the microplate reader (ELX800). The results were expressed as GI₅₀ values (concentration of the sample, which inhibits 50% of cell growth). Ellipticine was used as a positive control (**Figure 25**).



Figure 25. Cytotoxicity: A- Cell suspension; B- Cell counting; C- Cells with sample; D- Absorbance at 540 nm.

2.5.2. *In vitro* cell transport assay in Caco2 cell cultures

The cholesterol absorption assay was carried out according to Gil-Ramírez et al., 2014. Briefly, the CaCo2 cell line was maintained in RPMI-1640 containing without FBS, glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 µg/mL) and were incubated at 37 °C with humidified air and 5% CO₂. Afterwards, the cells were placed onto a 44 cm² insert membrane with 0.4 µm pore size at a density of 5*10⁵ cell per insert. The cells plate was

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incubated at 37 °C in humidified atmosphere containing 5% CO₂. The culture medium was replaced every 3 days and cells were allowed to differentiate for 21 days before further experimental procedures. The integrity of the cell layer was evaluated by measuring the transepithelial electrical resistance (TEER) (EVOM2; World Precision Instruments, Sarasota, FL). Only inserts with values above 400 Ω were utilized. The samples were applied to CaCo2 cell monolayers at the concentrations in 975 μL of incomplete medium (medium without added cholesterol) at the upper compartment. After these processes, the microplate was incubated at 37 °C with 5% CO₂ during 1h. Therefore, the upper solution as well as the solution underneath the cell's monolayer were collected for cholesterol and ergosterol quantification, to evaluate the content of cholesterol/ergosterol absorbed by the CaCo2 cells (**Figure 26**).



Figure 26. Addition of samples in Caco2 cells for cholesterol absorption

2.7. Microorganisms analysis

2.7.1. General sample preparation

The preparation of cheese samples was performed according to the International Organization for Standardization procedure (ISO) 6887-1:2003 as described by Carocho et al., 2019.. Cheese samples (10 g) were mixed with 90 mL of peptone water (PW) in stomacher bags and further homogenized in a stomacher equipment (ECN 710-0873, Italy) for 1 min at 300 units (**Figure 27**). The obtained suspensions were further diluted to obtain dilutions from 10^{-1} to 10^{-12} . Each dilution was analysed in duplicate.

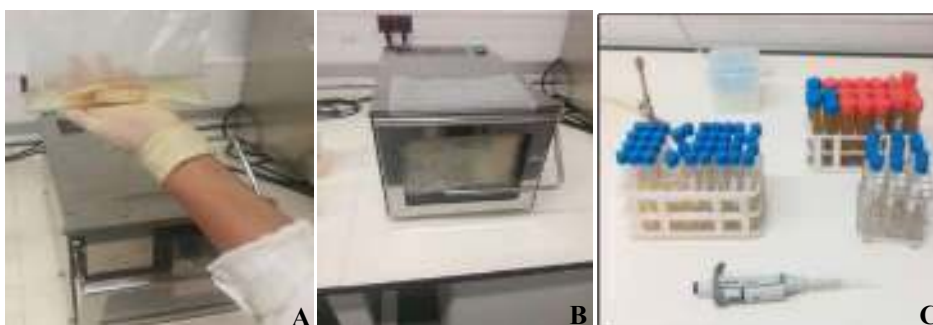


Figure 27. Sample preparation: A- Addition of peptone water to the cheese samples; B- Homogenization in the Stomacher; C- Serial dilutions

2.7.2. Microorganisms analysis

Different microorganisms were analysed in the cheese samples according to the data related with the most important microorganisms that usually appear in cheese samples. Thus, aerobic mesophilic microorganisms, coliforms, yeasts, moulds and *Psychrotrophic bacteria* were analysed by selective culture media (**Figure 28**).

Aerobic plate count (APC): 1 mL of each prepared suspension was mixed with 20 mL of Plate Count Agar (PCA) by the pour plate method, in duplicate (LOQ = 1 log UFC/g). The plates were further incubated in reversed position at 30 °C for 72h and counted according to ISO 4833-2:2013.

Coliforms: For the coliforms counting, 1 mL of each suspension was mixed with 20 mL of Violet Red Bile Lactose Agar (VRBLA), by the plate method, in duplicate (LOQ = 1 log UFC/g). The plates were further incubated in reversed position at 37 °C for 48h and counted according to ISO 4832:2006.

Yeasts and molds: 0.2 mL of each suspension was spread in petri dishes containing 20 mL of Agar Dicloran Rosa Bengala Cloranfenicol Base (DRBC), in duplicate (LOQ = 1.7 log UFC/g).

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The plates were further incubated in upright position at 25 °C for: 72h for yeast counting and 120h for mould counting, according to ISO 21527-2:2008.

Psychrotrophic bacteria: 1 mL of each suspension was mixed with 20 mL of Man, Rogosa and Sharpe agar (MRS), using the plate method, in duplicate. The medium was allowed to solidify and another 5 mL of medium was added to create anaerobiosis (LOQ = 1 log UFC/g). The plates were further incubated in upright position at 22 °C for 5 days and counted according to ISO 4832:2006 (ISO 2006).



Figure 28. Cheese samples in different culture media

After the incubation period, the colonies of each microorganism were counted and the UFC/g of sample were calculated according to the following formula: $N = \frac{\Sigma c}{(v(n1+0.1n2)/d)}$, in which N= number of colonies per g or mL of sample; Σc – sum of the colonies in the counted plates; V – Volume of the suspension used; n1– number of plates counted in the first countable dilution; n2– number of plates counted in the second countable dilution; d- first countable dilution.7

2.8. Statistical analysis

Throughout the whole document, all data was expressed as mean \pm standard deviation. Furthermore, in order to better understand the effect of the addition of the pure ergosterol and mushroom extract to the cheeses, for the nutritional profile, fatty acids, lactic acid, external colour and texture, the samples were analyzed by a two-way ANOVA with type III sums of squares using the SPSS Software, version 25. This multivariate general linear model treats the two factors, maturation time (MT) and incorporation type (IT) as independent, thus allowing the effect of each one to be analyzed independently, thus providing more insight into their contribution towards the sample. If a significant interaction (<0.05) was recorded among the

two factors (ST×IT), these were evaluated simultaneously, and some general conclusions and tendencies were extracted from the estimated marginal means (EMM). If there was no significant interaction (>0.05), each factor was evaluated independently using a simple Student's T test (for MT) or a Tukey's multiple comparison test (IT) when the means were homoscedastic, and a Tamhane's T2 for non-homoscedastic samples. Homoscedasticity was evaluated using a Levene's test. All analyses were carried out using a significance level of 0.05.

3. Results and discussion

3.1. Nutritional profile

As stated in the previous section, for some analyzed parameters, a 2-way ANOVA was used, allowing for an individualized assessment of each factor. **Table 3** represents the nutritional profile of each cheese in g/100 g of fresh weight. The upper section displays the two analyzed times (0 and 2 months), while the lower section shows the three cheese types, namely the control cheese (Control), the cheese incorporated with the mushroom extract (Extract) and finally the cheese incorporated with the pure ergosterol (Ergosterol). The values are represented as means of each maturation time (MT) including all three incorporation types (IT) in the upper section, and for the lower one, the means of MT include all maturation times. This type of representation allows the aforementioned independent analysis of each factor individually and thus, the standard deviations should not be regarded as an accuracy of an individual analysis as it includes the variation of the non-fixed factor (MT or IT). If a significant interaction among these two factors is detected (MT×IT $p<0.05$), no multiple comparisons can be carried out, meaning that both factors, MT and IT, contributed to the changes in the cheeses for each parameter, hindering concrete conclusions, although general tendencies can be concluded from the Estimated Marginal Means (EMM) plots. Inversely, if this value is higher than 0.05, each factor was classified individually using either Tukey's or Tamhane T2 tests depending on the homoscedasticity of the distribution, for IT, and a Student's T test for MT.

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Table 3. Nutritional profile of the cheeses represented as g/100 g of fresh weight during the maturation period of two months.

		Moisture	Fat	Proteins	Ash	Carbohydrates	Energy
Maturation time (MT)	0 months	49.6±0.1*	19±1	10.8±0.5	2.6±0.3	18±3	290±14
	2 months	42.3±0.1	20±2	14±2	2.7±0.6	21±3	309±17*
<i>p</i> -value (n=15)	Student T test	<0.001	0.031	0.001	<0.001	<0.001	0.020
Incorporation Type (IT)	Control	45.4±0.4	19.5±0.7	13±4	2.1±0.05	20±2	312±12
	Extract	46.1±0.5	18±2	13±3	2.9±0.3	20±5	308±11
	Ergosterol	47.0±0.3	18±2	12±1	3.0±0.7	21±5	289±9
<i>p</i> -value (n=10)	Tukey's HSD test	0.145	<0.001	<0.001	<0.001	0.648	0.421
MT×IT (n=30)	<i>p</i> -value	0.392	<0.001	<0.001	<0.001	<0.001	0.260

In each row, for the maturation time, an asterisk (*) means different statistical differences among the two periods, while for the incorporation types, different letters also mean significant statistical differences, with an overall significance value of 0.05. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values.

It can be seen on **Table 3** that while moisture decreased over time, from the unmatured cheese (0 months) to the matured cheese (2 months), all other nutrients increased, which is explained by the loss of moisture over the maturation time. This is a normal occurrence in unpasteurized cheese, provided that maturation is a security process that allows cheese to become edible and free from putative dangerous microorganisms. The only exception to this increase was recorded for ash, which did not vary, provided it is not susceptible to variations due to ash being composed of the elements that compose the cheese. Regarding the statistical approach to analyze the influence of each factor independently, there was a significant interaction for fat, proteins, ash and carbohydrates, while it is clear that time had a critical effect in moisture changes, which is explained by the 0.932 value of interaction and a significative difference between its quantity in the two different time stamps (represented by an *). Interestingly, for energy there was a same behavior, with time having the influence on the changes, which was expected, provided that the energy is just the reflection of the other individual nutrients summed up. Overall, the incorporation of the extract and pure ergosterol did not have significant changes in the nutritional profile of the cheese, as expected.

3.2. Fatty acids

The individual fatty acids can be found in **Table 4**, having been quantified through GC-FID, and represented as relative percentage of themselves. Overall, 28 individual fatty acids were quantified, although the table only represent the 11 most abundant ones. Furthermore, the total saturated fatty acids (SFA), monounsaturated fatty (MUFA) and polyunsaturated fatty acids (PUFA) are also represented in order to provide a general idea of the distribution of fatty acids in the cheeses. These lipidic molecules represent a very large portion of cheese, and thus changes to their profile are critical to the overall flavor, odor and aroma of the cheeses. The most abundant fatty acid in all cheeses was C16:0 (palmitic acid), a SFA, followed by C18:1 (oleic acid) a MUFA. Overall, the most abundant fraction of fatty acids was SFA with roughly 70%, followed MUFA (21 to 22%) and PUFA (5 to 6%), rendering cheese as a food with high amounts of saturated fat. Interestingly, for C18:1, the effect of incorporation showed a higher influence in the quantities of this fatty acid in the cheeses. It is clear that the incorporation of ergosterol was paramount in preserving this unsaturated fatty acid over the maturation time. The same was registered for C18:2, although the mushroom extract seem to have a preserving action, hindering the autoxidation of this fatty acid.

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Table 4. Fatty acids profile of the cheeses, including SFA, MUFA and PUFA, represented as relative percentage.

		C6:0	C8:0	C10:0	C12:0	C14:0	C15:0	C16:0	C18:0	C18:1c	C18:2t	C18:2c	SFA	MUFA	PUFA
Maturation	0 months	4.6±0.6	3.6±0.2	10.1±0.4	4.8±0.8	11±1	1.0±0.1	25.3±0.9	11.8±0.8	20±3	0.8±0.2	3±2	74±3	21±3	5±2
Time (MT)	2 months	4.1±0.4	3.3±0.2	9.9±0.6	4.5±0.3	20.2±0.4	1.0±0.1	25.1±0.5	12.3±0.3	20±3	1.2±0.3	3±2	73±2	22±3	6±2
<i>p</i> -value (n=15)	Student T test	<0.001	0.647	0.632	<0.001	<0.001	0.203	0.174	0.001	0.770	0.055	0.952	0.005	0.473	0.823
Incorporation	Control	4.8±0.2	3.9±0.2 ^b	10.3±0.3	5.4±0.5	12±1	1.12±0.02 ^c	26.0±0.4 ^c	11.6±0.9	17.7±0.2 ^a	1.0±0.4	2.36±0.04 ^b	76.3±0.8 ^c	19.1±0.2 ^a	4.5±0.7
Type (IT)	Extract	4.5±0.7	3.7±0.2 ^b	9.7±0.2	4.5±0.1	20.1±0.2	1.05±0.05 ^b	25.0±0.4 ^b	12.0±0.2	18.2±0.7 ^a	1.1±0.1	5.5±0.1 ^c	72.6±0.9 ^b	19.5±0.7 ^a	7.9±0.2
	Ergosterol	3.8±0.1	3.4±0.1 ^a	10.0±0.6	4.1±0.2	9.8±0.2	0.855±0.001 ^a	24.2±0.7 ^a	12.4±0.2	24.3±0.6 ^b	0.83±0.02	1.50±0.05 ^a	70.6±0.7 ^a	25.8±0.6 ^b	3.49±0.07
<i>p</i> -value (n=10)	Tukey's HSD test	<0.001	0.001	0.077	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
MT×IT (n=30)	<i>p</i> -value	<0.001	0.045	0.413	<0.001	<0.001	0.055	0.041	<0.001	0.151	<0.001	0.930	0.26	0.082	<0.001

In each row, for the maturation time, an asterisk (*) means different statistical differences among the two periods, while for the incorporation types, different letters also mean significant statistical differences, with an overall significance value of 0.05. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values.

These two interesting occurrences seem to render both the use of the mushroom extract and pure ergosterol as preserving agents for the lipid fraction of the cheese. This has previously been tried with plant extracts (Carocho et al., 2016; Carocho et al., 2015), and while the purpose of this work is not the preservation of cheese with the mushroom extract it is interesting to retain this functionalization feature of the *A. bisporus* extract.

3.3. Lactose and lactic acid

Table 5 reports the amount of lactic acid and lactose found in the cheeses. Lactic acid was analyzed through UFLD-DAD and expressed as g/100 g of fresh weight. On the upper section of the table, it can be inferred that there was an increase in lactic acid over time for all samples, which rose from an average of 0.08 to 0.5 g/100 g. This occurrence was quite expected due to the conversion of lactose to lactic acid by lactic bacteria during the maturation of the cheese. Lactose was also quantified in this work, having been analyzed by HPLC-RI, obtaining very similar values for all three cheeses, which decreased from 2.8 at T0 to 0.2 g/100 g after two months of maturation. As expected, lactose was only quantified in the unmaturred samples, having been converted overtime to lactic acid.

Table 5. Representation of the lactose and lactic acid variations among maturation process and in different incorporation types, represented as g/100 g of fresh weight.

		Lactose	Lactic Acid
Maturation time (MT)	0 months	2.8±0.3	0.08±0.08
	2 months	0.2±0.3	0.5±0.1
<i>p</i> -value (n=15)	Student T test	<0.001	<0.001
Incorporation Type (IT)	Control	1.5±1.6	0.3±0.3
	Extract	1.4±1.6	0.4±0.2
	Ergosterol	1.5±1	0.2±0.1
<i>p</i> -value (n=10)	Tukey's HSD test	0.587	<0.001
ST×IT (n=30)	<i>p</i> -value	<0.001	<0.001

In each row, for the maturation time, an asterisk (*) means different statistical differences among the two periods, while for the incorporation types, different letters also mean significant statistical differences, with an overall significance value of 0.05. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values.

3.4 Ergosterol and cholesterol quantification

Preliminarily to the bioactive assays, the cholesterol and ergosterol were identified and quantified in the cheese samples, so that, it was possible to infer the amount of cholesterol present in the sheep cheese and monitor the amount of ergosterol that was effectively incorporated in the cheese samples (**Table 6**).

Table 6. Cholesterol and ergosterol quantification in the matured sheep cheeses.

	Control Cheese	CPEM	CEXTM
Cholesterol (mg/100g fw)	56.64±0.04	58.23±0.02	57.58±0.02
Ergosterol (µg/100g fw)	-	195±2	114±2
Ergosterol losses (%)	-	35%	21%

fw- fresh weight

From the obtained results it was possible to infer that the sheep cheese has a cholesterol content of 56.64±0.04 - 58.23±0.02 mg/100g fw. Regarding the ergosterol analysis it is possible to conclude that in the cheese incorporated with pure ergosterol (CPEM), from the incorporated content of pure ergosterol (300 mg/100g fw), only 195±2 mg/100g fw were quantified, meaning that the incorporation process suffered a loss of 35%. Regarding the ergosterol identified in the cheese with *A. bisporus* extract (CEXTM), from the 144 µg/100g fw (ergosterol content present in 4 g of *A. bisporus* extract/100g cheese), only 113.7±1. µg/100g fw were found, representing a loss of 21% in the incorporation process.

The losses in the incorporation process can be due to the manual pressing in which the excess of whey is removed, removing some ergosterol from the cheese. The lower loss in the cheese with the *A. bisporus* extract can be due to the fact that the ergosterol can combine itself with other molecules and thus resist to being washed out in the aqueous whey. It is also possible to infer that the cheese incorporated with the *A. bisporus* extract is the most viable formulation for the industry, not only because the pure ergosterol translates into higher costs, but also because the *A. bisporus* extract present lower incorporation losses, and the amount can be increased to offset the loss.

3.5 A_w and Salt Contents

Water activity (A_w) was also analyzed and is an important feature for food analysis by reporting the amount of unbound water there is in the sample, which can be available for unwanted microorganisms to use as substrate and promote food spoilage. There was no difference among samples, and over the two months, a small decrease was found for all samples, which varied from an average of 0.99 to 0.97, while the temperature (expressed in °C) and dew point (expressed in minutes) rose from 19,7 to 20 °C, and from two minutes to two minutes and a half, respectively. The salt content was, as expected, equal in all samples, due to it being added to the surface of the cheese after its preparation. Due to water loss, the average amount of salt in g/100 g rose from 0.6 to 0.7 during the maturation time.

3.6. Texture analysis

In **Table 7** the different dimensions of the cheese texture which were measured with a Stable Micro Systems TA.XT texturometer are presented. Three TPA's were carried out on each cheese, which after running a macro allowed to obtain the six dimensions displayed on **Table 7**, such as hardness, adhesiveness, springiness, cohesiveness, chewiness and resilience.

It is clear that there was a significant interaction between the time and incorporation, which only allowed for general tendencies to be extracted from the EMM plots. The first dimension, hardness, is defined as the force the teeth have to apply on the food, and is measured in grams (Di Monaco et al., 2008) and by analyzing **Table 7**, it appears clear that there was an evident increase in hardness, fact that is corroborated in **Figure 29**, where it is clear that all cheeses increased in this dimension, although the control sample increased beyond the incorporated cheese. Still, the cheese with the extract was the one which after maturation showed the lowest values. This could be an interesting feature for the marketing of the hypocholesterolemic cheese, in which beyond having remarkable cholesterol lowering features, the cheese is creamier than normal cheese. The second analyzed dimension is adhesiveness, which is defined as the capacity that food has to adhere to the teeth while chewing, and is expressed in negative values due to the measuring force being applied from bottom to top in the texturometer (Paula and Conti-Silva, 2014).

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Table 7. Texture profile of the cheeses represented by six dimensions; hardness, springiness, cohesiveness, chewiness and resilience.

		Hardness	Adhesiveness	Springiness	Cohesiveness	Chewiness	Resilience
		(g)	(g.sec)	(%)	(%)		(%)
Maturation time (MT)	0 months	2441±520	-4±3	1.4±0.8	0.89±0.07	2443±1425	0.4±0.1
	2 months	22063±8516	-0.6±0.2	1.2±0.4	0.9±0.02	27954±19821	0.7±0.1
<i>p</i> -value (n=15)	Student T test	<0.001	0.031	0.001	<0.001	<0.001	<0.001
Incorporation Type (IT)	Control	16625±15915	-2±2	2.2±0.4	0.91±0.05	28684±27325	0.6±0.1
	Extract	7019±5390	-4±4	0.8±0.1	0.95±0.04	5582±5251	0.4±0.2
	Ergosterol	13112±11131	-0.7±0.1	0.99±0.01	0.88±0.06	11330±9806	0.6±0.1
<i>p</i> -value (n=10)	Tukey's HSD test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
MT×IT (n=30)	<i>p</i> -value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

In each row, for the maturation time, an asterisk (*) means different statistical differences among the two periods, while for the incorporation types, different letters also mean significant statistical differences, with an overall significance value of 0.05. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values.

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As expected, all the values were quite low, and further decreasing over time. Another dimension of texture is the springiness, defined by the rate at which a deformed food reverts to the undeformed state after removing the deforming force (Faber et al., 2017), and is measured in percentage.

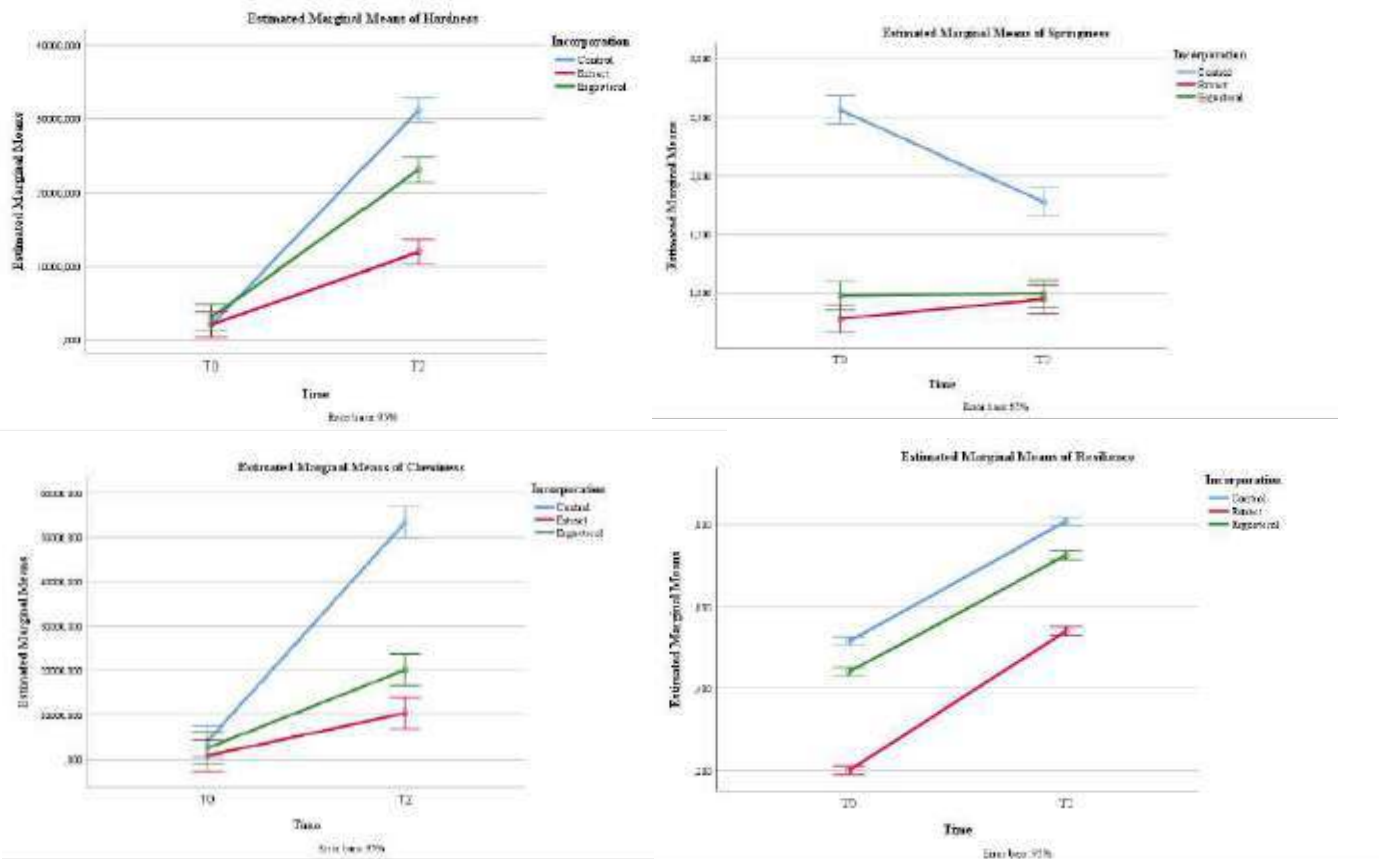


Figure 29. EMM plot of Hardness, Springiness, Chewiness and Resilience during the maturation time of the cheeses.

According to **Figure 29**, and although there was virtually no change during the maturation time, the EMM shows an interesting tendency. At T0, the control cheese showed a higher springiness, which could be explained by the need to disassemble the breads to incorporate both the extract and the ergosterol in their respective cheese, and thus, a maintenance of the springiness during the maturation interval, while the control sample reduced its springiness during this time. Once again, there seems to be a physical change induced by the incorporation of the extract and pure compound. Cohesiveness is considered as the success of a food to withstand a second deformation relative to its resistance to the first deformation and is also expressed in percentage. Cohesiveness did not show any changes among the maturation time or be influenced by the incorporation of the extract or ergosterol. Chewiness is the product of hardness, cohesiveness

and springiness, and usually defined as the energy required to masticate food, although it does not have units (Chandra and Shamasundar, 2015). Once again, through the EMM plots in **Figure 29**, it is clear that there was a tendency for the control sample to increase beyond the normal increase detected for the other two samples, making the cheese chewier after the 2 months, which means the control cheese needs more energy to masticate, further contributing the overall interest an incorporated cheese could have in terms of mouth sensation when eaten. Finally, resilience is similar to springiness, although it measures both the speed and forces involved in the recovery of a food when a deforming force is removed, being also measured in percentage. Some describe it as how a food “fights” to regain its original height (Chandra and Shamasundar, 2015). Once again, and as per the other dimensions of texture, there was a significant interaction among the two factors and general conclusions could be extracted from the EMM plots. **Figure 29** clearly shows an increase in resilience over time, in which the control sample shows higher values than the incorporated counterparts, which also tend to increase over time. Thus, it seems that the incorporated samples are “easier” to eat and “feel better” in the mouth, with less force needed to chew them, also contributing to a general acceptance pattern in terms of texture. Texture was, as expected, one of the few analyses in which there were visible and measurable differences among the control and incorporated samples.

3.7. External Colour

In **Table 6** it is possible to analyze the external colour of the cheese samples, using a portable colorimeter that measured the L*, a* b* coordinates of the CIELab. L* measures the lightness of samples, and varies from -100 to 100, being the latter absolute white, and -100 the black.

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Table 7. External colour profile of the cheeses represented by the CIELab coordinates

		L*	a*	b*
Maturation time (MT)	0 months	87±1	-2±1	16±2
	2 months	68±3	0±4	27±14
<i>p</i> -value (n=15)	Student T test	<0.001	0.031	0.001
Incorporation Type (IT)	Control	78±10	-2.4±0.3	17±2
	Extract	79±10	2±3	30±17
	Ergosterol	76±13	-2.8±0.3	17.8±0.8
<i>p</i> -value (n=10)	Tukey's HSD test	0.117	<0.001	<0.001
MT×IT (n=30)	<i>p</i> -value	0.012	<0.001	<0.001

In each row, for the maturation time, an asterisk (*) means different statistical differences among the two periods, while for the incorporation types different letters also mean significant statistical differences, with an overall significance value of 0.05. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values.

a* measures the interval between the red and green, where the values from 0 to +100 represent the red, and 0 to -100 represent the green color. Finally, b* represents the blueness of a sample, where positive values show an increase in blue, and negative ones show an increase in yellow. It is clear that there was an increase in all color parameters along the maturation time, although there was a significant interaction among the two factors (time and incorporation). Still, some general conclusions can be extracted from the EMM plots, namely for a* (**Figure 30**), in which a general tendency for increase in the values over time is evident, especially for the cheese with the mushroom extract, representing a shift to redness, while the other two cheeses also increased the redness, but at a much lower rate. This increase in color intensity was also verified for L* and b*.

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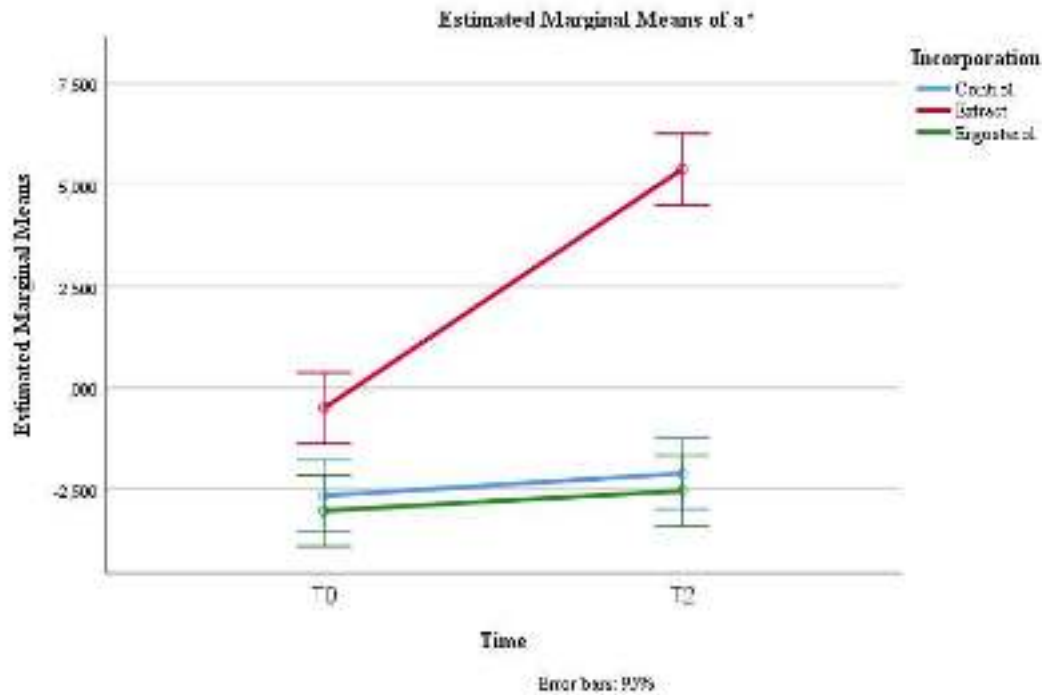


Figure 30. EMM plot of a* over the 2 month maturation time

Figure 30 shows the different colors of the cheese, obtained from the conversion of L*, a* and b* to RGB colors. Furthermore, the total difference (ΔE) was calculated between the same cheeses at the two different times and also between the two incorporated samples and the control (only for T2), in order to verify the changes the incorporations excerpted in the cheese. During the maturation, as expected, all cheeses showed a high difference, with the extract showing the highest, namely 36.35, while the control sample only showed 17.89 of total difference from T0 to T2. This was quite expected, provided that the mushroom extract had its own darker color which changed during the 60 days. Furthermore, at T2, there was a higher difference between the control sample and the extract incorporated cheese, namely 4.05, while the cheese with ergosterol only showed a 1.65 difference to the control one. The colors displayed in **Figure 31** can differ somewhat from the expected colour of the cheese, and thus should be regarded as a composition of all the colors that compose the cheese surface.

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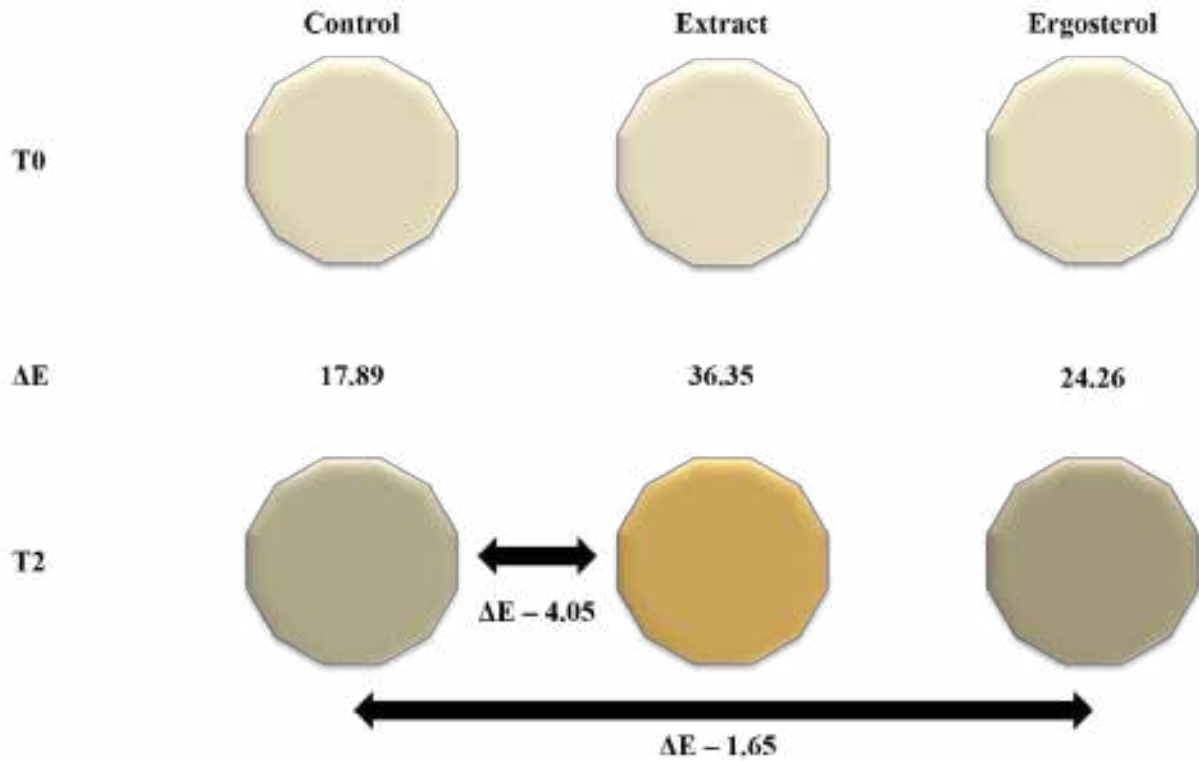


Figure 31. Representation of the cheese colours and the ΔE between the same cheeses at the two different maturation times, and also the ΔE between the control sample and the two incorporation types.

The cheese with *A bisporus* extract showed a high difference when compared to the control, which can be an appealing feature of this cheese mushroom incorporated cheese. It is common for consumers to buy foods that have different colors to the ones they would normally expect for food, and thus, innovation is also a good driver in consumer acceptance. Allied to the cholesterol reducing capability of this cheese, it could become an interesting product for the dairy industry, especially for the different colour it has.

3.8. Cytotoxicity and cholesterol absorption

The cytotoxicity of the *A. bisporus* extract, pure ergosterol and cholesterol was carried out in the normal cell line PLP2 and in the tumor CaCo2 cell line in order to evaluate the safety of these agents for normal cells and also to select a subtoxic concentration to apply in the CaCo2 cells in the cell transport assay. From the obtained results, for the PLP2 cell line, the *A. bisporus* extract presented no effect with a $GI_{50} > 500 \mu\text{g/mL}$, the pure ergosterol presented a GI_{50} of $86 \pm 23 \mu\text{g/mL}$ and cholesterol a GI_{50} of $120 \pm 8 \mu\text{g/mL}$. These results agree with Francisco et al., 2018 that obtained similar results for the cytotoxic effects of *A. bisporus* extract and pure ergosterol.

Regarding the cytotoxic effects on the CaCo2 cell line, the *A. bisporus* presented a GI_{50} value of $237 \pm 10 \mu\text{g/mL}$, followed by cholesterol ($GI_{50} = 91 \pm 3 \mu\text{g/mL}$) and ergosterol ($GI_{50} = 66 \pm 2 \mu\text{g/mL}$).

According to the results obtained from the cytotoxic properties, a concentration of $50 \mu\text{g/mL}$ of ergosterol was selected as the subtoxic concentration to be applied in the cell transport assay. The same concentration was selected as the subtoxic concentration of cholesterol, based on the fact that these molecules are competitors in the absorption assay, so that the same concentration was applied. Therefore, for the cell transport assay, different samples were prepared with culture medium as follows: i) control samples: ergosterol ($50 \mu\text{g/mL}$); cholesterol ($50 \mu\text{g/mL}$); sheep cheese in a volume that allows a concentration of $50 \mu\text{g/mL}$ of cholesterol; *A. bisporus* extract in a volume that allows a concentration of $50 \mu\text{g/mL}$ of ergosterol; ii) sheep cheese with pure ergosterol (CPEM); sheep cheese with *A. bisporus* extract (CPEXTM); the last two samples were dissolved in a volume that allows the concentration of $50 \mu\text{g/mL}$ of ergosterol.

The results obtained in the cell transport assay can be observed in **Figure 32**. Regarding the control samples, the cholesterol solution, it can be seen that 38% of the applied ergosterol was detected in the upper compartment, and 30.4% was detected in the underneath compartment. For the sheep cheese a detection of 46% of the cholesterol present in the cheese was achieved in the upper compartment while a detection of 31% was obtained in the underneath compartment. These results mean that the cholesterol present in the cheese is not all released by the cells.

Analyzing the control samples with ergosterol, both samples of ergosterol and *A. bisporus* extract revealed an upper quantification of 45% and a percentage of 14% in the underneath compartment. This suggests that similarly to the cholesterol behavior, the ergosterol is not all released by the CaCo2 cells. Furthermore, it can be seen that ergosterol is less absorbed than

the cholesterol by the Caco2 cells. These results agree with other studies performed using ergosterol (Gil-Ramírez et al., 2014), and phytosterols (Calpe-Berdiel et al., 2009), corroborating the similar behavior of ergosterol and phytosterols.

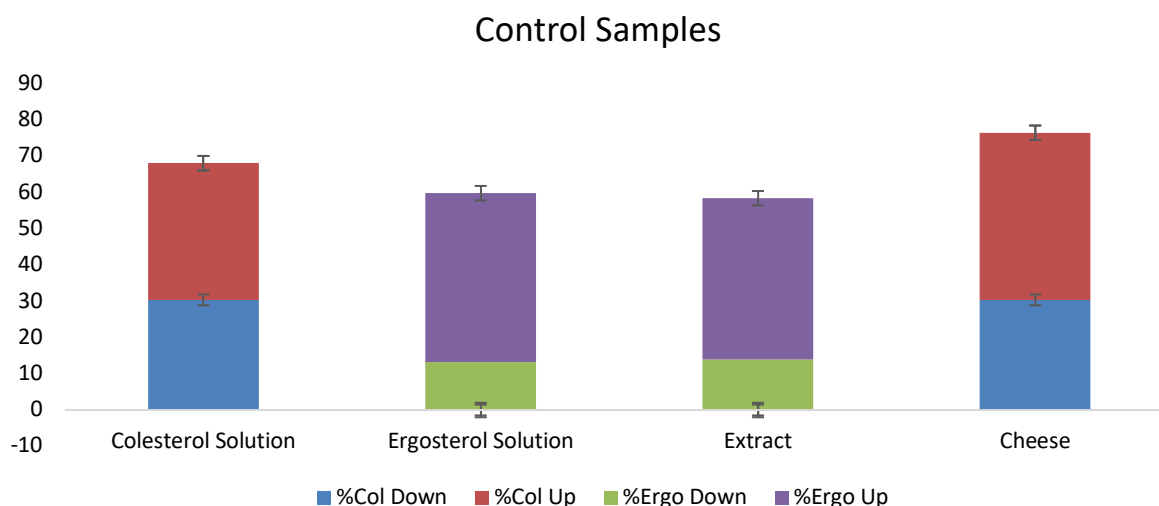


Figure 32. Cholesterol and ergosterol quantification in the upper and underneath compartments of the control samples applied to the CaCo2 cell line

The results obtained for the cholesterol absorption in the presence of ergosterol are present in **Figure 32**. Regarding the cholesterol content, it can be observed in the cheese sample with pure cholesterol, a quantification of 42% in the upper compartment and 18.5% in the underneath one. Similar results were obtained for the cheese sample with *A. bisporus* extract, in which 40% of the present cholesterol was quantified in the upper compartment and 14.7% in the underneath one. These results are important and satisfactory since it was possible to obtain a reduction of the cholesterol absorption in the presence of pure ergosterol of 11.85% and a reduction of 15.55% in the presence of *A. bisporus* extract.

Regarding the ergosterol content in the cheese samples it can also be seen from **Figure 33** that in the cheese with pure ergosterol, the upper compartment presented a percentage of 43.5% and the underneath compartment a percentage of 13.73%. Similar results were also obtained for the cheese with *A. bisporus* extract that presented a percentage of ergosterol of 40.1% in the upper compartment and 17.3% in the underneath one. It is possible to infer that the cheese sample with *A. bisporus* extracted incorporation revealed a higher capacity in decreasing the cholesterol absorption. This fact may be due to the presence of other molecules in the extract besides

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ergosterol, that may link to the cholesterol and increase its molecular mass, and thus avoiding the passing through the cell membrane.

Clearly, more studies are needed to better understand this process, but, these results focus the most important, that is the capacity in reducing the cholesterol absorption.

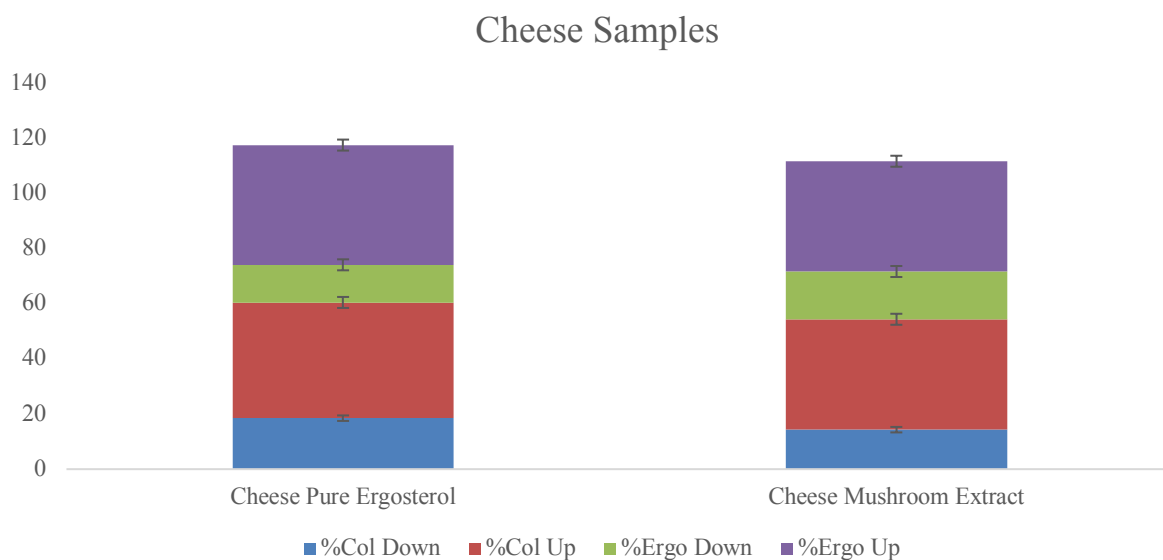


Figure 33. Cholesterol and ergosterol quantification in the upper and underneath compartments of the cheese samples applied to the Caco2 cell line

These results clearly corroborate the ergosterol capacity in the reduction of cholesterol absorption as well as its ability to compete with cholesterol. Similar results were obtained by Gil-Ramírez et al., 2014 that also applied an enriched ergosterol extract to the CaCo2 cell line and verified that in the presence of the extract, the cholesterol content released by the cells was lower.

Nevertheless, from the 50 µg added to the CaCo2 cells, not all the amount was quantified in the assay (by the sum of the upper and underneath contents). This situation was verified for both cholesterol and ergosterol. As stated by other authors, these can be due to the accumulation of cholesterol and ergosterol in the intracellular phase or to the transformation of ergosterol in other ergosterol derivatives that were not analyzed (Gil-Ramírez et al., 2014).

3.9. Microbial analysis

The microbial analysis was performed only in the cheese with *A. bisporus* extract at T0 (CEXT) and after two months of maturation (CEXTM) and in the control cheese (CC and CCM). This formulation was selected considering the costs of the pure ergosterol, that would significantly increase the final price of the cheese.

Thus, the most viable formulation was the cheese with the *A. bisporus* extract, since the extract is obtained from mushroom bio residues with no added value meaning significantly lower costs than the use of pure ergosterol. Moreover, it was possible to see from the results of the cholesterol absorption in the CaCo2 cells that the formulation with *A. bisporus* extract revealed higher activity than the formulation with pure ergosterol, highlighting the viability of this formulation and turning it in the most economic choice.

Table 8 represents the microbial load found for the different cheese types and is divided into two sections. The upper section refers to the unmaturation cheese (T0), while the bottom section pertains to the matured cheese (T2). A comparison between times and incorporation types was performed, thus, when the * symbol is present, it means a significant difference between different incorporation within the same time, while the † symbol states a significant difference between the same type of incorporation among the two-time stamps.

Briefly, the * details significant differences among the same section of the **Table 8**, while † shows significant differences between the same incorporation types between the two sections.

Table 8. Microbial analysis in the cheese samples over the maturation period.

		Yeasts	Moulds	Aerobic mesophilic	Psychrotrophic bacteria	Enterobacteria
T0	Unmatured Control	6.74±0.04*, †	2.84±0.04†	12.22±0.04	8.17±0.05	7.81±0.07†
	Unmatured Extract	6.160±0.007	2.7±0.1†	12.3±0.1	8.28±0.02*	7.80±0.04
T2	Matured Control	5.76±0.01	-	14.15±0.01†	12.31±0.04†	7.41±0.07
	Matured Extract	6.644±0.007*, †	-	14.17±0.01†	12.2±0.1†	7.78±0.05*

* and † both represent significant differences among samples using a Student's T test, but while * represents the differences among the same sections of the table (upper or lower), † represents significant differences between the same incorporation types (control or extract) between the two sections. The symbols are always placed in the higher value

Thus, for the yeasts and psychrotrophic bacteria, a significant difference was found in T0, with the extract incorporated cheeses showing lower amounts of yeasts, but higher amounts of psychrotrophic bacteria. For T2, between the two incorporations, a significant difference was found for yeasts and enterobacteria, with higher amounts found in the extract incorporated cheese.

Regarding the significant differences between the same incorporation types along the maturation time (†), significant differences were found for all studied microorganisms except for the enterobacteria in the samples with the mushroom extract.

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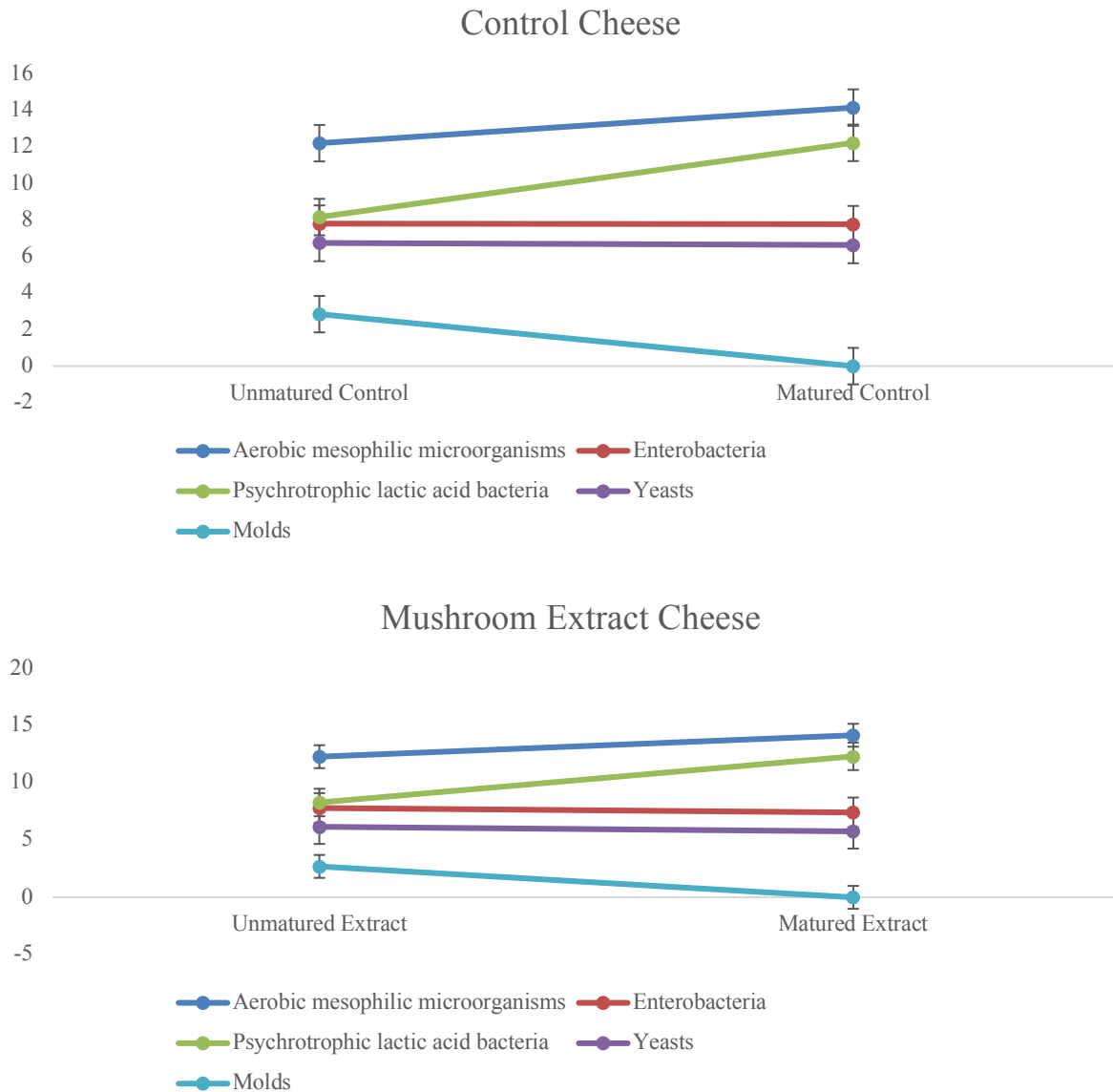


Figure 34. Microorganisms analysis along the cheese maturation process: A- control cheese; B- Cheese with *A. bisporus* extract.

Generally, for all microorganisms, their amounts increased significantly from T0 to T2, which is quite expected due to normal occurrence of microorganisms in the maturation chambers, and the ones needed to produce the cheese (**Figure 34**).

The psychrotrophic bacteria are very important in the fermentation process of the cheese, being its increase predictable in the final stage of the maturation time. The only major exception was recorded for the moulds, which were not detected at T2.

These results agree with other authors that analysed the maturation process of the cheese regarding the content in lactic bacteria. A group of Portuguese researchers found that on the

artisanal cheese the lactic acid bacteria were the most abundant microbial group during the experiments (3-4 log₁₀ cycles over all residual groups) and reached their highest value in the order of 10⁹ CFU/g at 7 days of cheese ripening (Vasek et al., 2013). In another study, also after identification and characterization of the microbial composition of traditional cheese it was concluded that during the ripening process of (0, 45, 90 days) the lactic bacteria was the abundant microorganisms in the cheese (Fuka et al., 2013). Moreover in 2015 a group of researchers controlled the microbial load on the sheep and goat cheese from the first day of preparation until 360 days of ripening, and the lactic bacteria also became predominant by exceeding 8 log CFU/g and due to this microorganisms, the fermentation and ripening was achieved (Pappa et al., 2016). Furthermore, and also as expected, there were minimum effects of the mushroom extract on the normal microbial load of the cheese.

The main objective of this analysis was to verify if there was some effect in the normal maturation process of the cheese. The developed cheese will be also analysed during different times along the normal shelf life of the cheese with the *A. bisporus* extract and the behaviour of the different microorganisms will be monitored.

4. Conclusion and Perspectives

The number of people with high cholesterol levels and suffering of cardiovascular diseases is drastically increasing worldwide, being a major global concern. These people usually have their dietary habits controlled and consuming foods with high cholesterol levels can cause serious health problems that can lead to morbidity and mortality.

In this perspective, foods with hypocholesterolemic agents have been developed, so they can be consumed without negative side effects and with the ability to decrease the cholesterol absorption, making these foods an option for people with high levels of cholesterol in the organism.

The present dissertation, was focused on the development of sheep cheese with hypocholesterolemic agents, namely the pure ergosterol and the *A. bisporus* extract enriched in ergosterol, in collaboration with two local enterprises: The "Mogaricus mushrooms - Sociedade Unipessoal Lda" that kindly provided the mushroom bioresidues, and the "Queijaria Vaz" that was a partner in the development of the final cheese formulation.

From the obtained results, it is important to highlight that the mushroom material was a mushroom bio-waste, meaning the mushroom samples that had no commercial value were used and valued. It was possible to obtain high value molecules from this residue, helping the mushroom enterprise in giving an economic destination to this material. Furthermore, it was also possible to verify that the incorporation of pure ergosterol and *A. bisporus* extract in the cheese was successful, presenting no significant changes in the nutritional profiles, while the changes in terms of physical analysis was slight and towards a better acceptability of these cheeses by the consumer. Furthermore, the normal microbiological maturation process was also untouched.

The selected final formulation was the one with the *A. bisporus* extract, that also had a high acceptability from the enterprise due to its appearance. Moreover, this formulation was chosen based on the lower production costs comparatively with the pure ergosterol sample, and high cholesterol lowering activity.

Overall it was possible to produce a differentiated product with hypocholesterolemic effect by the incorporation of bioactive agents obtained from a bio-waste that was transformed into a high valuable material to obtain hypocholesterolemic molecules.

More studies will be conducted, namely in order to understand the mechanism by which the ergosterol decreases the cholesterol absorption (for example the quantification of the intracellular cholesterol and ergosterol levels), as well as *in vivo* studies.

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Sensorial analysis will also be conducted in order to evaluate the consumer acceptance of the developed cheeses.

Other types of cheese as well as other foods can also be used as a medium for the incorporation of this bioactive ingredient.

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Ângela Fernandes

Sumário

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1. Identificação

<i>Deliverable</i>	5.1.7
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2. Informação

A publicação relativa aos ingredientes com melhor capacidade de aumento da absorção de cálcio e sem toxicidade são:

UV-irradiated mushrooms as a source of vitamin D₂: A review

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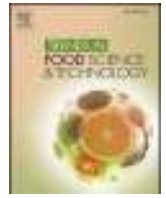
Trends in Food Science & Technology, 70, 82-94, 2017

Flour fortification for nutritional and health improvement: A review

Cardoso R. V. C., Fernandes A., González-Paramás A. M., Barros L., Ferreira I. C. F. R.



3. Anexos



Review

UV-irradiated mushrooms as a source of vitamin D₂: A review

Oludemi Taofiq^{a,b,c}, Ângela Fernandes^{a,b}, Lillian Barros^a, Maria Filomena Barreiro^b, Isabel C.F.R. Ferreira^{a,*}

^a Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal

^b Laboratory of Separation and Reaction Engineering - Laboratory of Catalysis and Materials (LSRE-LCM), Bragança Polytechnic Institute, 5301-857 Bragança, Portugal

^c GIP- USAL, Unidad de Nutrición y Bromatología, Faculty of Pharmacy, University of Salamanca, Campus Miguel de Unamuno, 37007 Salamanca, Spain

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ABSTRACT

Background: The deficiency of vitamin D has been widely reported all over the world and linked to several chronic diseases. Mushrooms are valuable nutritional foods with recognized bioactive properties, leading the application of UV irradiation to the production of significant amounts of vitamin D₂. In this context, cultivated species such as *Agaricus bisporus*, *Lentinula edodes* and *Pleurotus ostreatus* have been widely studied.

Scope and approach: However, there is still gap considering the knowledge of the most appropriate irradiation procedures (dose, intensity, distance between source and sample, exposure time) in order to maximize the content of vitamin D₂ in the mushrooms. This strategy will enable vitamin D₂-enhanced mushrooms to be commercially available at affordable costs. Considering the interest and potential of application, this review mentioned some of the physiological roles and sources of vitamin D, while the major focus was on mushroom's UV irradiation as a source of vitamin D₂. Also, topics related to its bioavailability and clinical studies evidencing the health benefits reported so far were also addressed.

Key findings and conclusions: UV-irradiated mushrooms present a high rate of conversion from ergosterol to vitamin D₂ at short treatment time and have the potential to increase serum 25-hydroxyvitamin D levels. Even though irradiated mushrooms exhibit some promising advantages, there is still a huge knowledge gap to allow for extraction, separation, recovery and purification of vitamin D₂ from irradiated mushroom at minimal process cost and high purity percentage to be utilized as bio-based ingredient to reduce vitamin D deficiency as well as present other health promoting benefits.

1. Introduction

Vitamin D, popularly referred to as “sunshine vitamin”, plays an important role in several human metabolic processes such as calcium and phosphorus metabolism, and skeletal and neuromuscular homeostasis. It is mainly obtained endogenously after UV exposure, from dietary supplements and food sources (Elangovan, Chahal, & Gunton, 2017). The most well reported symptoms of vitamin D deficiency are rickets and osteomalacia arising from poor calcium and phosphorus mineralization; but other diseases such as cardiovascular disease, cancer, hypertension, stroke, diabetes, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, periodontal disease, muscular degeneration, liver diseases, mental illness, and chronic pain have been also reported to be associated with the lack of vitamin D (Kalaras, Beelman, & Elias, 2012a). In this context, studies on vitamin D have received considerable attention over the years supported by the increasing number of reports of vitamin D deficiency, now prevalent in

Europe, Middle East and North America.

There are various forms of this vitamin, but the most physiologically relevant ones are vitamin D₃ or cholecalciferol, which is the most biologically active form found in animals and humans produced after skin exposure to UVB radiation, and vitamin D₂ (ergocalciferol) found in some phytoplankton, invertebrates, yeast and mushrooms in response to UV radiation (Chen et al., 2015; Malaeb, Hallit, & Salameh, 2017). Populations from countries with temperate climate (> 30°N and > 30°S), i.e. regions where exposure to sunlight is limited especially during winter season, are subjected to little or no synthesis of vitamin D₃, and as such, dietary intake in the form of supplements or fortified foods is needed (Schoenmakers, Gousias, Jones, & Prentice, 2016).

Both forms of vitamin D have shown to be responsible for maintaining serum levels of 25-hydroxyvitamin D in humans (Koyyalamudi, Jeong, Pang, Teal, & Biggs, 2011). Vitamin D from sunlight, or dietary sources, is biologically inactive and undergoes two-step hydroxylation

* Corresponding author.

E-mail address: iferreira@ipb.pt (I.C.F.R. Ferreira).

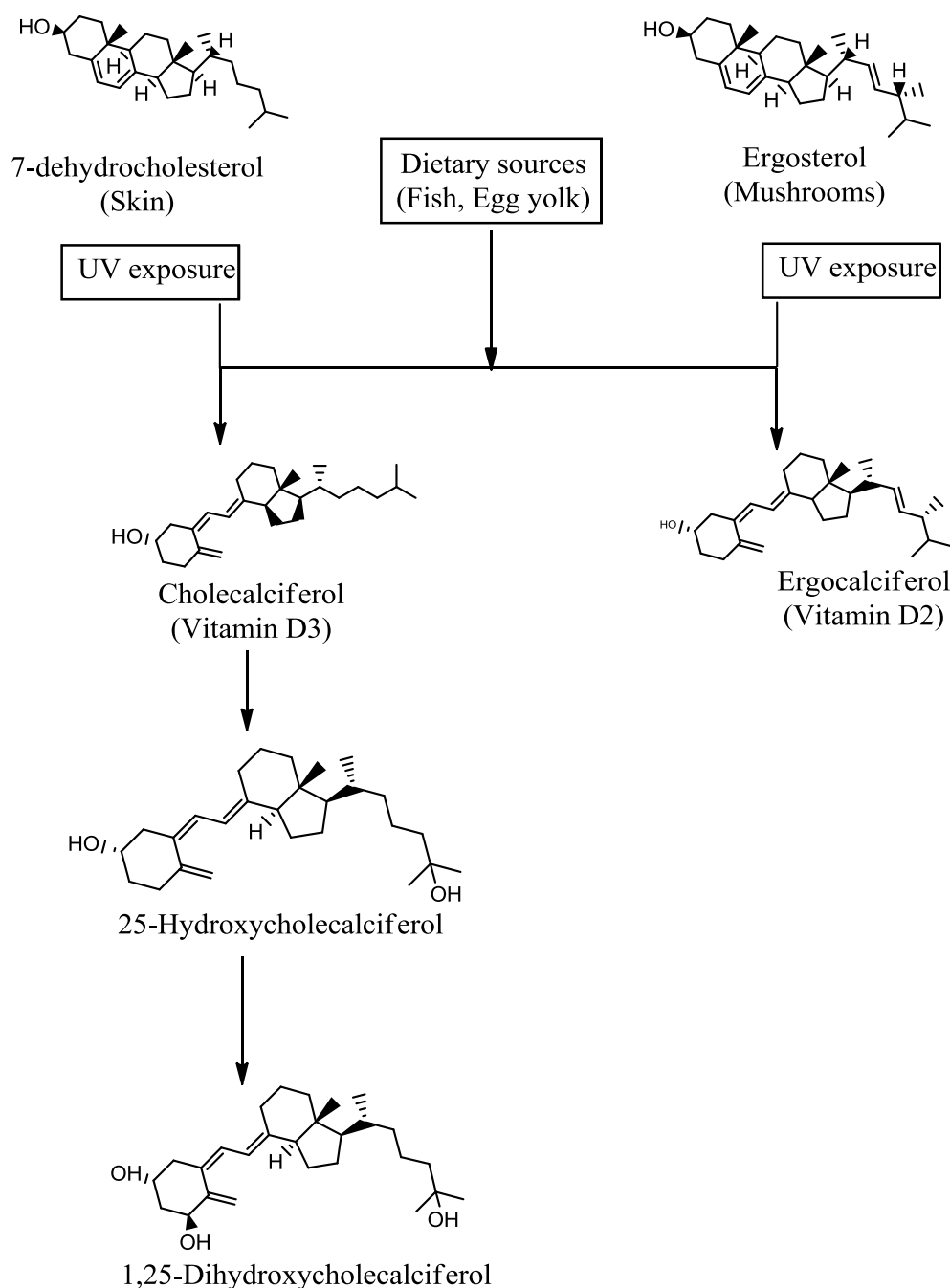


Fig. 1. Chemistry of vitamin d (adapted from Vuolo, Di Somma, Faggiano, & Colao, 2012).

to form 25-hydroxyvitamin D (calcidiol) and metabolically active 1,25-dihydroxyvitamin D (calcitriol), as shown in Fig. 1 (Harika & Eilander, 2013).

After vitamin D intake, it enters blood circulation, being transported to the liver where it becomes hydroxylated to form 25-hydroxyvitamin D [25(OH)D], the major circulating form of vitamin D. In the kidney, a second hydroxylation of the 25-hydroxyvitamin D occurs, resulting in the formation of 1,25-dihydroxyvitamin D, which is the most potent form of vitamin D. In fact, most of the physiological effects of vitamin D in the body are related to the activity of 1,25-dihydroxyvitamin D (Harika & Eilander, 2013).

Even the contribution of food ingestion to vitamin D levels is relatively not significant, some important dietary sources of vitamin D are fish, beef liver, cod liver oil, egg yolks; additionally, mushrooms exposed to sunlight have shown to be rich in vitamin D₂ (Guan et al., 2016; Itkonen et al., 2016; Kim & Bae, 2016).

Facing to the described importance of vitamin D, and identified health problems related to its deficiency, the present review will address topics such as vitamin D and its main roles, main sources and strategies for their production. An emphasis will put on mushroom UV-irradiation as a promising technique for vitamin D obtainment.

2. Vitamin D and its main roles

Vitamin D deficiency is still an unrecognized epidemic, especially among the elderly people. Nevertheless, some reported data are noteworthy, namely the one concerning United States (US), where over 50 % of elderly people are lacking from vitamin D (Jasinghe, Perera, & Sablani, 2007). In general, vitamin D deficiency is mostly prevalent in the Middle East and South Asia, while low levels have also been described across the USA and Canada. Individuals living in some European countries located at higher latitudes experience low 25(OH)D

serum concentrations due to reduced UV exposure especially during winter months. In the south of Europe, severe hypovitaminosis D have been reported among elderly people, even though these countries presented lower latitudes and increased UV exposure (O'Mahony, Stepien, Gibney, Nugent, & Brennan, 2011).

Among others, vitamin D has impact on bone and muscle health, cancer disease, cardiovascular diseases, liver function, atopic dermatitis, obesity, depression and diabetes.

2.1. Bone and muscle health

The most significant role of vitamin D has been related to musculoskeletal health where deficiency is known to cause rickets in children and osteomalacia in adults, both characterized by aching muscles and bones (Tomlinson, Joseph, & Angioi, 2015). The role of vitamin D as a nutritional factor responsible for promoting bone formation and mineralization is due to its potential to stimulate osteoblasts cells and inhibit osteoclast responsible for bone resorption. Several controlled trials have shown a positive dose-response correlation between vitamin D supplementation and prevention of fracture upon fall, mainly attributed to the potential of vitamin D to provide adequate levels of calcium and phosphate (Bikle, 2014). Several signs and symptoms of bone disease, namely, growth retardation, muscle weakness, skeletal deformities, stunted growth and bowed legs have been associated with vitamin D deficiency, being the most common bone pathologies affecting Europe, the US and Nordic countries (Keegan, Lu, Bogusz, Williams, & Holick, 2013).

2.2. Cancer disease

Several studies reported in the past, have linked sun exposure and vitamin D levels to cancer disease, both prevention and prevalence. In fact, cancer mortality appeared to be significantly reduced among farmers and individuals working in agricultural businesses characterized by a constant exposure to sunlight. Even though there is a high chance to develop nonmelanoma skin cancer, a less dangerous and easy to treat disease, sun exposure also boosts the immune system against some cancer forms such as breast, colon and prostate cancers. Additionally, years of research have found a direct relationship between exposure to solar UVB radiation and premature death among Americans and British (Grant, 2009; Lim et al., 2006).

Inferior rates of prostate and ovarian cancer incidence have been linked to inhabitation at lower latitudes, while some reports have shown that cancer therapy applied in summer season resulted in better survival rates, comparatively with the ones undergone in winter, fact mainly attributed to seasonal issues, among them vitamin D levels (Tang et al., 2012).

The mechanism behind the observed antitumor properties involves activation of some specific kinases by vitamin D that tend to keep cell cycle in G1/S phase, prevent DNA synthesis and avoid growth of the malignant cells (Holick, 2014). One major limitation that hinders vitamin D and its analogues to be effectively used to treat cancer is their potential to cause calcemic toxicity (Mattila, Valkonen, & Valaja, 2011). Even though certain strategies are being utilized by these cells to produce vitamin D destructive enzymes, unconfirmed reports have suggested that improving vitamin D levels, by the intake of either vitamin D rich or fortified foods, may help to improve chemotherapy and reduce the risk of malignancy.

2.3. Cardiovascular diseases

Vitamin D has attracted recent attention due to their cardioprotective functions in several cardiovascular diseases (CVD). The mechanism behind some of its cardioprotective roles involves promoting expression of vascular endothelial growth factor (VEGF) in the endothelial cells lining the heart valves, which activates several cell signal transduction

pathways responsible for endothelial cell proliferation, cell survival, migration and vascular permeability. Some studies have reported that vitamin D supplementation can serve as an adjuvant therapy to reduce serum total cholesterol levels, thereby contributing to a better cardiovascular function (Qin, Zhao, Chen, Yin, & Wang, 2015; Skaaby et al., 2012).

Vitamin D plays a major role in suppressing Rheumatic Heart Disease (RHD), the major cause of cardiac related deaths (Sarkar, Chopra, Rohit, Banerjee, & Chakraborti, 2016). The mechanism through which vitamin D helps to suppress the severity of cardiovascular diseases involves reducing the expression of genes responsible for renin production, thus down regulating the renin-angiotensin system (RAS) that primarily leads to increased blood pressure (Papandreou & Hamid, 2015).

2.4. Liver function

The role of vitamin D in preventing liver diseases is associated with its potential to down regulate several signal transduction pathways, which allows for expression of interferons, chemokines and pro-inflammatory genes such as TNF- α , IL-4, IL-6 and the toll-like receptors, reducing the risk and providing protection against hepatitis B, liver inflammation, liver cirrhosis and hepatocellular carcinoma (HCC) (Elangovan et al., 2017).

The liver has regenerative capability even when facing an acute or chronic injury; however, the mechanism of regeneration may become powerless leading to the development of acute liver failure, cirrhosis, hepatic failure and/or HCC. Vitamin D and vitamin D receptor (VDR), in liver, play a significant role ensuring that the organ is apt to perform its functions. Even though the anti-inflammatory and immune-modulatory properties of vitamin D are the mechanisms behind its hepatoprotective properties, further studies are needed to fully understand the mechanism behind vitamin D synthesis, activity and bioavailability (Kitson & Roberts, 2012).

2.5. Atopic dermatitis

Atopic dermatitis (AD) is a chronic inflammatory disorder characterized, mainly, by dry and itchy skin. The physiological mechanism conducting to this status, is still not fully understood, but has been associated with environmental and dietary factors that may cause over expression of inflammatory mediators, such as nitric oxide (NO), TNF- α , interleukins and irritants, causing redness, pain and edema.

Studies have reported that deficiency of vitamin D is involved in AD development. Additionally to nutritional supplementation with to vitamin D, omega-3 fatty acids, and vitamin E are recognized as beneficial in reducing the symptoms and severity of AD (Kim & Bae, 2016). The reduction on AD severity has also been linked to the potential of vitamin D to induce expression of antimicrobial peptides like cathelicidin, filaggrin and β -defensin, which prevent skin infection and also support the barrier properties of the *Stratum corneum* (Kim & Bae, 2016; Reinholz, Ruzicka, & Schaubert, 2012).

2.6. Obesity, depression and diabetes

Epidemiological studies have shown a close co-existence of vitamin D deficiency with the occurrence of obesity and diabetes. Studies have also pointed out the chance of low vitamin D levels in obese individuals due to their low appetite to participate in outdoor activities. Whether food fortification with vitamin D could help reducing the tendency to develop obesity and diabetes, this is still not fully established (Sadiya et al., 2016).

The presence of vitamin D receptors in other body tissues aside from the bone, and linked with other pathological conditions, is now being studied. Some clinical studies conducted in individuals with pre-diabetes, showed that a vitamin D supplemented diet improves and

prevents the development of full blown diabetes (Bikle, 2014).

Studies have also reported a link between some genetic factors and diabetes (Papandreou & Hamid, 2015). Nutritional and dietary products such as omega-3 fatty acids, vitamin D and vitamin B have been used as a natural supplement with antidepressant effects. Even though the mechanism behind this action is still unknown, some studies have shown that vitamin D receptors are responsible for activating some brain neurotransmitters responsible for addressing mood disorders. Thus, efforts towards more clinical studies are needed to better understand the physiological function of vitamin D in the brain and stress out more conclusive remarks (Parker, Brotchie, & Graham, 2017).

3. Sources of vitamin D

The exact concentration of serum vitamin D levels defining deficiency and sufficiency is subjected to some contradictions because of the wide range of inconsistencies reported by several bodies and governmental councils. However, the recommended daily allowance (RDA) according to the US Institutes of Medicine (IOM) has been recommended as 400 IU/d (10 µg) for infants, 600 IU/d (15 µg) for children, adolescents and adults, and 800 IU/d (20 µg) for adults aged over 70 years. The RDA values sometimes depend on clinical and environmental factors like latitude of residence, level of exposure to sun, skin pigmentation, dietary practices, clothing and cultural habits and health care system (Pludowski et al., 2017).

There are various ways in which vitamin D can be supplied to meet recommended daily intake, some of which include sunlight, and ingestion of some naturally occurring and fortifying foods. Examples include fortified milk, cheese, soy drink and fish.

3.1. Sunlight

The skin is able to synthesize vitamin D from 7-dehydrocholesterol after UV exposure from sunlight. This source becomes depleted during periods where sunlight is lacking, demanding the use of alternative sources in the form of dietary supplements, as naturally occurring or fortified foods (Jakobsen, 2007).

Exposure to the sun during the last few years has been reduced due to cultural and behavioural issues. These include advices to the general public to minimize sun exposure because of the potential danger to cause skin cancer, together with over expression of collagen and elastin associated with skin ageing. This has increased the use of sunscreen products that tend to hinder vitamin D production (Spiro & Buttriss, 2014).

3.2. Naturally occurring food sources

Very few natural occurring food sources contain vitamin D. The most common sources of vitamin D are cod liver oil, salmon, herring, kipper fillets, mackerel, sardines, tuna, anchovy, cod, trace sole, mushrooms, milk, cheese, yoghurt, meat, eggs, liver, beef, pork, cured bacon, ham, chicken and turkey. Their vitamin D levels varies from source to source; 250 µg/100 g in cod liver oil to 8–30 µg/100 g in fatty fish such as salmon, eel and mackerel and 3–9 µg/100 g in lean fish such as halibut, sole and tuna (Barnkob, Argyraki, Petersen, & Jakobsen, 2016).

Vitamin D in the form of D₃ is present in animal foods such as eggs and fish species while the one in the form of D₂ can be found in some mushrooms and phytoplankton, invertebrates, yeast and plants. In the latter case, it is formed after exposure to UV radiation that allows for photo conversion of ergosterol to ergocalciferol.

Data taken across Europe shows some variability in the dietary intake of vitamin D, with the Scandinavian countries (Sweden, Denmark, Finland and Norway) presenting higher intake when compared with Mediterranean regions such as Italy, Spain and Portugal (O'Mahony et al., 2011). Dietary sources of vitamin D vary from country to country;

fish and fats in Norway, milk and dairy products in Finland, oily fish, meat and meat products, cereal and cereal products in the UK, meat, fish and spreads in Ireland, fortified milk in Canada and US, fish/shellfish and eggs in Japan, and fish in Spain (O'Mahony et al., 2011).

3.3. Fortified foods

Fortified food products are foods to which one or more essential nutrients have been added in order to restore a deficiency typical a society or specific target groups (Hashemi, Eskandari, Mesbahi, & Hanifpour, 2015). The added nutrient may be naturally absent or lost during processing. There are several different classes of fortified foods and variability exists from country to country. Food fortification has shown to be an effective alternative to surpass the lack of vitamin D in few countries such as Canada, US, Australia, New Zealand, Sweden and Finland, either in the form of D₂ or D₃.

Food fortification is a cost-effective strategy that has not only been used to restore nutrient deficiency but also to improve nutrient quality and quantity (Hashemi et al., 2015). Among the existing food types fortified with vitamin D, dairy products such as milk products, margarines and breakfast cereals are the most common (O'Mahony et al., 2011). Also, cheese, some orange juices and yoghurts are reported as vehicles for Vitamin D fortification, while due to the generalized consumption of bread, several countries have encouraged its fortification with vitamin D (Lu et al., 2007).

Considering individuals that are intolerant to lactose, and countries with low dairy product consumption, new and improved foods need to be developed as vehicles for vitamin D fortification (O'Mahony et al., 2011).

3.4. Food fortification as an alternative source of vitamin D

Because foods that are rich in vitamin D are not frequently consumed, low abundance of food sources rich in vitamin D and sometimes UV availability is low or unavailable due to environmental factors, several strategies have been proposed to reduce low levels of vitamin D. The food fortification strategy is meant to address current dietary gaps that exist among several populations as a means to increase intake and prevent vitamin D deficiency.

This strategy tends to enhance vitamin D levels across all population groups. While some countries like Canada have made fortification of milk mandatory, there are evidences that a large percentage of the population still consume less than the estimated average requirement (EAR) for vitamin D. These low levels have raised a lot of concerns over the years and several strategies are being suggested to increase the range of food vehicles to be used as well as encourage voluntary fortification practice. The US, Australia New Zealand Sweden and Finland have made fortification of some food mandatory either in the form of D₂ or D₃. Among various food types fortified with vitamin D, dairy products such as milk products, margarines and breakfast cereals, infant foods are the most common (Cashman, 2015; Wagner et al., 2008).

As very few reports are describing food fortification in processed foods due to concerns regarding the stability in terms of pH, temperature and water availability, further studies need to be conducted to examine the long-term retention and heat stability of these vitamin D fortified foods (Wagner et al., 2008). Some limitations to fortification of foods include consumer preference, cultural traditions regarding food and cost of the products especially in vegan based foods (Calvo & Whiting, 2013). A recent trend that is now routinely used involves improving the nutritional composition of animal feeds by bio-fortifying with vitamin D either by selective breeding, or through genetic engineering by which the vitamin D content in animals such as cultured fish, beef, pork, lamb and chicken, are increased. Mattila et al. (2011) studied the effect of vitamin D fortified chicken feed on vitamin D content of eggs and chicken meat by comparing with known commercial feeds.

The mentioned authors reported that the effect of vitamin D₃ fortified feed in the control group was effectively transferred from the hens' diet to egg yolk. An alternative biofortification strategy is to expose excised skin of animals to UV light in order to increase the conversion of 7-dehydrocholesterol (7-DHC) to vitamin D₃ (Barnkob et al., 2016). This strategy was also followed by Kühn et al. (2015) while exposing chicks to UVB radiation.

Due to the extremely high amounts of 7-DHC in the exposed skin, especially in the legs where feathers are absent, exposed chicken produced eggs that contain 5 times higher vitamin D content than non-exposed chicks that were on normal diet. Biofortification process is cost effective and sustainable, and is currently being practiced in industrialized and developing countries (Barnkob et al., 2016). Studies on developing novel approaches to vitamin D enrichment of foods have increased in recent years. Bio-addition is another popular term used to describe the process of enriching food directly with one or more nutrient. This strategy involves exposing mushrooms to UV light in order to increase vitamin D₂ (ergocalciferol) content (Calvo & Whiting, 2013).

4. Current approaches for vitamin D production

At industrial level, vitamin D can be obtained from two main sources: fish oil and lanolin, a greasy substance that can be obtained as a byproduct of the wool industry.

4.1. Fish oil

Fish oil, produced from fish and its by products, apart from vitamin D, contains high levels of omega-3s in triglycerides, diglycerides and monoglycerides. Currently, there are some concerns about fish oil supplements since they may contain unsafe and illegal levels of polychlorinated biphenyls (PCBs), mercury, lead, and other environmental toxins (Nascimento et al., 2015; Pike & Jackson, 2010).

Fish oil supplements have been used to suppress the severity of cardiovascular disease, stroke, high blood pressure, renal injury, rheumatoid arthritis and autoimmune disorders. The major draw-back of these products is the presence of environmental contaminants through atmospheric deposition, run-off and pollution from shipping. These contaminants tend to accumulate in the liver, and fish oil-rich muscles and dietary products formulated from such tissues, tend to retain very high levels. Series of refining steps such as steam stripping, cold filtration and charcoal filtration, are industrially used to remove these high molecular weight compounds but might not be completely effective (Fernandes, Rose, White, Mortimer, & Gem, 2006).

Industrial production of fish oil comprises the next steps; after fish is sourced, it is boiled and pressed to extract the raw oil. Thereafter, the obtained raw-oil is subjected to various forms of washing and molecular distillation to remove impurities such as heavy metals, pesticides, dioxins, polybrominated diphenylethers (PBDEs), polycyclic aromatic hydrocarbons (PAHs) and (PCBs), before undergoing enzymatic or supercritical CO₂ extraction for concentration.

4.2. Lanolin

Industrial production of vitamin D can be also carried out from an animal based ingredient, lanolin, a greasy, waxy substance secreted by the sebaceous gland of wool bearing animals. These wools are present mainly as thermo-regulators to maintain a healthy and productive flock's life and protect them from the external environment.

Different methods are being used to shear the wool from sheep and other wool producing animals, generating lanolin and other wool products. Briefly, after the fleece has been collected from the animal, it is submerged in hot detergent to remove dirt and sweat salt, before collect crude lanolin from the solution by high-speed centrifugation. Then, the produced lanolin undergoes a series of chemical reactions to remove

lanolin derivatives such as aliphatic alcohols, cholesterol, isopropyl lanolate, laneth, lanogene, lanolin alcohols, lanosterols, sterols, and triterpene alcohols (Jover, Moldovan, & Bayona, 2002). The lanolin is then subjected to a series of purification steps to form 7-dehydrocholesterol before being converted, by UV radiation, to a final dried product thereafter concentrated to form vitamin D supplement.

The whole process suggests that vitamin D supplement produced from lanolin might be cruelty-free in terms of non-animal testing but it contains an animal based ingredient, and as such, consumers that are vegans, and those that are allergic to wool products, must find alternative sources for vitamin D.

5. Mushroom's UV-irradiation for vitamin D production

Mushrooms have been consumed since earliest history because of their nutritional and medicinal properties. Their consumption has been reported to present interesting health promoting benefits such as antioxidant, antitumor, antimicrobial, anti-inflammatory, antityrosinase, immunomodulator, antiatherogenic and hypoglycemic activities (Alves et al., 2013; Carcho & Ferreira, 2013a, 2013b; Taofiq, González-Paramás, Martins, Barreiro, & Ferreira, 2016, 2015). They can be used as food, dietary supplements, cosmeceutical ingredients, and serve as matrices to produce novel pharmacological active compounds.

The fruiting body of mushrooms, either in their fresh or processed forms, is rich in sterols, mainly ergosterol, form that can be converted into vitamin D₂ by UV-radiation. The amount of vitamin D varies among mushroom species, and also within the same species. Among them, mushrooms belonging to the genera *Agaricus*, *Lentiuola* and *Pleurotus* have been reported to contain interesting amounts of vitamin D after exposure to UV.

5.1. Mushrooms irradiation to photoconvert ergosterol into vitamin D₂

Mushrooms' irradiation using techniques like Gamma-irradiation and electron-beam is a safe and cost effective method that has been widely used to enhance their shelf life during pre- or post-harvesting, preserving their quality, nutritional (proteins, sugars and vitamins) and bioactive (phenolics including flavonoids and flavour compounds) compositions (Fernandes, Antonio, Oliveira, Martins, & Ferreira, 2012). Mushrooms contain very low levels of vitamin D, or are sometimes vitamin D₂ absent; but following exposure to UV radiation, ergosterol undergoes series of ring rearrangement forming previtamin D and, lastly, the active form of Vitamin D₂ (Sapozhnikova, Byrdwell, Lobato, & Romig, 2014; Slawinska et al., 2016).

Numerous studies have reported the potential of using UV-treated mushrooms as an alternative source of vitamin D, with wild species presenting higher vitamin D levels when compared with their cultivated counterparts (Kohn, 2016). Thus, irradiated mushrooms not only provide an alternative to animal source derived vitamin D, but also provide an attractive option for vegans or lactose intolerant individuals, taking into account that most vitamin D fortified foods are based on dairy products (Huang, Lin, Mau, Li, & Tsai, 2015).

The kinetics of the photo-conversion of ergosterol into vitamin D₂ in mushrooms has not been fully understand, but in the work of Jasinghe et al. (2007) factors such as temperature, moisture content, UV radiation type (UVB or UVC) and irradiation dose, are described to influence vitamin D yield. During photo-conversion, temperature of irradiation is significant in controlling thermal rearrangement of pre-vitamin D₂ to vitamin D₂. Also, the use of high or moderate temperatures sometimes leads to formation of by-products such as lumisterol and tachysterol diminishing the final yield, while high moisture content causes ergosterol dilution and subsequently lowering conversion rate (Jasinghe et al., 2007).

Studies assessing the effects of radiation on ergosterol conversion into vitamin D₂, in edible mushrooms, are mostly available for cultivated species, namely the ones with high production value. Examples

Table 1
Irradiated mushroom species and irradiation conditions.

Species	Origin	Sample	Radiation source	Doses	Irradiation procedure	Time of exposure	References
<i>Auricularia auricula-judae</i> (Bull.) J.Schröt.	Thailand	Fresh	UVB	45.86–550.32 J/cm ²	15 cm	15-180 min	(Banlangsawan & Sanoamuang, 2016)
<i>Agaricus bisporus</i> (J.E.Lange) Imbach	Denmark	Freeze-dried Mycelium	UVB	0–2.400 mJ/cm	0.25 m	1 min 46 s	(Kristensen et al., 2012)
	Korea	Fresh	UVB	1.36 W/m ²	–	10 min	(Lee & Aan, 2016)
	Canada	Fresh	UVC	0.5, 1.0 and 2.0 kJ/m ²	–	50, 100 and 200 s,	(Guan et al., 2016)
	Poland	Fresh	UVB	411 mJ/cm ²	–	30 min	(Slawinska et al., 2016)
	USA	Fresh	UVB	1 J/cm ² , 492 W/m ²	–	–	(Bilbao-Sainz et al., 2017)
	USA	Fresh	UVB	80–90 mJ/cm ² , 2800–2900 mJ/cm ²	–	25 s, 10 min	(Sapozhnikova et al., 2014)
	Germany	Fresh	UVB	1.5 J/cm ² (2.54 mW/cm ²)	–	20 min	(Nölle, Argyropoulos, Ambacher, Muller, & Biesalski, 2016)
	Australia	Fresh	UVC	1700 mW/cm ²	30 cm	12 and 30 s,	(Bennett et al., 2013)
	USA	Fresh	Pulsed UV	0.791 J/cm ² /pulse	3.18 cm	–	(Kalaras et al., 2012b)
	Sweden	Fresh	UVA, UVC	94.7, 189.5 and 379.0 J/cm ²	–	30 min, 1hr, and 2 h	(Teichmann et al., 2007)
	Singapore	Fresh	UVA, UVB and UVC	3.5, 4.9, 3.2 W/m ² and 25.2, 35.3, and 23.0 kJ/m ²	15 cm	1 h	(Jasinghe & Perera, 2006)
	Singapore	Fresh	UVA	3.5 W/m ² , 0.21 kJ/m ²	15 cm	2 h	(Jasinghe et al., 2007)
	Germany	Fresh	UVB	1.5 J/cm ²	–	25 min	(Urbain et al., 2011)
	USA	Fresh	UVB	1.08 J/cm ²	10-15 cm	–	(Simon et al., 2011)
	USA	Fresh	Pulsed UV	0.791 J/cm ²	3.18 cm	–	(Kalaras et al., 2012a)
	Ireland	Fresh	UVA, UVB	0.13–2.80 J/cm ²	–	15 - 360 min.	(Urbain & Jakobsen, 2015)
	Ireland	Fresh	UVA, UVC	0.53 J/cm ²	–	–	(Urbain, Valverde, & Jakobsen, 2016)
	Singapore	Fresh	UVC	0.125 and 0.25 J/cm ²	30, 40, and 50 cm	2.5–60 min	(Koyyalamudi, Jeong, Song, Cho, & Pang, 2009)
	Korea	Fresh	UVA	25.2 kJ/m ²	15 cm	2 h	(Jasinghe & Perera, 2005)
	Australia	Fresh	UVB	10, 20, and 30 kJ/m ²	–	–	(Ko, Lee, Lee, and Park, 2008)
	Denmark	Fresh	Pulsed UV	1.150 J/cm ²	–	–	(SKoyyalamudi et al., 2011)
	Taiwan	Fresh	UVB	0–2.400 J/cm ²	0.25 m	1 min 46 s	(Kristensen et al., 2012)
Taiwan	Fresh	UVB, UVC	0.247, 0.493, and 0.986 J/cm ²	30 cm	0.5, 1, and 2 h	(Mau, Chen, & Yang, 1998)	
Taiwan	Fresh	γ-irradiated	0.5, 2, 5 and 10 kGy	–	–	(Tsai et al., 2014)	
USA	Fresh	UVB	0.5, 1.0, and 1.5 J/cm ²	–	< 2 h	(Roberts et al., 2008)	
<i>Agaricus bisporus</i> Portabella (J.E.Lange) Imbach	Sweden	Fresh	UVA, UVC	94.7, 189.5 and 379.0 J/cm ²	–	30 min, 1hr, and 2 h	(Teichmann et al., 2007)
<i>Agaricus bitorquis</i> (Quélet) Sacc.	Taiwan	Fresh	UVB, UVC	0.247, 0.493, and 0.986 J/cm ²	30 cm	0.5, 1, and 2 h	(Mau et al., 1998)
<i>Agrocybe cylindracea</i> , (Pers.) Fayod	Taiwan	Fresh	UVB	0.36 mW/cm ² , 25.9 kJ/m ²	19 cm	2 h	(Huang, Lin, & Tsai, 2015)
<i>Auricularia polytricha</i> (Mont.) Sacc.	Taiwan	Fresh	UVB	0.36 mW/cm ² , 25.9 kJ/m ²	19 cm	2 h	(Huang, Lin, & Tsai, 2015)
<i>Boletus edulis</i> Bull.	Sweden	Fresh	UVA, UVC	94.7, 189.5 and 379.0 J/cm ²	–	30 min, 1hr, and 2 h	(Teichmann et al., 2007)
<i>Cantharellus tubaeformis</i> .	Sweden	Fresh	UVA, UVC	94.7, 189.5 and 379.0 J/cm ²	–	30 min, 1hr, and 2 h	(Teichmann et al., 2007)
<i>Cordyceps militaris</i> (L.) Fr.	Taiwan	Fresh	UVB	0.36 mW/cm ² , 25.9 kJ/m ²	19 cm	2 h	(Huang, Lin, Mau, et al., 2015)
<i>Flammulina velutipes</i> (Curtis) Singer	Thailand	Fresh	UVB	45.86–550.32 J/cm ²	15 cm	15-180 min	(Banlangsawan & Sanoamuang, 2016)
<i>Hericium erinaceus</i> (Bull.) Persoon	Singapore	Freeze-dried Mycelium	UVA	25.2 kJ/m ²	15 cm	2 h	(Jasinghe & Perera, 2005)
	Thailand	Fresh	UVB	45.86–550.32 J/cm ²	15 cm	15-180 min	(Banlangsawan & Sanoamuang, 2016)
<i>Hypsizygus marmoreus</i> (Peck) Bigelow	Taiwan	Freeze-dried Mycelium	UVB	0.36 mW/cm ² , 25.9 kJ/m ²	19 cm	2 h	(Huang, Lin, & Tsai, 2015)

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Table 1 (continued)

Species	Origin	Sample	Radiation source	Doses	Irradiation procedure	Time of exposure	References
<i>Lentinula edodes</i> (Berk.) Pegler	Thailand	Fresh	UVB	45.86–550.32 J/cm ²	15 cm	15-180 min	(Banlangsawan & Sanoamuang, 2016)
		Freeze-dried Mycelium					
	Taiwan	Fresh	UVB	0.36 mW/cm ² , 25.9 kJ/m ²	19 cm	2 h	(Huang, Lin, & Tsai, 2015)
	Poland	Fresh	UVB	411 mJ/cm ²	–	30 min	(Slawinska et al., 2016)
	USA	Fresh	UVB	80–90 mJ/cm ² , 2800–2900 mJ/cm ²	–	25 s, 10 min	(Sapozhnikova et al., 2014)
	Taiwan	Fresh	UVB	0.36 mW/cm ² , 25.9 kJ/m ²	19 cm	2 h	(Huang, Lin, & Tsai, 2015)
	Sweden	Fresh	UVA, UVC	94.7, 189.5 and 379.0 J/cm ²	–	30 min, 1hr, and 2 h	(Teichmann et al., 2007)
	Singapore	Fresh	UVA, UVB and UVC	3.5, 4.9, 3.2 W/m ² and 25.2, 35.3, and 23.0 kJ/m ²	15 cm	1 h	(Jasinghe & Perera, 2006)
	Singapore	Fresh	UVA	3.5 W/m ² , 0.21 kJ/m ²	15 cm	2 h	(Jasinghe et al., 2007)
	Singapore		UVB	35.3 kJ/m ²	15 cm	1 h	(Jasinghe, Perera, & Barlow, 2005b)
	Singapore	Fresh	UVA	25.2 kJ/m ²	15 cm	2 h	(Jasinghe & Perera, 2005)
	Korea	Fresh	UVB	25, 50, and 75 kJ/m ²	–	–	(Ko et al., 2008)
	Taiwan	Fresh	UVB, UVC	0.247, 0.493, and 0.986 J/cm ²	30 cm	0.5, 1, and 2 h	(Mau et al., 1998)
	Korea	Fresh	UVB	0.6–1.8 W/m ²	–	60-180 min	(Zhang, Wu, Song, & Ahn, 2015)
	Spain	Supercritical extracts	UVA, UVC	–	–	0, 15, 30, 60, 120 min	(Morales et al., 2017)
<i>Lentinus squarrosulus</i> (Mont.)	Thailand	Fresh	UVB	45.86–550.32 J/cm ²	15 cm	15-180 min	(Banlangsawan & Sanoamuang, 2016)
		Freeze-dried Mycelium					
<i>Lentinus polychrous</i> Lév.	Thailand	Fresh	UVB	45.86–550.32 J/cm ²	15 cm	15-180 min	(Banlangsawan & Sanoamuang, 2016)
		Freeze-dried Mycelium					
<i>Pholiota nameko</i> (T. Itô) S. Ito & S. Imai	Taiwan	Fresh	UVB	0.36 mW/cm ² , 25.9 kJ/m ²	19 cm	2 h	(Huang, Lin, & Tsai, 2015)
<i>Pleurotus citrinopileatus</i> Singer	Taiwan	Mycelium	UVB	0.36 mW/cm ² , 25.9 kJ/m ²	19 cm	2 h	(Huang, Lin, & Tsai, 2015)
	Taiwan	Fresh	UVB	0.36 mW/cm ² , 25.9 kJ/m ²	19 cm	2 h	(Huang, Lin, & Tsai, 2015)
<i>Pleurotus cystidiosus</i> O.K. Mill.	Singapore	Fresh	UVA, UVB and UVC	3.5, 4.9, 3.2 W/m ² and 25.2, 35.3, and 23.0 kJ/m ²	15 cm	1 h	(Jasinghe & Perera, 2006)
	Singapore	Fresh	UVA	3.5 W/m ² , 0.21 kJ/m ²	15 cm	2 h	(Jasinghe et al., 2007)
	Singapore	Fresh	UVA	25.2 kJ/m ²	15 cm	2 h	(Jasinghe & Perera, 2005)
<i>Pleurotus eryngii</i> var. <i>ferulae</i>	Thailand	Fresh	UVB	45.86–550.32 J/cm ²	15 cm	15-180 min	(Banlangsawan & Sanoamuang, 2016)
		Freeze-dried Mycelium					
	Taiwan	Mycelium	UVB	0.36 mW/cm ² , 25.9 kJ/m ²	19 cm	2 h	(Huang, Lin, & Tsai, 2015)
	Taiwan	Fresh	UVB	0.36 mW/cm ² , 25.9 kJ/m ²	19 cm	2 h	(Huang, Lin, & Tsai, 2015)
	Taiwan	Fresh	UVB	0.36 mW/cm ² , 25.9 kJ/m ²	19 cm	2 h	(Huang, Lin, & Tsai, 2015)
	Taiwan	Fresh	Pulsed UV	–	–	18 s	(Chen et al., 2015)

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Table 1 (continued)

Species	Origin	Sample	Radiation source	Doses	Irradiation procedure	Time of exposure	References
<i>Pleurotus ostreatus</i> (Jacq. ex Fr.) P.Kumm.	Budapest	Mycelium	UVB	–	32 cm	15 to 90 min	(Szabó & Gyórfi, 2012)
	Thailand	Fresh	UVB	45.86–550.32 J/cm ²	15 cm	15–180 min	(Banlangsawan & Sanoamuang, 2016)
		Freeze-dried Mycelium					
	Poland	Fresh	UVB	411 mJ/cm ²	–	30 min	(Slawinska et al., 2016)
	Taiwan	Fresh	UVB	0.36 mW/cm ² , 25.9 kJ/m ²	19 cm	2 h	(Huang, Lin, & Tsai, 2015)
	Singapore	Fresh	UVA, UVB and UVC	3.5, 4.9, 3.2 W/m ² and 25.2, 35.3, and 23.0 kJ/m ²	15 cm	1 h	(Jasinghe & Perera, 2006)
	Germany	Fresh	UVB	11.5 W/m ²	10–11 cm	60 min	(Maximilian Wittig, Krings, & Berger, 2013)
	Germany	Fresh	UVB	11.5 W/m ²	10 cm		(Krings & Berger, 2014)
	Singapore	Fresh	UVA	3.5 W/m ² , 0.21 kJ/m ²	15 cm	2 h	(Jasinghe et al., 2007)
	Kenya	Fresh	UVA, UVC	3.5 W/m ² , 0.0327 W/m ² 0.21 kJ/m ² , 1.96 J/m ²	–	10 - 60 min	(Edward et al., 2015)
	Korea	Fresh	UVA, UVB and UVC	0.3–1.2 W/m ²	–	2 h	(Wu & Ahn, 2014)
	Singapore	Fresh	UVA	25.2 kJ/m ²	15 cm	2 h	(Jasinghe & Perera, 2005)
Indonesia	Fresh	UVA, UVC	–	10 cm	15, 30, 60, 90, and 120 min	(Ruslan, Reza, & Damayanti, 2011)	
<i>Pleurotus pulmonarius</i> (Fr.) Quel.	Thailand	Fresh	UVB	45.86–550.32 J/cm ²	15 cm	15–180 min	(Banlangsawan & Sanoamuang, 2016)
		Freeze-dried Mycelium					
<i>Pleurotus djamor</i> (Rumph. ex Fr.) Boedijn	Taiwan	Fresh	UVB	0.36 mW/cm ² , 25.9 kJ/m ²	19 cm	2 h	(Huang, Lin, & Tsai, 2015)
<i>Volvariella volvacea</i> (Bul. ex Fr.) Singer	Taiwan	Fresh	UVB, UVC	0.247, 0.493, and 0.986 J/cm ²	30 cm	0.5, 1, and 2 h	(Mau et al., 1998)

Table 2
Bioaddition of Vitamin D₂ in mushrooms after undergoing UV radiation.

Mushroom	Source of radiation	Vitamin D ₂		Reference
		Before radiation	After radiation	
<i>Agaricus bisporus</i>	UVB	0.18 µg/100 g fw	491 µg/100 g fw	(Urbain et al., 2011)
	UVB	5.5 µg/100 g dw	410.9 µg/100 g dw	(Simon et al., 2011)
	Pulsed UV	0.005 µg/g	27 µg/g dw	(Kalaras et al., 2012a)
	UVA-UVB	0.1 µg/g dw	3.9 µg/g dw	(Urbain & Jakobsen, 2015)
	UVB	–	141.32 µg/g dw	(Wittig et al., 2013)
	Pulsed UV	–	32.2 µg/g dw	(Kalaras et al., 2012b)
	UVB	–	13.10 µg/g dw	(Slawinska et al., 2016)
	UVB	179 IU/g	6292 IU/g	(Sapozhnikova et al., 2014)
	UVB	0.0003–0.0008 µg/g dw	157 µg/g dw	(Bilbao-Sainz et al., 2017)
	UVB	–	406 µg/g dw	(Nölle et al., 2016)
	Pulsed UV	–	20.9 µg/g dw	(Koyyalamudi et al., 2011)
	UVA, UVC	0.07 µg/g dw	10.14 µg/g dw	(Teichmann et al., 2007)
	UVA, UVB and UVC	–	56.5 µg/g dw	(Jasinghe & Perera, 2006)
	UVC	0.79 mg/100 g dw	1.34 mg/100 g dw	(Guan et al., 2016)
	UVB	0.01 µg/g dw	7.28 µg/g dw	(Roberts et al., 2008)
	UVB	–	67.1 µg/g dw	(Paul Urbain et al., 2016)
	UVB	–	491 µg/100 g	(Urbain et al., 2011)
	UVC	–	30 µg/kg	(Louise Bennett et al., 2013)
	UVB	–	741.50 µg/g	(Lee & Aan, 2016)
	<i>Agaricus bisporus</i> var. <i>Portobello</i>	UVC	0.43 mg/100 g dw	0.95 mg/100 g dw
<i>Agaricus blazei</i>	UVB	–	22.13 µg/g dw	(Huang, Lin, & Tsai, 2015)
<i>Agrocybe cylindracea</i>	UVB	0.95 µg/g dw	42.36 µg/g dw	(Huang, Lin, & Tsai, 2015)
<i>Auricularia polytricha</i>	UVB	–	60.29 µg/g dw	(Huang, Lin, & Tsai, 2015)
<i>Cantharellus tubaeformis</i>	UVA, UVC	1.55 µg/g dw	14.03 µg/g dw	(Teichmann et al., 2007)
<i>Cordyceps militaris</i>	UVB	0.22 mg/g dry weigh	1.11 mg/g dw	(Huang, Lin, & Tsai, 2015)
<i>Hypsizygus marmoreus</i>	UVB	1.62 µg/g dw	15.06 µg/g dw	(Huang, Lin, & Tsai, 2015)
<i>Lentinula edodes</i>	UVB	–	29.33 µg/g dw	(Slawinska et al., 2016)
	UVB	–	1036 IU/g	(Sapozhnikova et al., 2014)
	UVB	0.35 µg/g dw	15.10 µg/g dw	(Huang, Lin, & Tsai, 2015)
	UVA, UVB and UVC	–	53.9 µg/g dw	(Jasinghe & Perera, 2006)
<i>Pholiota nameko</i>	UVB	–	61.78 µg/g dw	(Huang, Lin, & Tsai, 2015)
<i>Pleurotus ostreatus</i>	UVB	1.78 µg/g dw	27.89 µg/g dw	(Banlangasawan & Sanoamuang, 2016)
<i>Pleurotus citrinopileatus</i>	UVB	3.93 µg/g dw	208.65 µg/g dw	(Huang, Lin, & Tsai, 2015)
<i>Pleurotus cystidiosus</i>	UVA, UVB and UVC	–	79.5 µg/g dw	(Jasinghe & Perera, 2006)
<i>Pleurotus djamor</i>	UVB	2.13 µg/g dw	93.29 µg/g dw	(Huang, Lin, & Tsai, 2015)
<i>Pleurotus eryngii</i> var. <i>ferulae</i>	UVB	1.65 µg/g dw	52.30 µg/g dw	(Huang, Lin, & Tsai, 2015)
	UVB	1.56 µg/g dw	28.71 µg/g dw	(Huang, Lin, & Tsai, 2015)
<i>Pleurotus ostreatus</i>	UVB	–	239.67 µg/g	(Wu & Ahn, 2014)
	UVB	–	56.60 µg/g dw	(Slawinska et al., 2016)
	UVB	–	4411 IU/g	(Sapozhnikova et al., 2014)
	UVB	0.83 µg/g dw	69.00 µg/g dw	(Huang, Lin, & Tsai, 2015)
	UVA, UVB and UVC	–	184.65 µg/g dw	(Jasinghe & Perera, 2006)
	UVB	32.07 µg/g dw	141.32 µg/g dw	(Wittig et al., 2013)
	UV	–	48.19 µg/g	(Ruslan et al., 2011)

include *Agaricus bisporus* (J.E.Lange) Imbach, *Lentinula edodes* (Berk.) Pegler and *Pleurotus ostreatus* (Jacq. ex Fr.) P.Kumm. Nevertheless, other species such as *Auricularia auricula-judae* (Bull.) J.Schröt., *Agaricus bitorquis* (Quélet) Sacc., *Agrocybe cylindracea* (Pers.) Fayod, *Auricularia polytricha* (Mont.) Sacc., *Boletus edulis* Bull., *Cantharellus tubaeformis*, *Cordyceps militaris* (L.) Fr., *Flammulina velutipes* (Curtis) Singer, *Hericium erinaceus* (Bull.) Persoon, *Hypsizygus marmoreus* (Peck) Bigelow, *Lentinus squarrosulus* (Mont.), *Lentinus polychrous* Lév., *Pholiota nameko* (T. Itô) S. Ito & S. Imai, *Pleurotus citrinopileatus* Singer, *Pleurotus cystidiosus* O.K. Mill., *Pleurotus djamor* (Rumph. ex Fr.) Boedijn, *Pleurotus eryngii* var. *ferulae* (Lanzi) Sacc., *Pleurotus pulmonarius* (Fr.) Quel., and *Volvariella volvacea* (Bull. ex Fr.) Singer., have been also reported to present considerable amounts of vitamin D after exposure to UV radiation.

Mushroom species evaluated for photo conversion to vitamin D are from different parts of the world; they include Asia (Korea, Singapore, Taiwan, Thailand and Indonesia); North America (Canada, USA); Africa (Kenya); Europe (Poland, Germany, Sweden, Ireland, Denmark) and Australia (Australia) (Table 1 and cited references).

Different UV irradiation sources such as Ultraviolet A (UVA),

Ultraviolet B (UVB), and Ultraviolet C (UVC) were applied, mostly to fresh samples (Table 1 and cited references), but also to freeze-dried ones (Banlangasawan & Sanoamuang, 2016) and mycelium samples (Banlangasawan & Sanoamuang, 2016; Huang, Lin, & Tsai, 2015; Kristensen, Rosenqvist, & Jakobsen, 2012; Szabó & Györfi, 2012). Pulsed UV (Kalaras et al., 2012a; Kalaras, Beelman, Holick, & Elias, 2012b; Koyyalamudi et al., 2011), mushroom supercritical extracts (Morales et al., 2017) and γ -irradiation (Tsai, Mau, & Huang, 2014) were also applied to fresh samples. The applied UV doses, irradiation procedure and exposure time were variable among the documented studies (Table 1).

Agaricus is a genus of saprobic basidiomycetes comprising over 400 species distributed all over the world, with *A. bisporus* (J.E. Kange) Imbach as the most cultivated one with an annual production, in Europe, of one million metric tons (Rzymiski et al., 2017). Considering the number of reports available in literature concerning *A. bisporus*, it can be considered not only a valuable source of nutrients and non-nutritional components but also, in the present context, a source of vitamin D (Kohn, 2016).

According to Table 1, *A. bisporus* has been treated with UVA, UVB,

UVC and Pulsed UV following distinct irradiation conditions and giving rise to different levels of bioadded vitamin D₂ (Table 2). Simon, Phillips, Horst, and Munro (2011) reported that the level of vitamin D₂ present in *A. bisporus* exposed to UVB radiation at a dose of 1.08 J/cm² increased significantly, namely from 5.5 µg/100 g dw to 410.9 µg/100 g dw, which represents an increase of 747%. Other authors have reported increases after exposure to UVB of 0.1 µg/g dw to 3.98 µg/g dw (Urban & Jakobsen, 2015), 0.0003 µg/g dw to 157 µg/g dw (Bilbao-Sainz et al., 2017) and 0.01 µg/g dw to 7.28 µg/g dw (Roberts, Teichert, & McHugh, 2008). *Agaricus bisporus* var. *Portobello* treated with UVC showed a slight increase in vitamin D₂ content at 2.0 kJ/m² dose, namely from 430 µg/100 g dw to 950 µg/100 g dw (Guan et al., 2016). Considering the variable irradiation time, contradicting reports have been published suggesting that longer times did not increase vitamin D₂ levels (Ruslan et al., 2011). Szabó and Györfi (2012) also reported that shorter irradiation time gave higher vitamin D₂ levels in pre-harvest *A. bisporus* after UV treatment.

The conversion of ergosterol to ergocalciferol in crude mushrooms is highly reliant on the applied energy source as sufficient energy is required (at 254 nm) to break the diene bond of the B-ring of ergosterol (occurs dimerization and ring cleavage) to photo-convert it into pre-vitamin D (Ruslan et al., 2011). The most significant reports on vitamin D₂ bioaddition in mushrooms have been carried out on the fruiting bodies of mushrooms in their fresh or freeze-dried forms. However, Huang, Lin, Mau, et al. (2015) have demonstrated the advantages of UV radiated mycelia and submerged culture, mainly due to the increased surface area that allows an amplifies the exposure to the applied UV radiation. The mentioned authors reported an increase in vitamin D₂ content in *Cordyceps militaris* from 0.22 to 1.11 mg/g, and the presence of several physiologically active substances with well reported bioactive properties (adenosine, cordycepin, ergothioneine, and polysaccharides), thereby presenting a high potential of application for healthy food development, adding to high vitamin D₂ contents.

After application of Pulsed UV, with 12 pulses, the vitamin D₂ levels in *A. bisporus* samples, reach a maximum concentration of approximately 27 µg/g dw (Kalaras et al., 2012a), whereas the unprocessed control mushrooms contained no measurable levels of vitamin D₂.

Vitamin D₂ levels approached a maximum concentration of 124 µg/g dw upon treatment with 60 pulses (20 s total exposure) (Kalaras et al., 2012b). The amounts of vitamin D₂ produced in mg/g dw using pulsed UV light were directly proportional to the number of applied pulses. The yield of vitamin D₂ using 9 pulses of UV light (161 µg/g dw) was higher comparatively other study, that using continuous UV-C light with an intensity of 0.25 J/cm² and an exposure time of 10 min at a distance of 30 cm (141 mg/g dw) according to Koyyalamudi et al. (2011).

Application of UVC in *A. bisporus* has also resulted in an increase of vitamin D₂ levels from 0.07 µg/g dw to 10.14 µg/g dw (Teichmann, Dutta, Staffas, & Jägerstad, 2007), 790 µg/100 g dw to 1340 µg/100 g dw (Guan et al., 2016), and a level up to 56.5 µg/g dw was also reported by Jasinghe and Perera (2006). The irradiation conditions were different in each of the above case studies supporting the dissimilarity encountered in the reached vitamin D₂ levels.

Pleurotus genus is made up of over 200 saprophytic mushrooms, widely distributed in temperate and tropical regions, and with well reported nutritional and bioactive properties. *Pleurotus ostreatus*, popularly referred to as oyster mushroom, is the most common species among the *Pleurotus* genus and significant works are available reporting their potential for photo-convert ergosterol to vitamin D by exposure to UVA (Jasinghe & Perera, 2005), UVB (Banlangsawan & Sanoamuang, 2016; Aneta Slawinska et al., 2016), UVC (Jasinghe & Perera, 2006) and pulse irradiation (Chen et al., 2015). Mushrooms irradiation aiming at increase vitamin D₂ content on *Pleurotus* genus was reported by Huang, Lin, and Tsai (2015). The vitamin D₂ content after exposure was found to be 93.29 µg/g dw, 69.00 µg/g dw, 52.30 µg/g dw and 28.71 µg/g dw for *P. salmoneostramineus*, *P. ostreatus*, *P. ferulae* and *P. eryngii*,

respectively. A significant increase was observed in *P. cystidus*, namely up to 79.5 µg/g dw (Jasinghe & Perera, 2006).

Lentinula edodes, popularly called shiitake, is a saprobic, wood-colonizing mushroom with a huge commercial production, only second behind *A. bisporus*. After exposure to UVB irradiation, vitamin D₂ content in *L. edodes* reached values up to 29.33 µg/g dw (Slawinska et al., 2016) or 53.9 µg/g dw (Jasinghe & Perera, 2006), with the latter corresponding to higher irradiation doses and a longer exposure times. Other species of *Lentinus* such as *Lentinus squarrosulus* (Mont.) and *Lentinus polychrous* Lév. have also been reported to contain significant amounts of vitamin D₂ after irradiation (Banlangsawan & Sanoamuang, 2016). Several other examples of species where significant amounts of vitamin D₂ have been developed after irradiation can be found in Table 2.

To be able to apply the irradiation techniques at a productive level it is important to identify the best irradiation conditions to increase vitamin D₂ content in cultivated mushrooms. In this context, some authors have already conducting optimization studies by evaluating the effects of intensity (0.5, 0.75, and 1.0 mW/cm²), dose (0.5, 1.0, and 1.5 J/cm²), and postharvest time (1 and 4 days) on the vitamin D₂ formation in *Agaricus bisporus* (Roberts et al., 2008). These authors identify shorter times of exposure and high intensities to be beneficial to vitamin D₂ development. Also, the distance of the UV source to mushrooms samples need to be optimized to determine the most adequate position for irradiation. From Table 1 it can be noted that very dissimilar distances between irradiation source and sample have been used (from 3.18 up to 50 cm). Morales et al. (2017) reported that the vitamin D₂ content in *L. edodes* supercritical extracts was 5-fold higher if UV source was placed 4 cm distant from the sample, comparatively with 24 cm after 1 h exposure. In a different study, Banlangsawan and Sanoamuang (2016) reported that with UV-B irradiation the amount of vitamin D₂ in the *P. ostreatus* was increased from 1.78 µg/g dw (unexposed) to 27.89 µg/g dw after exposures of 180 min.

Because of the observed dissimilarities in the irradiation conditions needed to maximize ergosterol conversion into vitamin D₂ in mushrooms, Lee and Aan (2016) have recently carried out an optimization study using the response surface methodology (RSM) considering as independent variables the exposure time, temperature, and irradiation intensity. The authors reported that an exposure time of 10.4 min, a temperature of 26.33 °C, and a UVB irradiation intensity of 1.36 W/m² was ideal to achieve a content of vitamin D₂ of 741.50 µg/g. In another work, Wu and Ahn (2014) identified 28.16 °C, UVB intensity of 1.14 W/m², and exposure time of 94.28 min as the ideal conditions to maximize vitamin D₂ content in *P. ostreatus* up to 239.67 µg/g dw. Also, an important variable is the contact surface area. This topic was treated by Ko et al. (2008) where different parts of shiitake mushroom were exposed to UVB at 25 kJ/m². The authors suggested that sliced mushrooms (higher surface area) resulted in higher vitamin D₂ levels (106.4 µg/g).

Irradiation studies to improve vitamin D₂ content have been carried out mainly on *Pleurotus*, *Agaricus* and *Lentinula* genera, but Huang, Lin, and Tsai (2015) conducted studies on *Agrocybe*, *Auricularia*, *Hypsizigus*, and *Pholiota* species using a UVB source placed 19 cm far from the samples. The irradiation intensity and dose was 0.36 mW/cm² and 25.9 kJ/m², respectively, for 2 h. The vitamin D₂ content after exposure was found to be 42.36 µg/g dw, 60.29 µg/g dw, 15.06 µg/g dw and 61.78 µg/g dw in *Agrocybe cylindracea*, *Auricularia auricular*, *Hypsizigus marmoreus* and *Pholiota nameko*, respectively. The authors reported *in vitro* antioxidant properties for the irradiated species due to the contribution of ergothioneine and phenolic compounds, thus reinforcing their potential to be used as food ingredients/supplements with health promoting properties.

5.2. Bioavailability of vitamin D from UV irradiated mushrooms

Some studies comparing the bioavailability of the various forms of vitamin D by monitoring serum 25(OH)D (25-hydroxyvitamin D) levels

have been reported. Intake of supplements in the form of D₃, mushroom derived D₂, fortified foods or dietary sources have shown differences but yet effective in maintaining serum 25(OH)D levels (Jakobsen, 2007). Very few studies have been able to determine the fate of vitamin D in the human gastrointestinal tract. A randomized controlled trial (RCT) data on the level of serum 25-hydroxyvitamin D in healthy individuals that consumed UV light treated edible mushrooms was reported by Cashman, Kiely, Seamans, and Urbain (2016). The above authors reported an increase in serum 25(OH)D when baseline vitamin D status was low but when baseline vitamin D status is high, there was no significant effect on serum 25(OH)D levels.

Vitamin D from several sources, firstly need to be released from the food matrix in which it is embedded, before undergoing a series of fate that becomes influenced by the food composition, presence and type of digestive enzymes, and the transport mechanism across intestinal cell (Borel, Caillaud, & Cano, 2013). One of the early studies on the bioavailability of vitamin D₂ from vitamin D₂-biofortified *A. bisporus* were conducted by Urbain, Singler, Ihorst, Biesalski, and Bertz (2011). Serum levels of 25(OH)D in those ingesting treated mushrooms (3.9 nmol/l) were similar to the ones of the control group receiving vitamin D supplements (4.7 nmol/l), making the former as effective as the vitamin D supplements itself in improving vitamin D levels.

Human studies were conducted by Ozzard, Hear, Morrison, and Hoskin (2008) in order to evaluate the potential of UVB-irradiated mushrooms to increase serum 25(OH)D levels. This case study was conducted on 30 years old man by administering stir-fried UVB irradiated mushrooms. Blood samples were taken and an increase in serum 25(OH)D level up to 129 % was observed coupled with an increase in calcium serum levels.

A contrasting report on vitamin D₂ bioavailability after ingestion of UV exposed mushrooms (fresh, sliced and cooked) was reported by Mehrotra et al. (2014). Forty-three prediabetic and vitamin D deficient adults were exposed to 100 g of these diets daily for 16 weeks. The authors reported little or no significant increase in 25(OH)D levels, mainly attributed to the longer exposure of fresh UV irradiated mushroom to cooking times. Nevertheless, Stephensen et al. (2012) reported a lack of difference between cooked (11.6 ± 1.1 µg/serving) and uncooked (10.9 ± 0.70 µg/serving) mushrooms.

UVB irradiated *A. bisporus* containing 15 µg of vitamin D₂ per gram of dried mushrooms was fed on female rats, and the control was fed with a vitamin D deficient diet. After 15 weeks following the above diets, there was no effect on the registered weight; UVB exposed mushroom fed rats had 30-fold of the recommended National Research Council (NRC) vitamin D level in its serum; furthermore, plasma levels of inflammatory mediators namely TNF-α and IL-1β were greatly reduced thereby suppressing the incidence of inflammation (Babu, Balan, Garthoff, & Calvo, 2014).

Chen et al. (2015) evaluated the potential of pulsed irradiated *Pleurotus eryngii* var. *ferulae*, a mushroom commonly grown in the far east, fed to female mice to enhance vitamin D synthesis and analyzed the serum osteoblast and osteoclast metabolites as indicators for bone density using NMR spectroscopy. The mice fed with irradiated mushrooms showed an increase in bone density, when compared with the mice fed with non-irradiated, with higher osteoblast and lower osteoclast suggesting that there was an increased bone formation rather than bone resorption. Blood was collected from time to time to measure ALP, osteocalcin, procollagen I C-terminal propeptide (PICP) and pyridinoline (PYD) levels. The results reveal that pulse irradiated mushrooms can exert proper resistance against bone resorption, thus maintaining bone health. The enhanced bone metabolism is due to increased vitamin D levels, which stimulate calcium and phosphorus absorption necessary for bone mineralization.

The bioavailability of biofortified UV-treated *A. bisporus*, was analyzed in thirty-eight volunteers by Stephensen et al. (2012). They have received treated mushrooms for 6 weeks and the results showed that the ergocalciferol formed after UV exposure was absorbed and showed

similar levels to the ones of the control group that have received ergocalciferol supplements.

Dietary factors contribute significantly to bone mineralization and femur density has been reported by Calvo et al. (2013) to show higher bone mineralization in rats fed with UVB irradiated mushrooms, when compared with control fed rats. These authors reported a fairly consistent average body weight among tested rats while plasma levels of creatinine were used as an indicator to analyze renal function/hypercalcemia. All tested samples showed no significant difference in plasma creatinine levels suggesting that no renal impairment was observed, even after weeks of high vitamin D mushroom diet. Histological studies were conducted on the tissues of the liver and spleen of the randomized fed rat, and the results also showed a negative tendency for the vitamin enriched mushroom diet to cause hyperparathyroidism.

The potential of UVC fortified *A. bisporus* to maintain vitamin D level was also reported by Bennett et al. (2013), and the authors exploited the *in vivo* potential of the above sample to prevent memory impairment and the incidence of neurological disorders.

To the authors best knowledge, Monaghan Mushrooms, Ireland is the only producer of UV treated mushroom as a novel food to increase vitamin D content in the EU, in agreement to Regulation (EC) No. 258/97 Article 4.2. It was initially assessed on the 10th July 2015 by the Food Safety Authority of Ireland (FSAI) before undergoing a 60 days comment period by member states of the European commission to ascertain its safety and potency. UV light treated commercially grown *A. bisporus* became the first irradiated mushroom specie to be approved and authorized as a source of vitamin D capable of yielding ≤ 10 µg/100 g fresh weight.

6. Concluding remarks and future perspectives

Several studies have been conducted measuring the effects of gamma irradiation and three subtypes of UV (UVA, UVB and UVC) on vitamin D₂ generation in several mushrooms. The method by which mushrooms have been treated, mostly with UV light, diverges significantly amongst studies not only by UV type and applied dose but also other factors, namely, origin and cultivar of the mushroom, time after harvest, positioning of the mushrooms to the light source, fresh or dried, among others. Trying to identify the irradiation conditions needed to maximize ergosterol conversion to vitamin D₂ in mushrooms still needs to be optimized based on some contradicting reports that have been presented so far.

The consumption of D₂ enriched mushrooms is maybe an exclusive case since irradiated mushrooms are one of the only products that would possibly contain not only D₂ but also its photoisomers. Recent studies have shown the huge potential that irradiated mushroom present as a source of vitamin D₂. The research studies have been mainly focused on commercial species such as *Agaricus* sp., *Lentinula edodes* and *Pleurotus* sp. The data showed a high rate of conversion from ergosterol to vitamin D₂ at short treatment time, which is required by the mushroom industry. Most studies have shown significant amount of vitamin D content in UV irradiated mushrooms as well as their efficacy to increase serum 25-hydroxyvitamin D levels and as such, because these mushrooms contain high bioactive and nutritional components, they can be utilized as multifunctional bio-based ingredient to reduce vitamin D deficiency as well as present other health promoting benefits.

There is still a huge knowledge gap as regards the interaction between the food matrix in which these irradiated mushrooms are embedded and their bioavailability. This report shows that irradiated mushrooms are an alternative source of vitamin D₂ whose large-scale production is commercially viable at low cost. Further studies still need to be conducted to allow for extraction, separation, recovery and purification of vitamin D₂ from irradiated mushroom with minimal process cost and high purity percentage to be utilized as fortified food ingredient or as bio-based supplements. Also, considering vitamin D can be degraded during the process of extraction and purification, another

potential possibility is to isolate ergosterol from mushrooms and then irradiate the isolated ergosterol for conversion into vitamin D₂.

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1 **Flour fortification for nutritional and health improvement: A review**

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3 Rossana V. C. Cardoso^{a,b}, Ângela Fernandes^a, Ana M. González-Paramás^b, Lillian Barros^a,

4 Isabel C.F.R. Ferreira^{a,*}

5
6 ^aCentro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança Campus de
7 Santa Apolónia, 5300-253 Bragança, Portugal.

8 ^bGrupo de Investigación en Polifenoles (GIP), Unidad de Nutrición y Bromatología, Facultad
9 de Farmacia, Universidad de Salamanca, Campus Miguel de Unamuno E-37007, Salamanca,
10 Spain.

11
12 *Corresponding author. Tel.+351 273 303219; fax +351 273 325405. E-mail address:
13 iferreira@ipb.pt (I.C.F.R. Ferreira).

16 **ABSTRACT**

17 Deficiencies of micronutrients, essentially vitamins and minerals, have mainly cause several
18 illnesses, especially in children and women worldwide. Governments and world organizations
19 have focused great efforts to address these shortcomings and improve the health of the
20 populations. This malnutrition can be combated by fortifying basic foods that are consumed
21 daily. Thus, flours, especially wheat, maize and rice, are a popular and common food in the
22 world's populations diet and are gaining considerable attention as a suitable vehicle for
23 micronutrient fortification. There are some studies that show the effectiveness of flour
24 fortification in combating micronutrient deficiencies and several diseases and the flour
25 fortification strategies are directed to meet nutritional and health needs of the populations. The
26 main points discussed in this review are food fortification, with great focus in flour
27 fortification, health benefits, and legislative issues. This review also highlights multifaceted
28 issues related to flour fortification to meet nutritional needs and to improve the health of
29 vulnerable populations.

30

31 **Keywords:** Flours; fortification, micronutrients; health benefits

32 **1. Introduction**

33 Vitamins and minerals are essential elements for the growth, metabolism and maintenance
34 of a healthy body and deficiencies of these nutrients have resulted in irreversible physical and
35 cognitive consequences, since these compounds play an important role in the normal
36 functioning of almost all organs. The FAO, IFAD, UNICEF, WFP & WHO, (2018) have
37 estimated that billions of people are deficient in different types of vitamins and minerals.
38 Revealing that in several countries, women and children suffer from severe deficiencies caused
39 by inadequate amounts of vitamins, amino acids and minerals, more precisely vitamin A,
40 vitamin D, iron, zinc and probably other micronutrients. It has also been related that several
41 micronutrient deficiencies cohabit and are more prevalent in developing countries (Black et al.,
42 2013; Blumfield, Hure, Macdonald-wicks, Smith, & Collins, 2013; Ramakrishnan, 2002). A
43 study in pregnant Nepalese women showed vitamin B6 deficiency and in villages in India
44 showed zinc, iron and folate deficiency (Jiang, Christian, Khatry, Wu, & West, 2005; Pathak,
45 Kapoor, Saxena, Kumar, & Gupta, 2004). Moreover, according to WHO, millions of pre-school
46 children worldwide and pregnant women suffer from vitamin A deficiency (WHO, 2009);
47 otherwise, it is also estimated that, globally, about million people are affected by iodine
48 deficiency (Li & Eastman, 2012).

49 Micronutrient deficiencies can lead to unfavourable health consequences, such as growth
50 problems, immune competence, mental and physical development, and poor reproductive
51 outcomes. It has also been reported to be related to an increased occurrence, severity and
52 mortality of infectious diseases such as malaria, diarrhoea, pneumonia, among others. These
53 deficiencies have numerous unfavourable results across all populations and age groups, with
54 children and women of reproductive age being more vulnerable (Black et al., 2013; Gibson &
55 Hotz, 2002).

56 Many strategies have been used to combat micronutrient deficiencies, such as exclusive
57 breastfeeding during babies first six months, control of parasitic infections, food fortification,
58 food diversification, and nutritional supplementation (Black et al., 2013; Hemery et al., 2018;
59 WHO, 2000). Particularly, fortification is a method of incorporating nutrients or non-nutritive
60 bioactive components into food products (Dwyer et al., 2015). Food fortification is one of the
61 methods that has been applied increasingly and addressed to all age groups, being widely used
62 to minimize micronutrient deficiency. By placing micronutrients in food products consumed
63 daily, it reaches target populations, from which daily dietary requirements of micronutrients
64 are scarcely satisfied (WHO & FAO, 2006). It is an impressive public health strategy with
65 interesting cost-effectiveness ratios and has the advantage of being installed in the usual dietary
66 patterns, without a major change in eating or health practices and is generally well accepted by
67 the populations (Berner, Keast, Bailey, & Dwyer, 2014; WHO & FAO, 2006).

68 As a staple food common in many countries, flour is often considered one of the most
69 suitable vehicles for multi-micronutrient fortification (Hemery et al., 2018). In addition,
70 fortified flours can be a major source of bioactive compounds, since flour can be fortified with
71 many micronutrients, reducing the risk of multiple deficiencies where they exist and improve
72 health benefits (Akhtar, Anjum, & Anjum, 2011; Oghbaei & Prakash, 2012; Serdula, 2010a,
73 2010b).

74 Wheat and maize flours can be fortified with several micronutrients and reduce vitamin,
75 mineral and other micronutrients deficiencies when identified as health public problem. It is
76 estimated that the amount of industrial flour fortified is 97% in the Americas, 21% in Southeast
77 Asia, 6% in Europe, 4% in the Western Pacific, 31% in Africa, and 44% in the Mediterranean
78 area in 2007-2008 (WHO, 2009). Efforts made by some countries to adopt mandatory
79 fortification of flours with some micronutrients are useful in combating these deficiencies.
80 These initiatives are an excellent example for other countries, considering their own programs,

81 however, when it comes to food fortification, appropriate legislation is needed to ensure impact
82 and safety, and the intended benefits to health (Luthringer, Rowe, Vossenaar, & Garretta, 2015;
83 Serdula, 2010a). Therefore, it is necessary to explore how nutrient fortification contributes in
84 the context of the current nutrient intakes. The aim of this review is to evaluate the impact of
85 flour fortification for nutritional and health improvement, detailing the prevalence of
86 micronutrient deficiencies, health consequences, and global trends and experiences regarding
87 flour fortifications.

88

89 **2. Food fortification**

90 Food fortification has a long history of use in some countries and has successfully controlled
91 micronutrient deficiencies (WHO & FAO, 2006). World Health Organization (WHO) and the
92 Food and Agriculture Organization of the United Nations (FAO), defines fortification as the
93 practice of deliberately increasing the amount of a vital micronutrient, i.e. vitamins, minerals,
94 amino acids among others, in a food regardless of whether the nutrients are initially in the food
95 before processing or not, with the intention of improving the nutritional quality of the food and
96 providing a public health benefit with little health risk (Whiting, Kohrt, Warren, Kraenzlin, &
97 Bonjour, 2016; WHO & FAO, 2006). This strategy can lead to relatively rapid improvements
98 in the number of people with food deficiencies within a population, since the benefits are
99 potentially large and consists of very cost-effective public health interventions (Dwyer et al.,
100 2015; WHO, 2009).

101 The advantages of fortification to public health has been demonstrated over time by the
102 scientific community, helping in the micronutrients deficiency correction and prevention or
103 reduction of these deficiencies in a population, having the capacity to improve nutritional status
104 and dietary intake and consequently improving eating habits and lifestyles (FAO et al., 2018;
105 WHO & FAO, 2006).

106 To have consolidated results in food fortification, the micronutrient used must have a good
107 availability of absorption by the organism, ideal organoleptic characteristics (not changing the
108 colour and the flavour of the fortified food), be easily accessible, should belong to the usual
109 diet of the population and have a good acceptance.

110 The most common fortified foods are cereals and cereal products, milk and dairy products,
111 fats and oils, tea and other beverages and various condiments such as salt, soy sauce, sugar and
112 infant formulas (Pacho, Spohrer, Mei, & Serdula, 2015; Whiting et al., 2016; WHO & FAO,
113 2006). In the early 1920s salt iodization was adopted in Switzerland and the United States of
114 America and today it is used in most countries. From the 1940s, fortification of cereal products
115 with thiamine, riboflavin, and niacin became a common practice. In Denmark, margarine was
116 fortified with vitamin A and in the United States milk with vitamin D. Also, one procedure that
117 significantly reduced the risk of iron deficiency anaemia in young children, was the
118 fortification of foods with iron (Vlaic et al., 2019; WHO & FAO, 2006). Nowadays,
119 fortification of foods with different types of micronutrients has spread throughout the world,
120 for example, folic acid fortification has spread in the Americas, a tactic adopted by the United
121 States and Canada and approximately 20 Latin American countries (FAO et al., 2018; Vlaic et
122 al., 2019).

123

124 2.2. *Types of fortification*

125 According WHO and FAO, food fortification includes different forms (**Fig. 1**): i) mass
126 fortification (fortify foods that are quite consumed by the general population), ii) targeted
127 fortification (fortify food targeted to specific population groups, *e.g.* small children), and iii)
128 market-driven fortification (ensure that food is available in the market). There are other types
129 of fortification such as household fortification (addition of micronutrients to homemade foods,
130 namely a fusion of supplements and fortification) and biofortification of staple foods (breeding

131 and genetic modification of plants to improve the content and the absorption of nutrients)
132 (WHO & FAO, 2006).

133

134 [Insert Figure 1]

135

136 Generalizing, mass fortification is often mandatory, targeted fortification is mandatory or
137 voluntary and depends on the importance for public health, and market-driven fortification is
138 always voluntary, but controlled by regulatory limits (Liyanage & Hettiarachchi, 2011;
139 Marques, Marques, Xavier, & Gregório, 2012; WHO & FAO, 2006).

140 According to Liyanage & Hettiarachchi, (2011), commercial and industrial fortification
141 includes available products, such as flour, rice, cooking oils, sauces, butter, etc. and the
142 procedure takes place throughout the manufacturing process. On the contrary, the
143 biofortification encompasses the creation of micronutrient cultures using traditional breeding
144 and/or biotechnology techniques (for example, transgenic "Golden Rice" containing higher
145 amounts of iron and significant levels of beta-carotene (Lonnerdal, 2003). There is also
146 microbial biofortification, which includes the use of probiotic bacteria, which ferment to
147 produce β -carotene in foods or directly in the intestine. For example, the use of animal feed
148 enriched with these bacteria (more specific lactic acid bacteria), so that meat, milk and
149 bioproducts are enriched with vitamin A (Liyanage & Hettiarachchi, 2011; Sasson, 2005).
150 Moreover, there is home fortification, where micronutrients obtained from packages or tablets
151 can be incorporated when cooking and or consumed in a homemade meal to fill micronutrient
152 deficiencies in the populations (Liyanage & Hettiarachchi, 2011; Marques et al., 2012; WHO
153 & FAO, 2006).

154

155 *2.3. Issues and challenges of nutrient deficiencies*

156 The greatest modifiable threat to global health and survival is represented by malnutrition,
157 especially among children in the world's poorest countries (Prentice et al., 2008). Given the
158 increasing demands of pregnant and lactating women and young children, this group are among
159 the most vulnerable to micronutrient deficiencies (Black et al., 2013).

160 WHO reports that worldwide approximately 19.1 million pregnant women are deficient in
161 vitamin A and it is estimated that 82% of these women have zinc intake inappropriate to meet
162 the normal needs of pregnancy; 100 million women of reproductive age have iodine deficiency;
163 190 million pre-school children are also deficient in vitamin A; iron deficiency is globally
164 found worldwide and approximately 1.62 billion people are anaemic (mostly among pre-school
165 children and pregnant women). Deficiencies of vitamin B6 and B12 have also been observed
166 in many countries (McLean, Benoist, & Allen, 2008; WHO, 2000, 2009).

167 Expansion of infectious diseases and mortality from pneumonia, malaria, diarrhoea and
168 measles are linked to micronutrient deficiency. Proper nutrition influences health status and
169 helps prevent many diseases, being responsible for providing nutrients (including vitamins,
170 minerals and others nutrients), and contributing to the proper functioning of the body
171 (Jakubowska & Staniewska, 2015). The implications of these shortcomings are not only limited
172 to health standards, but also have effects on the economy, through secondary physical and
173 mental disabilities and modified work productivity (Das, Salam, Kumar, & Bhutta, 2013).
174 Currently the approach to nutritional issues has undergone considerable changes.

175 Nowadays solutions are already known (such as fortified foods), but require some
176 challenges like political dedication, economic advancement, and scientific research, in order to
177 range a plausible solution. In this sense, several agencies worldwide are focusing their attention
178 on the fight against micronutrient deficiencies, since they are a short-term resolution (FAO,
179 2017; Prentice et al., 2008). Some challenges, such as choosing appropriate fortification
180 vehicles, having a good policy, targeting populations, avoiding excessive consumption in non-

181 target groups, and monitoring nutritional status, are among relevant factors in improving health
182 and will have the possibility to save billions of lives (Dwyer et al., 2015; Osendarp et al., 2018;
183 Prentice et al., 2008; WHO & FAO, 2006).

184

185 **3. Fortification of flour around the world**

186 Worldwide, millions of tons of flours are used for human consumption each year, they are
187 consumed as noodles, breads, pasta and other flour products (Pacho et al., 2015; Serdula,
188 2010b). In 2016, according to the Food Fortification Initiative, of the 250 metric tons of
189 industrially milled wheat flour, 26 metric tons of industrially milled maize flour, and 171 metric
190 tons of industrially milled rice, 34%, 57%, and 1%, respectively for each one of the flours was
191 fortified. Between 2016 and 2017, 87 countries have decided to fortify at least one of these
192 cereals (FFI, 2016, 2018; Marks et al., 2018).

193 The first cereal product to be largely fortified was wheat flour, and the first
194 recommendations about cereals fortification from the World Health Organization (WHO)
195 referred to maize and wheat flour. At the beginning of 2015, 83 countries demanded the
196 fortification of wheat flour, of which 14, required simultaneously the fortification of maize
197 flour. Most of these countries require fortification of wheat flour and maize with at least iron
198 and folic acid, excluding Australia that does not require fortification of flour with iron, and
199 Congo, Nigeria, Philippines, United Kingdom, and Venezuela do not require flour fortification
200 with folic acid (Pacho et al., 2015; WHO, 2009).

201 Today, just 86 countries worldwide have legislation requiring the fortification of at least one
202 grain of industrially milled cereal, depending on the country the flour is fortified with vitamins,
203 minerals, amino acids and other micronutrients. Within these, 85 countries fortify wheat flour
204 alone or in combination with other grains, taking away the country Papua New Guinea which
205 is mandated only for rice flour fortification (**Fig. 2**) (FFI, 2019c).

206

207 [Insert Figure 2]

208

209 Each country adopts its fortification standard varying the geographic region, income status,
210 food vehicle(s) and nutrient(s). The total number of nutrients fortified in flours are determined
211 according to the fortification pattern (**Table 1**) (GFDx, 2019).

212

213 [Insert Table 1]

214

215 Nutrition International directs and supports flour fortification efforts in developing countries
216 through various programs and partners such as: Global Alliance for Improved Nutrition
217 (GAIN), UNICEF, World Food Program (WFP), Food Fortification Initiative (FFI), United
218 State Centres for Disease Control and other organizations, working within different countries
219 (South Africa, Yemen, Iran, India, Pakistan, Nepal, Bolivia, Central and South America and
220 the Middle East, Indonesia, Nigeria, among others countries). They support and expand
221 fortification programs of flours with different micronutrients to combat the deficiency of these
222 nutrients and improve health disorders (Nutrition International, 2019).

223

224 *3.1. Types of fortified flours*

225 Since the early days, cereals and cereal products have been the main components of the
226 human diet throughout the world. Major cereal crops include wheat, rice, maize and barley.
227 Maize (or corn) is the most produced, but it is less important than wheat and rice, which are
228 the most important cereals for human nutrition (Preedy, Watson, & Patel, 2011).

229 Cereals are common staple foods since they are versatile, tasty, always available on the
230 market, accessible and culturally acceptable, are consumed every day by all age groups, being
231 a great fortification vehicle.

232 Cereals are commonly consumed after being processed by milling industries. When the
233 flours are fortified at the industrial level (flour production), the different produced food
234 products in the food preparation industries (e.g. bakeries) are easier to prepared. This makes
235 the cereal industrial unit much more powerful in this type of fortification, than the a bakery, or
236 even supplements (Johnson, Mannar, & Ranum, 2004; Pacho et al., 2015).

237 Wheat flour was the first cereal grain product to be extensively fortified and the
238 recommendations of the World Health Organization (WHO) is that fortification of wheat,
239 maize and rice flour are an opportunity to improve health (WHO, 2009). **Table 2** presents some
240 types of flours or derived products that have already been fortified and it is found that the work
241 on fortifications has been made mostly in wheat flour.

242

243 [insert Table 2]

244

245 In the market there are many flours that can be fortified or used to fortify other flours, the
246 following topics highlight the main produced and/or consumed cereals flours worldwide.

247

248 *3.1.1. Wheat flour*

249 The third largest cereal production in the world is wheat, after maize and rice, and is the
250 second most consumed by the populations after rice. Wheat flour is considered one of the most
251 appropriate vehicles for multi-micronutrient fortification because of its worldwide
252 consumption and given the high consumption of bread and pasta worldwide (Awika, 2011;
253 Peña-Rosas, Field, Burford, & De-Regil, 2014).

254 The industrial processed fortification of wheat flour, when properly implemented, is an
255 effective, simple and inexpensive strategy to provide vitamins and minerals to the world
256 population, thereby improving the nutritional quality of food supply and providing a public
257 health benefit (Cardoso et al., 2019; FFI, 2019b; Nuria Mateo Ansón, 2010; Peña-Rosas et al.,
258 2014).

259 Globally the effort to begin fortifying wheat flour was launched during the 1940s as a way
260 to improve the health of populations (Bishai & Nalubola, 2002). Wheat flour has been fortified
261 with different micronutrients such as iron, folic acid, B-complex vitamins, vitamin A, D and
262 C, zinc, calcium among others, in different parts of the world (Akhtar et al., 2011; FFI, 2019b,
263 2019c; Johnson et al., 2004; Jungjohann et al., 2015; Marks et al., 2018; Pacho et al., 2015;
264 Peña-Rosas et al., 2014; Rebellato et al., 2017; Serdula, 2010b; WHO, 2009).

265

266 3.1.2. Rye flour

267 Rye is essentially a European cereal and globally the largest production is also centred in
268 Europe. In the baking industry, it is the second most used cereal worldwide, after wheat. In the
269 Middle East, rye is developed as a secondary crop, it has a great wintering capacity and high
270 tolerance to drought, cold, and develops well on low fertility soils. With these characteristics,
271 it becomes a low-risk and economical crop (Cardoso et al., 2019; Carena, 2009; Kaminski,
272 Silva, Nascimento Júnior, & Ferrão, 2011; Redant, Buggenhout, Brijs, & Delcour, 2017).

273 The interest in rye has been increasing because of its nutritional profile, due to its high levels
274 in dietary fibre. Beside its excellent nutritional quality, it also has many health benefits,
275 decreasing the absorption of triglycerides and blood cholesterol levels; reduction in blood
276 glucose; prevention of constipation, and prebiotic effects, among others. These characteristics,
277 allow the classification of rye products as functional foods, proving a relationship between diet
278 and health (Carena, 2009; EFSA Panel on Dietetic Products Nutrition and Allergies (NDA),

279 2011; Grossmann & Koehler, 2016; Kaminski et al., 2011; Moniz et al., 2018; Preedy et al.,
280 2011; Redant et al., 2017). There are some studies that have presented the fortification of rye
281 flour with cellulose fibre (Fuckerer, Hensel, & Schmitt, 2015, 2016) and with folic acid (Gujska
282 & Majewska, 2005) to increase its health benefits.

283

284 3.1.3. *Rice flours*

285 Rice is the staple food for more than half the world's population and is the main cereal in
286 many developing countries. In most Asian countries, rice provides between 50% and 80% of
287 the caloric intake. In South and Southeast Asia, most women and children are anaemic and the
288 nutritional value of rice has a significant impact in their health (FFI, 2019e; Preedy et al., 2011;
289 Sasson, 2005; WHO, 2018a).

290 Such as wheat and maize flour fortification, rice fortification with vitamins, minerals and
291 other micronutrients is a public health opportunity, in order to prevent deficiencies in these
292 compounds and severe diseases (de Pee, 2014; FFI, 2019e; Forsman, Milani, Schondebare,
293 Matthias, & Guyondet, 2014).

294 According to GFDx, (2019) some countries like Costa Rica, Nicaragua, Panama, Papua New
295 Guinea, Philippines, United States of America, Bangladesh, India and Venezuela fortify rice
296 with zinc, vitamin A, vitamin B1, B2, B3, B6, B9, and B12, iron, calcium, and vitamin D.

297 However, rice is very difficult to fortify because most of the grains are not processed.
298 Therefore, the alternative is to fortify rice flour instead of rice grain, which can be fortified
299 with the same methods used for the other flours. In Sri Lanka, rice flour is processed, and its
300 cost is approximately equal to wheat flour, leading to a growing interest by the population.
301 Fortification of rice flour has been proposed in countries such as the Philippines and Guyana,
302 which have a very significant consumption of this product (Hettiarachchi, Hilmers, Liyanage,
303 & Abrams, 2004; Johnson et al., 2004; Marks et al., 2018).

304

305 *3.1.4. Maize flours*

306 Maize is grown all over the world and millions of tonnes are produced, actually, different
307 types of maize, varying colours, are cultivated globally. The United States, China, India,
308 France, Brazil, Argentina, and Indonesia are the main maize producing countries. It is also the
309 main food preferred by billions of consumers in sub-Saharan Africa and Latin America. Tons
310 of maize flours are milled annually, and its consumption is performed in many forms. After
311 wheat and rice, maize is the third most frequently consumed cereal in the world (Sasson, 2005;
312 WHO, 2009).

313 Around 65% of industrially processed maize in the world is fortified (FFI, 2018, 2019d) and
314 this fortification has been practiced for several years in many countries, where this ingredient
315 is used for the preparation of many common dishes. Between the African and American
316 continent about 16 countries have mandatory legislation to fortify maize flour (Enzama, Afidra,
317 Johnson, & Verster, 2017; FFI, 2019d). Maize flour can be fortified with several
318 micronutrients, such as iron, folic acid, vitamin A and B, zinc, among others; some of them are
319 used to replace nutritional contents and others are used to prevent deficiencies of certain
320 micronutrients relevant to health (GFDx, 2019; WHO, 2016).

321

322 **3.2. Flour fortification as a supplement**

323 Supplementation is a set of substances (vitamins, minerals or other nutrients) that serve to
324 complement your diet when the natural intake of these components is insufficient; this term is
325 used to describe large doses of micronutrients, usually in the form of capsules, tablets or syrups.
326 It has the advantage of offering the ideal amount of specific micronutrients in an absorbable
327 form and is often the fastest way to combat nutrient deficiency (Bailey, Fulgoni, Keast,
328 Lentino, & Dwyer, 2012; Dwyer et al., 2015; WHO & FAO, 2006).

329 Worldwide, supplementation programs have been widely used to provide iron, folic acid,
330 vitamin A among other nutrients to pregnant women, infants, children under 5 years, among
331 other groups of individuals (Datta & Vitolins, 2016). However, as for the more water-soluble
332 vitamins and minerals, the populations or target populations need to consume the supplements
333 more often for the control and combat for this type of micronutrient deficiencies. Flour
334 fortification with micronutrients (**Table 2**) has been widely implemented worldwide, due to
335 the extensive consumption of these foods in different forms and for being a cost-effective and
336 sustainable strategy (Datta & Vitolins, 2016; Marks et al., 2018; Pacho et al., 2015).

337 As it has been described earlier, there are different types of micronutrients that can be used
338 to supplement food, the following topics highlight the major micronutrients used to fortify
339 flours worldwide.

340 *3.2.1. Vitamins*

341 Vitamins are essential nutrients for growth and maintenance of life, since our organism does
342 not have the capacity to synthesize them, we must guarantee their ingestion through food or
343 supplements. These compounds are present in very small quantities and are linked to various
344 processes related to the transfer and storage of energy, protection and strengthening of the
345 body's defences, protection against various diseases, bone and tissue formation, formation and
346 maintenance of cellular structure and functions, visual system, activity of other nutrients, etc.
347 (Das et al., 2013; Johnson et al., 2004; Verma, 2015).

348 It has been reported the use of fortified flours with different vitamins to reduce nutritional
349 deficiencies and to prevent and control various diseases, for example, rice has been fortified
350 with vitamin A, to improve the iron status and vitamin A nutrition of populations, while maize
351 flour has been fortified with B vitamins, contributing in the elimination of beriberi and pellagra
352 in many countries, vitamin D has been used to fortified chapattis consumed by residents of
353 private sunbeds in Romania, showing higher serum vitamin D levels and significantly increased

354 bone density (Akhtar et al., 2011; Allen, Dangour, Chalabi, & Tedstone, 2015; Das et al., 2013;
355 Johnson et al., 2004; Peña-Rosas et al., 2014; Ritu G & Gupta, 2015; Serdula, 2010a; WHO,
356 2016, 2018a).

357 3.2.2. *Minerals*

358 Minerals (sodium, potassium, calcium, iron etc.) are nutrients important to the body and are
359 responsible for the proper functioning of metabolism. They favour the balance and maintenance
360 of basic bodily functions such as conduction of nerve impulses, cellular activity and
361 maintenance, and structural functions in the body, nail, tooth, and bone formation (Akhtar et
362 al., 2011; Peña-Rosas et al., 2014; WHO & FAO, 2006; WHO, 2009). The different essential
363 minerals are classified according to their concentration in the body and the requirement in the
364 diet. The differences in the required mineral quantity has a great influence on the cost and on
365 other aspects of mineral fortification of the flours. The main minerals for our organism are
366 calcium, phosphorus, sodium, potassium, chloride and magnesium, while those that are needed
367 in smaller quantities are iron and zinc, and the trace elements needed are iodine, copper and
368 selenium (BNF, 2019; Peña-Rosas et al., 2014; Serdula, 2010a; WHO, 2016).

369 There are several studies demonstrating the use of minerals to strengthen different types of
370 flours in order to reduce their deficiencies and prevent/control some diseases. Iron deficiency
371 leads to anaemia and is one of the most prevalent public health problems in the world, and for
372 example, in Iran, the fortification of flour with iron is considered a strategy to combat this
373 deficiency (Blanco-rojo & Vaquero, 2018; Sadighi et al., 2008; WHO & FAO, 2006). Zinc is
374 being added to maize flour in South Africa and Mexico and in wheat flour in Mexico, South
375 Africa, Central Asia and Indonesia to address the problem of their deficiency (delayed growth
376 and increased risk of disease), especially in children (Johnson et al., 2004; WHO & FAO, 2006;
377 WHO, 2017). The fortification of calcium in whole wheat flour in Asia has been performed in

378 order to increase the amount of calcium in the body and to combat some diseases, such as
379 osteoporosis (Muhammad et al., 2016; WHO, 2017).

380 3.2.3. *Other nutrients*

381 There are many other micronutrients with health benefits that can be used to fortify flours.
382 Fibre deficiency has a negative impact on quality of life and can lead to serious health
383 problems. Food fibres can be classified as soluble and insoluble fibres. Both types of fibre have
384 several health benefits, including maintaining intestinal integrity and overall health, lowering
385 blood cholesterol levels, controlling blood sugar levels, and providing non-caloric volume that
386 may help in the loss of weight ratio by replacing caloric components. In this sense, fortification
387 of flours with fibre can bring many health benefits (Anderson et al., 2009; Salmean, Zello, &
388 Dahl, 2013).

389 Proteins belong to a category of compounds that are essential to life through its specific
390 action. They are important for cell walls, muscles, blood, hair, internal organs, such as the heart
391 and brain, among others, also for hormones, enzymes and antibodies, and replacement of waste
392 cells. Essential amino acids can only be acquired through food, because the body cannot
393 produce them by itself. One advantage may be the fortification of products with plant proteins,
394 for example, the fortification of wheat flour with chickpeas, amaranth, quinoa, lentils and
395 mushroom powder, etc. (Preedy et al., 2011; Prodhan, Linkon, Al-Amin, & Alam, 2015).

396 Essential fatty acids are also an important nutrient, especially for the cardiovascular system.
397 Essential fatty acid deficiency is uncommon, but include scaly dermatitis, alopecia,
398 thrombocytopenia and, in children, intellectual disability. Diets in the West are deficient in
399 omega-3 fatty acids. Although there is not much mentioned about fortified flours with essential
400 fatty acids, there are products derived from flour that have been incorporated with these
401 compounds (*e.g.*: breads fortified with oils containing omega-3). Nevertheless, there are other

402 products that have been fortified with essential fatty acids that include meat, oil, butter, jelly,
403 different types of sauces, etc. (Preedy et al., 2011; Vlaic et al., 2019).

404

405 **3.3. Flour fortification with bioactive compounds**

406 The usefulness of food fortification is mostly given to bioactive compounds such as
407 vitamins, minerals, phenolic compounds, essential amino acids, dietary fibre, among other
408 group of compounds. By fortifying day-to-day foods we can guarantee the necessary
409 micronutrients ingestion in order to stay healthy (Del Pino-García, Rico, & Martín-Diana,
410 2018).

411 There are many natural sources with bioactive compounds that can be used to fortify flours,
412 a good example could be mushrooms, which can provide relevant amounts of B vitamins,
413 ergosterol (precursor of vitamin D2) and minerals such as selenium, potassium, copper and
414 zinc. Mushrooms are nutritious foods and an excellent source of bioactive compounds, and
415 they have already been used to fortify wheat flour in order to increase the availability of
416 different micronutrients (Cardwell, Bornman, James, & Black, 2018; Prodhan et al., 2015).
417 Moreover, wheat and maize flours have been fortified with egg shell powder as a source of
418 calcium, with finger millet (excellent source of bioactive compounds), due to its high
419 concentration in fibres, minerals, protein, and calcium (Naves, Fernandes, Prado, & Telxeira,
420 2007; Oghbaei & Prakash, 2012; Preedy et al., 2011).

421 Several non-wheat cereal flours are also used for substitution of a portion of wheat flour to
422 improve the micronutrients content in breads, *e.g.*: rice, maize, amaranth, barley, oat, rye,
423 emmer, buckwheat, spelt and sorghum. There are also other natural source that can be used to
424 fortify flour and develop different types of products, as they contain a large number of bioactive
425 compounds, such as: chicken bones, quinoa, apple pomace, moringa, okra flour, banana and
426 mango flours, chempedak, soy proteins, sweet potato, lupine kernel fibre, brewer's spent grain,

427 among others; these natural products, help to decrease micronutrient deficiency, and
428 control/prevent several diseases (Berner et al., 2014; Muhammad et al., 2016; Oyeyinka &
429 Oyeyinka, 2018; Preedy et al., 2011).

430

431 **3.4. Health benefits of fortified flour**

432 Flour fortification in the field of public health is a very attractive tactic and has the advantage
433 of reaching massively a risk populations through existing food distribution systems, without
434 widely changing existent patterns of consumption (Das et al., 2013; Preedy et al., 2011).

435 The benefits of fortification of flour or derivative flour products (**Table 2**), act in a positive
436 way over the entire life cycle of the population, especially in children and pregnant women,
437 preventing the birth of children with intellectual disabilities or malformations or deficiencies
438 (Vlaic et al., 2019). It is one of the most efficient ways of combating malnutrition and
439 controlling various diseases linked to vitamin or mineral deficiency (Santos & Pereira, 2007;
440 Vlaic et al., 2019). For example vitamin A fortified cereal flours can be effective to reduce this
441 vitamin deficiency (Ranum, 2001); as previously described, flour fortification with iron is a
442 strategy to combat iron deficiency in Iran (Sadighi et al., 2008); folic acid fortification
443 programs in Chile have resulted in significant declines in the occurrence of pregnancies
444 affected by neural tube defect (Berry, Bailey, Mulinare, Bower, & Dary, 2010).

445 If consumed regularly, many are the health benefits that can be gained from fortified flour
446 consumption, especially because it can help to maintain body reserves of nutrients in a more
447 efficient way than supplements. Since fortified flours provide nutrients that are similar to those
448 provided by an effective and balanced diet, fortified foods will contain “natural” amounts of
449 nutrients and this does not happen with supplements by its own (Dwyer et al., 2015; Preedy et
450 al., 2011; Whiting et al., 2016).

451 Flour fortification has the potential to improve the nutritional status of a large portion of the
452 population, regardless of the social class, as they are a staple food widely distributed and
453 consumed worldwide, and, this fortification does not require changes in existing food patterns
454 of populations, being a very cost-effective method. It is also more efficient in reducing the risk
455 of multiple deficiencies that can result from deficits in food supply or a poor diet. It is a major
456 benefit primarily for women of childbearing age, during periods of pregnancy and lactation
457 (increasing the rate of vitamins and minerals in breast milk and reducing the use of
458 supplements) that need adequate amounts of micronutrients, as well as growing children, which
459 need nutrients daily for growth and development (Berner et al., 2014; Das et al., 2013; Datta
460 & Vitolins, 2016; Dwyer et al., 2015; FFI, 2018; Johnson et al., 2004; Preedy et al., 2011).
461 Moreover, it is also usually possible to add one or more micronutrient fortification (multi-
462 nutrient fortification) without adding significantly to the total cost of the flour at the point of
463 manufacture (Das, Salam, Kumar, Lassi, & Bhutta, 2014; Dwyer et al., 2015; Whiting et al.,
464 2016; WHO, 2009).

465

466 **4. Legislation and major issues regarding quality control**

467 The main objectives of food law are to protect consumer's health, facilitate trade, and protect
468 consumer's fraud. In the case of fortified meals, the population should be protected from
469 consuming toxic levels of micronutrients or nutritionally ineffective. Legislations are applied
470 to require appropriate control in the fortification process to ensure that micronutrient levels are
471 adequately within acceptable limits. The legislation also serves to prevent fortification with
472 nutrients from unsafe or nutritionally unnecessary products. (Marks et al., 2018).

473 The wide variety in each country's particularities and the public health goals worldwide have
474 resulted in the development of many different approaches in the regulation of fortified foods.
475 In most countries, fortification standards (in this case flour) are established by law or through

476 cooperative arrangements. In some countries the fortification of food is achieved without any
477 form of management guidance or quality control. Quality control is performed to evaluate if
478 the fortified product is following the established technical standards, using objective and
479 measurable indicators. It typically consists of collecting samples of fortified food, depending
480 on the production system, and determining its micronutrient content, since it is important to
481 routinely collect and analyse the samples in order to verify and control whether the technical
482 standards are being met. Quality control focuses on purely public health optics and, in this case,
483 concentrates mainly on indicators and criteria that are relevant to the food fortification process
484 (Johnson et al., 2004; Nestel & Nalubola, 2002; Verma, 2015; WHO & FAO, 2006).

485 The management tools available in order to establish an appropriate level of control over
486 food fortification are food laws and related measures, as well as a broader food control system.
487 This management has the function of protecting public health, being generally recommended
488 that all forms of fortified foods be adequately regulated, to ensure that food fortification is safe
489 and effective for certain population groups, mostly those with micronutrient deficiency risks
490 (Marques et al., 2012; WHO & FAO, 2006). Food fortification techniques follow the principles
491 established by the Codex Alimentarius to ensure food security (FAO & WHO, 2015). Any
492 legislation on food fortification should also include the World Trade Organization (WTO)
493 Agreement on the Application of Sanitary Measures (SPS) and the WTO Agreement on
494 Technical Barriers to Trade (TBT), which have added new values to standards, guidelines,
495 Codex codes and recommendations (FAO & WTO, 2017; Orriss, 1998; WHO & FAO, 2006).

496 In the legal context, fortification can be classified as mandatory or voluntary, which refers
497 to the level of obligation imposed on producers of fortified foods to comply with the
498 government's purposes evidenced by law (Datta & Vitolins, 2016; WHO & FAO, 2006).
499 Mandatory fortification is where a manufacturer is legally obliged to add one or more
500 micronutrients to a specific food or food product. Compulsory fortification can reach the

501 general population or a specific group, depending on the consumption criterion of that food.
502 For example, fortification of a staple food, such as flours, would increase consumption of a
503 micronutrient in the general population, while fortification of complementary infant foods
504 would only increase the intake of micronutrients from a target group. In controversy voluntary
505 fortification occurs when a producer freely decides to fortify food, however, it varies depending
506 on the micronutrient and the socio-political and legal environment present. Governments are
507 advised to impose an appropriate degree of control over voluntary fortification, it should not
508 only be consistent with general regulatory objectives but should also consider the General
509 Principles of Codex for the addition of essential nutrients to food. Voluntary fortification is
510 moved by a desire of the industry to promote trade and the consumer to increase consumption
511 of micronutrients as a means to gain health benefits (FAO & WTO, 2017; Hennessy, Walton,
512 & Flynn, 2013; Marks et al., 2018; WHO & FAO, 2006).

513 In general, and according to the FFI, for wheat flour 82 countries have mandatory
514 fortification and 10 countries have voluntary fortification, for maize flour 16 countries have
515 mandatory fortification and 4 countries have voluntary fortification, while for rice 7 countries
516 have mandatory fortification and 11 countries have voluntary fortification. The list of countries
517 that have mandatory or voluntary fortification can be consulted in FFI, (2019a) and WHO,
518 (2018b).

519

520 **5. Concluding remarks and future perspectives**

521 Micronutrient deficiency is a serious problem that has adverse consequences and leads to
522 economic and health tragedies for populations that are in risk around the world. Food
523 fortification programs are extremely important to overcome and ensure the correct intake of
524 micronutrients by these groups of population, so fortification of food is seen as an excellent
525 methodology in the correction of nutritional deficiencies.

526 In general, flours are a potential vehicle for fortification, because of their high consumption
527 worldwide. Nevertheless, the success of flour fortification is based on the correct evaluation of
528 the prevalence of micronutrient deficiency, political opinions and their implementation,
529 selection of fortifiers, levels of fortification, usual level of flour consumption and products
530 derived from that staple food, fortification of other food vehicles, feasibility, cost and
531 acceptability studies. Systems of monitoring and inspection of fortified products are also
532 necessary.

533 Despite being fortification a mandatory priority for the international corporations to
534 eliminate micronutrient deficiency, it remains a problematic issue in several countries. Thus,
535 food fortification should be included into the national health and nutrition plans of each
536 country, as a strategy to overcome micronutrients deficiency. These fortifications should
537 include the incorporation of different micronutrients in the staple foods, in order to meet
538 different purposes of millions of people around the world, being considered a low-cost,
539 effective strategy with a low toxicity risk.

540 Moreover, the studies that have been performed until now, clearly show the high potential
541 of fortifying flours as an alternative to combat micronutrient deficiencies, and consequently to
542 control and/or eliminate various diseases, thus bringing nutritional benefits and improving
543 health in general.

544

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554

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557

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667 processed food products of animal origin, plus bread and panification products (ID 1193,
668 1194), herbal yeast plasmolysate (ID 1815, 1816), apple polyphenols (ID 2713), rye flour
669 (ID 1266), tomato juice (ID 1202), whey protein and alpha-lactalbumin (ID 424, 430, 432,
670 725, 1433), “brocco shoots”, “broccoli sprout powder” and “*Brassica oleracea* var. *italica*
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Table 1

Nutrients in Food Fortification Standards of some country. (GFDx, 2019; WHO, 2013)

Country	Food Vehicle	Nutrients
Afghanistan	Wheat Flour	Vitamin B12, Folate (B9), Iron, Zinc
Bolivia	Wheat Flour	Niacin (B3), Riboflavin (B2), Thiamine (B1)
Costa Rica	Wheat Flour	Folate (B9), Iron, Niacin (B3), Riboflavin (B2), Thiamine (B1)
	Rice	Folate (B9), Vitamin B12, Niacin (B3), Selenium, Thiamine (B1), Vitamin E, Zinc
	Maize Flour	Folate (B9), Iron, Niacin (B3), Riboflavin (B2), Thiamine (B1)
Tanzania	Wheat Flour	Vitamin B6, Vitamin B12, Folate (B9), Iron, Niacin (B3), Riboflavin (B2), Thiamine (B1), Vitamin A, Zinc
	Maize Flour	Vitamin B12, Folate (B9), Iron, Zinc
Colombia	Wheat Flour	Calcium, Folate (B9), Niacin (B3), Riboflavin (B2), Thiamine (B1)
Philippines	Wheat Flour	Iron, Vitamin A
	Rice	Iron
Nigeria	Wheat Flour	Zinc, Vitamin A, B6, B12, Folate (B9), Iron, Niacin (B3), Riboflavin (B2), Thiamine (B1)
	Maize Flour	Zinc, Vitamin A, Thiamine (B1), Riboflavin (B2), Niacin (B3), Iron, Folate (B9), Vitamin B12 And B6
Nepal	Wheat Flour	Folate (B9), Iron, Vitamin A
United States of America	Maize Flour	Calcium, Folate (B9), Iron, Niacin (B3), Riboflavin (B2), Thiamine (B1), Vitamin D
	Rice	Thiamine (B1), Vitamin D

Table 2

Flour fortification with micronutrients and their health benefits.

Flours	Micronutrients ^a	Health benefits ^b	References
Wheat	Minerals	Increases the content or bioavailability of a specific micronutrients	(Chiplonkar et al., 1999) (Gujska & Majewska, 2005) (Towo, E., Mgoba, C., Ndossi, D. G., & Kimboka, 2006) (Butt, Arshad, Alam, & Nadeem, 2007) (Gigante & Victora, 2007) (Naves et al., 2007) (Akhtar, Anjum, Rehman, Sheikh, & Farzana, 2008) (Pachón, Stoltzfus, & Glahn, 2008) (Sadighi et al., 2008) (Anton, Lukow, Fulcher, & Arntfield, 2009) (Škrbić, Milovac, Dodig, & Filipčev, 2009)
	Vitamins	Prevention/reduction/ improvement of the incidence of anaemia or specific mineral deficiency	(Berry et al., 2010) (Kaminski et al., 2011)
	Proteins	Functional food with therapeutic protective effects against diabetes and cardiovascular diseases	(Barbosa, Taddei, Palma, Ancona-Lopez, & Braga, 2012) (Buzzo et al., 2012) (Fares & Menga, 2012) (Erukainure, Ebuehi, Adeboyejo, Aliyu, & Elemo, 2013) (Danza et al., 2014) (Peña-Rosas et al., 2014)
	Fibre	Effect on the prevention of colon cancer	(Gawlik-Dziki et al., 2015) (Pacho et al., 2015) (Prodhan et al., 2015)
	Fatty acids	Antioxidants activity Prevention of civilization diseases (<i>e.g.</i> : hypertension, obesity)	(Muhammad et al., 2016) (Sui, Zhang, & Zhou, 2016) (Bolarinwa, Aruna, & Raji, 2017) (Kurek, Wyrwicz, Karp, & Wierzbicka, 2017) (Rebellato et al., 2017) (Reshmi, Sudha, & Shashirekha, 2017) (Aranibar et al., 2018) (Armellini et al., 2018) (Benjakul & Karnjanapratum, 2018) (Emaleku, Omueti, & Emaleku, 2018) (Garrod et al., 2018) (Hemery et al., 2018)

			(Jakubczyk, Świeca, Gawlik-Dziki, & Dziki, 2018) (Moniz et al., 2018) (Rebellato, Klein, Wagner, & Lima Pallone, 2018) (Sheikholeslami, Karimi, Komeili, & Mahfouzi, 2018) (Bryszewska et al., 2019)
Rye	Fibre Vitamins	Increases the content of a specific micronutrients	(Gujska & Majewska, 2005) (Fuckerer et al., 2015) (Fuckerer et al., 2016)
Maize	Minerals Vitamins	Increases the content or bioavailability of a specific micronutrients Prevention/reduction/ improvement of the incidence of anaemia or specific mineral deficiency Antioxidants activity	(Chiplonkar et al., 1999) (Hansen, Bæch, Thomsen, Tetens, & Sandström, 2005) (Towo, E., Mgoba, C., Ndossi, D. G., & Kimboka, 2006) (Andang'o et al., 2007) (Gigante & Victora, 2007) (Naves et al., 2007) (Bilgi Boyaci et al., 2012) (Buzzo et al., 2012) (Pacho et al., 2015) (Giménez, Drago, Bassett, Lobo, & Sammán, 2016) (Kumar, Xavier, Lekshmi, Balange, & Gudipati, 2018)
Rice	Minerals Vitamins Fibre	Increases the content or bioavailability of a specific micronutrients Antioxidants activity	(Hettiarachchi et al., 2004) (Sharif, Rizvi, & Paraman, 2014) (Igoumenidis, Lekka, & Karathanos, 2016) (Kumar et al., 2018) (Mounjouenpou, Pauline Eyenga, Kamsu, Kari, Patience Bongseh Ehabe, & Ndjouenkeu, 2018)
Millet	Minerals Vitamins	Increases the content or bioavailability of a specific micronutrients	(Chiplonkar et al., 1999) (Tripathi & Platel, 2010) (Tripathi, Chetana, & Platel, 2010) (Tripathi & Platel, 2011)

			(Tripathi, Platel, & Srinivasan, 2012) (Tripathi & Platel, 2013) (Adetola, Oluyimika, Kruger, White, & Taylor, 2019)
Sorghum	Minerals Proteins Fatty acid Amino acids	Increases the content or bioavailability of a specific micronutrients Increases the content of protein and fatty acid Antioxidant activity	(Chiplonkar et al., 1999) (Tripathi et al., 2010) (Tripathi & Platel, 2011) (Tripathi et al., 2012) (Tripathi & Platel, 2013) (Abdualrahman et al., 2019)

^a in this section, the micronutrients have been used directly or they are derived from other foods that are used to fortify the flours.

^b some of the health benefits that can be found in referenced works.

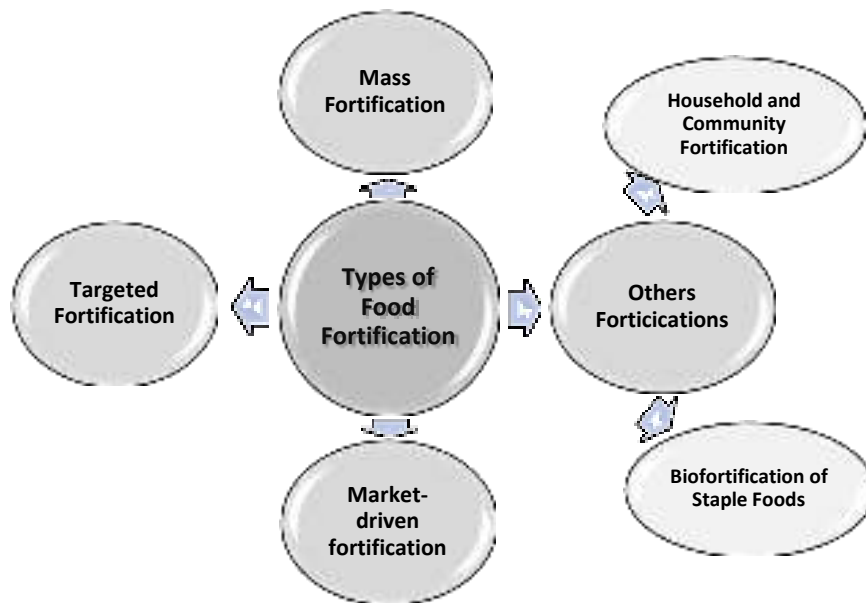


Fig. 1. Different types of food fortification.

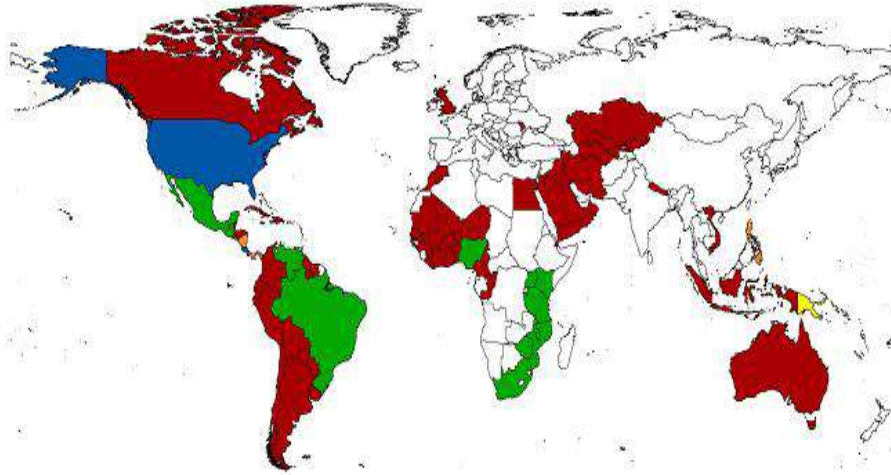


Fig. 2. Mandatory fortification of different types of flour around the world:

● 65 countries - **wheat flour**, ● 14 countries - **wheat flour and maize flour**, ● 4 countries - **wheat flour and rice** (Nicaragua, Panama, Philippines, Solomon Islands), ● 2 countries - **wheat flour, maize flour and rice** (Costa Rica and the United States), ● 1 country - **rice** (Papua New Guinea) (FFI, 2019).



ValorNatural – Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar.

Entregável nº 5.2.1.

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Responsável: IPB-CIMO

Nome do Documento: Folheto descritivo das condições que garantem a maior estabilidade dos ingredientes bioativos

Histórico de Revisão

Revisão	Data	Parceiros Envolvidos	Descrição



Lista de Autores

Ângela Fernandes

Sandrina Heleno

Sumário

Este entregável resume a estabilidade dos ingredientes bioativos ergosterol e vitamina D2 a diferentes pH, variações de temperatura, exposição à luz e incorporação em matrizes lipofílicas e hidrofílicas.

Índice

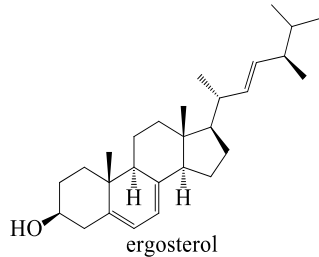
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1. Identificação

<i>Deliverable</i>	5.2.1. Folheto descritivo das condições que garantem a maior estabilidade dos ingredientes bioativos
Tipo de <i>deliverable</i>	Folheto
Nível de disseminação	Público
PPS	5. Bioativos naturais

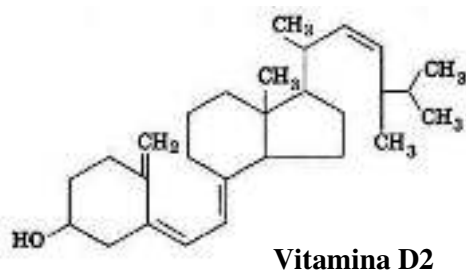
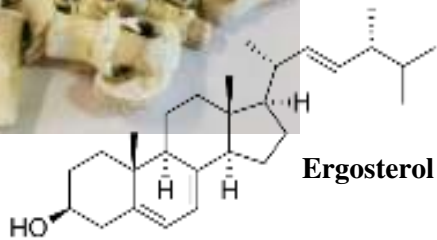
2. Informação

Ergosterol



- Solúvel em solventes apolares como o clorofórmio, azeite, ou óleo até 10 mg/mL.
 - Solúvel em solventes polares como o etanol, recorrendo a temperatura (50 °C) de um máximo de 2 mg/mL.
 - Estável a diferentes pH, suportando um intervalo de pH de 4-8.
 - Estável a temperaturas até 250°C sem decomposição.
 - Fraca estabilidade quando exposto à luz, uma vez que a exposição à luz promove a sua transformação em vitamina D2, pelo que deve estar ao abrigo da luz.
 - Fácil incorporação em alimentos lipofílicos, sendo resistente a variações de pH, temperatura, apresentando também estabilidade quando exposto a processos de fermentação.
 - A presença de outras moléculas como compostos (ex. compostos fenólicos) nos alimentos e a presença de microrganismos (quer contaminantes como bolores, quer benéficos como bactérias fermentativas), não prejudica a estabilidade do ergosterol nem a sua capacidade bioativa.
 - Difícil incorporação em meio aquoso devido à falta de homogeneidade da molécula no produto pela difícil dissolução, sendo necessário o recurso a técnicas de encapsulação.
-

Radiação Ultravioleta



- Vitamina lipossolúvel.
- Relativamente estável após incorporação nos alimentos que têm caráter lipofílico.
- A fermentação, a cozedura e o armazenamento têm pouco efeito na sua atividade.
- Relativamente robusta.
- Estável a pHs na gama de 4,5-8.
- Estável durante a cozedura até 200 °C.
- Degrada com a exposição à luz sendo necessária a sua estabilização por técnicas de encapsulação.



ValorNatural – Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar.

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Data de Submissão: 28/02/2019

Responsável: UP (FEUP-LSRE)

Nome do Documento: Relatório com a lista dos requisitos de funcionamento do sistema laboratorial de extração SFE-CO₂.

Histórico de Revisão

Revisão	Data	Parceiros Envolvidos	Descrição



Lista de Autores

José Carlos Lopes

Madalena Dias

Cláudia Almeida

Isabel Martins

Sumário

Este entregável apresenta os requisitos de funcionamento do sistema laboratorial de extração SFE-CO₂.

Índice

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1. Identificação

<i>Deliverable</i>	6.1.1 Relatório com a lista dos requisitos de funcionamento do sistema laboratorial de extração SFE-CO ₂ .
<i>Tipo de deliverable</i>	Relatório
<i>Nível de disseminação</i>	Confidencial
<i>PPS</i>	6. Inovação em processos de extração, refinação e técnicas de conservação.

2. Informação

No âmbito do projeto “Valor Natural” pretende-se construir um sistema laboratorial de extração SFE-CO₂ que seja versátil e facilmente adaptável a diferentes matérias-primas provenientes do setor agroalimentar (nomeadamente, a beterraba, a cereja, os cogumelos, entre outros). O sistema de extração será um equipamento inovador e versátil que deverá permitir a recirculação do dióxido de carbono durante a fase extração a pressão constante.

De modo a definir as especificações da instalação de extração em fluido supercrítico foi feita uma análise exaustiva às diferentes matérias-primas envolvidas no projeto. A Tabela 1 resume as diferentes condições de operação, nomeadamente a temperatura e pressão usadas no processo de extração usando CO₂ supercrítico.

Tabela 1 - Condições de operação do processo de extração com CO₂ supercrítico para diferentes matérias-primas.

Matéria-Prima	Nome Científico	Condições de Operação	Rendimento / % m/m	Ref.
Cereja	<i>Prunus avium</i> L.	T = 20 – 60 °C P = 25 – 250 bar Q = 20 – 40 L _{CO₂} /kg _{amostra} 0 – 20 % m/m EtOH	0.5 – 8	[1-3]
Sabugueiro	<i>Sambucus nigra</i> L.	T = 40 °C P = 200 bar		[4]
Medronho	<i>Arbutus unedo</i> L.	T = 40 – 80 °C P = 150 – 300 bar Q = 30 kg _{CO₂} /kg _{amostra} 0 – 20 % m/m EtOH		[1]
Hibisco	<i>Hibiscus sabdariffa</i> L.	T = 40 – 80 °C P = 200 – 400 bar		[1]
Rosa	<i>Rosa damascena</i> ‘Alexandria’ <i>R. gallica</i> ‘Francesa’ enxertada em <i>R. canina</i>	T = 30 – 80 °C P = 250 – 450 bar Q = 0.4 – 1.6 kg _{CO₂} /kg _{amostra}	5.72	[1, 5]
Cogumelos	<i>Agaricus bisporus</i> L.	T = 40 °C P = 90 – 300 bar Q _{CO₂} = 3.4 kg/h 0 – 10 % v/v EtOH	0.5 – 2	[6]
Mirtilo	<i>Vaccinium myrtillus</i> L.	T = 40 °C P = 150 – 250 bar Q _{CO₂} = 0.4 – 0.5 kg/h	1.84 – 2.19	[7]

Tendo em conta que a matéria-prima de interesse são cogumelos da espécie *Agaricus Bisporus* L. (em especial, os compostos ergosterol e o ergocalciferol) indica-se, na Tabela 2, a composição dos cogumelos desta espécie e os métodos de análise que foram usados para a sua quantificação.

Tabela 2 - Composição da matéria-prima (*Agaricus Bisporus* L.).

Matéria-Prima	Composição (mg / 100 g _{dry weight})	Método de Análise	Ref.
Cogumelos (incluindo <i>Agaricus Bisporus</i> L.)	<ul style="list-style-type: none"> ▪ Ergosterol: 602 – 654 ▪ Ergosta-7,22-dienol: 14.6 – 15.2 ▪ Ergosta-7,5-dienol: 47.1 – 94.0 ▪ Fungisterol: 13.5 – 25.8 	GS-MS	[8]
Cogumelos (incluindo <i>Agaricus Bisporus</i> L.)	<ul style="list-style-type: none"> ▪ Ergosterol: 671.5 ± 0.5 ($\approx 90\%$ da fração de esteróis) 	HPLC – UV	[9]

Assim, e de acordo com a pesquisa efetuada, ficou definido que o sistema de extração supercrítica deveria suportar temperaturas máximas até 80 °C e pressões até 450 bar. O caudal máximo de dióxido de carbono deverá estar na gama dos 0.1 – 5 litros/minuto e o extrator deverá ter uma capacidade compreendida entre os 0.5 – 5 litros. Esta gama de volumes foi definida com base na necessidade de se obter ca. 10 g de extrato para a sua caracterização e sabendo que cogumelos da espécie *Agaricus Bisporus* L. contêm cerca de 90 % m/m de água [10]. Estima-se que para um extrator com capacidade de ca. 2 litros, seja possível tratar numa amostra de 1 kg, sendo que 900 g correspondem a água e o restante a matéria-prima de base seca que contém os compostos extraíveis. Assumindo que 10 % desta matéria-prima seca é extraível, é possível obter aproximadamente 10 g de extrato.

Adicionalmente, a instalação deve permitir a recirculação do dióxido de carbono em ciclo fechado, de modo a atingir o limite de solubilidade dos compostos alvo no CO₂, ou seja permitir extrair o máximo de extrato durante a etapa de extração a pressão constante. Este sistema de extração deverá integrar um sensor em linha para monitorizar a concentração do extrato na corrente de CO₂ e, conseqüentemente, determinar o fim da etapa de extração. De referir que o método de espectroscopia por absorção UV/vis atualmente é utilizado com sucesso para pressões até 180 bar, sendo provável a possibilidade de operação a pressões superiores.

Para além disso, e com o objetivo de conservar todas as propriedades naturais da matéria-prima, será estudada a possibilidade de se utilizar a matéria-prima sem pré-tratamento, isto é, na sua forma hidratada. O uso da matéria-prima hidratada seria um passo inovador no design do processo de extração já que atualmente as matérias-primas passam por um processo de desidratação antes da etapa de extração. Na etapa de extração, a água seria arrastada pelo dióxido de carbono e, posteriormente, separada deste num separador com um sistema de purga para a remoção da água. Dado que o ergosterol (composto de interesse) é cerca de 1000 vezes mais solúvel em dióxido de carbono supercrítico do que em água, este composto seguirá na corrente de CO₂.

Após a secção de extração, o sistema terá dois separadores, com controlo independente de temperatura e pressão para permitir o fracionamento do extrato (produto extraído). Será estudado

o efeito da injeção de co-solvente no extrator e/ou nos separadores com o objetivo de aumentar o rendimento de extração e/ou recuperar a totalidade do extrato.

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ValorNatural – Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar.

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Nome do Documento: Relatório com a lista dos requisitos de funcionamento do sistema laboratorial de refinação com recurso à tecnologia NETmix.

Histórico de Revisão

Revisão	Data	Parceiros Envolvidos	Descrição



Lista de Autores

José Carlos Lopes

Madalena Dias

Cláudia Almeida



Sumário

Este entregável apresenta os requisitos de funcionamento do sistema laboratorial de extração e refinação com recurso à tecnologia NETmix.

Índice

1. Identificação.....	5
2. Informação.....	6

1. Identificação

<i>Deliverable</i>	6.2.1 Lista dos requisitos de funcionamento do sistema laboratorial de extração e refinação.
Tipo de <i>deliverable</i>	Relatório
Nível de disseminação	Confidencial
PPS	6. Inovação em processos de extração, refinação e técnicas de conservação.

2. Informação

No âmbito do Projeto Valor Natural (PPS6, atividade 19), pretende-se desenvolver uma unidade refinação inovadora com recurso a tecnologia NETmix. O NETmix será utilizado para extração líquido/líquido com dióxido de carbono (CO₂) em condições líquida e/ou supercrítica. Vantagens inerentes a uma instalação deste tipo incluem uma melhoria na eficiência da mistura entre as diferentes fases e, conseqüentemente, uma melhor eficiência de extração. Para além disso, o dióxido de carbono na fase líquida/supercrítica irá substituir o uso de solventes orgânicos frequentemente utilizados na indústria para este tipo de extrações e que podem ser nocivos para a saúde.

O conceito de funcionamento desta instalação consiste na mistura de água (ou outro solvente) com uma corrente de dióxido de carbono líquido/supercrítico que contém substâncias resultantes de um processo de extração com dióxido de carbono em condições supercríticas (PPS6 – Atividade 1). A mistura entre estas duas correntes, promovida na rede do NETmix, irá permitir o fracionamento dos componentes do extrato por ambas as fases. Deste modo, será esperado que as substâncias apolares permaneçam na fase orgânica (CO₂) e que os compostos mais polares criem uma maior afinidade com a fase aquosa e, portanto, se dissolvam na água. A seguir ao NETmix é necessário a existência de um separador com um tempo de residência suficientemente elevado para que as duas fases se separem. No final do processo o extrato será fracionado, obtendo-se dois extratos distintos.

De acordo com o procedimento descrito, existe a necessidade de dois reservatórios para o armazenamento dos solventes (água e dióxido de carbono líquido contendo extrato), um NETmix que irá promover a mistura entre as duas correntes, um separador que permita um tempo de residência da mistura água-CO₂ suficientemente elevado para que se dê a segregação das duas fases, de seguida dois reservatórios para a recolha dos produtos da extração. Note-se que, após a extração líquido-líquido, é possível gaseificar o dióxido de carbono e, conseqüentemente, obter o extrato apolar na sua forma sólida e livre de solvente. Na Figura 1, apresenta-se um diagrama detalhado da instalação de extração/refinação idealizada.

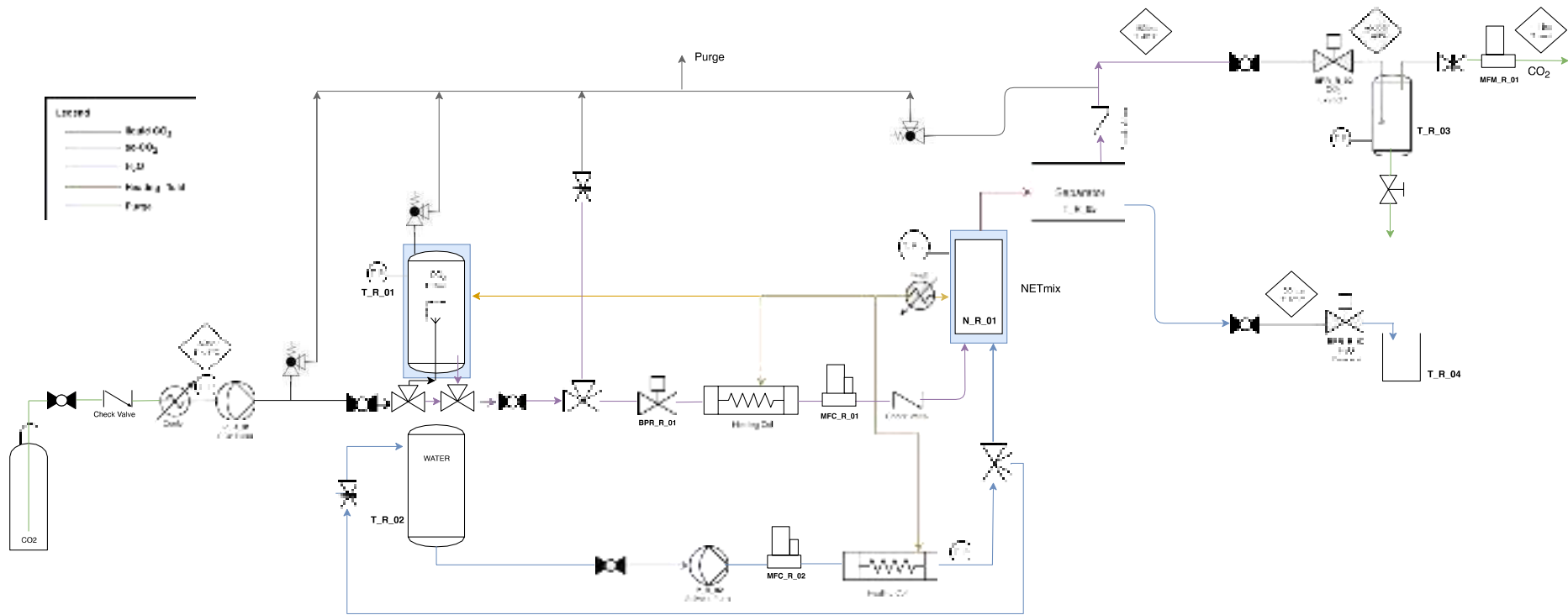


Figura 1 - Diagrama do processo da instalação de refinação.

De modo a definir as condições de operação das principais peças da instalação, foi necessária uma análise pormenorizada ao diagrama de fases do dióxido de carbono (apresentado na Figura 2). Concluiu-se que, para evitar a formação de hidratos, se deverá limitar a temperatura mínima de operação a 15 °C e a pressão do dióxido de carbono deverá ser sempre superior a 80 bar (de modo a evitar a gaseificação deste solvente e conseqüente alteração da solubilidade do extrato no dióxido de carbono).

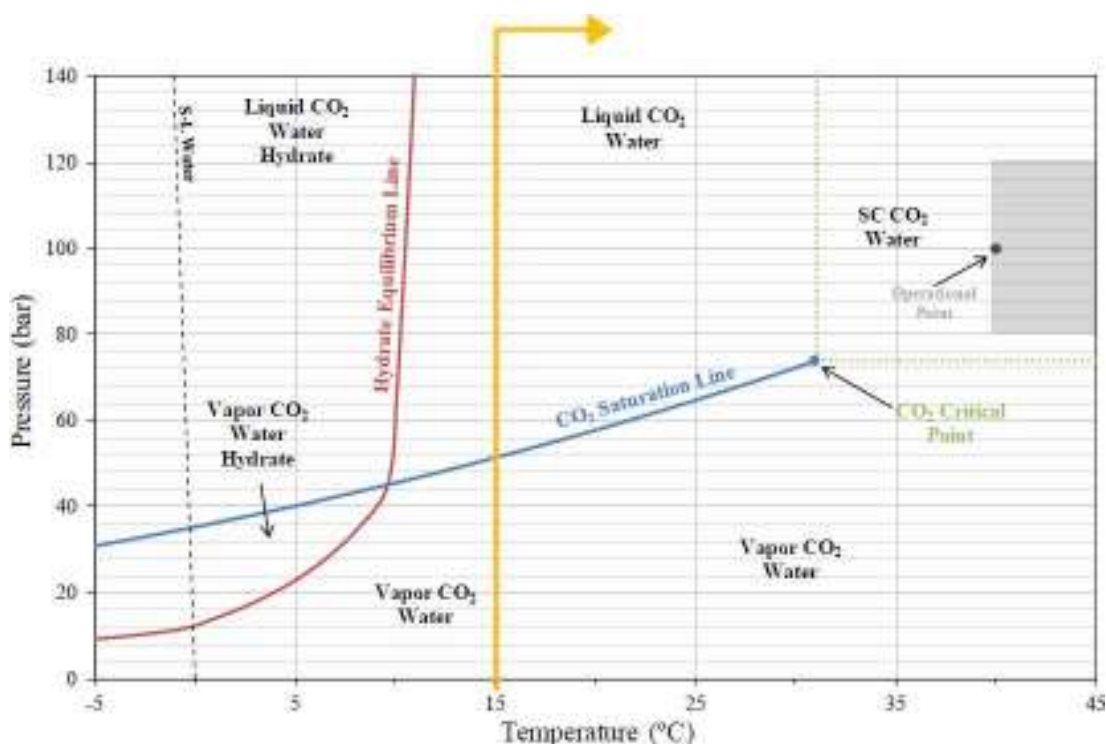


Figura 2 - Diagrama de fases do dióxido de carbono.

Adicionalmente e tendo em conta a alteração da densidade do dióxido de carbono com a temperatura e pressão (Figura 3), conclui-se que um aumento da temperatura, para uma mesma pressão, irá originar uma diminuição da densidade do dióxido de carbono. Deste modo, a utilização de temperaturas elevadas implicava uma diminuição da solubilidade dos compostos no dióxido de carbono, pelo que se limitou a temperatura máxima de operação a 40°C. Por outro lado, para uma mesma temperatura, um aumento da pressão resulta num aumento da densidade do dióxido de carbono e da sua capacidade de dissolução de compostos. A pressão de operação foi definida para uma gama de 80-100 bar (Figura 3).

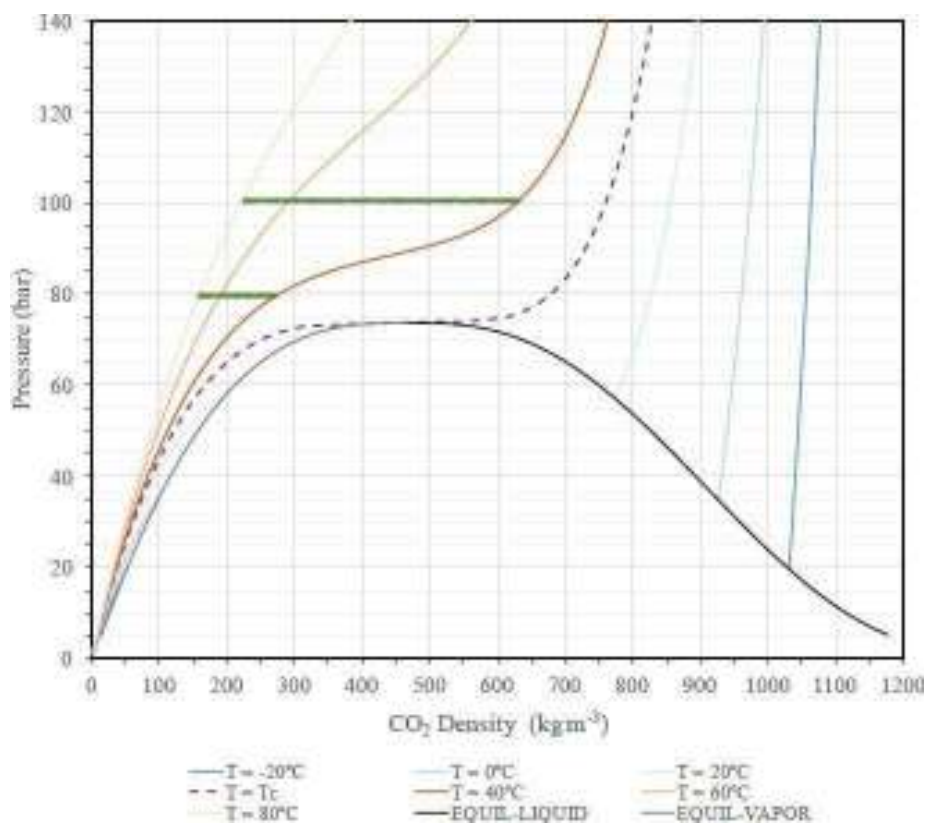


Figura 3 – Diagrama de PVT do dióxido de carbono.

Na Tabela 1, apresenta-se uma lista das principais peças da instalação de refinação e as respectivas condições de operação definidas.

Tabela 1 - Lista dos principais equipamentos da instalação de refinação e respectivas condições operatórias.

Referência	Peça/Equipamento	Temperatura (°C)	Pressão (bar)	Volume (L)
T_R_01	Reservatório CO ₂ líquido	20 - 40	80 - 120	1 - 2
T_R_02	Reservatório água	20 - 40	Atmosférica	20 - 40
N_R_01	NETmix/Separador	20 - 40	80 - 100	Sep > 0.07
T_R_03	Coletor da fase orgânica	Ambiente	50	0.20 - 0.5 L
T_R_4	Coletor da fase aquosa	Ambiente	Atmosférica	20 - 40

Dado que as condições de operação do NETmix e do separador são as mesmas (temperatura entre 20 e 40 °C e pressões desde 80 a 120 bar) foi proposta a hipótese de incorporar uma placa a seguir a rede do NETmix que cuja função seja separar a fase orgânica e aquosa. O separador será desenhado de maneira que exista de uma zona onde a velocidade da água é tal que permite a separação do CO₂.

Relativamente ao NETmix, definiu-se um diâmetro hidráulico de ~1 mm para o NETmix, sendo que o diâmetro da câmara é de 6.75 mm. De forma a minimizar o efeito da parede no escoamento foi definido um mínimo de 8 colunas e que o comprimento do NETmix deve ser entre 3 e 5 vezes superior à sua largura (para a garantir uma mistura completa das fases). Através do cálculo dos números de Reynolds para diferentes misturas de CO₂/H₂O, foi definido que o Reynolds nos canais do NETmix deve ser superior a 300. É ainda de referir que o NETmix deverá possuir um permutador de calor com alhetas.



ValorNatural – Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar.

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Histórico de Revisão

Revisão	Data	Parceiros Envolvidos	Descrição



Lista de Autores

José Carlos Lopes

Madalena Dias

Cláudia Almeida



Sumário

Este entregável apresenta os requisitos de funcionamento do sistema laboratorial de produção de hidratos com recurso à tecnologia NETmix.

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2. Informação.....	6

1. Identificação

<i>Deliverable</i>	6.3.1 Lista dos requisitos de funcionamento do sistema laboratorial de produção de hidratos.
Tipo de <i>deliverable</i>	Relatório
Nível de disseminação	Confidencial
PPS	6. Inovação em técnicas de conservação.

2. Informação

No âmbito do Projeto Valor Natural (PPS6, atividade 3), pretende-se desenvolver uma unidade de produção de hidratos de dióxido de carbono com recurso a tecnologia NETmix. O NETmix será utilizado para promover a mistura dos reagentes e, favorecendo elevadas taxas de transferência de calor, permite uma reação controlada obtendo os melhores resultados. Vantagens inerentes a uma instalação deste tipo incluem uma melhoria na eficiência da mistura entre as diferentes fases como também, elevadas taxas de transferência de calor entre os reagentes e o fluido de transferência de calor.

O conceito de funcionamento desta instalação consiste na mistura de água com uma corrente de dióxido de carbono gasoso que, às condições de pressão e temperatura predefinidas para a formação de hidratos, reagem numa reação do tipo exotérmica. O calor libertado tem de ser então removido pelo fluido de transferência de calor.

A mistura entre estas duas correntes, promovida na rede do NETmix, irá permitir uma correta mistura melhorando a interface de contacto entre ambos os reagentes permitindo uma produção eficiente de hidratos. O extrator de hidratos irá permitir remover o excesso de água e obter os hidratos sólidos. O intuito será o fornecimento de hidratos de dióxido de carbono que possam ser bons substitutos do gelo na conservação de alimentos.

De acordo com o procedimento descrito, existe a necessidade de um reservatório para o armazenamento de água (com o intuito de permitir variações no consumo de água), um NETmix que irá promover a mistura entre as duas correntes e a reação entre os dois reagentes, um separador que permita extrair o excesso de água líquida e a obtenção de hidratos sólidos que permitam a sua utilização final e um chiller de arrefecimento que irá permitir atingir as temperaturas necessárias no reator NETmix, para além dos componentes principais, componentes como bombas, válvulas, sensores, medidores, controladores e outros serão necessários.

Na Figura 1, apresenta-se um diagrama detalhado da instalação de produção de hidratos idealizada.

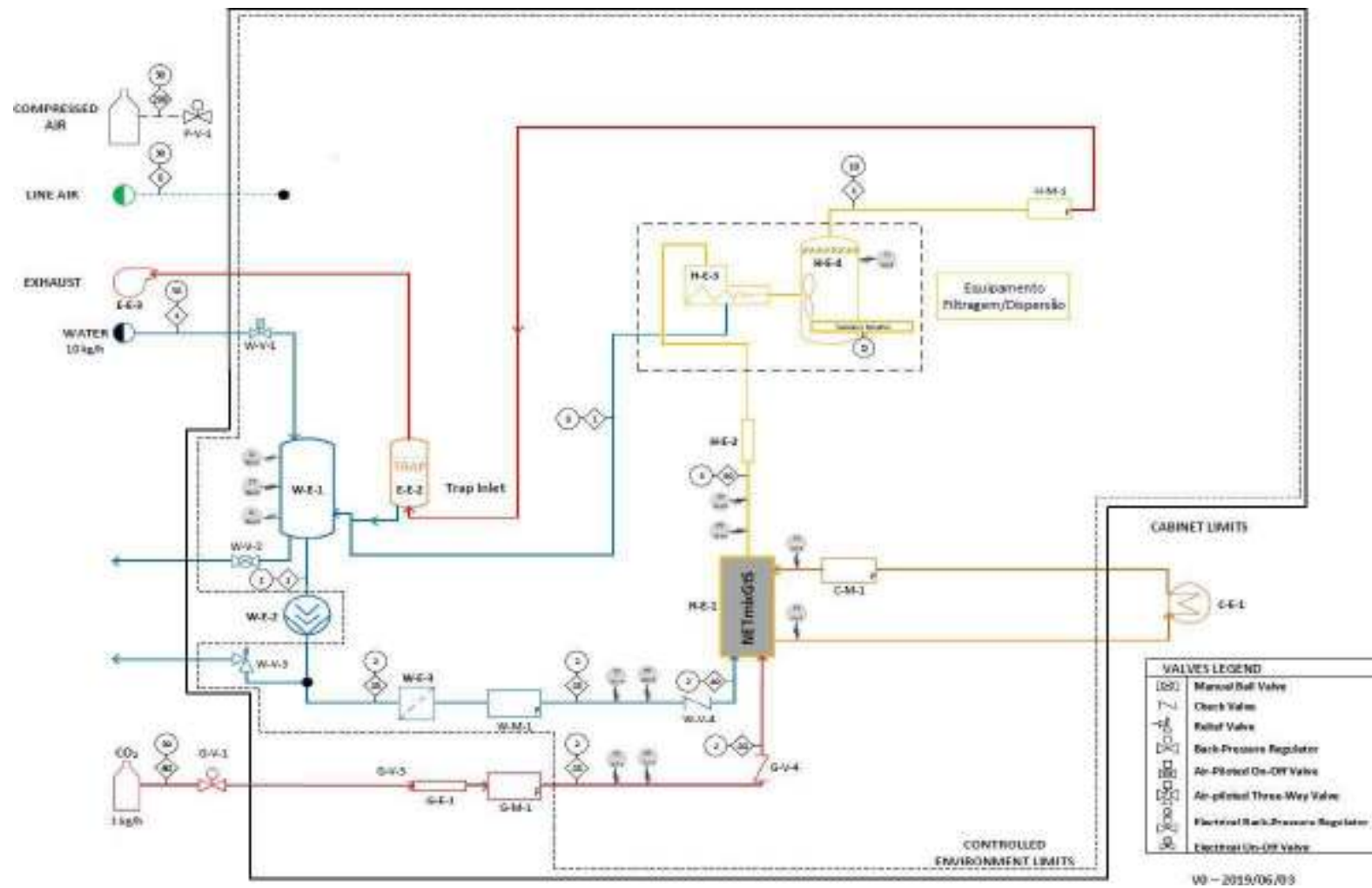


Figura 1 - Diagrama do processo da instalação de refinação.

De modo a definir as condições de operação das principais peças da instalação, foi necessária uma análise pormenorizada ao diagrama de fases do dióxido de carbono (apresentado na Figura 2). Concluiu-se que, para promover a formação de hidratos, se deverá limitar a temperatura mínima de operação a 0 °C, evitando o congelamento da água e que a pressão do dióxido de carbono deverá ser sempre superior a 10 bar.

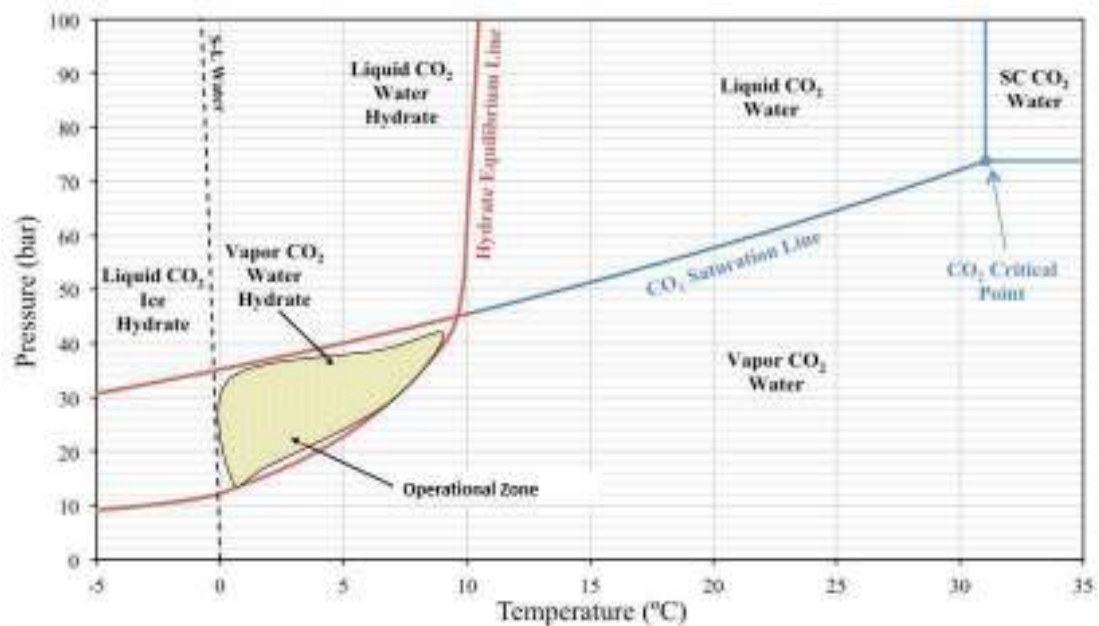


Figura 2 - Diagrama de fases do dióxido de carbono.

Tendo em conta o diagrama de fases presente na Figura 2 rapidamente se observa que a zona de trabalho é limitada não apenas por limites mínimos de funcionamento como também por limites máximos. No caso da temperatura não se torna possível a formação de hidratos de dióxido de carbono acima dos 10°C. No que diz respeito à pressão, o valor máximo aceite serão, aproximadamente, os 45 bar. Resumindo, a gama de temperatura de funcionamento situa-se entre os 0°C e os 10°C. A nível de pressões de funcionamento trabalhar-se-á entre os 10 e os 45 bar.

Na Tabela 1, apresenta-se uma lista das principais peças da instalação de produção de hidratos e as respectivas condições de operação definidas.

Tabela 1 - Lista dos principais equipamentos da instalação de produção de hidratos e respectivas condições operatórias.

Referência	Peça/Equipamento	Temperatura (°C)	Pressão (bar)
H-E-1	NETmix	0-10	10-45
H-E-3	Separador/Difusor	0-10	10-45
H-E-4	Difusor/Distribuidor	0-10	Atmosférica

Relativamente ao NETmix, definiu-se um diâmetro hidráulico de ~0.5 mm, sendo que o diâmetro da câmara é de 3.3 mm. De forma a minimizar o efeito da parede no escoamento foi definido um mínimo de 7 colunas e que o comprimento do NETmix deve ser entre 3 e 5 vezes superior à sua largura (para a garantir uma mistura completa das fases permitindo uma maior taxa de reação). Através do cálculo dos números de Reynolds para diferentes misturas de CO₂/H₂O, foi definido que o Reynolds nos canais do NETmix deve ser superior a 300. É ainda de referir que o NETmix deverá possuir um permutador de calor com alhetas. O caudal de produção deverá rondar o 1 kg/h.

No que diz respeito ao separador, o caudal de produção deverá ser idêntico ao caudal de produção do reator com o intuito de evitar acumulações no equipamento ou falta de produção. O equipamento apresentará um elemento de filtragem que irá permitir a remoção do excesso de água líquida e um elemento compressivo que irá forçar este excesso de água pelo elemento de filtragem, comprimindo os hidratos sólidos, produto final a obter.



Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor
Acrescentado para Aplicações na Indústria Alimentar

Entregável nº 8.1.1

Versão do Documento: 1.0

Data de Submissão: 30/10/2018

Responsável: IPB

Nome do Documento: Plano de Comunicação

Histórico de Revisão

Revisão	Data	Parceiros Envolvidos	Descrição
0.0	01/10/2018	IPB	Estruturação do documento
1.0	19/10/2018	IPB	Acréscimo de informação ao documento (7. Indicadores)
2.0	24/10/2018	IPB	Informação adicionada ao documento (9. Anexos; Anexo I)
3.0	30/10/2018	IPB	Informação adicionada ao documento (9. Anexos; Anexo I)



Lista de Autores

Ana Saldanha (IPB)

José Santos (IPB)

Sumário

O Plano de Comunicação tem como objetivo assegurar o impacto pretendido do projeto, assegurar a exploração e divulgação de resultados relativos a todos os PPS, suscitar interesse nos diferentes públicos-alvo, bem como desenvolver um plano informativo para toda a comunidade. Assim, é um documento flexível sujeito a atualização e monitorização sempre que necessário.

Índice

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3. Objetivos	7
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5. Meios e Ferramentas	9
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1. Identificação

Tabela 1. Identificação do *deliverable*.

<i>Deliverable</i>	Plano de Comunicação
Tipo de <i>deliverable</i>	Documento
Nível de disseminação	Público
PPS	PPS8 – Disseminação de informação e exploração de resultados

2. Introdução

A participação de um elevado número de intervenientes no projeto de contextos profissionais distintos, em conjunto com a equipa de profissionais experientes em comunicação e divulgação, assegurará uma eficaz comunicação e exploração dos resultados do projeto.

O Plano de Comunicação é um instrumento flexível e dinâmico, pelo que será objeto de contínua monitorização através da utilização de um conjunto de indicadores. Estes permitirão ajustar e repensar os diversos meios e técnicas disponíveis, a fim de se atingirem mais eficazmente os objetivos a que o Plano de Comunicação se propõe.

3. Objetivos

O Plano de Comunicação tem os seguintes objetivos específicos:

- Assegurar a prioridade das atividades de divulgação e exploração de resultados em todos os PPS;
- Assegurar que o pleno impacto do projeto seja alcançado;
- Despertar interesse sobre o público-alvo relativamente ao projeto;
- Criar uma imagem credível e de confiança relativamente ao projeto junto do público em geral;
- Gerar um plano informativo que seja compreendido por toda a comunidade.

4. Segmentação dos Público-Alvo do Projeto

No âmbito do projeto ValorNatural foram identificados diferentes públicos-alvo que serão abordados de forma distinta de acordo com os seus diferentes interesses. Os grupos beneficiários identificados foram:

- Tecido empresarial, nomeadamente PMEs;
- Comunidade científica;
- Consumidores;
- Entidades reguladoras;
- Agências de desenvolvimento local;
- Meios de comunicação social;
- Público em geral.

5. Meios e Ferramentas

Relativamente aos instrumentos de divulgação a utilizar no projeto vão ser empregadas uma variedade de ferramentas dirigidas, a todos os públicos-alvo pretendidos (mencionados na Seção 4). Foi dada especial atenção ao facto de a comunidade científica utilizar como principais canais de informação as publicações, *sites* de informação científica especializada e congressos científicos. Por outro lado, o público em geral obtém conhecimento científico através da televisão, jornais, revistas, rádio e Internet. Para além destes dois extremos serão também utilizados instrumentos de comunicação B2B (*business-to-business*) (Tabela 2).

Tabela 2. Meios de comunicação e divulgação do Projeto.

Sites de informação científica especializada
Congressos científicos
Televisão, Jornais, Rádio
Internet (<i>Facebook, Twitter</i>)
Publicações
Receitas inovadoras desenvolvidas por um <i>Chef</i> de culinária
“Laboratórios de demonstração”
Materiais informativos em papel
Página web do projeto, Intranet
Feiras, exposições, congressos
Blog, tutoriais e vídeos demonstrativos de tecnologias inovadoras
Portal Web dinâmico com dados de cada aditivo e comparação entre aditivos sintéticos e naturais
Portal com plataforma de vigilância tecnológica e inteligência competitiva
<i>Show Cooking</i>
Boletins semestrais relativos a tecnologias, patentes, normas/legislações
Publicações comerciais, técnicas, financeiras e industriais
<i>Press releases e press kit</i>
Feiras e seminários técnicos e comerciais

6. Ações de Comunicação

A divulgação dos resultados e a divulgação do Projeto será feita através da realização de diferentes ações de comunicação com o intuito de disseminar informação junto de todos os públicos-alvo pretendidos. As ações a realizar estão descritas na Tabela 3.

Tabela 3. Ações de comunicação.

Ação	Descrição	Objetivos	Público-Alvo
Criação da identidade corporativa e produção de estacionário e de material de divulgação	Através dos serviços de imagem desenvolver a identidade do Projeto e produzir materiais de divulgação (brochuras, materiais informativos em papel); Procedeu-se ao registo da marca “ValorNatural”.	Divulgar por todo o público-alvo o projeto, bem como conferir-lhe uma entidade própria reconhecida por todos.	<u>Consumidores</u> <u>Agências de desenvolvimento local</u> <u>Meios de comunicação social</u> <u>Público em geral</u>
Desenvolvimento e manutenção da intranet e da página web do projeto	Desenvolvimento de ferramentas de comunicação externa e interna ao projeto, onde se encontra toda a informação e conteúdos. As plataformas serão atualizadas frequentemente para todos os interessados.	A página web será utilizada para comunicar com os <i>stakeholders</i> externos e a intranet permitirá a comunicação eficiente e eficaz entre copromotores.	<u>Tecido empresarial</u> <u>Consumidores</u> <u>Agências de desenvolvimento local</u> <u>Meios de comunicação social</u> <u>Público em geral</u>
	Serão publicados regularmente <i>press</i>		

<p>Divulgação do projeto e dos resultados das atividades</p>	<p><i>releases</i> e <i>press kits</i>, <i>newsletters</i>, notícias no site, nas redes sociais. Os membros do consórcio participarão em eventos como feiras, exposições, congressos e encontros. Os novos ingredientes naturais e produtos alimentares serão usados em receitas inovadoras por um <i>chef</i> de culinária. As receitas desenvolvidas serão amplamente divulgadas nos média e em eventos “<i>show cooking</i>”. Os resultados serão todos apresentados num evento de encerramento do Projeto.</p>	<p>Divulgar junto da imprensa e da opinião pública os resultados do projeto, divulgar apoios e cativar os diferentes públicos-alvo.</p>	<p><u>Tecido empresarial</u> <u>Comunidade científica</u> <u>Consumidores</u> <u>Agências de desenvolvimento local</u> <u>Meios de comunicação social</u> <u>Público em geral</u></p>
<p>Recolha, sistematização e disseminação de informação tecnológica e estratégica</p>	<p>O sistema incluirá: boletins semestrais relativos a tecnologias, patentes, normas/legislação aplicáveis a potenciais fontes de financiamento; blog; tutoriais e vídeos demonstrativos de tecnologias inovadoras. Todos os serviços estarão na página <i>web</i>.</p>	<p>Identificação de patentes; esclarecimento de questões técnicas, partilha de experiências, necessidades e oportunidades; divulgação de resultados e de informações.</p>	<p><u>Tecido empresarial</u> <u>Comunidade científica</u> <u>Consumidores</u> <u>Entidades reguladoras</u> <u>Agências de desenvolvimento local</u> <u>Público em geral</u></p>
<p>Base de dados relativa a aditivos alimentares naturais e sintéticos, e a projetos de I&I e a tecnologia e conhecimento relevantes existentes no sistema nacional de I&I</p>	<p>Será disponibilizada uma base de dados para a temática em causa, com repositório aberto relativo a tecnologia e conhecimento existente no sistema nacional de I&I no tema do projeto. Será desenhada e implementada uma base de dados normalizada relativa aos aditivos alimentares aprovados pela EFSA e FDA. Portal <i>web</i> dinâmico com todos</p>	<p>Normalização de dados de forma a permitir um armazenamento consistente e um acesso eficiente aos mesmos; garantir uma busca de informação rápida e acessível ao público em geral.</p>	<p><u>Tecido empresarial</u> <u>Comunidade científica</u> <u>Consumidores</u></p>

	os dados relevantes de cada aditivo e a comparação entre aditivos naturais e sintéticos.		
Fomento e apoio à Inovação Colaborativa	Desenvolvimento de um portal que reúna para além de uma plataforma de vigilância tecnológica e inteligência competitiva, funcionalidades como: registo de ideias, desafios e oportunidades; registo de provedores de soluções, sistema de comunicação entre “clientes” e “fornecedores”, sistema de reconhecimento da performance dos “fornecedores”.	Operacionalização de ferramentas de gestão que permitam uma melhor comunicação, cooperação e interação entre parceiros.	<u>Tecido empresarial</u> <u>Comunidade científica</u> <u>Consumidores</u> <u>Entidades reguladoras</u>
Demolabs	Eventos com duração de um dia, a decorrer nas instalações do Parque de Ciência e Tecnologia Brigantia EcoPark, com uma componente demonstrativa e outra indutiva. Prevêem-se quatro encontros.	Disseminar e demonstrar os resultados do projeto; potenciar a geração de ideias; envolver grupos de investigadores, empresários, empreendedores e alunos.	<u>Tecido empresarial</u> <u>Comunidade científica</u> <u>Consumidores</u> <u>Entidades reguladoras</u> <u>Agência de desenvolvimento local</u> <u>Público em geral</u>
Publicação de Artigos em Revistas	Publicação dos resultados do projeto em Artigos e Revistas (<u>Anexo I</u>).	Apresentar os resultados do Projeto a um público alargado nacionalmente e internacionalmente.	<u>Tecido empresarial</u> <u>Comunidade científica</u> <u>Consumidores</u>

7. Indicadores

De acordo com o objetivo do Plano de Comunicação, este mesmo utiliza um conjunto de indicadores com o intuito de monitorizar a eficácia da comunicação do projeto e dos seus resultados aos públicos-alvo pretendidos. Os indicadores a utilizar são os seguintes:

- Número de visitas ao *site*, *blog*;
- Número de participantes em eventos;
- Número de publicações em revistas científicas;
- Número de publicações em revistas não científicas;
- Número de comunicados para a comunicação social;
- Número de reportagens na comunicação social;
- Número de utilizadores da base de dados relativa aos aditivos, bem como do portal com os dados de cada aditivo;
- Número de seguidores nas redes sociais;
- Número de visualizações dos tutoriais e vídeos;
- Nível de satisfação relativo aos eventos organizados, através do preenchimento do documento “Inquérito de Satisfação”.

8. Cronograma

De acordo com as ações de comunicação mencionadas na Tabela 2 foram estabelecidas datas para cumprimento das mesmas, descritas na Figura 1.

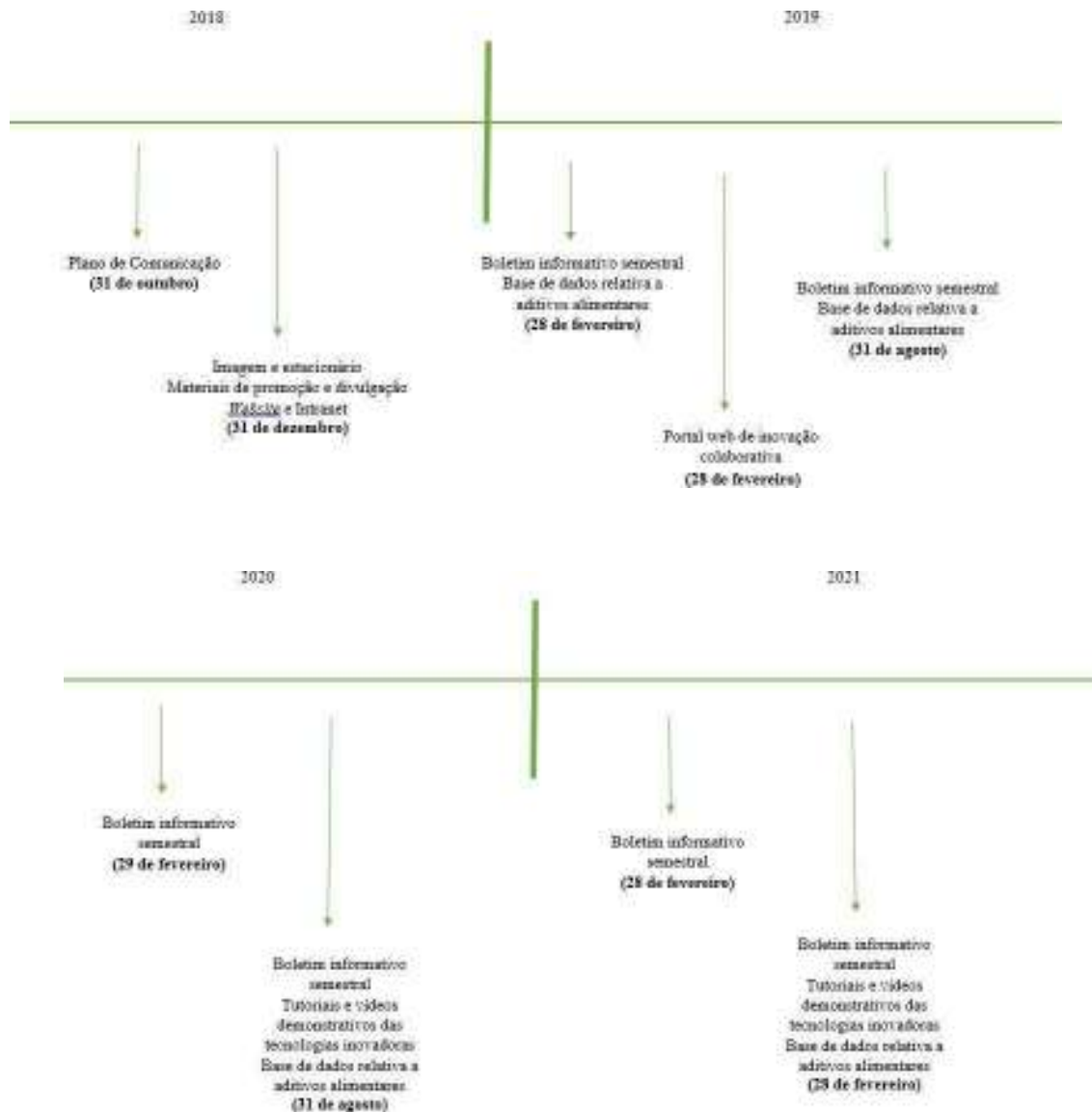


Figura 1. Cronograma geral.

9. Anexos

Anexo I – Exemplos de Eventos e Revistas

Exemplos de eventos: Biofach (Nuremberga, Fevereiro 2019), IPM Essen (Alemanha, Janeiro 2019), Portugal Agro (Braga, Maio 2019), Feira Agrícola Nacional (Santarém, Junho 2019), XVIII Congresso de Nutrição e Alimentação da Associação Portuguesa de Nutrição (Porto, Maio 2019), SISAB Portugal (Lisboa, Fevereiro 2019), Alimentaria & Horexpo (Lisboa, Março 2019), SIAL (Canada, Maio 2019), Nutraceuticals Europe (Madrid, Fevereiro 2019), XII CIBIA – Iberoamerican Congress of Food Engineering (Algarve, Julho 2019), Agroglobal, 22nd World Congress on Nutrition and Food Sciences (Brisbane, Junho 2019), ICNFF 2019: 21st International Conference on Nutraceuticals Foods (Sydney, Janeiro 2019), 21st International Conference on Bioprocessing and Food Engineering (Paris, Abril 2019), 15th Internacional Conference on Renewable Resources and Biorefineries (Toulouse, Junho 2019); International Symposium on Agricultural and Agroindustrial Waste Management (Florianópolis, Maio 2019), 5th Internacional Conference on Environment and Bio-Engineering (Singapura, Janeiro 2019), International Congress Ethnopharmacology (Alemanha, Junho 2019), International Conference on Medicinal and Aromatic Plants, Anuga FoodTec (Colônia, Outubro 2019), Fi Europe & Ni (Paris, Dezembro), International Conference on Green Chemistry (Paris, Dezembro 2019), Nordic Wood Biorefinery Conference.

Exemplos de revistas técnicas e científicas de referência: Food Chemistry, Food Research International, Journal of Agricultural and Food Chemistry, Food & Function, Journal of Functional Foods, Industrial Crops and Products, Innovative Food Science and Emerging Technologies, LWT – Food Science and Technology, Food and Bioprocess Technology, Bioresource Technology, Chemical Engineering, Journal of Environmental Management, Bioresource Technology, Journal of Separation and Purification Technology, Flavour and Fragrance Journal, Carbohydrate Polymers, Chemical Engineering Journal, Journal of Applied Polymer Sciences, Industrial Crops and Products, Chemical Engineering Research and Design, Proceedings of the Institution of Mechanical Engineers, Part I: Journal of Systems and Control Engineering, The International Journal of Advanced Manufacturing Technology, Journal of Cleaner Production, Journal of Renewable Materials, Journal of Microencapsulation, Journal of Functional Polymers, Processed Food Industry e Food Safety Magazine.





ValorNatural – Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar.

Entregável nº 8.1.2

Versão do Documento: 1

Data de Submissão: 31-12-2018

Responsável: IPB

Nome do Documento: Imagem e Estacionário

Histórico de Revisão

Revisão	Data	Parceiros Envolvidos	Descrição



Lista de Autores

Ana Saldanha

Sumário

O IPB, no âmbito da PPS 8 – Comunicação, desenvolveu a identidade corporativa (imagem), material de divulgação e promoção, e procedeu à criação do material estacionário do projeto.

Índice

1. Identificação	5
2. Informação	6

1. Identificação

<i>Deliverable</i>	E 8.1.2 – Imagem e Estacionário
<i>Tipo de deliverable</i>	Documento
<i>Nível de disseminação</i>	Público
PPS	8

2. Informação

A identidade corporativa (marca) do projeto ValorNatural foi desenvolvida pelos serviços do IPB (Figura 1), bem como o estacionário: modelo de envelope (Figura 2) e modelo de ofício (Figura 3).



Figura 1. Logotipo do projeto ValorNatural.



Figura 2. Modelo de envelope do projeto ValorNatural.



Figura 3. Modelo de ofício do projeto ValorNatural.



ValorNatural – Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar.

Entregável nº 8.1.3

Versão do Documento: 1

Data de Submissão: 31/12/2018

Responsável: IPB

Nome do Documento: Materiais de promoção e divulgação

Histórico de Revisão

Revisão	Data	Parceiros Envolvidos	Descrição



Lista de Autores

Ana Saldanha

Sumário

No âmbito da comunicação do projeto ValorNatural foi desenvolvida a identidade corporativa (imagem) e produzidos materiais de promoção e divulgação. A divulgação do projeto é de extrema importância para que se consiga atingir todos os públicos-alvo pretendidos, e contribuir assim para o alcance eficaz dos objetivos do projeto.



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1. Identificação.....	5
2. Informação.....	6

1. Identificação

<i>Deliverable</i>	E 8.1.3. Materiais de promoção e divulgação
<i>Tipo de deliverable</i>	Publicação
<i>Nível de disseminação</i>	Público
PPS	PPS 8 - Comunicação

2. Informação

No período de tempo a que este entregável se reporta, foi desenvolvido diverso material de divulgação e promoção do projeto ValorNatural. Através dos Serviços de Imagem do IPB foi possível a realização de um cartaz A3 (ver Figura 1), um desdobrável (ver Figura 2), um folheto (ver Figura 3) e um *roll-up* (ver Figura 4). Este material será utilizado para a promoção do projeto, para se conseguir atingir o máximo de público-alvo.



Figura 1. Cartaz A3 desenvolvido no âmbito do projeto ValorNatural.



Figura 2. Desdobrável desenvolvido no âmbito do projeto ValorNatural.



Os consumidores têm questões.

Nós temos soluções!



Ingredientes naturais e inovadores para a indústria, da classe dos corantes, aromas e bioativos.

Novos processos de extração, refinação e conservação.

O projeto Valor Natural dedica-se à investigação e desenvolvimento de ingredientes naturais de elevado valor acrescentado para aplicações na indústria.

Possuímos fortes competências e capacidades nas áreas agroalimentares, e um interesse crescente nas áreas têxtil, cosmética e farmacéutica.

www.valornatural.pt
 Email: geral@valornatural.pt - Tel: 233 200 830

<https://bit.ly/2R08020>
<https://bit.ly/2960yP6>
<https://bit.ly/2R08020>





Os consumidores têm questões.
Nós temos soluções!

Principais resultados

- Corantes de origem natural por extração de matrizes vegetais e bio-resíduos;
- Aromas naturais e modelos de aromas;
- Bioativos de origem natural por extração de bio-resíduos de cogumelos;
- Processos inovadores para extração e refinação de ingredientes a partir de matérias-primas naturais;
- Novos processos de conservação de matérias-primas naturais.
























Figura 3. Folheto desenvolvido no âmbito do projeto ValorNatural.



Os consumidores têm questões.

Nós temos soluções!

VALOR NATURAL

Ingredientes naturais e inovadores para a indústria, da classe dos cosméticos, aromas e bioativos.
Novos processos de extração, refinação e conservação.

O Valor Natural dedica-se à investigação e desenvolvimento de ingredientes naturais de elevado valor acrescentado para aplicações na indústria.

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Partners: 

Figura 4. Roll-up desenvolvido no âmbito do projeto ValorNatural.



ValorNatural – Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar.

Entregável nº8.1.4

Versão do Documento: 1

Data de Submissão: 6-02-2019

Responsável: IPB

Nome do Documento: Comunicação – *Website e Intranet*

Histórico de Revisão

Revisão	Data	Parceiros Envolvidos	Descrição
0.0	6-02-2019	IPB	Adição de Anexos



Lista de Autores

Ana Saldanha

Sumário

A página *Web* tem como finalidade a comunicação com todas as partes interessadas externas ao projeto, a divulgação de assuntos relacionados com o tema do ValorNatural e ainda a promoção de resultados do projeto. Para além da comunicação externa ao consórcio conseguida pelo *Website*, a *Intranet* permite a comunicação entre copromotores.

Índice

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1. Identificação

<i>Deliverable</i>	E 8.1.4. <i>Website e Intranet</i>
<i>Tipo de deliverable</i>	<i>Website</i>
Nível de disseminação	Público
PPS	PPS 8 - Comunicação

2. Informação

O *Website* e a *Intranet* têm ambos como finalidade a comunicação do projeto, o primeiro com *stakeholders* externos, e o segundo entre os próprios copromotores. Estas ferramentas, que foram desenvolvidas e que serão mantidas pelo IPB, terão o contributo de todos os parceiros para serem otimizadas de forma a contribuírem para uma comunicação ágil e fluida.

O *Website* é composto por diferentes Menus que permitem ao utilizador ter um conhecimento mais aprofundado do projeto, ter contacto com oportunidades relacionadas com o tema do ValorNatural, e posteriormente estar a par dos resultados do mesmo.



Figura 1. Imagem ilustrativa do *Website* do projeto ValorNatural.

“**O Projeto**”: são apresentados os objetivos gerais do projeto, bem como todos os parceiros que fazem parte do consórcio.

“**Investigação e Inovação**”: são apresentadas e descritas as diferentes áreas de investigação desenvolvidas no projeto (corantes naturais, aromas naturais e modelos de aromas, bioativos naturais, inovação em processos de extração e refinação de ingredientes, inovação em processos de conservação).

“**Atualidades**”: é uma seção que pretende reunir notícias, projetos, tecnologias e publicações científicas no âmbito do tema do ValorNatural, para que possam ser consultados por todos os interessados na temática.

“**Contactos**”: reúne os principais contactos com a equipa de gestão do *site*.

“**Newsletter**”: esta seção permite a subscrição da *Newsletter*, que conta com atualidades relacionadas com o projeto ou com o tema do mesmo.

A *Intranet* é também composta por diferentes Menus que permitem para além de uma comunicação ágil entre parceiros, a gestão de documentos de uma forma muito clara e rápida. As diferentes funcionalidades da *Intranet* foram compiladas e explicadas no “Manual de utilização da Intranet do projeto ValorNatural” (ver Anexo I).



Figura 2. Imagem ilustrativa da *Intranet* do projeto ValorNatural.

“**Notificações**”: reúne a lista de informações prioritárias.

“**Repositório de Documentos**”: é a seção onde estão concentrados todos os documentos que foram submetidos pelos utilizadores e que podem ser consultados;

“**Ações**”, “**Questões**” e “**Riscos**”: são diferentes menus onde se consegue aceder à lista das diferentes ações, questões e riscos criadas pelos utilizadores, com indicação do prazo para a execução da mesma, do estado de desenvolvimento, do responsável e da prioridade.

“**Entregáveis**” e “**Marcos**”: são seções que reúnem a lista de entregáveis e marcos, a data limite para serem finalizados, a prioridade atribuída a cada um, o estado de execução e o responsável.

“Fórum”: o fórum permite o contacto rápido e fácil entre parceiros para “discussão” de uma determinada dúvida ou assunto;

“Contacto”: permite o contacto com a equipa de gestão da plataforma, e ainda a possibilidade de anexar qualquer documento que seja necessário.

“Criar conteúdo”: permite ao utilizador submeter documentos como entregáveis, criar algum tipo de ação, marco, questão, risco ou notificação. A criação destes conteúdos será feita tendo em conta a PPS a que se quer referir.

3. Anexos

Anexo I – Manual de Utilização da *Intranet* do projeto ValorNatural



Manual de utilização da *Intranet* do projeto ValorNatural



Histórico de Revisão

Revisão	Data	Parceiros Envolvidos	Descrição
0.0	6-02-2019	IPB	Alteração da estrutura

Em caso de dúvida contactar:

E-mail: geral@valornatural.pt

Telefone: 273 303 382

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Acesso à Intranet

Para aceder à **Intranet**, cada utilizador terá um “Nome de utilizador” e uma “Palavra-Passe” que lhe dará acesso a todos os conteúdos da sua/das suas áreas de trabalho. Sendo assim, terá que efetuar o “Login” (ver Figura 1) no *site* do ValorNatural (www.ValorNatural.pt).



Figura 1 - Login do utilizador.

Grupos

A *Intranet* do projeto ValorNatural está organizada em diferentes grupos de trabalho, de acordo com a organização do presente projeto (PPS). Em cada grupo existem conteúdos e serviços de acesso exclusivos a membros desse grupo, sendo que cada utilizador pode pertencer a um ou mais grupos de trabalho.

PPS1 – Gestão do Projeto

PPS3 – Corantes naturais

PPS4 – Aromas naturais e modelos de aromas

PPS5 – Bioactivos naturais

PPS6 – Inovação em Processos de extração, refinação e técnicas de conservação

PPS8 – Disseminação de informação e exploração de resultados

Geral – Grupo com informações gerais sobre o Projeto, todos os membros têm total acesso à informação

O utilizador após fazer *Login* com as suas credenciais pode:

-Visualizar Ficheiros disponíveis no Repositório de Documentos;

- Visualizar Notificações, Ações planeadas, Marcos e Entregáveis planeados, Riscos identificados, Questões colocadas no grupo e itens do fórum;
- Inserir Notificações, Ações, Ficheiros, Tópicos no fórum, Questões e Riscos;
- Contactar a equipa gestora do projeto.

Área de utilizador

Quando o utilizador realiza *Login* na sua área de trabalho acede à sua página pessoal. Na página inicial consegue ter acesso às seguintes informações (ver Figura 2):

- Na coluna da direita, os **últimos ficheiros que foram submetidos**, e as **últimas notificações inseridas**;
- Na coluna da esquerda, um **calendário** onde constam as Ações, os Entregáveis e os Marcos dos grupos a que pertence.

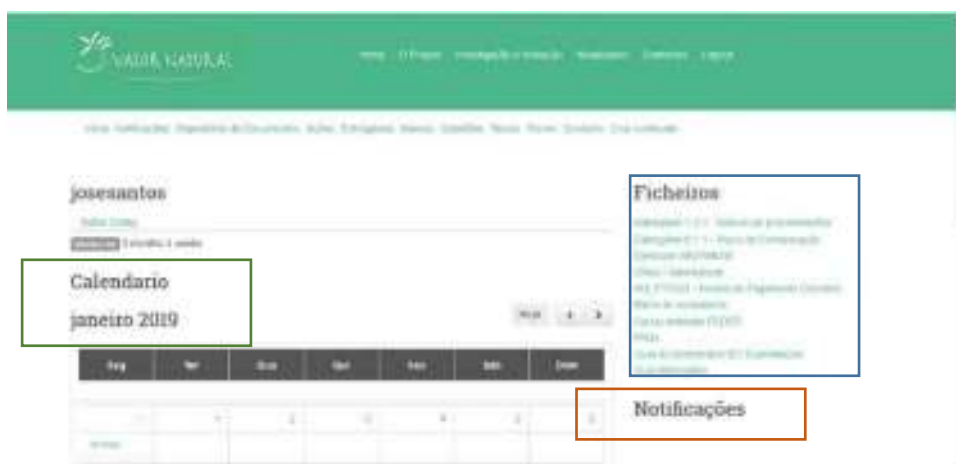


Figura 2 – Página inicial da área do utilizador.

Na página inicial da área do utilizador é possível:

- Alterar os dados pessoais do utilizador (ex: Palavra-Passe) utilizando o *link* “Editar Conta”;
- Visualizar o menu com os itens representados na Figura 3.



Figura 3 – Menu do utilizador.

Itens do menu

Início – Este menu permite ao utilizador voltar à página inicial da sua área de trabalho (página onde visualiza o calendário e os *links* para os últimos ficheiros e para as últimas notificações).

Notificações – Este menu permite visualizar as notificações existentes no grupo ou nos diferentes grupos a que pertence o utilizador (ver Figura 4).



Figura 4 – Página de notificações do utilizador.

Repositório de documentos – Este menu apresenta todos os ficheiros que o utilizador tem disponíveis (em todos os grupos em que participa) para fazer o *download* (ver Figura 5).

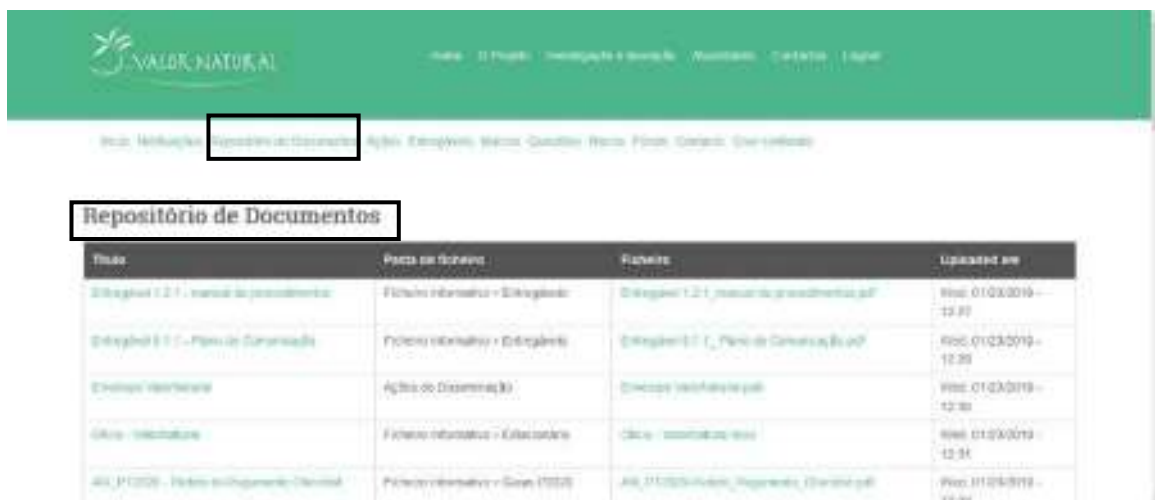


Figura 5 – Página acessível no menu “Ficheiros disponíveis”.

Ações – Este menu permite visualizar Ações planeadas existentes em todos os grupos a que pertence o utilizador (ver Figura 6).



Figura 6 – Página de ações do utilizador.

Entregáveis – Este menu permite visualizar o planeamento de Entregáveis existentes em todos os grupos a que pertence o utilizador (ver Figura 7).

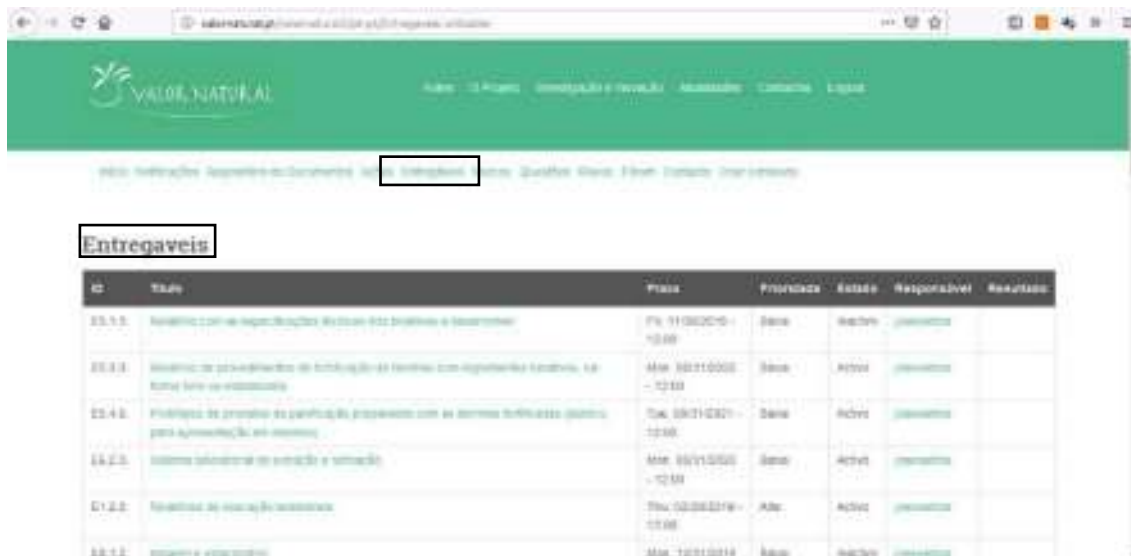


Figura 7 – Página de Entregáveis.

Marcos – Este menu permite visualizar os Marcos existentes em todos os grupos a que pertence o utilizador (ver Figura 8).

ID	Título	Detalhes	Plano	Prorrida	Estado	Responsável	Resultado
M007	Implementação do Sistema		SI	Ativa	Ativa	gestor	
M008	Realização de reuniões para a criação de documentos		SI	Ativa	Ativa	gestor	
M009	Realização de reuniões de trabalho para a criação de documentos		SI	Ativa	Ativa	gestor	
M010	Realização de reuniões de trabalho para a criação de documentos		SI	Ativa	Ativa	gestor	

Figura 8 – Página de Marcos.

Questões – Este menu permite visualizar qualquer tipo de questões ou eventuais problemas que possam surgir. Esta questão será visualizada por todos os membros que pertencem ao mesmo grupo de trabalho que o membro que colocou a questão (ver Figura 9).

Figura 9 – Página com questões colocadas pelos grupos a que pertence o utilizador.

Riscos– Este menu permite visualizar os Riscos identificados em todos os grupos a que pertence o utilizador (ver Figura 10).

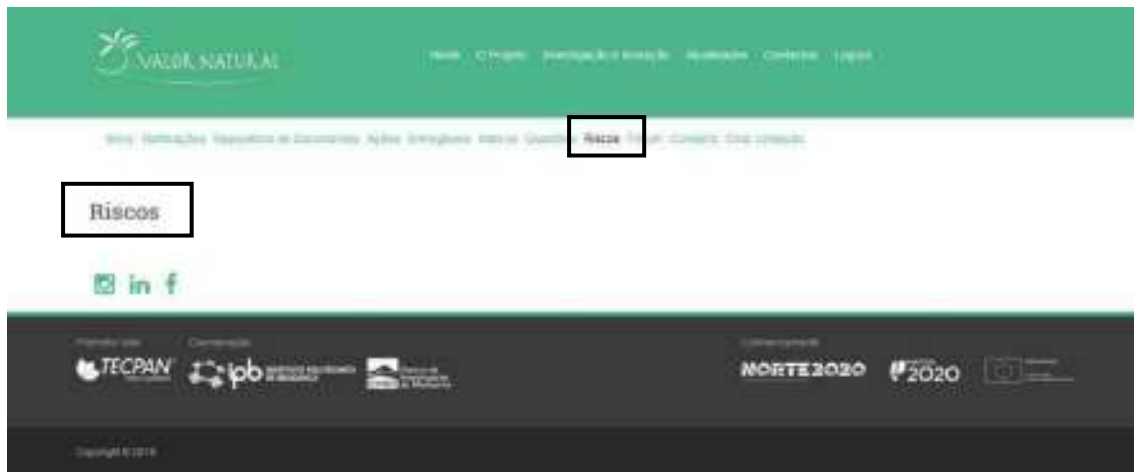


Figura 10 – Página de riscos identificados para grupos a que pertence o utilizador.

Fórum – Este menu permitirá visualizar as conversações mantidas nos grupos a que pertence o utilizador, como por exemplo, o tópico de fórum denominado “Funcionalidades *site web*”. O utilizador poderá subscrever esse fórum e inserir comentários (ver Figura 11).

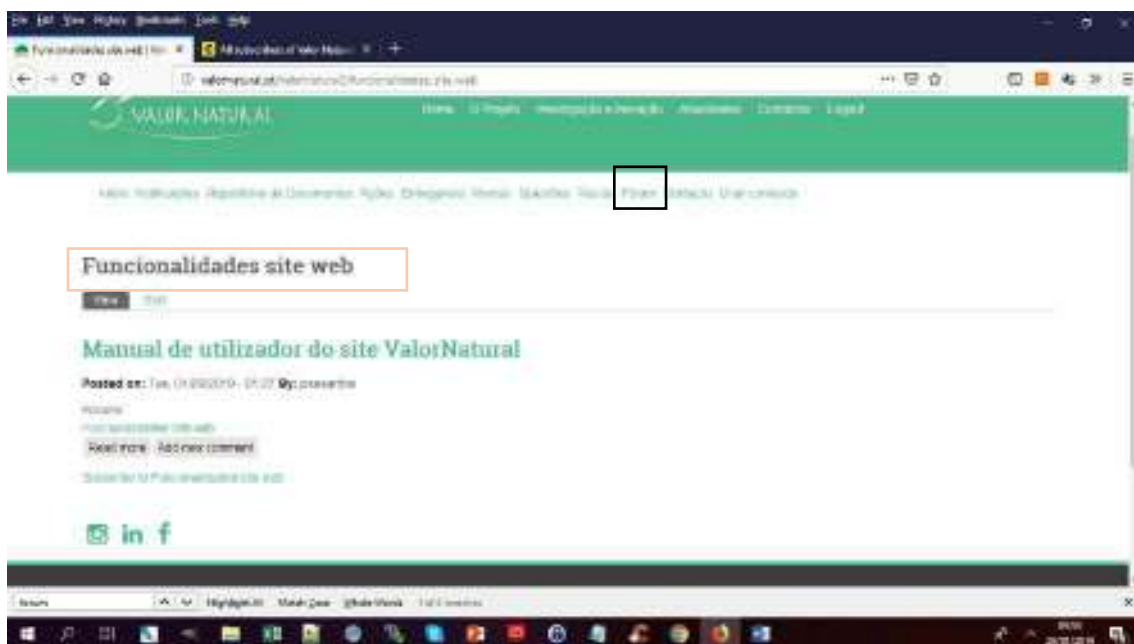


Figura 11 – Página com tópicos colocados pelos grupos a que pertence o utilizador.

Contacto – Este menu permite que o utilizador contacte a gestão do projeto sobre diversos assuntos.



Figura 12 – Janela de contactos.

Criar Conteúdo – Este menu possibilita que o utilizador insira conteúdos nos grupos a que pertence. O utilizador deve, em primeira instancia, escolher em que grupo vai introduzir conteúdo (ver Figura 13).



Figura 13 – Página para inserir conteúdos.

Após ser seleccionado o grupo em que vai ser inserido o conteúdo, surge então uma página em que deve escolher o tipo de conteúdo que deve ser criado (ver Figura 14).



Figura 14 – Tipos de conteúdos a criar.

E, por fim, após ter sido seleccionado o tipo de conteúdo a submeter, preenchem-se os campos necessários a cada tipo de conteúdo. Todos os tipos de conteúdo são preenchidos em dois passos, no primeiro passo é inserido um título e no segundo passo são inseridos os campos que lhe são característicos.

Criar Ações

Para criar uma “Ação” o utilizador deverá:

1. Seleccionar o Menu “Criar Conteúdo”, onde surgirá a janela com os diferentes grupos onde o utilizador pode criar uma Ação (ver Figura 15).

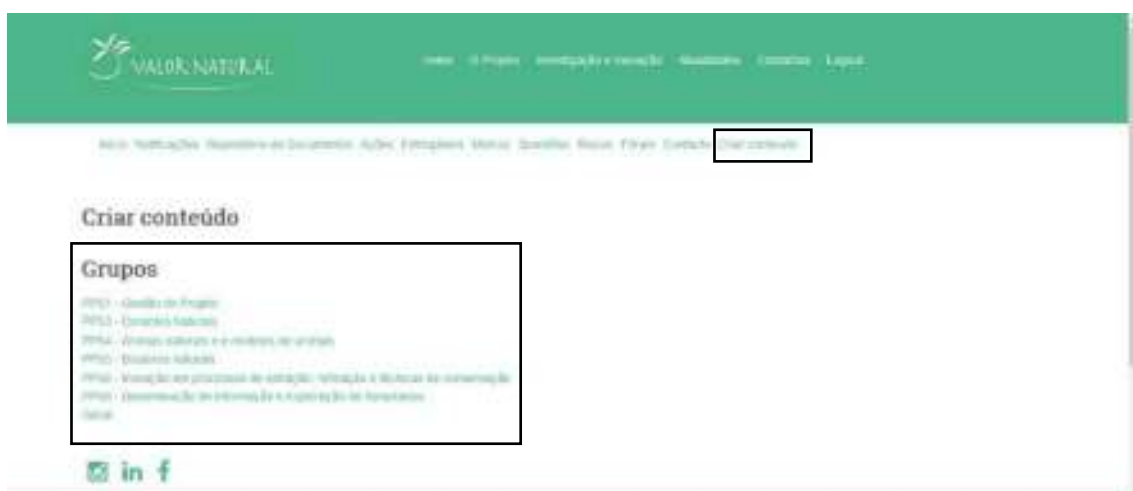


Figura 15 – Criar Ação (janela 1).

2. Selecionar o grupo em que pretende criar a “Ação”, e posteriormente surgirá a janela com os diferentes conteúdos, neste caso o conteúdo a criar será uma Ação (ver Figura 16).

**Figura 16** – Criar Ação (janela 2).

3. Selecionar o *link* “Ações” e de seguida surgirá a primeira janela de inserção da ação. Neste campo deve ser colocado o título da Ação, e selecionar o botão “Guardar/Save” (ver Figura 17).

**Figura 17** – Criar Ação – primeira janela de inserção da Ação.

4. Posteriormente surge a última janela que irá permitir finalizar a submissão da Ação. Neste espaço deverão ser preenchidos todos os campos com as informações pedidas e finalizar selecionando o botão “Criar conteúdo no grupo...” (ver Figura 18).



Figura 18 – Criar Ação – última janela de inserção da Ação.

Inserir Ficheiros

Os Ficheiros a serem submetidos deverão estar identificados de forma clara, ou seja, aconselha-se que os nomes sejam sistematizados da seguinte forma: ID_Nome do ficheiro_Versão (exemplo: Entregável 1.2.1_Manual de Procedimentos_Versão 1).

Para submeter um Ficheiro o utilizador deverá:

1. Selecionar o Menu “Criar Conteúdo”, onde surgirá a janela com diferentes Grupos que podem ser selecionados pelo utilizador (ver Figura 19).



Figura 19 – Inserir Ficheiro (janela 1).

2. Selecionar o grupo em que pretende criar conteúdo, neste caso será a submissão de um ficheiro. Posteriormente surgirá a janela de conteúdos que são possíveis criar, neste caso será selecionado o *link* “Ficheiros” (ver Figura 20).



Figura 20 – Inserir Ficheiro (janela 2).

3. A primeira janela para submissão do Ficheiro requer que seja mencionado o nome do mesmo, e de seguida seleccionar o botão “Guardar/Save” (ver Figura 21).



Figura 21 – Inserir Ficheiro – Nome do Ficheiro.

4. Na última janela para submissão de um Ficheiro é necessário preencher todos os campos solicitados, e através do botão “Browse” pode ser inserido o Ficheiro pretendido. Por último, deve ser selecionado o botão “Criar Conteúdo no grupo...” para ser finalizada a submissão do Ficheiro ao Grupo pretendido (ver Figura 22).



Figura 22 – Inserir ficheiro – última janela para submissão do ficheiro.

Criar tópico de Fórum

Para inserir um tópico no Fórum o utilizador deverá:

1. Selecionar o Menu “Criar Conteúdo”, onde surgirá a janela com os diferentes Grupos que podem ser selecionados pelo utilizador (ver Figura 23).



Figura 23 – Inserir tópico no Fórum (janela 1).

2. Selecionar o Grupo em que pretende criar o tópico, e posteriormente selecionar o conteúdo que quer criar, neste caso “Tópico de Fórum” (ver Figura 24).



Figura 24 – Inserir tópico de Fórum (janela 2).

3. Após ter selecionado “Tópico de Fórum” irá surgir a primeira janela para a inserção do tópico. Nesta janela deverá colocar o assunto do tópico que vai ser criado, preencher todos os campos solicitados pelo formulário e terminar selecionando o botão “Criar conteúdo no grupo...” (ver Figura 25).



Figura 25 – Inserir tópico de Fórum – última janela para criação de tópico.

Inserir Questões

Para inserir uma Questão o utilizador deverá:

1. Selecionar o Menu “Criar Conteúdo”, e posteriormente surgirá a janela de grupos em que pode ser criada a Questão (ver Figura 26).

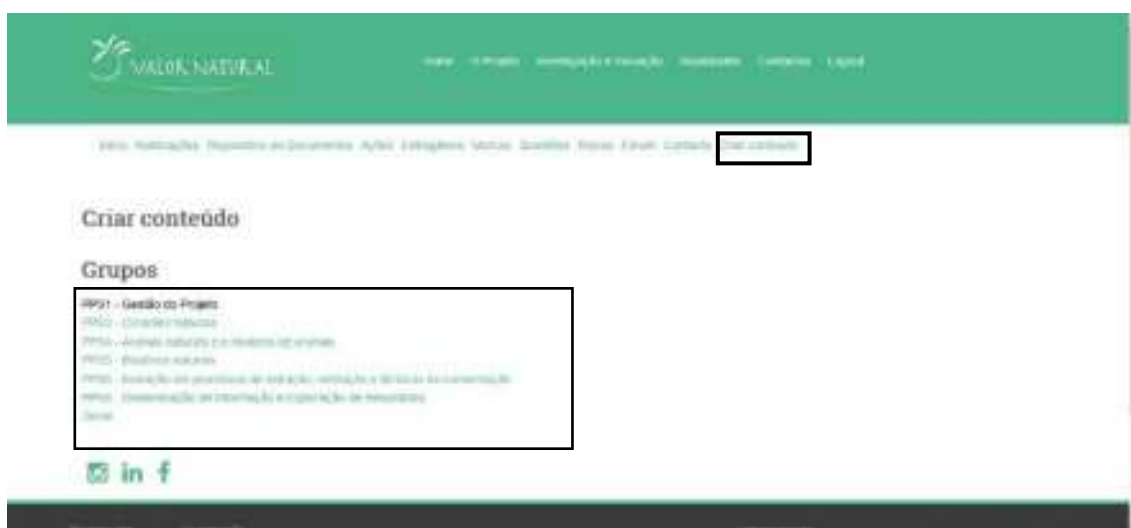


Figura 26 – Inserir Questão (janela 1).

2. Após seleccionar o Grupo pretendido, surgirá a janela de Conteúdos e terá de seleccionar o *link* “Questões” (ver Figura 27).



Figura 27 – Inserir Questão (janela 2).

3. Na janela que permite criar a Questão, é necessário preencher todas as informações requeridas pelo formulário, e finalizar seleccionando o botão “Criar conteúdo no grupo...” (ver Figura 28).



Figura 28 – Inserir Questão – última janela para finalizar a submissão de uma Questão.

Inserir Riscos

Para inserir um Risco o utilizador deverá:

1. Selecionar o Menu “Criar Conteúdo”, onde surgirá a janela dos Grupos que podem ser selecionados para ser inserido um risco (ver Figura 29).



Figura 29 – Inserir Risco (janela 1).

2. Selecionar o grupo em que pretende adicionar um Risco, e posteriormente selecionar o Conteúdo que quer introduzir, neste caso “Riscos” (ver Figura 30).



Figura 30 – Inserir Risco (janela 2).

3. Após selecionar o *link* “Riscos” irá surgir a primeira janela de inserção do Risco, onde terá que inserir o Título do Risco a assinalar (ver Figura 31).



Figura 31 – Inserir Risco – Inserir título do Risco.

4. Após ter inserido o título do Risco, será necessário preencher todas as informações requeridas no formulário e finalizar a submissão através do Botão “Criar Conteúdo no grupo...” (ver Figura 32).



Figura 32 – Inserir Risco – última janela para submissão do Risco.



ValorNatural – Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar.

Entregável nº 8.2.1

Versão do Documento: 2

Data de Submissão: 24-05-2019

Responsável: IPB

Nome do Documento: Publicação de boletins Informativos

Histórico de Revisão

Revisão	Data	Parceiros Envolvidos	Descrição
1.0	21-03-2019	IPB	2º edição da <i>Newsletter</i>
2.0	24-05-2019	IPB	3º edição da <i>Newsletter</i>



Lista de Autores

Ana Saldanha

Sumário

A *Newsletter* do projeto ValorNatural tem como intuito a promoção da comunicação com os seus subscritores, sendo utilizada a caixa de correio eletrónico de cada um para envio da informação. A informação que consta na *Newsletter* é diversificada, atual e está sempre relacionada com o próprio projeto ou com os diferentes temas que ele abrange, será enviada trimestralmente. No decorrer do primeiro ano de execução do projeto foram publicadas 3 edições da *Newsletter*.

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1. Identificação

<i>Deliverable</i>	8.2.1
<i>Tipo de deliverable</i>	Publicação
Nível de disseminação	Público
PPS	8

2. Informação

A primeira *Newsletter* do projeto ValorNatural foi realizada no mês de janeiro de 2019 (Imagem 1) onde foram abordados diferentes temas atuais, relacionados com o próprio projeto e com o tema do mesmo.



Imagem 1. Newsletter mês de janeiro de 2019.

No decorrer do segundo semestre foram publicadas mais duas edições da *Newsletter*: mês de março (Imagem 3) e mês de maio (Imagem 4) de 2019.



Imagem 2. Newsletter mês de março de 2019.



Imagem 3. Newsletter mês de maio de 2019.