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Incorporação de ingredientes naturais em diferentes matrizes alimentares como potenciadores de conservação e promotores de saúde

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Tese de Doutoramento

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“No mesmo instante em que recebemos pedras no nosso caminho,
flores estão a ser plantadas mais à frente.
Quem desiste não as vê.”

William Shakespeare

Para as minhas princesas

Resumo

Os alimentos funcionais representam, atualmente, uma das áreas mais promissoras, com impacto e inovadoras da indústria alimentar. A preocupação dos consumidores na procura de alimentos rotulados como mais saudáveis e mais naturais é uma tendência crescente. É evidente um olhar cada vez mais crítico em relação ao rótulo dos alimentos, por parte dos consumidores, cada vez mais informados e conscientes das suas escolhas, e da sua influência na sua saúde e bem-estar. Assim, alimentos sem aditivos artificiais ou com uma adição mínima destes, têm vindo a ganhar a preferência dos consumidores. Tal tendência e exigência tem exercido influência direta na indústria alimentar a qual tenta ir ao encontro das expetativas dos consumidores. São vários os componentes adicionados aos alimentos com o intuito de os tornar funcionais. É o caso dos compostos fenólicos, componentes que ocorrem naturalmente na natureza e que estão associados a uma elevada capacidade antioxidante e a benefícios para a saúde humana. Apesar do grande número de estudos científicos e patentes sobre este tema, e a sua presença natural em alimentos, nomeadamente em fontes vegetais, são ainda muito poucos os produtos comercializados enriquecidos com estes compostos. Para que este tipo de produtos funcionais possa ser comercializado têm de cumprir vários regulamentos, de forma a comprovar a sua segurança e benefícios de saúde, e assim conquistarem a confiança do consumidor. Vários estudos descrevem diferentes propriedades bioativas dos compostos fenólicos, garantindo o seu potencial como ingredientes bioativos em alimentos. No entanto, dúvidas acerca da sua estabilidade e biodisponibilidade têm surgido, sendo necessário o seu esclarecimento de modo a que a sua adição se torne viável na funcionalização de alimentos.

Este trabalho teve por objetivo estudar extratos fenólicos vegetais, visando a sua adequação para incorporar em diferentes matrizes alimentares. Simultaneamente, visou obter alimentos funcionais e aumentar o seu prazo de validade, evitando a utilização pouco desejada de aditivos artificiais/conservantes.

Em todos os extratos avaliou-se o teor e a composição em compostos bioativos e a atividade biológica. Os compostos fenólicos foram determinados por cromatografia de alta eficiência acoplada a deteção por íodos e a espetrometria de massa (HPLC-DAD-ESI/MS). Avaliaram-se as atividades antioxidante, antimicrobiana (antibacteriana e antifúngica) e hepatotoxicidade. Para a determinação da atividade antioxidante total, realizaram-se os ensaios do radical 2,2-difenil-1-picril-hidrazilo (DPPH) e do poder redutor (PR). Para a avaliação da peroxidação lipídica realizaram-se os ensaios de inibição da descoloração do β-caroteno e da inibição de espécies reativas do ácido tiobarbitúrico

(TBARS). Na avaliação da atividade antimicrobiana, testaram-se oito bactérias e oito fungos selecionados segundo a sua importância ao nível da saúde pública; determinou-se a concentração mínima inibitória (MIC), a concentração mínima bactericida (MBC) e a concentração mínima fungicida (MFC). Relativamente aos ensaios de hepatotoxicidade, testaram-se os efeitos dos extratos vegetais em culturas primárias de células de fígado de porco.

Após a incorporação dos extratos nas diferentes matrizes alimentares foi necessário testar os seus efeitos no perfil nutricional, características físico-quimicas (pH e cor) e atividade antioxidante das diferentes amostras e nos diferentes tempos de armazenamento. Em todas as amostras foi determinado, a humidade, hidratos de carbono, proteínas, gorduras, cinzas e energia seguindo os métodos oficiais AOAC. Determinou-se o perfil de ácidos gordos por cromatografia gasosa com deteção de ionização de chama (GC-FID) e os açúcares por cromatografia de alta eficiência com deteção por índice de refração (HPLC-RI). A atividade antioxidante foi avaliada recorrendo aos dois ensaios referidos anteriormente (DPPH e PR).

Foeniculum vulgare Mill. (funcho) e *Matricaria recutita* L. (camomila) são plantas cujo consumo está associado às suas propriedades benéficas. Após caracterização química dos extratos obtidos por decocção, comprovaram-se as suas propriedades antioxidantes e antimicrobianas. A incorporação dos seus extratos, diretamente ou após encapsulação, ocorreu em amostras de requeijão. Comprovou-se que a adição dos extratos não altera o perfil nutricional, confere atividade antioxidante e prolonga o tempo de prateleira dos requeijões. Estes são alimentos com um tempo de vida muito reduzido, não sendo utilizados aditivos artificiais a nível industrial. Para ultrapassar esta situação, os referidos extratos foram incorporados em iogurtes, e o seu efeito comparado com o sorbato de potássio (E202). Neste estudo concluiu-se que as decocções das plantas referidas podem ser utilizadas para desenvolver novos iogurtes, substituindo conservantes artificiais e melhorando as propriedades antioxidantes do produto final, sem alterar o perfil nutricional. O mesmo tipo de estudo foi realizado num produto de pastelaria (biscoitos) onde se verificou que, os extratos naturais e o aditivo artificial (hidroxianisole butilado, BHA) conferiram uma atividade antioxidante similar, sem alterarem as propriedades físico-químicas ou perfil nutricional dos biscoitos. Após um estudo sensorial, apesar de os consumidores encontrarem poucas diferenças entre os biscoitos testados, os aditivos naturais foram associados a uma solução mais benéfica para os consumidores que preferem alimentos "livres" de aditivos artificiais.

Melissa officinalis L. (cidreira) é uma planta conhecida pela população desde os tempos ancestrais pelos seus benefícios reconhecidos, apresentando quimicamente um elevado teor de ácido rosmarínico. Assim, neste trabalho pretendeu-se obter as condições

que maximizam a extração deste composto comparando três técnicas diferentes: extração assistida por calor, micro-ondas e ultrassons. A extração por ultrassons provou ser o método mais eficaz, com capacidade de produzir 86,3 ± 4,1 mg de ácido rosmarínico/g planta (dw, massa seca) nas condições ótimas de extração (33,0 ± 3,2 min, 371,7 ± 19,3 W e 39,9 ± 1,4% de etanol). Tendo em vista a sua aplicação a nível industrial, o efeito da razão sólido/líquido nos valores ótimos num formato dose-resposta foi também estudado. Após avaliar as atividades antioxidante, antibacteriana e antifúngica do extrato de cidreira, bem como o seu potencial de hepatotoxicidade, o extrato rico em ácido rosmarínico, foi incorporado em *cupcakes* e os seus efeitos conservantes, composição química, parâmetros de cor e atividade antioxidante comparados com os obtidos com o E202. Apesar de a incorporação deste extrato alterar o aspeto visual dos *cupcakes* dando-lhes um aspeto mais "integral", os resultados comprovam a sua possível aplicação como ingrediente natural em produtos de pastelaria uma vez que se comprovou uma forte bioatividade (atividades antioxidante, antibacteriana e antifúngica) do extrato, fornecendo desta forma propriedades benéficas ao produto final e à saúde dos consumidores.

Para encerrar este projeto de doutoramento aliou-se o *ex libris* da região com um produto de pastelaria icónico do nosso país. Assim, após a preparação de um extrato rico em compostos fenólicos extraído por maceração a partir de flores masculinas de castanheiro, este foi testado de forma idêntica aos anteriores (atividade antioxidante e antimicrobiana) e incorporado em pastéis de nata. Tal como no estudo anterior com *cupcakes*, a incorporação do extrato de flores de castanheiro alterou a cor original do creme conferindo-lhe uma cor acastanhada. No entanto, os outros parâmetros não foram significativamente alterados, com exceção da atividade antioxidante.

De uma forma geral, os extratos obtidos a partir das diferentes plantas estudadas, quando incorporados em diferentes produtos alimentares, acrescentaram valor, aumentando o tempo de prateleira relativamente ao produto controlo (produto sem qualquer tipo de aditivos). Adicionalmente, conferem atividade antioxidante semelhante ou superior à demonstrada por produtos com aditivos artificiais. A avaliação do perfil nutricional revela que esta incorporação não altera significativamente a sua composição, ainda que altere um pouco o aspeto visual no caso dos *cupcakes* e pastel de nata. Contudo, o aspeto "integral" conferido é interpretado como "mais saudável", podendo constituir um fator atrativo aos olhos dos consumidores.

Em conclusão, os resultados demonstram o potencial das plantas estudadas e dos extratos preparados como ingredientes naturais para alimentos funcionais. Além de mais saudáveis, demonstraram ainda capacidade conservante com potencial para substituir os aditivos artificiais usados tradicionalmente na indústria alimentar.

Palavras-chave: Alimentos funcionais, ingredientes naturais, aditivos naturais/artificiais, compostos fenólicos, propriedades bioativas, consumidores.

Abstract

Today, functional foods represent one of the most promising, impacting and innovative areas of the food industry. There is a growing concern from several consumers in the search for foods labeled as healthier and more natural. In this way, there is an increasingly critical sight of the food label, coupled with the fact that consumers are more informed and aware of their choices and of the influence of these healthier choices on their health and well-being. Thus, foods without artificial additives, or with a minimal addition, have gained preference over those including them. Such a trend and demand from consumers, has exerted a direct influence on the food industry, which tries to meet the expectations of consumers. There are several components traditionally added to foods in order to make them functional. Phenolic compounds are an example of such naturally occurring components, which are associated with high antioxidant capacity and human health benefits. Despite the large number of scientific and patented studies on this topic, and their natural presence in foods, particularly in vegetable sources, there are still very few marketed products enriched with these compounds. The commercialization of this type of functional products needs to comply several regulations, in order to prove their safety and attributed health benefits, and thus conquer the consumer. There are several studies that describe different bioactive properties of phenolic compounds, thus ensuring their potential as bioactive ingredients in foods. However, some problems of inherent stability and lost of bioavailability have been identified, which need to be solved in order to become effective solutions to render foods functional.

In this work, phenolic extracts obtained from plants were studied to be incorporated in different food matrices aiming at obtaining functional foods, and at the same time, increase their shelf life by substitution of artificial additives, namely preservatives. For all the studied extracts, the characterization comprised the identification of bioactive compounds, their quantification and biological activity evaluation. The phenolic compounds were determined by high performance chromatography coupled with ion detection and mass spectrometry (HPLC-DAD-ESI / MS). Regarding biological activities, the antioxidant, antimicrobial (anti-bacterial and antifungal) activities and hepatotoxicity were evaluated. For the determination of the antioxidant activity, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and reducing power (PR) assays were carried out to evaluate the total antioxidant activity, whereas for the evaluation of lipid peroxidation the inhibition of β-carotene discoloration and inhibition of reactive thiobarbituric acid species (TBARS). For the evaluation of the antimicrobial activity, eight bacteria and eight fungi, selected according to their importance in public health, were tested and the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum

fungicidal concentration (MFC) determined. For the hepatotoxicity assays, they were tested in primary cultures of pig liver cells.

After the incorporation of the extracts in the different food matrices, it was necessary to test, for the different samples and storage times, the effects on the nutritional profile, physico-chemical characteristics (pH and color) and antioxidant activity. For all the produced samples, moisture, carbohydrates, proteins, fat, ash and energy were determined following the AOAC official methods. The fatty acid profile was determined by gas chromatography with flame ionization detection (GC-FID), and sugars by high performance chromatography with refractive index (HPLC-RI) detection. The antioxidant activity was evaluated using the two tests previously mentioned (DPPH and PR).

The work started with the study of two plants widely consumed due to their well recognized beneficial properties. After chemical characterization of the extracts obtained by decoction of *Foeniculum vulgare* Mill. (fennel) and *Matricaria recutita* L. (chamomile), their antioxidant and antimicrobial properties were evaluated. Their incorporation in cottage cheese was carried by using its free and encapsulated forms. The evaluation of the effects of this incorporation showed that the addition of the extracts does not change the nutritional profile of the cottage cheese, providing antioxidant activity and extending product's shelf life. Since artificial additives are not industrially used in cottage cheese, a food with a very short shelf life, the same extracts were tested in yogurts and compared with the effect of using potassium sorbate (E202). In this study, it was concluded that the decoctions of these plants can be used to develop new yoghurts, replacing artificial preservatives and improving the antioxidant properties of the final product, without modifying the nutritional profile. The same type of study was carried out in a pastry product (biscuits) where it was verified that the natural extracts and the artificial additive (butylated hydroxyanisole, BHA) conferred similar antioxidant activity to the biscuits without changing their physicochemical properties or nutritional profile. After a sensory study, although consumers find few differences among the tested biscuits, the natural additives were associated to a more convenient solution since consumers prefer foods "free" of artificial additives.

Melissa officinalis L. (lemon balm), a plant known by the population since ancient times, has recognized benefits, and chemically presents a high amount of rosmarinic acid. Thus, in this work the conditions maximizing the extraction of this compound was studied by comparing three different extraction techniques: heat-assisted, microwave and ultrasound. Ultrasond-based extraction proved to be the most efficient method, capable of producing 86.3 ± 4.1 mg of rosmarinic acid / g plant (dw, dry mass) under the optimal extraction conditions (33.0 ± 3.2 min, 371.7 ± 19.3 W and 39.9 ± 1.4% ethanol). In view of an industrial application, the effect of the solid / liquid ratio on the optimum values in a dose-response format was also inspected. After evaluating the antioxidant, antibacterial and antifungal activities of the lemon balm

extract, as well as the hepatotoxicity, the extract rich in rosmarinic acid was incorporated in cupcake formulations, and the preservative effect, chemical composition, color parameters and antioxidant activity compared with those obtained with E202 addition. Although the incorporation of this extract changes the visual aspect of the produced cupcakes, giving them a more "integral" aspect, the results corroborate the application of this extract as a natural ingredient in pastry products due to its strong bioactivity (antioxidant, antibacterial activities and antifungal), thus providing beneficial properties to the final product and consumer's health.

To conclude this study, a combination of the *ex libris* of the region with a pastry product representative of the country was explored. Thus, after preparation of an extract rich in phenolic compounds, obtained by maceration from chestnut flowers, its testing was performed similarly to the previously studied, i.e. characterization of the antioxidant and antimicrobial activity followed by its incorporation in *pastel de nata*. As in the previous study with cupcakes, the incorporation of the chestnut flower extract changed the original color of the cream imparting a brownish color. However, the other parameters were not significantly modified with the exception of the antioxidant activity.

In general, the extracts obtained from the studied plants, when incorporated into different food products, confer an added value by increasing the shelf life relative to the control product (product without any additives), while conferring an antioxidant activity similar, or even superior, to the one of the control product, as demonstrated by products added with artificial additives. The evaluation of the nutritional profile reveals that this incorporation does not significantly alter the composition of the final product. Although it slightly changes the visual appearance in the case of cupcakes and *pastel de nata*, the given more "integral" aspect is interpreted as "healthier" making the products more attractive to consumers.

As a main conclusion, these results demonstrate the potential of the tested plants, and respective extracts, to act as natural ingredients for functional foods, providing health benefits. Moreover, they have shown preservative capacity with potential to replace the artificial additives traditionally used in the food industry.

Keywords: Functional foods, natural ingredients, natural / artificial additives, phenolic compounds, bioactive properties, consumers.

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Lista de abreviaturas e símbolos

Visto tratar-se de um documento bilingue, a explicação da abreviatura/símbolo aparece na língua correspondente ao texto em que aparece mencionada.

a*	*Red-green*
A	*Additive*
ALT	Alanina transaminase
ANOVA	Análise de variância
AOAC	Associação Oficial de Químicos Analíticos/*Association of Oficial Analytical Chemists*
AST	Aspartato transaminase
b*	*Yellow-blue*
BHA	Hidroxianisol butilado / *Butylated hydroxyl anisole*
C	Control
$CaCl_2$	*Calcium chloride*
CCCD	*Circumscribed central composite design*
CCL_4	Tetracloreto de carbono
CDOA	*Caffeoyl-2,7-anhydro-3-deoxy-2-octulopyranosonic acids*
CF	*Cupcake formulation*
Ch80	*Biscuits with 80 mg of chamomile extract*
Ch800	*Biscuits with 800 mg of chamomile extract*
Cham	*Chamomile in the same amount of BHA*
Cham10	*Chamomile ten times more than BHA*
CHD	Doença cardíaca coronária
DAD	*Diode array detetor*
diCDOA	*Dicaffeoyl-2,7-anhydro-3-deoxy-2-octulopyranosonic acid*
DMEM	*Dulbecco's modified Eagle's medium*
DPPH	2,2-difenil-1-picril-hidrazilo/ *2,2-Diphenyl-1-picrylhydrazyl*
Dw	Massa seca/ *Dry weight*
E202	Sorbato de potássio/ *Potassium sorbate*
E392	*Rosemary Extract*
EC_{50}	*Effective concentration achieving 50% of antioxidant activity or 0.5 absorbance in reducing power assay*
EE	*Encapsulation efficiency*

EFSA	Autoridade Europeia para a Segurança Alimentar/ *European Food Safety Authority*
EMM	*Estimated marginal means*
ERA	*Lemon balm extract rich in rosmarinic acid*
ESI	*Electrospray ionization*
EUA	Estados Unidos da América
F	*Fortification*
F80	*Biscuits with 80 mg of fennel extract*
F800	*Biscuits with 800 mg of fennel extract*
FA	*Functionalizing agente*
FAME	*Fatty acids methyl ester*
FAO	Organização das Nações Unidas para a alimentação e a agricultura/ *Food and Agricultural Organization*
FDA	*Food and Drug Administration*
Fen	*Fennel in the same amount of BHA*
Fen10	*Fennel ten times more than BHA*
FID	*Flame ionization detetor*
FOSHU	*Foods for Specified Health Use*
FT	*Functionalization type*
FTIR	*Fourier transform infrared spectroscopy*
FUFOSE	*Functional Food Science in Europe*
Fw	*Fresh weight*
GC	*Gas-chromatography*
GI_{50}	*Sample concentration that inhibited 50% of the net cell growth*
GLM	*General linear model*
HAE	*Heat-assisted extraction*
HPLC	Cromatografia líquida de alta eficiência/ *High-performance liquid chromatography*
L*	*Lightness*
LAB	Bactérias do ácido láctico
LDA	*Linear discriminant analysis*
LDL	Lipoproteínas de baixa densidade
m/z	*Mass-to-charge ratio*
MA	*Malt agar*
MAE	*Microwave-assisted extraction*
MBC	Concentração mínima bactericida / Minimum bactericidal concentration

MDA–TBA	*Malondialdehyde–thiobarbituric acid*
MFC	Concentração mínima fungicida / *Minimum fungicidal concentration*
MH	*Mueller-Hinton agar*
MIC	Concentração mínima inibitória/ *Minimum inhibitory concentration*
MS	*Mass spectometry*
MS^2	*Second stage of mass spectrometry*
MUFA	*Monounsaturated fatty acids*
NADPH	Nicotinamida Adenina Dinucleotídeo Fosfato
Nd	*Not detected*
OM	*Optical microscopy*
OMS	Organização Mundial da Saúde
P/ X_2	*Utrasound power*
PDA	*Photodiode array detector*
PUFA	*Polyunsaturated fatty acids*
R	*Total extracted residue*
RA	*Rosmarinic acid*
RI	*Refraction índex*
RNS	Espécies reativas de nitrogênio
ROS	Espécies reativas de oxigênio
RP	Poder redutor/ *Reducing power*
RSM	*Response surface methodology*
Rt	*Retention time*
S/ X_3	*Ethanol solvent proportion*
SD	*Standard deviation*
SFA	*Saturated fatty acids*
ST	*Storage time*
t/ X_1	*Time*
T/ X_2	*Temperature*
TBARS	Espécies reativas do ácido tiobarbitúrico/ *Thiobarbituric acid reactive substances*
Trolox	*6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid*
UAE	*Ultrasound-assisted extraction*
UE	União europeia
UHT	*Ultra high temperature*
UV	Radiação ultravioleta
v/v	*Volumetric percentage*

w/w	*Percentage solution*
λ_{max}	*Wavelength of maximum absorption*

1.

Motivação, objetivos e estrutura da tese

No presente capítulo identificam-se as principais motivações para o desenvolvimento desta tese, apresentando a problemática do uso de aditivos artificiais e a crescente procura por parte da indústria alimentar, por substitutos naturais efectivos. Enumeram-se também os principais objetivos, apresentam-se as tarefas a executar para os atingir, bem como os artigos científicos resultantes do trabalho desenvolvido nesta tese.

1.1. **Motivação da Tese**

O uso de aditivos alimentares surgiu no passado devido à necessidade de aumentar o tempo de prateleira e/ou acrescentar alguma característica específica a determinado alimento (Aun et al., 2011). Atualmente, os alimentos processados necessitam muitas vezes de percorrer longas distâncias até chegarem aos consumidores e, portanto, é necessário assegurar que chegam ao destino nas melhores condições de qualidade e segurança (Carocho et al., 2014). Neste sentido, para prevenir a contaminação e deterioração dos alimentos começaram a introduzir-se aditivos alimentares artificiais. Apesar de existirem muitos aditivos autorizados para aplicação na indústria alimentar, são vários os estudos que têm revelado uma relação entre o consumo excessivo de determinados aditivos e vários problemas de saúde nomeadamente, a nível gastrointestinal, respiratório, dermatológico e neurológico (Branen, Davidson, Salminen, & Thorngate, 2001; Randhawa & Bahna, 2009; Wilson & Bahna, 2005).

Pelo referido, existe atualmente, por parte da indústria alimentar uma preocupação crescente pela substituição deste tipo de aditivos artificiais por compostos naturais, ao mesmo tempo que se responde às novas tendências e exigências por parte do consumidor, cada vez mais informado e sensibilizado para o consumo de opções mais saudáveis (Bearth, Cousin, & Siegrist, 2014). Desta forma, procura-se evitar o potencial tóxico dos aditivos alimentares desenvolvendo novos alimentos funcionais com ingredientes naturais promotores de saúde. São vários os compostos antioxidantes naturais estudados e apresentados como benéficos para a saúde, nomeadamente na prevenção de várias doenças crónicas (Carocho & Ferreira, 2013a). Adicionalmente, verificou-se que a atividade antimicrobiana destas moléculas pode retardar e/ou inibir o crescimento de microrganismos patogénicos e/ou produtores de toxinas nos alimentos. Consequentemente, a sua utilização reduz a incidência de toxicoinfeções alimentares (Beuchat, 2001).

Vários estudos atribuiram propriedades medicinais à presença de vários compostos com ação definida sobre a qualidade microbiológica, química e sensorial dos alimentos (Gurib-Fakim, 2006; Phillipson, 2007). Esta ação está maioritariamente associada à presença de elevadas quantidades de compostos fenólicos, os quais são conhecidos por serem fortes captadores de radicais livres e por desempenharem funções preventivas em diversas doenças, nomeadamente doenças cardiovasculares, neurodegenerativas ou cancro (Procházková, Boušová, & Wilhelmová, 2011; Weng, & Yen, 2012). A elevada disponibilidade de espécies vegetais com potencial aplicação é uma grande vantagem, considerando a necessidade de um fornecimento consistente de produtos bioativos de qualidade.

Neste trabalho, foi proposta a incorporação de extratos fenólicos de diferentes plantas (flores de *Matricaria recutita*, partes aéreas de *Foenicullum vulgare*, folhas de *Melissa*

officinalis e flores masculinas de *Castanea Sativa*), com atividade antioxidante e antimicrobiana, em diferentes produtos lácteos e de pastelaria. Com dois dos extratos, foi testado e comparado o efeito da sua incorporação na forma livre *versus* microencapsulada. Estudos comparativos de diferentes técnicas de extração e estudos de estabilidade foram também realizados. Todos os alimentos funcionais foram avaliados através da caracterização físico-química, nutricional e biológica, para garantir a qualidade e segurança destes produtos e garantir a preservação do produto final, após a incorporação dos ingredientes bioativos. A avaliação das propriedades bioativas foi também realizada para garantir que os ingredientes naturais preparados e incorporados no produto alimentar mantêm a sua bioatividade no produto final. Esta avaliação foi realizada ao longo do tempo de armazenamento (específico para cada produto testado tendo em conta o tempo de prateleira) e os resultados comparados com formulações convencionais incorporando aditivos artificiais.

1.2. **Objetivos**

Os principais objetivos deste estudo foram:

1) Selecionar diferentes plantas para obtenção de extratos ricos em compostos fenólicos (**Tabela 1**) para posterior incorporação em alimentos de forma a torná-los funcionais e aumentar o seu tempo de prateleira;
2) Estudar o efeito da incorporação destes extratos naturais em diferentes matrizes alimentares e comparar a sua performance com a de aditivos artificiais utilizados na área alimentar, visando avaliar o seu potencial na substituição destes a nível industrial.

Numa primeira abordagem, selecionaram-se duas espécies de plantas: *Foeniculum vulgare* Mill. (funcho) e *Matricaria recutita* L. (camomila). Após a preparação de extratos por decocção, procedeu-se à avaliação da atividade antioxidante e antimicrobiana (utilizando um painel de bactérias e fungos selecionados de acordo com a sua importância para a saúde pública) e à sua caracterização química através de HPLC-DAD-ESI/MS. Os extratos obtidos foram incorporados em requeijão, um produto lácteo muito apreciado pelos consumidores, mas com um tempo de prateleira muito reduzido. Neste produto foram estudados vários parâmetros:

i) a cor ao final de 7 e 14 dias para amostras de requeijão incorporando diretamente a planta, amostras controlo (sem qualquer tipo de aditivo) e amostras incorporando os extratos microencapsulados;
ii) a composição nutricional e a atividade antioxidante para os mesmos tempos.

O requeijão industrial é um alimento que não tem quaisquer aditivos incorporados para aumentar o tempo de prateleira. Assim, estudou-se o efeito da incorporação deste tipo de extratos, como substitutos de aditivos, em iogurtes, também um produto lácteo, mas que, normalmente apresenta o sorbato de potássio (E202) como aditivo. Nesta fase, comparou-se o efeito da adição dos extratos aquosos de funcho e camomila relativamente ao E202 a nível físico-químico (pH e cor), parâmetros nutricionais e avaliação antioxidante ao longo do tempo (imediatamente após a incorporação e no final de 7 e 14 dias).

Seguidamente realizou-se a incorporação dos extratos aquosos das duas plantas anteriores num produto de pastelaria (biscoitos), comparando o efeito deste enriquecimento de acordo com os mesmos parâmetros acima indicados e face a um aditivo artificial muito utilizado na indústria da panificação e pastelaria, o hidroxianisol butilado (BHA). No final deste estudo foi ainda realizada uma prova sensorial para avaliar a opinião dos consumidores relativamente quanto aos produtos desenvolvidos.

Numa segunda fase do trabalho, selecionou-se a *Melissa officinalis* L. (cidreira) para um estudo em que se pretendeu comparar o teor de ácido rosmarínico no extrato utilizando três técnicas distintas (extração assistida por calor, micro-ondas e ultrassons). Para o efeito utilizou-se metodologia de superfície de resposta para obter as condições que maximizam a extração do ácido rosmarínico. Após selecionar a extração por ultrassons como a técnica mais eficaz, efetuou-se a produção do extrato e avançou-se para a incorporação deste em *cupcakes* visando verificar a sua eficácia como aditivo natural em substituição do E202. Mais uma vez, os parâmetros físico-químicos, nutricionais, e atividade antioxidante foram avaliados ao longo do tempo (0, 3 e 5 dias) para comparar os seus efeitos.

Na terceira fase deste trabalho, selecionou-se um “ex libris” da região, o castanheiro, aproveitando as flores caídas e recolhidas do chão para estudar as melhores condições para obter um extrato rico em compostos fenólicos. Após comprovar a sua atividade antioxidante, antimicrobiana e a ausência de hepatoxicidade o extrato foi incorporado num produto de pastelaria tradicional portuguesa reconhecido mundialmente, o pastel de nata. Os parâmetros físico-químicos, nutricionais e a atividade antioxidante foi testada ao longo do tempo (0 e 2 dias) para avaliar os efeitos desta incorporação comparativamente com o aditivo artificial (E202).

Tabela 1. Descrição das plantas utilizadas no presente trabalho.

Nome científico	Nome em português	Nome em inglês	Origem	Foto	Parte da planta estudada
Foeniculum vulgare Mill.	Funcho	Fennel	Comercial (Empresa ADP, Portugal)		Partes aéreas
Matricaria recutita L.	Camomila	Chamomile	Comercial (Empresa ADP, Portugal)		Flores
Melissa officinalis L.	Cidreira	Lemon Balm	Comercial (Empresa Mais Ervas, Portugal)		Folhas
Castanea Sativa Mill.	Flor de castanheiro	Chestnut flower	Silvestre (Bragança, Portugal, junho, 2013)		Flores

1.3. **Organização e estrutura**

O documento apresentado é bilingue e está dividido em cinco capítulos distintos, nos quais se abrangem todos os objetivos propostos para o trabalho. No presente capítulo, capítulo 1, escrito em português, faz-se uma descrição da motivação, dos objetivos da investigação e apresenta-se a organização e estrutura da tese.

No segundo capítulo, também escrito em português, é apresentada uma revisão do estado da arte. Esta inicia-se com o conceito, classificação e desenvolvimento de alimentos funcionais, bem como o uso de plantas como ingredientes naturais alimentares. Apresenta-se uma breve revisão sobre a caracterização e classificação de compostos fenólicos, a sua biodisponibilidade e as condicionantes sobre a sua aplicabilidade em alimentos funcionais. Relacionando os sub-capítulos apresentados é ainda feita uma revisão sobre o uso e aplicação de compostos fenólicos em alimentos funcionais, fazendo a referência a alguns estudos já realizados.

O capítulo 3, escrito em inglês, descreve todo o trabalho experimental de caracterização e incorporação de extratos aquosos de funcho e camomila como ingredientes naturais em diferentes matrizes alimentares. Os efeitos dessas incorporações no perfil nutricional e na atividade antioxidante foram avaliados e comparados com a adição de aditivos artificiais comumente utilizados pela indústria alimentar.

No capítulo 4, apresentado também em inglês, é descrito o trabalho desenvolvido na preparação de extratos ricos em compostos fenólicos obtidos a partir de folhas de cidreira e flores de castanheiro e a sua aplicabilidade em produtos de pastelaria como possíveis substitutos de aditivos artificiais.

Finalmente, no capítulo 5, escrito em português, são sintetizadas as principais conclusões globais do trabalho desenvolvido, dando destaque à sua contribuição para o desenvolvimento de novos produtos funcionais. São ainda apresentadas algumas sugestões para perspectivas futuras de trabalho.

1.4. **Plano de trabalho**

Para se alcançarem os objetivos propostos no subcapítulo 1.2 desta tese, o trabalho desenvolvido foi organizado em 3 fases, tal como é apresentado na **Figura 1**.

O trabalho foi desenvolvido em quatro laboratórios de investigação:

- Centro de Investigação de Montanha do Instituto Politécnico de Bragança (CIMO);

- Laboratório Associado Laboratório de Processos de Separação e Reacção – Laboratório de Catálise e Materiais (LA LSRE-LCM), pólo Instituto Politécnico de Bragança;

- REQUIMTE/LAQV, Departamento de Ciências Químicas da Faculdade de Farmácia da Universidade do Porto;

- Departamento de Fisiologia Vegetal, Instituto de Pesquisa Biológica “Siniša Stanković”, Universidade de Belgrado, Sérvia;

Os resultados experimentais são apresentados na forma de artigos científicos entre os capítulos 3 e 4 desta tese.

Tarefa 1 – Incorporação de *Foeniculum vulgare* e *Matricaria recutita*

1. Preparação de decocção
   - Avaliação da atividade antioxidante;
   - Avaliação da atividade antimicrobiana;
   - Caracterização química.
2. Incorporação em matrizes alimentares

Produtos lácteos

Requeijões

Biscoitos

Funcho | Camomila | Controlo

Planta

Decocção

Microesferas

Iogurtes

Controlo | E202 | Decocção Funcho | Decocção Camomila

Controlo | BHA | Decocção Funcho | Decocção Camomila

3 Tempos: 0, 7 e 14 dias

5 Tempos: 0,15,30,45,60 dias

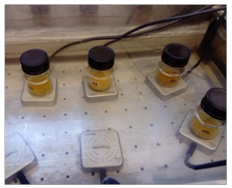
Avaliação:

- Parâmetros físico químicos;
- Perfil nutricional;
- Atividade antioxidante.

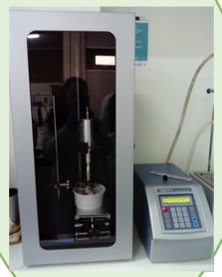
Tarefa 2 – Incorporação de *Melissa officinalis*

Otimização da extração de acido rosmarínico de *Melissa officinalis* comparando 3 técnicas de extração

Calor



Ultrassons



Micro-ondas

- Caracterização química;
- Avaliação da atividade antioxidante;
- Avaliação da atividade antimicrobiana
- Avaliação da hepatotoxicidade.

Incorporação em cupcakes

Controlo

E202

Extrato rico em ácido rosmarínico

Avaliação:

- Parâmetros físico químicos;
- Perfil nutricional;
- Atividade antioxidante.

3 Tempos: 0, 3, 5 dias

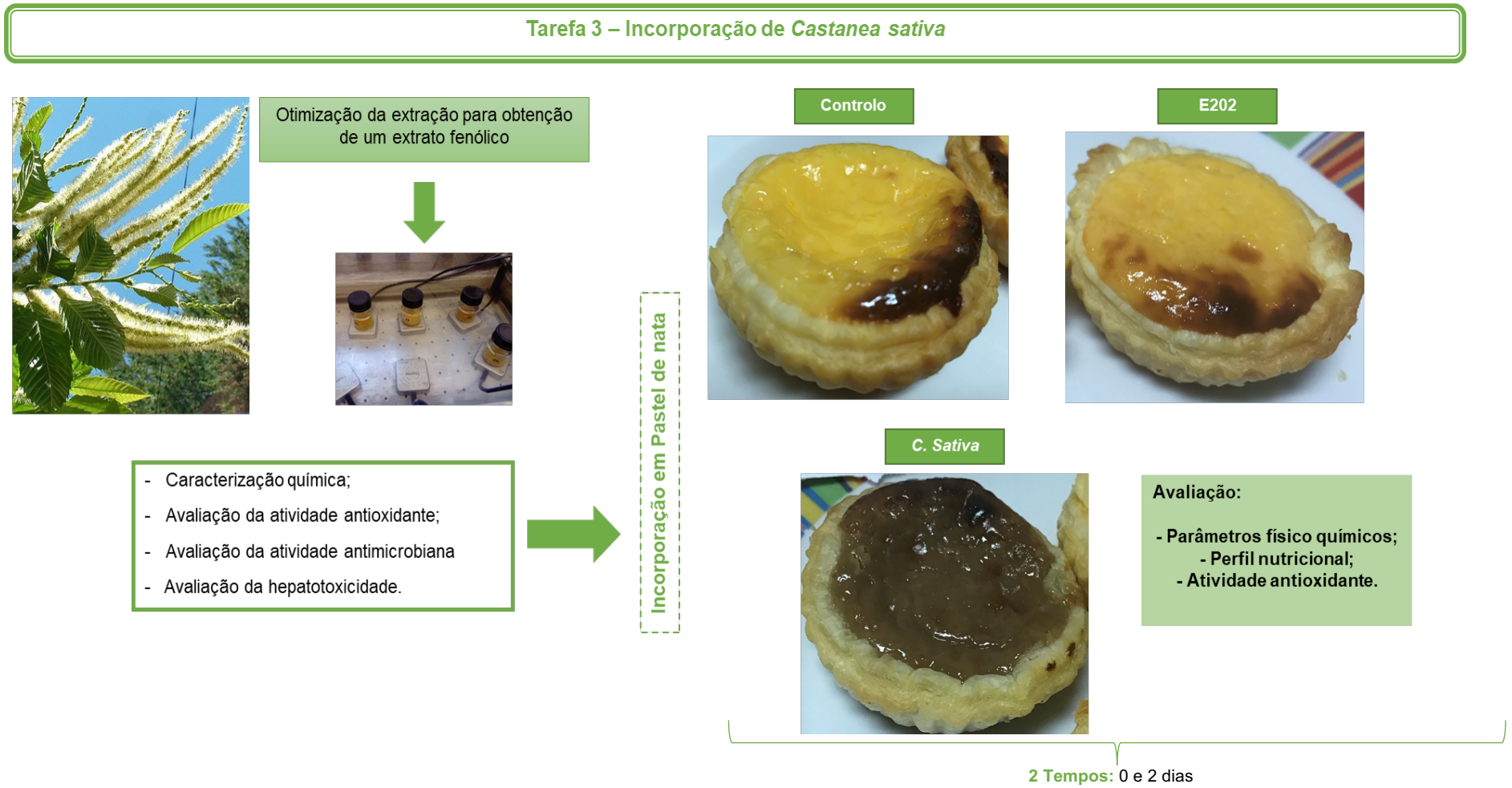


Figura 1. Descrição das diferentes tarefas.

1.5. **Bibliografia**

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Estado da arte

Neste capítulo apresenta-se o conceito, classificação, regulamentação e aceitabilidade dos alimentos funcionais; decreve-se o uso tradicional e ancestral de plantas como alimento funcional e como ingrediente natural de alimentos funcionais. Em seguida faz-se a revisão sobre a caracterização dos compostos fenólicos, principais componentes das plantas e responsáveis pelas funções benéficas que causam, sendo enumerados os factores que levam à sua degradação e apontando a técnica de microencapsulação como uma ferramenta de proteção útil para viabilizar a sua aplicação em alimentos. Por fim, apresenta-se uma revisão sobre os compostos fenólicos em alimentos funcionais.

2.1. **Plantas como alimentos funcionais**

2.1.1. O conceito e tendências atuais de alimentos funcionais

Hipócrates, um filósofo que viveu entre 460 e 370 aC, considerado o pai da medicina é o autor da célebre frase “Que o teu medicamento seja o teu alimento, e o teu alimento seja o teu medicamento”, ainda hoje muitas vezes citada. Apesar de ter vivido numa época em que a qualidade de vida era muito menor que atualmente conseguiu entender a necessidade e a importância de uma dieta saudável. Na primeira metade do século XX, essa visão começou a ser cada vez mais partilhada pelos consumidores e pela indústria alimentar que começou a focar-se nos requesitos mínimos de nutrientes essenciais (MMWR, 1999). Atualmente, esse conceito tem vindo a sofrer algumas alterações, progredindo de um conceito de "nutrição adequada" para uma "nutrição ideal" (Ashwell, 2003). Desta forma, os alimentos deixam de ser vistos apenas como uma forma de sobrevivência, satisfação da fome ou garantia da segurança alimentar, enfatizando o potencial dos alimentos para promover a saúde, tanto em termos de prevenção de doenças como de melhoria do bem-estar físico e mental (Nöthlings et al., 2007; Takachi et al., 2008). Na realidade, os consumidores estão, hoje em dia, cada vez mais conscientes e informados sobre este assunto e por isso, as expectativas de obter benefícios para a saúde a partir dos alimentos que ingerem são cada vez maiores (Diplock et al., 1999). Estas alterações de comportamento podem ser explicadas devido às tendências da sociedade atual, aos avanços rápidos da ciência e tecnologia, ao aumento dos custos com a saúde, aumento do número de pessoas idosas e da esperança média de vida, às mudanças na regulamentação alimentares que afetam o rótulo e reivindicações de produtos e, ao desejo das populações por uma maior qualidade de vida (Roberfroid, 2007).

A principal função dos alimentos é a de fornecer nutrientes para atender às necessidades do metabolismo humano e dar ao consumidor o sentimento de saciedade, satisfação e bem-estar através de atributos hedonistas como o gosto. Além disso, o alimento pode ter funções fisiológicas específicas no corpo humano (Li *et al*. 2014a; Zhang *et al*. 2015). Na verdade, a alimentação para além de poder ajudar a alcançar uma saúde e desenvolvimento ótimos, pode desempenhar um papel importante na redução ou prevenção do risco de algumas doenças. De acordo com a Organização Mundial da Saúde (OMS) e a Organização para a Alimentação e a Agricultura (FAO), diferentes padrões alimentares, aliados a hábitos e estilos de vida, são os principais fatores de risco em relação ao desenvolvimento de doenças coronárias, alguns tipos de cancro, diabetes, obesidade ou osteoporose (WHO 2003).

Os alimentos com essas propriedades foram inicialmente regulamentados no Japão em 1981 numa categoria específica designada por *Foods for Specified Health Use* (FOSHU) (Hasler 2002; Ohama et al., 2006). Mais tarde, na Europa, foi criado o projeto *Functional Food Science in Europe* (FUFOSE) com o objetivo de avaliar com base científica as evidências de que nutrientes e componentes alimentares específicos afetam de forma benéfica as funções alvo no corpo humano (Tijhuis et al., 2012). Apesar desta área ter vindo a crescer significativamente na Europa, na União Europeia não existem categorias específicas de alimentos sendo necessária uma série de regras e diretrizes para definir o produto em si, obedecendo ao Regulamento Geral de Legislação Alimentar sobre segurança alimentar (Coppens et al. 2006; Gulati & Ottaway 2006). Para além da legislação exigida para todos os alimentos, a evidência científica das alegações de saúde em relação à matriz alimentar em estudo é obrigatória para todos os novos produtos (Bech-Larsen & Scholderer 2007). Por outro lado, a *Food and Drug Administration* (FDA), é o órgão federal dos Estados Unidos responsável por definir as categorias de produtos, as quais dependem das suas características e obedecem a diversas questões de segurança, alegações de saúde e rotulagem (Milner 2000; Wrick 2005). Atualmente, esse tipo de alimento é geralmente referido como "alimento funcional".

Os alimentos funcionais são considerados mais um conceito do que um grupo de alimentos bem definido e consequentemente, não existe uma definição universalmente aceite para alimentos funcionais (Hasler 2002). De acordo com Diplock et al. (1999) um alimento pode ser considerado como "funcional" se demonstrar, de maneira satisfatória, que contribui de forma benéfica e relevante para uma ou mais funções alvo no corpo humano, de forma a melhorar a saúde e bem-estar e reduzindo o risco de doenças, além de apresentar efeitos nutricionais adequados. Estes alimentos devem, no entanto, preservar a aparência original e os seus efeitos benéficos devem ser demonstrados quando ingeridos em quantidades normalmente consumidas na dieta habitual, ou seja, não são comprimidos ou cápsulas, mas parte de um padrão alimentar normal. Além disso, um alimento funcional pode ser um alimento natural ou não modificado, ou um alimento ao qual foi adicionado ou removido um componente por meios tecnológicos ou biotecnológicos. Pode ainda ser considerado um alimento onde a natureza de um ou mais componentes foi modificada, a biodisponibilidade alterada ou qualquer combinação dessas possibilidades. Além disso, um alimento funcional pode ser funcional para todos os membros de uma população ou para grupos particulares da população. Para além de não exister uma definição universal, os mercados globais também não possuem os mesmos sistemas regulatórios para esses alimentos (Bagchi 2014).

Ainda que a ciência na área dos alimentos funcionais esteja num estado inicial do seu desenvolvimento, o conhecimento sobre os efeitos funcionais dos alimentos é crescente e a

funcionalidade de determinados alimentos e componentes alimentares é mais amplamente reconhecida e a tecnologia é um aliado para tornar estes alimentos e componentes alimentares mais amplamente disponíveis e acessíveis (Howlett, 2008). Por outro lado, sabe-se que os fatores genéticos influenciam a relação entre dieta e doença e as formas como diferentes fatores protetores e de risco podem atuar. Além disso, é possível visualizar as diferenças entre os perfis genéticos dos indivíduos a nível molecular e entender como eles se relacionam com as diferenças entre as respostas dos indivíduos aos fatores fisiológicos. Assim, num futuro próximo, o conhecimento adquirido nos campos da genómica, proteómica e metabolómica serão de extrema importância para o desenvolvimento de alimentos funcionais e para criar programas de dieta personalizados, bem como em verificar a influência de fatores alimentares na saúde e doença humana, o que pode levar à identificação de novas rotas de funcionalidade alimentar (Howlett, 2008).

2.1.2. Classificação e desenvolvimento de alimentos funcionais

Atualmente os alimentos funcionais representam uma das áreas de investigação que mais tem despertado o interesse e consequentemente o desenvolvimento na indústria alimentar (Annunziata & Vecchio 2011). Apesar do desenvolvimento e *design* destes produtos representarem um proceso bastante dispendioso (Betoret *et al*. 2011), esta é uma questão fundamental representando um enorme desafio científico. Deve basear-se em conhecimentos científicos fundamentais e procurar obter os alimentos funcionais tão desejados pelos consumidores (Diplock *et al*. 1999).

Os alimentos funcionais, dependendo da sua natureza, podem ser classificados como, alimentos funcionais naturais (ou não modificados) e modificados. Ainda que possam ser modificados, estes alimentos têm sempre de garantir a segurança para o consumidor não alegando qualquer benefício em compensação do risco para a saúde. Mais especificamente, e de acordo com a definição de alimentos funcionais apresentada anteriormente, estes podem ser classificados como: a) Produtos não alterados: alimentos que naturalmente apresentam um teor superior de nutrientes e/ou compostos promotores de saúde; b) Produtos fortificados: alimentos em que foi acrescentado o teor de certos componentes, normalmente já existentes; c) Produtos enriquecidos: alimentos aos quais é adicionado um componente normalmente não existente, para proporcionar benefícios; d) Produtos alterados: alimentos em que um componente é removido ou substituído por um componente alternativo com propriedades favoráveis; ou e) Produtos melhorados: a composição do alimento é alterada ou seja, um dos

componentes é melhorado através de condições especiais de crescimento ou de meios biotecnológicos.

O lançamento de novos produtos com alegações de benefícios para a saúde está em constante crescimento em todo o mundo (Bigliardi & Galati, 2013). Atualmente já é possível encontrar alguns produtos comerciais com funções benéficas para a saúde nas prateleiras dos supermercados; entre eles, os mais procurados pelos consumidores são: iogurte líquido probiótico contendo culturas *Lactobacillus casei Imunitass*® (marca Actimel® da Danone®, França), iogurte sólido com *Bifidus ActiRegularis*® (marca Activia® da Danone®, França), snacks e barras de cereais com fibras naturais e extra minerais e vitaminas (marca Snack Fibra® de Celigüeta®, Espanha), bebida láctea com *L. casei Shirota* (marca Yacult® de Yacult®, Japão) e iogurte com pré e probióticos e ómega-3 (marca Vitality®, Müller®, Alemanha).

Entre outros, os produtos lácteos têm muitos benefícios para a saúde. São largamente consumidos e apreciados pelos consumidores e, assim, representam um veículo importante para o desenvolvimento de alimentos funcionais. Este setor está em constante inovação no desenvolvimento de novos produtos com valor acrescentado. Consequentemente, verificou-se uma variedade crescente de novos produtos lácteos para satisfazer a procura do consumidor por produtos mais saudáveis, incluindo leite com baixo teor de gordura (por exemplo, leite magro ou meio-gordo) e com elevado teor proteico (Kanekanian, 2014).

Apesar de mundialmente a indústria de alimentos funcionais apresentar um crescimento exponencial, a comercialização bem-sucedida de novos alimentos funcionais continua a representar um grande desafio, especialmente devido à necessidade de uma abordagem estratégica para os processos de produção (Howlett 2008). Desta forma, a aceitação sensorial, conveniência, estabilidade, propriedades químicas e funcionais bem como o preço, são algumas das variáveis que são consideradas no desenvolvimento de alimentos funcionais modificados (Granato *et al*. 2010; Betoret *et al*. 2011). Um ponto-chave que deve ser tido em conta no *design* destes novos alimentos é o facto de o efeito funcional depender do acesso do componente ativo ao local-alvo funcional. No entanto, os alimentos são misturas complexas que podem interceptar o composto ativo, modular a sua libertação ou inibir sua atividade. Desta forma, a matriz alimentar, tanto no seu estado bruto, após a preparação culinária, ou armazenamento pode ter uma influência significativa sobre a atividade ou libertação dos principais componentes. Segundo Betoret et al. (2011), o desenvolvimento de veículos alimentares adequados para manter a forma ativa até ao momento do consumo, e entregar essa forma no local alvo desejado no organismo, é o passo crucial para o sucesso dos alimentos funcionais.

Num estudo apresentado por Betoret et al. (2011) as tecnologias disponíveis para o desenvolvimento de alimentos funcionais foram agrupadas em três grupos principais. O primeiro grupo é formado pelas tecnologias mais utilizadas para o desenvolvimento de alimentos funcionais, incluindo as tecnologias tradicionalmente utilizadas no processamento, formulação e mistura de alimentos, bem como para o cultivo e a reprodução. O segundo grupo, constituído por metodologias que formam uma estrutura para tentar evitar a deterioração de compostos fisiologicamente ativos, inclui a microencapsulação, utilização de filmes e revestimentos comestíveis e tecnologias de impregnação a vácuo. O terceiro e último grupo é formado por tecnologias recentes que visam projetar alimentos funcionais voltados para a nutrição personalizada, sendo este o que mais tem crescido nos últimos anos.

Atualmente, os alimentos que indicam melhoria para a saúde do consumidor têm que passar por um grande controlo, principalmente no que diz respeito à informação da rotulagem. A União Europeia (UE) enfrenta, desde 2012, uma nova legislação que exige um rigoroso padrão de evidência científica para fazer alegações de saúde sobre alimentos, bebidas e suplementos alimentares (Gulati & Ottaway, 2006; Wong, Lai, & Chan, 2015).

O mercado dos alimentos funcionais está em crescimento crescente em todo o mundo (Annunziata & Vecchio, 2010; Menrad, 2003; Verbeke, 2005). Estima-se que em 2012, o seu mercado global tenha sido cerca de 625 mil milhões de dólares (Khan et al., 2013), sendo os três principais mercados representados pelo Japão, seguidos dos Estados Unidos da América (EUA) e, finalmente, pela UE. Estes três mercados contribuem para 90% das vendas totais (Bigliardi & Galati, 2013). Além de seu papel pioneiro (os alimentos funcionais começaram a ser uma realidade neste país), o Japão também é o mercado mais forte do mundo; em apenas 10 anos (1988-1998) mais de 1700 produtos alimentares funcionais foram legalizados (Bigliardi & Galati, 2013; Hilliam, 2000). Este mercado apresentou uma taxa média de crescimento de 9,6% ao ano durante os anos 90, com uma receita de faturação estimada em aproximadamente 12 biliões de dólares em 2003 (Annunziata & Vecchio, 2010).

Os EUA correspondem ao segundo maior mercado de alimentos funcionais, com uma faturação de 10,5 biliões de dólares em 2003. Neste país, a cota do mercado para alimentos funcionais é de cerca de 2 a 3% e está em constante crescimento (Annunziata & Vecchio, 2010; Menrad, 2003). O crescente mercado de alimentos e bebidas funcionais aumentou, globalmente, 1,5 vezes tendo alcançando 24,2 biliões de dólares em 2010 e 29,8 biliões de dólares em 2014 (Wong et al., 2015).

Os europeus ainda apresentam algum ceticismo em relação aos alimentos funcionais, em parte devido à suspeita de segurança dos mesmos, mas também por causa do seu processo produtivo (Annunziata & Vecchio, 2010). A procura por alimentos funcionais na UE varia de acordo com as tradições alimentares e culturais, sendo maior no norte da Europa do

que nos países mediterrânicos. O Reino Unido (2,6 biliões de dólares), a Alemanha (2,4 biliões de dólares), a França (1,4 bilião de dólares) e a Itália (1,2 bilião de dólares) constituem os principais mercados (Bigliardi & Galati, 2013; Annunziata & Vecchio, 2010). No entanto, muitos outros mercados, como Holanda, Espanha, Polónia e Hungria, estão a atingir elevadas taxas de crescimento (Ezhilarasai, Indrani, Jena & Anandharamakrishnan, 2013; Annunziata & Vecchio, 2010; Monar, 2007; van Trijp, 2007; Makinen-Aakula, 2006).

Na UE, assim como no Japão, o mercado dos alimentos funcionais é dominado pelos probióticos, tendo sido lançados mundialmente, em 2005, cerca de 370 novos produtos (Ouwehand, 2007). As bactérias do ácido láctico (LAB) e bifidobactérias são as mais estudadas e utilizadas para o desenvolvimento de novos produtos; em Itália, por exemplo, o produto mais importante no mercado dos alimentos funcionais é o chamado "iogurte de saúde" que em 2008 representou uma taxa de crescimento de 6,3%, atingindo assim 560 milhões de euros de vendas, correspondendo a quase 4% de todo o setor italiano (Bigliardi & Galati, 2013).

Os alimentos funcionais devem ter benefícios adicionais quando comparados aos tradicionais. A funcionalidade desses alimentos é resultado da presença de ingredientes bioativos. Tipicamente, os ingredientes funcionais incorporados aos alimentos visam prevenir algumas doenças crónicas, para além de promover o bem-estar físico e psíquico. Portanto, este tipo de produtos destina-se essencialmente aos consumidores com preocupações com a saúde, bem-estar e envelhecimento saudável.

De acordo com Arvanitoyannis & Houwelingen-Koukaliaroglou (2005), populações de diferentes países procuram características diferentes nos alimentos. Assim, os americanos procuram principalmente alimentos funcionais para perda de peso e redução do colesterol; Na UE, a principal preocupação reside na prevenção de saúde através alimentos. Em particular, no Reino Unido há maioritariamente uma procura por alimentos para promover a saúde dos ossos e dentes; na Alemanha para reforçar/estimular o sistema imunitário e a saúde óssea; e na França, para estimular a redução do colesterol e, geralmente, aumentar a resistência a doenças.

Os alimentos funcionais foram desenvolvidos em praticamente todas as categorias de alimentos. Os componentes funcionais dos cereais, como o beta-glucano (principalmente de aveia e cevada), têm sido utilizados nas indústrias de produtos lácteos para produzir gelados com baixo teor de gordura (Brennan & Cleary, 2005). Os produtos de panificação ainda representam uma pequena participação na indústria de alimentos funcionais (pouco mais de 13% em 2000), comparativamente com produtos lácteos, que detêm a maior fatia do mercado de alimentos funcionais (Siró, Kápolna, Kápolna, & Lugasi, 2008; Menrad, 2003). No entanto, foi observado um crescimento neste setor, com alguns exemplos de sucesso no mercado.

Este é o caso da Unilever® que produziu em 2003 a "Blue Band Goede Start®", o primeiro pão branco enriquecido com elementos funcionais (fibra, vitaminas B1, B3 e B6, ferro, zinco e inulina) (Benkouider, 2005).

Os ovos, alimento rico em nutrientes muito apreciado pelos consumidores, são outro exemplo que despertou o interesse da indústria de alimentos para torná-los funcionais. No Reino Unido, a Freshlay Foods® produziu o VITA Eggs®, descrito como ovos enriquecidos com ácidos gordos ómega-3 em conjunto com antioxidantes e outras vitaminas (D, E, B12 e ácido fólico). Também na Bélgica, está descrito um caso de sucesso semelhante: o Columbus® da Belovo®, ovos enriquecidos em ómega-3 e vitamina E, exportados para todo o mundo (Siró et al., 2008).

Na UE, apesar de ainda representar uma fatia muito pequena no setor, o mercado de bebidas não alcoólicas enriquecidas com vitaminas (especialmente A, C e E) e outros componentes bioativos, está em constande desenvolvimento. Em particular, bebidas com soja e ómega-3 (para baixar o colesterol), bebidas com luteína (para melhorar a visão) e bebidas com cálcio e inulina (para melhorar os problemas de saúde), são alguns exemplos (Keller, 2006). A Alemanha é um representante de sucesso desta categoria de alimentos, principalmente devido ao sucesso das bebidas ACE®, com uma receita de vendas de muitos milhões de dólares na última década (Siró et al., 2008). Outro exemplo de sucesso, especialmente devido à grande contribuição para a economia da Estônia, são as bebidas Largo® (sumos enriquecidos com inulina, L-carnitina, vitaminas, cálcio e magnésio) (Tammsaar, 2007). Também os produtos lácteos líquidos funcionais, incluindo iogurtes, estão entre os produtos mais bem-sucedidos. Estes alimentos são geralmente enriquecidos com pré e/ou probióticos, de forma a transmitir algumas funções benéficas ao organismo humano. Este tipo de produtos lácteos apresenta um crescimento expressivo de muitos milhões de dólares em toda a UE. Nesta área, as marcas mais representativas são a Danone® e a Unilever®, com um crescimento exponencial (Siró et al., 2008). Um produto amplamente conhecido é o Benecol®, uma bebida láctea enriquecida com óleo de camelina como fonte de ácidos gordos, em particular do tipo ómega-3 associado à redução do colesterol. Os cremes funcionais para barrar são outro exemplo de produtos com grande expansão de mercado. Becel® pro-activ, nome comercial de Becel® (Unilever®), é um produto bem conhecido para reduzir o colesterol (Hopia, 2006).

Várias empresas são reconhecidas mundialmente como apostando grande parte de seu investimento em produtos referidos como "mais saudáveis". Alguns exemplos incluem empresas de alimentos como a Nestlé®, Danone® Group, Kraft Foods®, Unilever®, PepsiCo®, Coca-Cola® e Heinz®.

Dentre todos os mercados de alimentos funcionais, os principais lançamentos de produtos são: lácteos, pastelaria, panificação e alimentos para bebés (Bigliardi & Galati, 2013; Menrad, 2003). O mercado de produtos lácteos funcionais tem crescido rapidamente devido ao reconhecido investimento de grande porte na investigação direcionada ao setor, o que assegurou o desenvolvimento de novos produtos comercializáveis. Além disso, muitos autores explicam o grande sucesso dos produtos lácteos funcionais devido à boa imagem que os consumidores têm destes (Siró et al., 2008; Szakály, Szigeti, Máthé & Szente, 2007).

2.1.3. Plantas como ingredientes naturais em alimentos funcionais

O consumo de diversas plantas é uma tradição que permanece desde os tempos ancestrais em diversas culturas, seja devido aos seus benefícios nutricionais e de saúde, ou devido a um comportamento sociocultural que caracteriza muitas sociedades (Groot et al., 2002; Pardo-de-Santayana et al., 2007; Schulp et al., 2014). No entanto, as drásticas mudanças ambientais, sociológicas e económicas que ocorrem atualmente podem vir a alterar a forma como se olham e se valorizam as plantas. Tendo em conta que a saúde e a nutrição são os dois pilares que sustentam a sobrevivência humana, tem-se vindo a procurar no vasto ecossistema vegetal novas formas de garantir cuidados médicos (Heywood, 2011).

Os alimentos enriquecidos com propriedades funcionais parecem ser uma aposta da indústria alimentar do futuro que garante estar cada vez mais focada na nutrição e saúde dos consumidores (Bernal et al., 2011). Os alimentos funcionais à base de plantas, ainda que pouco explorados, parecem ser a resposta para as exigências atuais dos consumidores que, por relacionarem cada vez mais a sua saúde e bem-estar com a sua alimentação, procuram alimentos mais saudáveis e mais naturais. A indústria alimentar pretende tornar funcionais certos alimentos, de forma a acrescentar-lhe um potencial benefício para a saúde do consumidor quando incluídos numa dieta, sem, no entanto, alterar o aspeto tradicional a que o consumidor está habituado (Hasler, 2000; Gulati & Ottaway, 2006).

Apesar de apenas um pequeno número de alimentos vegetais estar suportado por documentação clínica substancial relativamente aos seus benefícios para a saúde, existe um número ainda mais restrito (que incluí apenas plantas cultivadas) que conseguiu superar o rigoroso padrão exigido pela FDA para autorização em produtos com reivindicação para a saúde (Hasler 2002). Por exemplo, a fibra solúvel de aveia, proteína de soja e creme para barrar enriquecido com éster de esterol e estanol, são alimentos à base de plantas atualmente conformes para receber uma alegação de saúde aprovada pela FDA (Hasler 2002). No entanto, há uma crescente pesquisa clínica que suporta os potenciais benefícios para a saúde de vários alimentos vegetais (incluindo plantas silvestres) ou componentes alimentares que,

atualmente não têm alegações de saúde aprovadas e, portanto, são descritos como tendo "evidências moderadamente fortes". Alguns exemplos apontados são bagas, alho, uvas e chocolate, entre outros listados na **Tabela 2**.

A **Tabela 2** apresenta uma listagem de plantas selvagens que foram estudadas devido às reinvindicadas propriedades funcionais que possuiam. Estas plantas silvestres comestíveis são fontes importantes de ingredientes fisiologicamente ativos que estão associados a diferentes efeitos benéficos para a saúde. Várias bagas, como sabugueiro, mirtilo, amora, framboesa e morango silvestre destacam-se como fonte de antocianinas, proantocianidinas, flavonóis, ácidos fenólicos e vitaminas, entre outros compostos bioativos. Estas moléculas, isoladas ou em extratos combinados, possuem propriedades antioxidantes, anti-inflamatórias, anticarcinogénicas, cardioprotetoras e antibacterianas (Madhavi et al., 1998; Singh et al., 2009; Bowen-Forbes et al., 2010; Barros et al., 2011; Sidor & Gramza-Michalowska, 2014; Najda et al., 2014). As frutas silvestres colhidas em habitats naturais foram destacadas por Najda et al. (2014) por apresentarem uma elevada atividade antioxidante, associada a um teor muito elevado de antocianinas. Da mesma forma, Lv et al. (2014) mostraram que a cultivar de lichias selvagens Hemaoli apresenta um elevado teor fenólico em comparação com uma das principais cultivares comerciais. Esses frutos possuem também elevados teores de carotenoides e vitamina C, que contribuem para os seus efeitos antioxidante, antiapoptótico e hepatoprotetor (Huang et al., 2010; Bhoopat et al., 2011; Lv et al., 2014). Atualmente, existe um *marketing* agressivo que aponta diversos benefícios para a saúde relacionados com o consumo de mangostão, açaí, acerola ou goji, exemplos também apresentados na **Tabela 2**, o que resultou na sua classificação como "super-frutos" ou "superalimentos".

Assim, é possível encontrar no mercado vários alimentos funcionais à base de plantas. A pesquisa científica realizada nos últimos anos comprova a sua eficácia como alimentos saudáveis, e as indústrias alimentar e farmacêutica estão cada vez mais interessadas em desenvolver novos produtos com base nestas plantas. Assim, as plantas medicinais e aromáticas desempenham um papel importante no desenvolvimento de alimentos funcionais novos ou melhorados. A nível de pesquisa, alguns extratos de plantas silvestres foram incorporados em produtos alimentares com a finalidade de aumentar as suas propriedades promotoras de saúde. Martins et al. (2014) formularam iogurtes utilizando extratos fenólicos de flores de amoreira silvestre (*Rubus ulmifolius* Schott). Os autores microencapsularam o extrato hidroalcoólico numa matriz à base de alginato e incorporam no iogurte de forma a obter benefícios antioxidantes. Recentemente, Caleja et al. (2015) melhoraram as propriedades antioxidantes de um requeijão com base na incorporação de um extrato enriquecido em compostos fenólicos obtidos por decocção de funcho (*Foeniculum vulgare* Mill.), adicionando não apenas funcionalidade ao produto final, mas também promovendo a

melhoria da preservação devido ao potencial antimicrobiano do funcho. Espera-se que no futuro, a combinação do conhecimento popular, da investigação e desenvolvimento tecnologico leve ao desenvolvimento de alimentos funcionais e de novas abordagens preventivas e terapêuticas.

Tabela 2. Plantas selvagens comestíveis que alegam apresentar propriedades funcionais (Pinela et al., 2016).

Espécie	Nome comum e parte estudada	Extrato avaliado	Compostos funcionais	Potenciais benefícios para a saúde	Referências
Allium ampeloprasum L.	Alho-porro bravo (bolbo)	Extratos aquoso e etanólico.	Fibra, zinco, ácidos gordos polinsaturados (principalmente ácido palmítico), polissacarídeos (glucofrutanos) e saponinas esteroidais.	Antioxidante, anti-inflamatório, antiulcerogénico e gastroprotetor.	Adão et al., 2011; García-Herrera et al., 2014; Malafaia et al., 2015
Beta spp.	Beterraba (raíz)	Extratos aquoso, hidro-etanólico, metanólico e sumo.	Ácidos fenólicos (ácidos ferúlico, vanílico, p-hidroxibenzóico, cafeico e protocatecuico), flavonoides (catequina, epicatequina, rutina e vitexina), betaínas (betanina, isobetanina e vulgaxantina I), minerais (potássio, magnésio, ferro, zinco, cálcio, sódio), ácido fólico, biotina e fibra solúvel.	Atividade antioxidante, hepatoprotetora, anticancerígena, antiproliferativa em linhagens celulares MRC5 e MCF-7, anti-hipertensivas e hipoglicémicas.	Wootton-Beard & Ryan, 2011; Ninfali & Angelino, 2013; Vulić et al., 2014
Capparis decidua (Forssk.) Edgew.	Abisga (fruto)	Extratos aquoso, metanólico, metanólico acidificado e etanólico	N-pentacosano, β-sitosterol, β-caroteno, alcaloides, compostos fenólicos incluindo flavonoides e minerais (manganês, cobre e ferro).	Antioxidante, antidiabético, diurético, hipercolesterolémico, anti-hipertensivo, anti-aterosclerótico, hipolipidémico, antimicrobiano e anti-helmíntico.	Rathee et al., 2010 ; Sharma et al., 2010; Zia-Ul-Haq et al., 2011; Shad et al., 2014
Dimocarpus longan Lour.	Longana ou olho-do-dragão (fruto)	Extratos aquoso, hidromeanólico (80%), acetona: etanol (1:1, v/v), hidroacetona (70%) e extrato rico em polifenóis.	Compostos fenólicos (corilagina, ácidos gálhico e elágico, flavonoglucósidos, glucosídos de quercetina e kaempferol e epicatequina), vitamina C, fibras e minerais.	Efeitos antioxidantes, anti-inflamatórios, anti-tirosinase, anticancerígenos e potenciadores da memória.	Huang et al., 2010; Yang et al., 2011; Huang et al., 2012
Eugenia uniflora L.	Pitanga (fruto)	Extrato etanólico e acetato de etilo.	Antocianinas, carotenoides e flavonóis.	Anti-diarreico, diurético, anti-reumático, anti-febril, antidiabético, antimicrobiano e anti-tripanossoma.	Costa et al., 2013
Euterpe oleracea Mart.	Açaí (fruto)	Extrato n-butanólico, hidrometanólico (50%), hidroacetónico (70%) e de acetato de etilo.	Antocianinas, flavonoides, ácidos fenólicos, procianidinas, lignanas e estilbenos.	Antioxidante, anti-alérgico, anticancerígeno, anti-inflamatório, melhora a função endotelial e a agregação plaquetária, vasodilatadora e previne doenças cardiovasculares.	Rufino et al., 2010 ; Kang et al., 2011; Costa et al., 2013

Espécie	Nome comum e parte estudada	Extrato avaliado	Compostos funcionais	Potenciais beneficios para a saúde	Referências
Ficus carica L.	Figo (fruto)	Extrato metanólico, hidrometanólico e hexano.	Ácidos fenólicos (ácido clorogénico), antocianinas, flavonóis, flavonas (luteolina), minerais (ferro, potássio, sódio e cálcio), fibras, açúcares e vitamina A.	Antioxidante, anticolinesterásico, anticarcinogénico, atividade antiproliferativa em várias linhagens celulares, digestivas, e antifúngicas.	Huang et al., 2010; Barolo et al., 2014; Shad et al., 2014
Fragaria vesca L.	Morango selvagem (fruto)	Extrato aquoso e extrato n-butanólico combinado com HCl (1 mol/dm^3).	Flavonoides, ácidos fenólicos, antocianinas e ácido salicílico.	Antioxidante.	Najda et al., 2014
Garcinia mangostana L.	Mangostão (fruto)	Extratos aquoso, metanólico etanólico, hidro-etanólico (40 e 50%) e sumo.	Xantonas (α-, β- e γ-mangostinas, garinona E, 8-desoxicardanina e gartanina).	Antioxidante, antitumoral, antiproliferativo, pró-apoptótico, anti-inflamatório, antialérgico, antibacteriano, antifúngico, antiviral, antimalárico, antidiabético, anti-hiperlipidémico e antiaterogénico, cardioprotector, hepatoprotetor, imunomodulador e antiulceroso.	Pedraza-Chaverri et al., 2008; Gutierrez-Orozco & Failla, 2013
Gardenia jasminoides J. Ellis	Gardénia (flor)	Extratos hidrometanólico (80%), hidro-etanólico (60%) e acetona: etanol (1:1, v/v).	Derivados do ácido cafeoilquínico (ácido clorogénico, ácido dicafoilquínico e outros derivados do ácido quínico conjugados com cafeoil), flavonoides (rutina), iridóides (geniposídeos) e carotenóides (crocina).	Antioxidante e anti-inflamatório.	Huang et al., 2010; Peng et al., 2013
Litchi chinensis Sonn.	Lichia (fruto)	Extratos metanólico (70 and 80%), acetona: etanol (1:1, v/v) e sumo.	Compostos fenólicos (ácido cinâmico e procianidinas), carotenoides e vitamina C.	Antioxidante, antiapoptótico e hepatoprotetor.	Huang et al., 2010; Bhoopat et al., 2011; Lv et al., 2014
Lycium barbarum L.	Goji (fruto)	Extratos aquoso e metanólico e extrato de polissacarídos bruto e purificado.	Polissacarídeos, carotenoides (zeaxantina), betaína, cerebrosídeo, beta-sitosterol, ácido p-cumárico e vitamina C.	Antioxidante, antienvelhecimento, anti-inflamatório, anticancerígeno, citoprotetor, neuroprotetor, estimulador do metabolismo, regulador da glicose em diabéticos, glaucoma (benefícios para a saúde ocular), imunomodulador, antibacteriano e cardioprotetor.	Amagase & Farnsworth 2011
Malpighia emarginata DC.	Acerola (fruto)	Extrato metanólico, hidrometanólico (50%),	Vitamina C, carotenoides (β-caroteno), riboflavina, tiamina,	Antioxidante, antienvelhecimento, anti-inflamatório e previne o ganho de peso.	Mezadri et al., 2008; Rufino et al.,

Espécie	Nome comum e parte estudada	Extrato avaliado	Compostos funcionais	Potenciais beneficios para a saúde	Referências
		hidroacetônico (70%), aquoso e sumo.	fibras, minerais (fósforo, cálcio e ferro) e flavonoides (antocianinas (cianidina-3-ramnosídeo e pelargonidina-3-ramnosídeo) e flavonóis (quercetina)).		2010 ; Delva & Goodrich-Schneider 2013; Dias et al., 2014
Myrciaria cauliflora (Mart.) O. Berg.	Jaboticaba (fruto)	Extratos metanol: ácido fórmico (9:1, v/v), metanol: água: ácido acético (85:15:0,5, v/v/v/), metanólico, hidrometanólico (50%), etanólico, acetónico e hidroacetónico (70%).	Antocianinas, ácido elágico e gálhico, carotenóides, depsides, taninos, rutina e vitamina C.	Antioxidante, anti-inflamatório, inibe a produção de IL-8, efeitos antiproliferativos contra células tumorais, efeito protetor na doença cardiovascular e *diabetes mellitus* tipo 2.	Rufino et al., 2010; Leite et al., 2011; Costa et al., 2013
Myrciaria dubia (Kunth) McVaugh	Camu-camu (fruto)	Extratos hidrometanólico (50%) e hidroacetónico (70%).	Antocianinas, miricetina e conjugados, ácido elágico e conjugados, elagitaninos, flavanóis, proantocianidinas e vitamina C.	Antioxidante e anti-inflamatório.	Rufino et al., 2010 ; Costa et al., 2013; Fracassetti et al., 2013
Nasturtium officinale W.T. Aiton	Agrião (parte aérea)	Extratos metanólico e hidrometanólico (70%).	Compostos fenólicos e minerais (fósforo, potássio, cálcio e manganês).	Antioxidante, anticarcinogénico e quimiopreventivo.	Pereira et al., 2011; Manchali et al., 2012
Physalis spp.	Fisális (fruto)	Extrato hidroetanólico (70%).	Fisalinas, esteróis, polissacarídeos e flavonas.	Anti-inflamatório, antioxidante, antitumoral, hipoglicémico e analgésico.	Li et al., 2014b
Prosopis cineraria (L.) Druce	Bardana (vagem)	Extratos aquoso e matanólico.	Triterpenoides (ácido 3-benzil-2-hidroxi-urs-12-en-28-óico e ácido maslínico-3-glucósido), ácido gordo (ácido linoleico), alcaloide de piperidina (prosofilina) e polifenóis (5,5'-oxibis). 1,3-benzenodiol, ter 2-hidroxietico do ido 3,4,5-tri-hidroxiciónico e 7-glucósido de 5,3 ', 4'- tri-hidroxiflavanona).	Antioxidante e anti-inflamatório.	Liu et al., 2012
Psidium cattleianum Sabine	Araçá (fruto)	Extrato aquoso, etanólico, metanólico, hexano e acetate de etilo.	Compostos fenólicos (ácido elágico, ácido desoxihexosídeo e galato de epicatequina), carotenoides, vitamina C e fibras.	Antioxidante, anti-inflamatório e antimicrobiano.	McCook-Russell et al., 2012; Ribeiro et al., 2014

Espécie	Nome comum e parte estudada	Extrato avaliado	Compostos funcionais	Potenciais beneficios para a saúde	Referências
Psidium guajava L.	Goiabeira (fruto)	Extratos metanólico, hidrometanólico (80%), acetona: etanol (1:1, v/v), hexano, acetato de etilo e etanol/ água/ ácido fórmico.	Ácidos fenólicos (ácido clorogénico), flavonoides (catequina), antocianinas (delfinidina-3-*O*-glucósido e cianidina-3-*O*-glucósido).	Antioxidante, anti-inflamatório e antimicrobiano.	Huang et al., 2010; McCook-Russell et al., 2012; Flores et al., 2015
Punica granatum L.	Romã (fruto)	Extratos aquoso, acetato de etilo, acetónico e metanólico.	Antocianinas, galotaninos, elagitaninos (ácido elágico, ácido gálhico e punicalagina), ésteres de galagila, ácidos hidroxibenzóico e hidroxicinâmico e diidroflavonol.	Antioxidante, anti-inflamatório, antialérgico, quimiopreventivo, anticancerígeno, cardioprotetor, gastroprotetor e antimicrobiano.	Ismail et al., 2012
Rhodomyrtus tomentosa (Aiton) Hassk.	Mirtilo peludo (fruto)	Extratos hexano, metanólico, hidrometanólico (80%), acetona: etanol (1:1, v/v), acetona: água: ácido acético (50:49:1, v/v/v) e extrato rico em flavonoides.	Flavonoides (gallocatequina galato, dihidromicetina, quercetina, kaempferol, antocianinas e vitexina), ácidos orgânicos, polissacarídeos, fibras, vitamina E (α-tocoferol), minerais (manganês e cobre) e ácidos gordos essenciais (principalmente ácido linoleico).	Antioxidante.	Huang et al., 2010; Lai et al., 2015; Wu et al., 2015
Rubus spp.	Amora e framboesa (frutos)	Extrato hexano, acetate de etilo e metanólico.	Antocianinas, flavonóis, ácidos fenólicos (ácido elágico), vitaminas C e E, ácido fólico e β-sitosterol.	Antioxidante, anti-inflamatório e quimiopreventivo.	Bowen-Forbes et al., 2010
Sambucus nigra L.	Sabugueiro (fruto)	Extratos metanólico, metanólico acidificado, etanólico e hidroetanólico (80%).	Polifenóis (antocianinas, flavonóis, ácidos fenólicos e proantocianidinas), terpenos, lectinas, ácidos gordos insaturados, fibras, vitaminas A, B, C e E e minerais.	Antioxidante, proteção cardiovascular, antidiabético e antiobesidade, reforça o sistema imunológico, antiviral, antibacteriano e protetor de radiação UV.	Barros et al., 2011; Sidor & Gramza-Michałowska 2014
Syzygium cumini (L.) Skeels.	Jamelão (fruto)	Extratos metanólico, hidrometanólico (50%), hidroacetônico (70%) e hexano.	Antocianinas, ácido elágico, quercetina, rutina, carotenóides, vitamina C e manganês.	Antioxidante, antiescorbútico, diurético e antidiabético.	Rufino et al., 2010; Costa et al., 2013; Shad et al., 2014
Theobroma cacao L.	Cacaueiro (semente)	-	Compostos polifenólicos (catequinas e antocianinas).	Antioxidante, anti-inflamatório, quimiopreventivo e anticancerígeno, aumenta o fluxo sanguíneo para os tecidos	Kim et al., 2011

Espécie	Nome comum e parte estudada	Extrato avaliado	Compostos funcionais	Potenciais benefícios para a saúde	Referências
				cutâneos e subcutâneos e contribui para a aparência e textura da pele, aumenta o fluxo sanguíneo cerebral, estimula o sistema nervoso, facilita a digestão e melhora a função renal e intestinal.	
Vaccinium myrtillus L.	Mirtilo (fruto)	Extrato metanólico acidificado, acetato de etilo, hexano e extratos ricos em antocianinas e proantocianidinas.	Flavonoides (proantocianidinas e antocianinas), carotenoides (luteína e zeaxantina) e esteróis.	Antioxidante, antibacteriano (inibição de infeção do trato urinário), atividade anticarcinogénica e antiproliferativa em duas linhagens celulares de cancro de mama humano MCF-7 e BT-20.	Madhavi et al., 1998
Vaccinium spp.	Arando (fruto)	Extrato hidroacetónico (80%), acetato de etilo e extrato fenólico.	Ácidos fenólicos e flavonoides (antocianinas, proantocianidinas e flavonóis).	Antioxidante, anti-inflamatório e protetor cardiovascular e do trato urinário.	Singh et al., 2009; Khoo & Falk, 2014
Zingiber officinale Rosoe	Gengibre (rizoma)	Extrato aquoso e metanólico.	6-gingerol, 6-shogaol, fibras e flavonoides.	Antioxidante, anti-inflamatória, antitrombótico e potenciador para baixar o colesterol, analgésico, antipirético e hipotensiva.	Thomson et al., 2002; Mojani et al., 2014
Ziziphus jujuba Mill.	Jujuba (fruto)	Extrato aquoso, hexano, metanólico e hidrometanólico.	Saponinas, taninos, terpenoides, flavonoides e ferro.	Antioxidante, anti-inflamatório e protetor gastrointestinal.	Yu et al., 2012; Shad et al., 2014

2.2. **Compostos fenólicos como ingredientes bioativos**

2.2.1. Caracterização química e biológica

Os compostos fenólicos são metabolitos secundários de plantas, geralmente envolvidos na defesa contra a radiação ultravioleta ou agressão por patogénios (Crozier, Clifford & Ashihara, 2006), sendo os principais antioxidantes da dieta humana (Scalbert, Manach, Morand, Rémésy & Jiménez, 2005).

As principais fontes alimentares deste tipo de compostos são as frutas e bebidas derivadas de plantas, como sumos de frutas, chás, café e vinho tinto. Os legumes, cereais, chocolate e leguminosas secas também contribuem para a ingestão total de compostos fenólicos (Scalbert, Johnson & Saltmarsh, 2005; Pandey & Rizvi, 2009). A ingestão total é de cerca de 1g / dia, sendo muito superior à de todos os outros antioxidantes da dieta, cerca de 10 vezes superior à de vitamina C e 100 vezes superior à de vitamina E e carotenoides (Scalbert et al., 2005b; Scalbert & Williamson, 2000).

Na natureza, os compostos fenólicos estão, geralmente, conjugados com açúcares e ácidos orgânicos (Pandey & Rizvi, 2009; Yamagata, Tagami, & Yamori, 2015). Estes compostos podem ser classificados de acordo com dois tipos principais, flavonoides e não flavonoides (**Figura 2**) e em classes diferentes, que dependem do número de anéis fenólicos e dos elementos estruturais que ligam esses anéis (Yamagata et al., 2015; Manach, Scalbert, Morand, Rémésy. & Jiménez, 2004). No entanto, ainda há alguma controvérsia associada às classes de compostos fenólicos que devem ser consideradas (O'Connell & Fox, 2001).

O grupo dos flavonoides tem uma característica estrutural comum, que consiste em 2 anéis aromáticos (A e B) unidos por 3 átomos de carbono que formam um heterociclo oxigenado (anel C). Este grupo pode ser dividido em seis subclasses de acordo com o tipo de heterociclo envolvido: flavonóis, flavonas, flavanóis, flavanonas, isoflavonas e antocianinas (Manach et al, 2004). As diferenças individuais dentro de cada grupo referem-se a variações no número e disposição dos grupos hidroxilo, bem como na sua extensão de alquilação e/ou glicosilação (Pandey & Rizvi, 2009). Entre os vários compostos, a quercetina e as catequinas destacam-se como as mais comuns. Vários estudos têm sugerido que os flavanóis, flavonas e flavanonas, os principais flavonoides das frutas cítricas, ocorrem em elevada concentração nos produtos ricos em cacau e chocolate escuro, e estão relacionados com a redução do risco de doença cardiovascular (Hooper et al., 2012; Shrime et al., 2011).

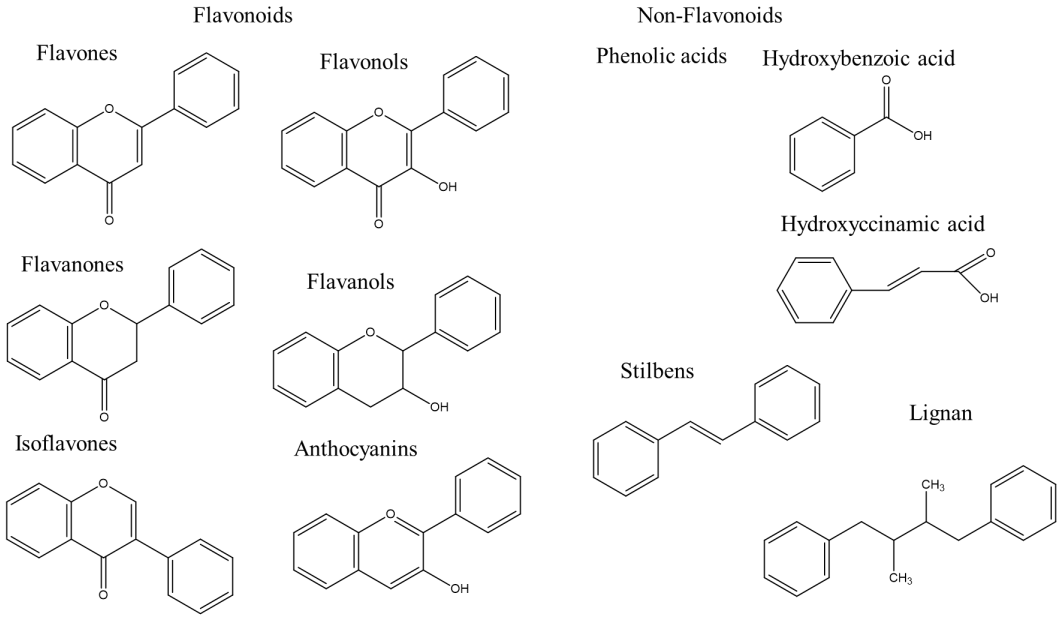

Figura 2. Estrutura química das sub-classes dos flavonoides e não-flavonoides (Caleja et al., 2017).

O grupo dos não flavonoides é composto por ácidos fenólicos, estilbenos e lignanos (Manach et al., 2004). Os ácidos fenólicos encontram-se abundantemente em alimentos e podem ser divididos em duas classes: os ácidos hidroxibenzoicos e os hidroxicinâmicos. O teor de ácido hidroxibenzoico em plantas comestíveis é geralmente baixo, com exceção de certas frutas vermelhas e cebola. Normalmente, estes frutos possuem grandes quantidades de ácido gálhico. Os ácidos hidroxicinâmicos são os mais comuns e consistem principalmente em ácidos cumárico, cafeico, ferúlico e rosmarínico (Pandey & Rizvi, 2009; Manach et al., 2004). Os estilbenos, normalmente, não são ingeridos em grandes quantidades, uma vez que as plantas apenas os sintetizam se sujeitos a episódios de infecção ou lesão. No entanto, as uvas e o vinho contêm quantidades significativas de resveratrol, tornando este estilbeno um dos mais estudados (Aun et al., 2011).

Segundo a literatura os compostos fenólicos têm uma ampla gama de bioactividades, tais como efeitos antioxidante, antimicrobiano, anti-inflamatório, antitumoral e hepatoprotector. Estes compostos estão envolvidos na neutralização de espécies reativas e na prevenção do stresse oxidativo, atuando desta forma em diferentes doenças tais como cancro, afeções cardiovasculares, aterosclerose, distúrbios neurológicos, hipertensão e *diabetes mellitus* (Carocho & Ferreira, 2013a).

Alguns destes compostos apresentam uma elevada atividade antioxidante como compostos individuais, enquanto outros, dependem de sinergismos para terem efeitos

bioativos (Carocho & Ferreira, 2013a). A apigenina, quercetina, kaempferol, miricetina, luteolina, isoramnetina, cafeína e ácido *p*-cumárico são dos compostos mais citados quando associados à atividade antioxidante (Sulaiman, Sajak, Ooi & Seow, 2011; Wojdylo, Oszmianski & Czemerys, 2007). Estes compostos demonstraram atividade antioxidante, levando a uma grande proteção das células endoteliais da lesão oxidativa (Chung, Kurisawa, Kim, Uyama & Kobayashi, 2004). Vários estudos comprovaram as propriedades antioxidantes das flavonas. A apigenina revelou forte atividade antioxidante *in vitro* e *in vivo* por meio de efeitos neuroprotetores contra o stresse oxidativo (Zhao, Wang, Wang & Fa, 2013; Li et al., 2014c). Outra flavona, o kaempferol, também revelou elevada atividade antioxidante, sendo recomendada como um bom antioxidante natural devido à sua tendência em doar eletrões e, portanto, estabilizar os alimentos (Dar et al., 2013). Esta atividade antioxidante está, normalmente, relacionada com a estrutura química destes compostos, dependendo do número e posição dos grupos hidroxílicos, glicosilação ou outros tipos de substituição (Cai, Sun, Xing, Luo & Corke, 2006).

Outros estudos descrevem também a atividade antifúngica de compostos fenólicos, expressa através de uma interação com a bicamada lipídica da membrana da célula fúngica (Martins, Barros, Henriques, Silva & Ferreira, 2015). Os compostos fenólicos mais estudados em termos de propriedades antifúngicas são os ácidos fenólicos, flavonoides e estilbenos (Martins et al., 2015). Os compostos fenólicos também demonstraram atividade antimicrobiana, comprovada por retardar ou inibir o crescimento de microrganismos patogénicos e/ou produtores de toxinas em alimentos, bem como minimizar a incidência de doenças transmitidas por alimentos causadas por bactérias e fungos (Beuchat, 2001). A atividade antimicrobiana dos ácidos fenólicos foi referida por vários autores que provaram que os ácidos clorogénico, ferúlico e gálhico têm a capacidade de inibir o crescimento de diferentes microrganismos, como *Staphylococcus aureus* e *S. epidermis*, *Escherichia coli*, *Pseudomonas aeruginosa* e *Listeria monocytogenes* (Chen, Liou, Tzeng, Lee & Liu, 2012; Borges, Ferreira, Saavedra & Simões, 2013; Chanwitheesuk, Teerawutgulrag, Kilburn & Rakariyatham, 2007; Sarjit, Wang & Dykes, 2015; Díaz-Gómez, López-Solís, Obreque-Slier & Toledo-Araya, 2013, Díaz-Gómez, López-Solís, Obreque-Slier & Toledo-Araya, 2014). A catequina, pertencente ao grupo flavan-3-ols, inibiu o crescimento de várias fungos e bactérias, como *Candida albicans* (Hirasawa, Takada, 2003), *Helicobacter pylori* e *Escherichia coli* (Díaz-Gómez et al., 2013). Também as flavanonas, hesperetina e narigenina, apresentaram fortes efeitos antifúngicos e antibacterianos contra *S. aureus*, *C. albicans* e *Lactococcus lactis* (Golfakhrabadi et al., 2016; Mandalari et al., 2007; Zhang et al., 2013). A elevada atividade frente à *P. aeruginosa*, *Salmonella typhimurium*, *S. epidermis*, *Bacillus subtilis* e *Saccharomyces cerevisiae* foi demonstrada para duas flavonas, apigenina e luteolina (Nayaka, Londonkar, Umesh &

Tukappa, 2014; Akhavan, Jahangirib & Shafaghat, 2015). Estudos com flavonóis (kaempferol e rutina) revelaram atividade antibacteriana para *Enterococcus faecalis* e *S. aereus* (Valle et al., 2016; Orhan et al., 2010). Por último, o resveratrol, um estilbeno, demonstrou atividade antibacteriana para várias bactérias, como *E. faecalis* (Valle et al., 2016), *Campylobacter* spp., *Arcobacter butzleri* (Duarte, Alves, Ferreira, Silva & Domingues, 2015), *S. cerevisiae*, *Aspergillus ninger*, *Penicillum expansum* (Filip et al., 2003) e *H. pylori* (Paulo, Oleastro, Gallardo, Queiroz, & Domingues, 2011), e também contra fungos como *C. albicans* (Li et al., 2012).

Diversos estudos científicos levados a cabo com compostos fenólicos descreveram também as suas propriedades anti-inflamatórias (Vulcano, Halabalaki, Skaltsounis & Ganzera, 2015; Mizgier et al., 2016). Durante o processo de inflamação observa-se a formação de várias espécies reativas de oxigênio (ROS) e espécies reativas de nitrogênio (RNS) que resultam no aumento da atividade de agentes pró-inflamatórios. Os compostos fenólicos mostraram capacidade de reduzir ou prevenir os referidos efeitos prejudiciais no organismo humano, através da redução dessas enzimas pró-inflamatórias (Bowen-Forbes, Zhang & Nair, 2010). Esta atividade foi relatada para a hesperetina, uma flavanona (Wang et al., 2016) e para duas importantes flavonas, a apigenina e luteolina (Funakoshi-Tago, Nakamura, Tago, Mashino & Kasahara, 2011; Jia et al., 2015).

A atividade antitumoral de alguns compostos fenólicos foi também comprovada em algumas investigações. Alguns autores demonstraram uma inibição da atividade da redutase em células tumorais, tratando linhas celulares leucémicas com ácido gálhico (Madlener et al., 2007). Outro estudo também demonstrou a eficiência deste ácido contra linhagens celulares de glioma, diminuindo a viabilidade celular, proliferação e angiogénese (Lu et al., 2010). Outros autores relataram que, ao induzir a apoptose e ativar as caspases, o ácido gálhico também teve efeitos eficientes nas linhagens celulares de cancro do pulmão (Ji et al., 2009). Alguns autores demonstraram o efeito anticancerígeno das antocianidinas, cianidina e delfinidina, contra o carcinoma colorretal (Cvorovic et al., 2010). Em particular, a delfinidina inibiu o crescimento celular e diminuiu o tamanho do tumor (Hafeez, et al., 2008). Ensaios *in vivo* revelaram que a catequina, uma flavan-3-ol, inibiu a formação de tumores e aumentou o tempo de vida dos animais. Por sua vez, testes *in vitro* confirmaram que este composto inibia a migração de células de melanoma (Menon, Kuttan, & Kuttan, 1999). Dentro do grupo flavanona, a hesperetina é sugerida como capaz de inibir o crescimento celular e induzir a apoptose, resultando na inibição das linhas celulares de carcinoma mamário humano (Natarajan, Thamaraiselvan, Lingaiah, Srinivasan & Periyasamy, 2011). Alshatwi et al. (2013) revelaram que a hesperetina exibe atividade anticancerígena *in vitro* contra linhagens celulares de cancro cervical humano através da redução da viabilidade celular e indução de

apoptose. No grupo flavona, a apigenina e a luteolina surgem como os compostos fenólicos mais estudados. A apigenina demonstrou efeitos antimutagênicos e apoptóticos na linha de células do linfoma humano (Hashemi, Long, Entezari, Nafisi & Nowroozii, 2010) e inibiu o crescimento e a migração de células do cancro pancreático (He et al., 2015). Por outro lado, a luteolina exibiu atividades pró-apoptóticas em células de cancro colorretal (Had et al., 2015) e demonstrou efeito anticancerígeno na linhagem celular de carcinoma de pulmão humano (NCI-H460) e a inibição da migração celular (Ma et al., 2015). Quercetina e kaempferol são os dois flavonóis mais estudados e que demonstraram atividade antitumoral com resultados eficazes contra células de diferentes carcinomas (Wei et al., 1994; Tan et al., 2003; Nair et al., 2004; Mylonis et al., 2010; Zhang et al., 2008; Lou et al., 2012; Kim et al, 2016). Os efeitos da genisteína, um isoflavona, no carcinoma da próstata foram avaliados e os resultados demonstraram a sua capacidade em inibir a proliferação celular, bem como de regular positivamente a glutationa peroxidase e regular negativamente os agentes inibidores da apoptose (Wang, Eltoum & Lamartiniere, 2002). No grupo dos estilbenos, o resveratrol representa o composto fenólico mais estudado e com atividade antitumoral direta comprovada contra muitos tipos de carcinomas, incluindo leucemia, neuroblastoma, carcinoma pancreático, de mama, próstata e renal (Simoni et al., 2006; Fulda & Debatin, 2004).

Os ácidos fenólicos, nomeadamente os ácidos cafeico, clorogénico e gálhico, demostraram efeitos hepatoprotetores através de diferentes mecanismos (Pereira, Calhelha, Barros & Ferreira, 2013; Nabadi et al., 2013; Zhao et al., 2014; Maheshwari et al., 2011). Dentro do grupo das flavanonas, a hesperetina e a naringenina surgem com propriedades hepatoprotetoras. A hesperetina é responsável por combater o dano hepático induzido por CCL_4 (tetracloreto de carbono) através da diminuição de várias enzimas (Cheng et al., 2013). A naringenina apresentou propriedades hipoglicémicas, vasorelaxantes e hepatoprotetoras através da diminuição das enzimas envolvidas nas doenças do fígado (Sánchez-Salgado et al.,2007). Em relação ao grupo das flavonas, a apigenina e a luteolina são descritas por vários autores como compostos com excelentes propriedades hepáticas, incluindo a diminuição de diferentes enzimas prejudiciais às células do fígado, bem como a capacidade de inibir a linhagem celular HepG2 (carcinoma hepático) (Pereira et al., 2013; Oh, Kim, Cho, & Kim, 2004 Jeyadevi et al., 2013). Kaempferol, miricetina e quercetina são alguns exemplos de flavonóis implicados em efeitos hepatoprotetores. Segundo vários autores, estes compostos são capazes de reduzir enzimas prejudiciais tais como a aspartato transaminase (AST) e a alanina transaminase (ALT) [Maheshwari et al., 2011; Cheng et al., 2013; Sintayehu et al., 2012). Além disso, Pereira et al (2013) descreveram o kaempferol como agente hepatoprotetor através da redução do crescimento da linhagem celular HepG2.

2.2.2. Biodisponibilidade e estabilidade de compostos fenólicos

A biodisponibilidade e estabilidade dos compostos fenólicos, após consumo, tornou-se um dos temas mais discutidos em artigos de investigação e revisão, com o objetivo de melhor compreender os mecanismos da ação dos fenóis no organismo (Crozier et al., 2010). A biodisponibilidade de qualquer composto é medida pela fração que atinge o sistema circulatório, que é dependente do seu grau de absorção (Carbonell-Capella et al., 2014). A maioria dos compostos fenólicos da dieta são agliconas ligadas à parede da matriz e também ligadas à porção de açúcar; no entanto, eles também podem ser encontrados como compostos livres (flavan-3-ol e proantocianidinas), como monómeros ou na forma polimérica (Crozier et al., 2010; Santos-Buelga et al., 2012). Existem milhares de compostos fenólicos, divididos em flavonoides e não flavonoides, que apresentam uma diversidade imensa de estruturas químicas, que influenciam o seu comportamento no organismo quando consumidos, mas também a sua estabilidade, funcionalidade e, consequentemente, biodisponibilidade, o que pode ser um fator limitante para a aplicabilidade como composto funcional e / ou aditivo em produtos alimentares (Holst & Williamson, 2008; Leong & Oey, 2012). Na **Figura 3** são descritos os principais pontos que caracterizaram a estabilidade e biodisponibilidade de compostos fenólicos.

A sua distribuição nos tecidos vegetais não é igual, o que afeta a sua estabilidade e limita o procedimento de protocolos para a sua extração (Santos-Buelga et al., 2012). Os fenólicos de baixa e média massa molecular são considerados polifenóis extraíveis, uma vez que a sua extração pode ser obtida por vários solventes; enquanto os fenólicos não extraíveis são aqueles com alta massa molecular e normalmente ligados às fibras e proteínas da dieta, diminuindo a sua solubilidade (Bravo, 1998). Tanto Martínez-Huélamo et al. (2015) num estudo em que usaram tratamentos mecânicos e térmicos em tomate, como Wang et al. (2014) que utilizaram tratamentos térmicos e de extrusão em grãos de cereais chegaram à conclusão, que esses tratamentos agressivos, apesar de permitirem a libertação dos compostos fenólicos, aumentando a sua biodisponibilidade, comprometem a sua estabilidade, alterando a sua estrutura química.

As características químicas estruturais dos compostos fenólicos são também um fator muito importante a ter em consideração quando se fala na sua biodisponibilidade. Karakaya (2004) pesquisou intensamente a biodisponibilidade de algumas classes de compostos fenólicos, tais como os ácidos fenólicos, flavonoides (flavonas, flavonóis, flavanóis) e antocianinas, apresentando uma série de fatores que podem afetar a sua biodisponibilidade e absorção. As moléculas de açúcar ligadas ao composto fenólico são um dos fatores que mais influenciam a absorção no intestino, em relação à sua natureza e número. Por exemplo,

os flavonoides acilados (epicatequina e epigalocatequinas) são bem absorvidos no intestino sem desconjugação e hidrólise. O autor também chegou à conclusão de que pequenas variações na estrutura dos compostos podem causar uma grande diferença no seu comportamento no organismo humano (Karakaya, 2004). A absorção de compostos fenólicos no intestino não é apenas influenciada pelas características intrínsecas dos compostos, podendo estar também relacionada com fatores externos, como o facto de o organismo humano carecer de muitos recetores específicos na superfície das células epiteliais do intestino delgado para vários compostos fenólicos que limitam a sua absorção sendo secretada na urina e nas fezes (Li et al., 2015). Adicionalmente, mudanças de pH do meio também podem afetar as antocianinas (Fernandes et al., 2014).

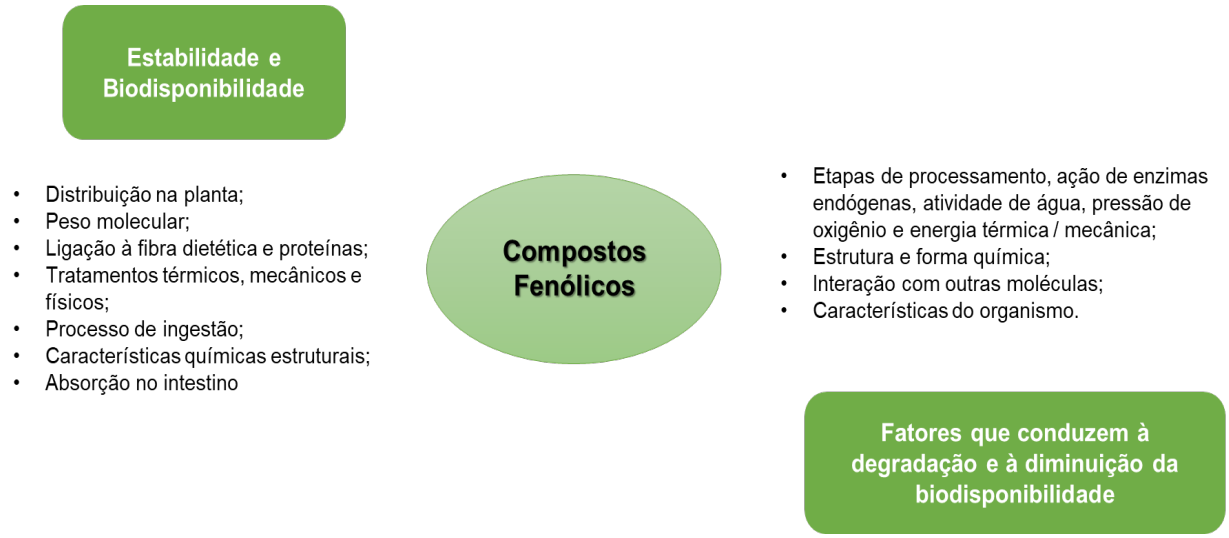

Figura 3. Características principais de estabilidade e biodisponibilidade dos compostos fenólicos e os pricipais fatores que conduzem à sua degradação e à diminuição da biodisponibilidade (Dias, Caleja, Ferreira & Barreiro, ISBN: 978-1-119-22879-0).

Existem vários métodos utilizados para avaliar a biodisponibilidade de compostos bioativos, neste caso, compostos fenólicos, que podem ser divididos em modelos *in vitro* (por exemplo: digestão gastrointestinal simulada, membranas artificiais), *ex vivo* (por exemplo: órgãos gastrointestinais em escala de laboratório), *in situ* (por exemplo: perfusão intestinal em animais vivos) e *in vivo* (por exemplo: ensaios em animais e humanos) (Carbonell-Capella et al., 2014). No entanto, muitos dos estudos realizados sobre o assunto resultam incompletos, exigindo estudos adicionais para compreender os mecanismos que levam à perda ou ganho da biodisponibilidade de compostos fenólicos (Pérez-Jiménez et al., 2009; Hole et al., 2012; Stalmach et al., 2012; Zhang et al., 2012; García-Villalba et al., 2014; Rubió et al., 2014).

2.2.3. Fatores que conduzem à degradação

A perda de bioatividade dos compostos fenólicos pode ocorrer devido a um conjunto de condições adversas em relação às etapas de processamento, ação de enzimas endógenas, atividade da água, pressão de oxigénio e também ação térmica/mecânica. Além disso, a estrutura química dos compostos fenólicos, bem como a sua interação com outras moléculas presentes no produto alimentar e no organismo, poderão influenciar a sua estabilidade e, consequentemente, a sua biodisponibilidade (**Figura 3**) (Holst & Williamson, 2008; Leong & Oey, 2012; Dias et al., 2015).

As etapas de processamento após a colheita, que incluem tratamentos térmicos e mecânicos, podem ser responsáveis pela perda da biodisponibilidade de compostos fenólicos. A maioria dos tratamentos pode levar à degradação dos compostos, modificando a sua estrutura química (Cermak et al., 2009). Por exemplo, a degradação de antocianinas é muito complexa, e o uso de tratamentos térmicos acima de 50ºC pode afetar os níveis desses compostos em frutas e vegetais, ou modificar a sua estrutura química (Patras et al., 2010). As antocianinas estão entre os compostos fenólicos mais propensos à conversão durante as etapas de processamento de produtos alimentares, levando à perda de estabilidade e cor (Cheynier, 2005). A estrutura química dos compostos fenólicos tem grande influência na sua biodisponibilidade (Karakaya, 2004). Além disso, alguns compostos fenólicos são suscetíveis à degradação e transformação durante o processo digestivo pela ação de várias enzimas que realizam uma série de reações químicas, levando à perda de algumas propriedades bioativas (Crozier et al., 2010; Heleno et al., 2015). As interações entre compostos fenólicos e outras moléculas nos produtos alimentares são também um fator importante conducente à degradação de compostos fenólicos, na medida em que alguns processos bioquímicos e químicos estão envolvidos nessas etapas. A oxidação enzimática é um dos principais fatores que conduz à perda da integridade estrutural dos compostos fenólicos, resultando também em detrimento da qualidade do produto (Cheynier, 2005).

2.2.4. A microencapsulação como proceso de proteção de bioativos

Para superar os problemas relacionados com o uso de bioativos na sua forma livre em matrizes alimentares, a técnica de microencapsulação, que corresponde ao desenvolvimento de sistemas partículados micro-dimensionáveis, garante proteção e a manutenção das propriedades funcionais do produto final.

A microencapsulação é a técnica segundo a qual um princípio ativo é revestido (encapsulado), usando um material encapsulante, de preferência um polímero natural. O objetivo principal é o de proteger o bioativo da ação do oxigénio, luz, hidrólise e outras condições externas (Gharsallaoui et al., 2007). Adicionalmente, é capaz de conferir uma maior estabilidade, compatibilidade, solubilidade e dispersão (Wang et al., 2014). A microencapsulação tem sido aplicada, maioritariamente, no setor farmacêutico (68%), enquanto que na indústria alimentar o seu uso representa uma fração de apenas 13%. No entanto, esta tendência tem vindo a ser alteranda sendo a microencapsulação cada vez mais aplicada no desenvolvimento de novos produtos alimentares funcionais (Dias et al., 2015). Além da vantagem de manter as características biológicas e bioativas dos extratos e compostos individuais, esta técnica permite mascarar algumas características organolépticas menos apreciadas (Martins et al., 2014). No entanto, devem ser seguidos parâmetros criteriosos para a produção de produtos microencapsulados para a área alimentar de forma a garantir a segurança para consumo humano (Nedovic et al., 2011). As técnicas de microencapsulação e materiais de encapsulamento devem ser selecionadas de acordo com o produto final a ser desenvolvido. As características específicas dos extratos/compostos bioativos também devem ser levadas em consideração (Lachman et al., 2001).

Como explicado anteriormente, os compostos fenólicos estão facilmente sujeitos à degradação. Assim, são vários os estudos que propõem a microencapsulação para superar estas limitações, protegendo e estabilizando os extratos e compostos individuais, mas também conferindo características de libertação controlada e/ou direcionada (Dias et al., 2015). Para entender melhor o potencial desta tecnologia, é essencial conhecer as principais características dos diferentes processos de microencapsulação, as propriedades dos materiais de encapsulação e também os mecanismos de libertação dos bioativos encapsulados (Lachman et al., 2001).

Os compostos são protegidos em pequenas cápsulas com tamanhos variáveis de 1 a 1000 μm, sendo libertados apenas em condições específicas, o que viabiliza diversas aplicações (Fang & Bhandari, 2010). A microencapsulação protege o material do núcleo (bioativo) e influencia o comportamento de entrega após a ingestão (Nedovic et al., 2011). A escolha do material de encapsulação é um passo muito importante, uma vez que a sua natureza pode influenciar a estabilidade do composto encapsulado. A escolha deve ter em conta as características físicas e químicas dos compostos/extratos bioativos, o método de microencapsulação e a aplicação pretendida (Suave et al., 2006). O material pode ser natural, semi-natural ou sintético (Gharsallaoui et al., 2007), mas também biocompatível e não tóxico. Os mais utilizados são os polímeros hidrosolúveis, seguidos dos polímeros não hidrossolúveis (Dias et al., 2015). São exemplo da primeira categoria os polissacarídeos (maltodextrina,

dextrose, alginato), gomas (goma arábica) e proteínas (gelatina e proteínas do leite) (Gharsallaoui et al., 2007).

Os alginatos, derivados de bactérias e algas (sendo a alga castanha a fonte mais comum), são dos polímeros mais utilizados em microencapsulação. As suas propriedades físicas e químicas permitem a aplicação comercial em diferentes campos. Na indústria alimentar, por exemplo, é utilizado como agente estabilizante e espessante para doces e bebidas (Goh et al., 2012). O uso de alginato na indústria alimentar é permitido pela FDA, que o classifica como não-tóxico para administração oral (George & Abraham, 2006). Para fins de microencapsulação, os alginatos são materiais encapsulantes atrativos, uma vez que, permitem a libertação controlada dos compostos protegidos em condições moderadas de pH alcalino, que normalmente ocorrem no trato intestinal. A libertação das moléculas bioactivas das micropartículas de alginato é evitada com pH ácido (por exemplo, o pH em ambiente gástrico). No entanto, ao transitar para um ambiente de pH superior (trato intestinal), a rede polimérica desagrega-se, adquirindo as propriedades de compatibilidade com a água, resultando na libertação dos compostos (George & Abraham, 2006).

Assim, as características morfológicas das microcápsulas tais como a textura e forma, não são os únicos parâmetros que influenciam a escolha da técnica de microencapsulação a utilizar, mas também o mecanismo responsável pela libertação dos compostos fenólicos deve ser tido em consideração (Bansode et al., 2010).

Existem inúmeras metodologias para o encapsulamento de compostos bioativos (Dias et al., 2015). Estes podem ser classificados de acordo com o mecanismo de formação das microcápsulas, o método de consolidação ou mesmo de acordo com o equipamento específico usado para a microencapsulação. Não há uma categorização clara, sendo geralmente referidos como métodos físicos, químicos ou físico-químicos. No entanto, uma nova abordagem foi feita por Dias et al. (2015) que propuseram coacervação, processos baseados em extrusão, processos baseados em spray, processos baseados em emulsões, lipossomas, processos baseados em fluidos supercríticos, processos baseados em ultrassons e outros, como categorias gerais para abranger o vasto leque de técnicas de microencapsulação existentes. Cada um apresenta um conjunto de parâmetros e características que determinam o seu uso de acordo com o objetivo pretendido (Suave et al., 2006).

Um dos principais objetivos da microencapsulação é a entrega dos compostos encapsulados na sua forma preservada a um tecido alvo, para assegurar a manutenção das propriedades bioativas. No caso da ingestão, a entrega deve ser direcionada (por exemplo, estômago ou intestino) e desencadeada, por exemplo, pelo efeito da temperatura ou estímulo ao pH. Assim, a libertação de compostos bioativos depende de vários fatores, como as

propriedades físicas e químicas do material da parede, a ligação molecular entre o núcleo e o material da parede e, finalmente, o estímulo externo (por exemplo, temperatura, humidade e pH) (Ko & Gunasekaran, 2014). Dessa forma, é necessário monitorizar o comportamento das microcápsulas após a ingestão, uma vez que a sua libertação pode ser afetada por vários fatores ao longo do tracto gastrointestinal (Li & McClements, 2010; Frank et al., 2012).

É importante reforçar que a libertação controlada de um composto bioativo com impacto na indústria alimentar deve ser estudada em meio aquoso, e sob diferentes condições de pH, mimetizando o trato gastrointestinal. A complexidade do mecanismo de libertação controlada permite uma variedade de aplicações envolvendo compostos bioativos na indústria farmacêutica e alimentar (Ko & Gunasekaran, 2014). Em relação à libertação controlada de compostos fenólicos encapsulados, há uma falta de informação considerável sobre o assunto. A maioria dos estudos de microencapsulação realizados refere-se à otimização do método de encapsulação ou à avaliação das propriedades bioativas dos compostos após o encapsulamento. Já foram realizados alguns estudos de libertação controlada envolvendo compostos encapsulados como vitaminas, riboflavina (Chen & Subirade, 2006; Wichchukit et al., 2013) e ácido fólico (Prasertmanakit et al., 2009), óleo de peixe, resveratrol e tributirina (Augustin et al., 2011), e até mesmo probióticos (Anal & Singh, 2007). Estes ensaios *in vitro* têm como principal vantagem a de permitir o estudo da absorção de compostos bioativos e, portanto, a de compreender os potenciais efeitos benéficos para a saúde que esses compostos podem apresentar após ingestão (Carbonell-Capella et al., 2014).

2.3. **Compostos fenólicos em alimentos funcionais**

A comunidade científica, bem como a indústria alimentar, têm demonstrado um interesse crescente pelas propriedades funcionais de alguns nutrientes e compostos naturais, sendo que a sua incorporação em diferentes alimentos lhe dará um valor acrescentado. Para este tipo de enriquecimento podem ser utilizadas diferentes fontes naturais de origem vegetal, animal ou micológica, mas também compostos sintéticos com atividades biológicas melhoradas. As vitaminas, aminoácidos, fibras, ácidos gordos e compostos fenólicos são alguns exemplos de ingredientes bioativos (Branen, Davidson, Salminen &Thorngate, 2001). Os ingredientes bioativos obtidos de fontes naturais são utilizados desde tempos ancestrais, em todo o mundo, com a convicção de que garantem propriedades medicinais e podem atuar na prevenção e melhoria de sintomas de determinadas doenças (Dai & Mumper, 2010; Rubió, Motilva & Romero, 2013).

O cancro é uma preocupação global que causa todos os anos milhões de mortes, mesmo nos países mais desenvolvidos (Jemal et al., 2011). Apesar dos grandes avanços no tratamento de pacientes com cancro, estes são ainda insuficientes para reduzir o sofrimento e a mortalidade (Foo & Michor, 2010). Na medicina tradicional, as plantas representam um importante aliado no tratamento de várias doenças. Atualmente, os compostos naturais representam uma importante fração na composição de novos fármacos antineoplásicos desenvolvidos pela indústria farmacêutica (Rocha, Lopes & Schwartsman 2001; Gordaliza,2007). Vários estudos permitiram concluir que os ingredientes bioativos de plantas podem ter efeitos anticancerígenos em linhas celulares, sendo mais eficientes que alguns compostos sintéticos (Carocho & Ferreira, 2013b). Existem vários problemas de toxicidade associados ao uso de moléculas sintéticas no tratamento do cancro. Alternativamente, alguns compostos naturais isolados de plantas podem ser administrados sem qualquer efeito colateral ou toxicidade para os pacientes (Carocho & Ferreira, 2013b). Os potenciais efeitos antitumorais de compostos fenólicos têm sido intensamente estudados, tanto com linhas celulares tumorais como com modelos *in vivo* e testes em humanos (Carocho & Ferreira, 2013b). Adicionalmente, estudos em relação à sua capacidade hepatoprotetora contra danos induzidos quimicamente, *in vivo* ou *in vitro* têm sido intensamente realizados. Estes compostos demonstram ter capacidade para superar lesões hepáticas, geralmente causadas por reações oxidativas, que promovem a peroxidação lipídica nos tecidos hepáticos. Entre esses constituintes, os flavonoides e os ácidos fenólicos têm recebido especial atenção devido à sua elevada atividade antioxidante, suportando os efeitos hepatoprotetores observados (Rice, Miller & Paganga, 1996).

Devido às suas características, os alimentos ricos em compostos fenólicos foram considerados alimentos funcionais. Apesar da existência de relatórios anteriores (1985-1999), observa-se um crescente interesse por este tipo de produtos, tanto da parte académica (através de artigos de pesquisa e revisões científicas) como industrial (através de patentes) desde 2000, indicando uma tendência crescente (**Figura 4**).

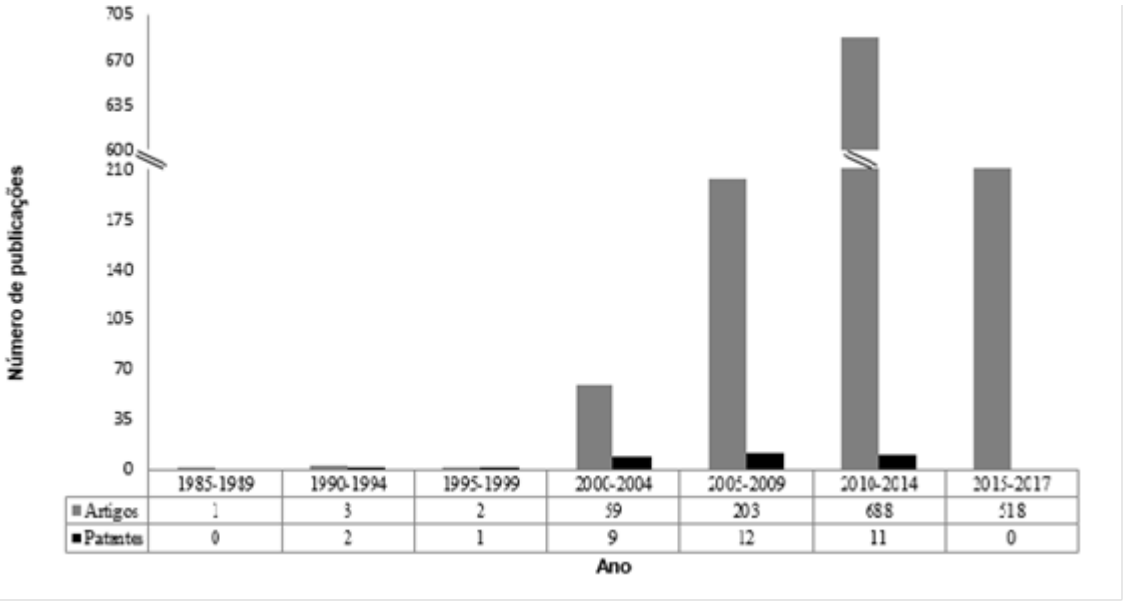

Figura 4. Número de artigos de pesquisa e de revisão e patentes publicados no período de 1985 a 2016 relativamente a alimentos funcionais e compostos fenólicos (Resultado de *web of science*, julho 2016; Palavras-chave: *functional food* e *phenolic compounds*). (Caleja et al., 2017).

A aceitação de um ingrediente funcional específico, por parte do consumidor, está intimamente relacionada com o conhecimento descrito sobre os seus efeitos benéficos para a saúde. Assim, os ingredientes funcionais conhecidos desde há algum tempo, como por exemplo os minerais, vitaminas, fibras e ácidos gordos, atingem índices de aceitação consideravelmente mais altos que os mais recentes, como os compostos fenólicos (Siró et al., 2008; Kanekanian et al., 2014). No entanto, vários estudos indicam um aumento no consumo de alimentos ricos em compostos fenólicos, o que pode estar associado à divulgação de efeitos na prevenção de diversas doenças mencionadas anteriormente (Scalbert et al., 2005; Quiñones, Miguel & Aleixandre, 2013; Khan & Mukhtar, 2007).

Atualmente, a indústria alimentar desenvolveu diferentes produtos alimentares fortificados para responder às necessidades e exigências dos consumidores, que estão cada vez mais preocupados com seus hábitos alimentares. Portanto, diversos produtos alimentares funcionais novos, muito apreciados pelos consumidores, foram desenvolvidos pela introdução do ingrediente ativo na dosagem correta, e sem alteração das propriedades organolépticas e de textura (Carocho, Barreiro, Morales & Ferreira, 2014; Aun et al., 2011).

A **Tabela 3** inclui alguns exemplos de alimentos ricos em compostos fenólicos com os respectivos efeitos biológicos reportados e associados à sua atividade antioxidante (prevenção de doenças cardiovasculares e cancro).

O vinho, particularmente o vinho tinto, é uma fonte importante de flavonoides, especialmente catequinas e procianidinas, que provaram ter um efeito protetor significativo contra a doença cardíaca coronária (CHD) (Teissedre & Landrault, 2000; Auger et al., 2004). Foi também observada uma correlação entre os níveis crescentes de ingestão de flavonoides a partir de frutas e vegetais, assim como a redução da CHD (Rimm, Katan, Ascherio & Stampfer, 1996). As catequinas e procianidinas demonstraram, *in vitro*, ser potentes inibidores da oxidação das LDL (Lipoproteínas de baixa densidade), superiores, por exemplo, ao α-tocoferol (Teissedre & Landrault, 2000). Além disso, foi descrito que o consumo moderado de vinho por humanos leva a um aumento na capacidade antioxidante do plasma (Fuhrman, Lavy & Aviram,1995).

A quercetina, também presente no vinho, quando ingerida em grandes quantidades reduz a incidência de cancro do pulmão (Knekt et al., 2002) e doença cardíaca isquémica (Auger et al., 2004). Teissedre & Landrault (2000) estudaram o efeito *in vivo* do ácido gálhico (originado do vinho tinto) em ratos, observando uma redução na taxa de mortalidade por CHD. As catequinas presentes no chá verde afetam o metabolismo lipídico por diferentes vias e impedem o aparecimento da placa aterosclerótica. A sua ingestão diminui a absorção de triglicéridos e colesterol devido ao aumento da excreção de gordura (Raederstorf, Schlachter, Elste & Weber,2003). O chá verde, devido aos seus componentes, também é apontado como auxiliar na prevenção da doença de Alzheimer (Wang & Li, 2015).

Sesso, Gaziano, Buring & Hennekens (1999) estudaram a relação entre o consumo de chá e café e o enfarte do miocárdio. Neste estudo verificou-se uma redução de 44% do risco cardiovascular nos indivíduos que bebem mais de uma chávena de chá por dia. Para o café, foi também observada uma correlação significativa. Todas as catequinas, procianidinas e metabolitos absorvidos podem contribuir para o aumento da capacidade antioxidante do plasma. Os seus efeitos cardioprotetores derivam da capacidade de inibir a peroxidação lipídica e atenuar alguns processos que envolvem ROS (Auger et al., 2004).

Tabela 3. Compostos fenólicos de fontes alimentares com efeitos biológicos relatados, relacionados com a capacidade antioxidante e prevenção de doenças cardiovasculares e cancro (Caleja et al., 2017).

Composto Fenólico		**Fonte**	**Referência**
Ácidos fenólicos	Ácido gálhico	Vinho tinto	Teissedre & Landrault, 2000
Antocianinas	Cianidina	Mirtilo, uva preta, cereja, morango, vinho tinto, ameixa	Mazza & Maniati, 1993; Clifford, 2000
	Pelargonidina		
	Peonidina		
	Delfinidina		
	Malvidina		
Taninos condensados	Procianidina	Vinho tinto, chocolate, sumo de arando e maçãs	Hammerstone et al., 2000
Flavan-3-ols	Catequina	Frutas, legumes, chocolate, lentilha, chá verde e preto, vinho, uva e ginkgo biloba	Mangiapane et al., 1992; Lotito & Fraga, 1998; Pascual-Teresa et al., 2000; Gu et al., 2004; Dwyer et al., 2005; Ding et al., 2006 ; Wang & Li, 2015.
Flavanonas	Hesperetina	Sumos de laranja, toranja e limão	Erlund et al., 2001; Gharras, 2009.
	Naringenina		
Flavonas	Apigenina	Salsa, aipo e uva	Gharras, 2009; Wang & Li, 2015.
	Luteolina		
Flavonóis	Quercetina	Frutas, vegetais e bebidas, como chá e vinho tinto	Hertog et al., 1995; Crozier et al., 1997 ; McDonald & Black, 1998 ; Wang & Li, 2015.
	Kaempferol		Hertog et al., 1993 ; Rimm et al., 1996 ; Justesen et al., 1997 ; Aziz et al., 1998.
Isoflavonas	Genisteína	Soja	Anderson et al., 1995; Anthony et al., 1996.

Composto Fenólico		Fonte	Referência
Estilbenos	Resveratrol	Legumes, uvas, vinho tinto, soja, amendoim e produtos de amendoim	Jang et al., 1997; Soleas et al., 1997; Sobolev & Cole, 1999 ; Sanders et al., 2000 ; Burns et al., 2000.

As principais fontes alimentares de estilbenos incluem uvas, vinho, soja, amendoim e produtos de amendoim (Cassidy, Hanley & Lamuela-Ravento´s, 2000). Os estilbenos, nomeadamente o resveratrol, mostraram ter efeitos na prevenção ou tratamento de certas doenças, como o cancro da mama (Kris-Etherton et al., 2002) e os tumores da pele (Jang et al., 1997). O sumo de laranja e o sumo de toranja contêm quantidades elevadas de hesperetina e naringenina e exibem efeitos anticarcinogénicos (Erlund, Meririnne, Alfthan & Aro, 2001). Tan et al (2003), também estudaram o potencial antiangiogénico da quercetina, que demonstrou inibir vários passos importantes da angiogénese, incluindo a proliferação, a migração e a formação de células endoteliais dérmicas microvasculares humanas. Outro estudo relatou o efeito sinérgico dos componentes da uva na prevenção de diabetes e doenças cardiovasculares e na promoção da saúde bucal (Wang & Li,2015).

As atividades biológicas dos compostos fenólicos no corpo humano são atribuídas, principalmente, à capacidade destes compostos exrcerem ações antioxidantes. A principal atividade antioxidante dos polifenóis consiste em potenciar os sistemas de desintoxicação celular, como os sistemas superóxido dismutase, catalase ou glutationa peroxidase, e inibir as enzimas geradoras de ROS, como a xantina oxidase e a NADPH oxidase (Nicotinamida Adenina Dinucleotídeo Fosfato-Oxidase) (Quiñones et al., 2013). No entanto, estudos recentes sugerem que os mecanismos pelos quais os compostos fenólicos exercem a sua ação protetora contra doenças cardiovasculares e cancro, não são simplesmente devido às suas propriedades redox, mas devido à capacidade de se ligarem diretamente a proteínas (Quideau, Deffieux, Douat-Casassus & Pouységu, 2011). Essa ação iria induzir a inibição de enzimas chave, a modulação de recetores celulares ou fatores de transcrição, bem como a perturbação de agregados proteicos, que podem regular as funções celulares relacionadas por exemplo, com o crescimento e a proliferação, a inflamação, a apoptose, a angiogénese, a metástase e respostas imunes, afetando as vias de transdução de sinal (Quiñones et al., 2013). No entanto, há uma grande diversidade de compostos fenólicos, e a maneira como eles interagem com as proteínas depende das propriedades físico-químicas de ambos (Quideau, et al., 2011).

Desta forma, os compostos fenólicos são considerados excelentes candidatos para a prevenção e tratamento de várias doenças e, como tal têm despertado tanto interesse a nível industrial para aplicação alimentar. As plantas medicinais e aromáticas desempenham um papel importante nesta área, devido à elevada quantidade que apresentam destes compostos. Atualmente, alguns alimentos funcionais que captam o interesse do consumidor podem ser encontrados comercialmente. A comunidade científica conseguiu comprovar os efeitos

benéficos para a saúde deste tipo de produtos, que as indústrias alimentar e farmacêutica têm disputado, viabilizando a produção de alguns novos produtos.

A **Figura 5** esquematiza possíveis processos de ingestão de compostos fenólicos através de alimentos fortificados e os seus efeitos no corpo humano. A incorporação desses compostos nos alimentos pode ser realizada diretamente na sua forma livre; no entanto, como referido anteriormente, a técnica de microencapsulação surgiu como uma estratégia muito eficaz e promissora para garantir a biodisponibilidade desses compostos, ajudando a superar os problemas de processamento e ingestão de alimentos (Dias, Ferreira & Barreiro, 2015). Após a ingestão, os compostos são absorvidos pela corrente sanguínea, causando alterações em vários mecanismos celulares, levando à prevenção de várias doenças.

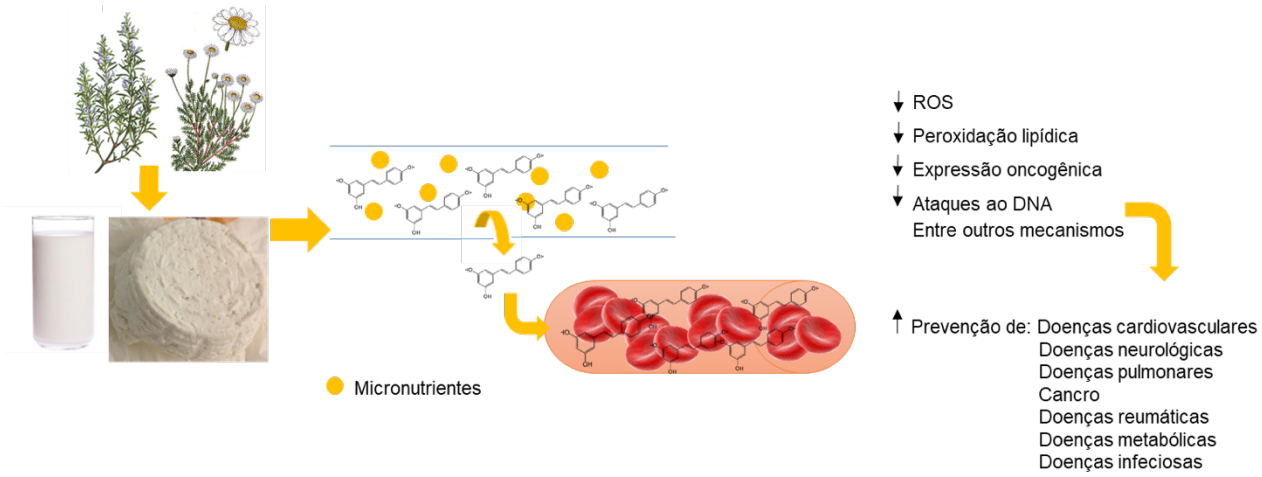

Figura 5. Efeitos benéficos dos compostos fenólicos (Caleja et al., 2017).

Apesar de existirem na literatura muitos artigos que demonstram a bioatividade de compostos fenólicos de diversas plantas e frutos, os estudos que descrevem a sua aplicação em alimentos funcionais ou formulações nutricionais, são ainda escassos. A **Tabela 4** lista alguns exemplos de compostos fenólicos usados como nutracêuticos ou compostos bioativos em alimentos funcionais. As isoflavonas de soja foram usadas em suplementos alimentares para controlar os sintomas da menopausa, enquanto antocianinas do arando foram usadas em cápsulas nutricionais para fins antioxidantes. Além disso, exemplos de estudos de caso com incorporação de extratos fenólicos em matrizes alimentares também são fornecidos na **Tabela 4**. Os extratos fenólicos de romã foram incorporados por Robert et al (2010) e Pillai et al (2012) em iogurte e massas, respetivamente, para melhorar a atividade antioxidante. Os extratos fenólicos de romã também foram incorporados por Çam et al (2014) em gelados, com o objetivo de aumentar as atividades inibidoras de antioxidantes e da α-glucosidase.

Ezhilarasai et al (2013) e Pasrija et al (2015), incorporaram extratos fenólicos de garcinia e chá verde, respetivamente, em pão, resultando num novo produto com maior atividade antioxidante. Chouchouli et al (2013). Martins et al (2014), usaram sementes de uva e flores de amoreira, respetivamente, para adicionar valor e atividade antioxidante a iogurtes funcionais. Mais recentemente, Caleja et al. (2015a, b) incorporaram extratos fenólicos de funcho e camomila em requeijão, relatando uma atividade antioxidante superior e um aumento do tempo de prateleira, comparativamente com o requeijão sem extratos. O mesmo grupo de investigação incorporou cogumelos (Ribeiro et al., 2015) e extratos de alecrim (Ribeiro et al., 2015) para melhorar as propriedades antioxidantes. Extratos de funcho e camomila foram também incorporados em iogurtes e apresentaram resultados superiores relativamente a iogurtes sem aditivos e iogurtes com um aditivo artificial (sorbato de potássio - E202) (Caleja et al., 2016). Outro estudo recente descreveu a incorporação de extratos ricos em compostos fenólicos obtidos a partir de goiabeira em pão, o que acrescentou valor antioxidante a este produto (Alves & Perrone, 2015).

A empresa Compal (Sumol + Compal, SA) desenvolveu produtos definidos como funcionais e comercializados sob a forma de néctar, como é o caso do Compal Vital AntiOx®, garantindo propriedades antioxidantes devido à presença de uma gama seleccionada de frutos vermelhos (http://www.compal.pt/#/pomar/nectares/vital/). Guimarães et al. (2013), provaram que os frutos vermelhos são ricos em compostos fenólicos, proporcionando atividade antioxidante. No entanto, quando adicionados aos alimentos, os compostos fenólicos não estão presentes na sua forma pura, sendo normalmente adicionados como extratos enriquecidos ou polpas de frutas.

Apesar de o desenvolvimento de alimentos funcionais e nutracêuticos ser considerado um processo rápido, esses produtos muitas vezes exigem evidências científicas sólidas para serem introduzidos no mercado. Os estudos clínicos são geralmente muito caros e, portanto, não são acessíveis para a maioria das empresas que lidam com a fabricação deste tipo de produtos alimentares, que não podem arcar com as despesas para realizar todos os testes (Roy, 2015; Wang & Li, 2015). Para cada estudo clínico, as empresas de pesquisa e/ou universidades precisam combinar centenas de milhares de tópicos. Em média, cada um destes estudos pode custar vários milhares de dólares (Betoret, Betoret, Vidal & Fito, 2011).

Apesar de a regulamentação associada a este tipo de produtos ser bastante diferente entre os países, as regras de rotulagem são semelhantes. É através da rotulagem que os consumidores têm acesso a todas as informações relativas à composição dos alimentos e respectivos benefícios para a saúde, o que no caso de alimentos funcionais e nutracêuticos se torna muito importante (Wang & Li, 2015). Alguns estudos indicam que as mulheres são mais influenciadas a comprar novos produtos, em parte porque estão mais preocupadas com

questões de saúde, mas também porque geralmente são responsáveis pelas compras de casa (Annunziata & Vecchio, 2009).

Os principais fatores que influenciam a compra de alimentos funcionais são o estilo de vida, preocupação com a saúde, gosto por alimentos mais saudáveis e o *design* da embalagens (Cox, Koster & Russell, 2004). Portanto, é importante que os consumidores tenham conhecimento e estejam familiarizados com os alimentos funcionais (Annunziata & Vecchio, 2009), com a segurança destes (Verschuren, 2002) e informados sobre o produto base (matriz alimentar) (Rams, 2002). Além disso, as alterações legislativas na UE no que respeita aos novos produtos também ajudaram a modificar as atitudes dos consumidores. Nesse contexto, uma maior confiança nas informações veiculadas pelos rótulos e a publicidade sobre as virtudes nutricionais e de saúde desses produtos alimentares é evidente (Annunziata & Vecchio, 2009).

Em geral, os produtos funcionais ainda precisam de superar alguns obstáculos para garantir o seu sucesso no mercado e conquistar a confiança do consumidor. Portanto, para apoiar a alta procura por alimentos funcionais, é necessário combinar esforços de várias áreas, como a ciência, indústria, mercado, meios de comunicação, educação e políticas governamentais, e assim conseguir desenvolver uma imagem de produto respeitável. A promoção do produto deve ser percebida por todos os grupos etários, dos mais jovens aos mais velhos, e, portanto, deve ser realizada de forma simples e clara, com vocabulário acessível (Wang & Li, 2015). No entanto, o sucesso dos alimentos funcionais também depende dos atributos organolépticos, especialmente sabor, aparência, preço e alegações de saúde (Routray & Orsat, 2012). Portanto, a indústria alimentar deve ter em consideração alguns fatores para o desenvolvimento/reestruturação de alimentos funcionais, destacando-se a aceitação sensorial, preço, estabilidade, propriedades químicas e funcionais (Betoret et al., 2011).

Sabe-se que os compostos fenólicos obtidos a partir de fontes naturais exibem várias bioactividades (Quiñones et al., 2013) e, assim, a sua introdução em matrizes alimentares proporcionará um maior valor acrescentado ao produto. No entanto, estes compostos estão associados a várias reações/instabilidades quando introduzidos em alimentos, decorrentes do seu processamento (por exemplo, temperaturas de fabricação), longos períodos de armazenamento em contacto com a matriz alimentar, entre outros fatores (Betoret et al., 2011; Dias et al., 2015). Assim, o progresso das tecnologias introduziu avanços na indústria alimentar, permitindo a inovação no desenvolvimento de novos produtos alimentares (Fang & Bhandari, 2012; Paini et al., 2015).

Tabela 4. Compostos fenólicos usados como nutracêuticos ou compostos bioativos em alimentos funcionais (Caleja et al., 2017).

Composto fenólico	Origem	Aplicação	Bioatividade	Referência
Ácidos fenólicos	Commercial	Cereais infantis	Propriedades antioxidantes e aroma	Li et al., 2010
Antocianinas	Arando (*Vaccinium macrocarpon* Ait.)	Cápsulas nutracêuticas	Atividade antioxidante	Bononi & Tateo, 2007
Isoflavonas	Soja (*Glycine max* L.)	Suplementos alimentares	Alívio dos sintomas da menopausa	Krenn & Pötsch, 2006
Extratos fenólicos	Flores de amora silvestre (*Rubus ulmifolius* Schott)	Iogurte	Atividade antioxidante e antimicrobiana	Martins et al., 2014
	Borragem (*Borago officinalis* L.)	Massa fresca	Atividade antimicrobiana	Miceli et al., 2015
	Flores de camomila (*Matricaria recutita* L.)	Requeijão e iogurte	Atividade antioxidante e antimicrobiana	Caleja et al., 2015b, 2016
	Partes aéreas de funcho (*Foeniculum vulgare* Mill.)	Requeijão e iogurte	Atividade antioxidante e antimicrobiana	Caleja et al., 2015a, 2016
	Frutos de garcinia (*Garcinia cowa* Roxb)	Pão	Atividade antioxidante	Ezhilarasai et al., 2013
	Semente de uvas (*Vitis vinífera* L.*)*	Iogurte	Atividade antioxidante	Chouchouli et al., 2013
	Chá verde (*Cammelia sinensis* L.)	Pão	Atividade antioxidante	Pasrija et al., 2015
	Flores de goiabeira (*Psidium guajava*)	Pão	Atividade antioxidante	Alves & Perrone, 2015
	Romã (*Punica granatum* L.)	Iogurte e massa	Atividade antioxidante	Robert et al.,2010; Pillai et al., 2012
	Casca de romã (*Punica granatum* L.)	Gelado	Antioxidant and aglucosidase inhibitory activities	Çam et al., 2014

Composto fenólico	Origem	Aplicação	Bioatividade	Referência
	Resíduos da indústria cervejeira	Carne	Antioxidant and antimicrobial activity	Barbosa-Pereira et al., 2014
	Alecrim (*Rosmarinus officinalis* L.)	Requeijão	Atividade antioxidante	Ribeiro et al., 2016
	Suillus luteus (L.: Fries) Gray *Coprinopsis atramentaria* (Bull.) Redhead, Vilgalys & Moncalvo	Requeijão	Atividade antioxidante	Ribeiro et al., 2015
	Veronica (*Veronica montana* L.)	Queijo creme	Atividade antibacteriana	Stojković et al., 2013

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3.

Caracterização e incorporação de *Foenicullum vulgare* Mill. e *Matricaria recutita* L. como ingredientes naturais em diferentes matrizes alimentares

O capítulo 3 compreende seis artigos resultantes da atividade experimental associada ao estudo das atividades antioxidante e antimicrobiana e à caracterização química de *Foeniculum vulgare* Mill. (funcho) e *Matricaria recutita* L. (camomila). Os resultados relativos à sua incorporação como ingrediente natural em diferentes matrizes alimentares, são também apresentados neste capítulo.

3.1. **Desenvolvimento de requeijões utilizando funcho e camomila como conservantes naturais**

Neste sub-capítulo apresenta-se a caracterização química, e as propriedades antioxidantes e antimicrobianas de extratos aquosos de *Foeniculum vulgare* Mill. e *Matricaria recutita* L obtidos por decocção. Neste mesmo sub-capítulo, é apresentado o estudo dos efeitos da incorporação destas decocções nas suas formas livre e microencapsulada em requeijões.

3.1.1. *Foeniculum vulgare* Mill. como conservante natural e promotor de saúde por incorporação em requeijão.

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Foeniculum vulgare Mill. as natural conservation enhancer and health promoter by incorporation in cottage cheese

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Abstract

Food industry is focused on the development of novel functional foods containing health promoting natural ingredients, avoiding the potential harm of synthetic food additives. In the present work, the antioxidant and antimicrobial potential of *Foeniculum vulgare* Mill. (fennel) decoction (phenolic-enriched extract) was evaluated; after chemical characterization of the extract by HPLC-DAD-ESI/MS, it was used as natural ingredient in cottage cheese samples for two purposes: increase shelf life and bring bioactive properties. The incorporation of fennel-based ingredients did not alter significantly the nutritional characteristics of control cottage

cheese (without fennel-based ingredients) but avoided the increase in yellowness (after 7 days of storage), and the decrease in lactose content (after 14 days of storage) observed in control samples. Control samples after 14 days of storage, were the only ones showing signs of degradation. Furthermore, the incorporation of the fennel decoction improved the antioxidant properties of cottage cheese, up to 14 days of storage. Overall, fennel decoction can be used as a natural conservation enhancer in cottage cheese, while bringing antioxidant properties to the final product.

Keywords: Foeniculum vulgare, Cottage cheese, Functional foods, Natural preservers.

Introduction

The demand for foods with high nutritional and bioactive value (functional foods), and longer shelf life, is a mandatory challenge for food science and industry (Carocho, Barreiro, Morales & Ferreira, 2014). Current interests of the food industry are focused on avoiding potential harmfulness of synthetic food additives and developing novel functional foods containing health promoting ingredients. Natural matrices/compounds with antioxidant and antimicrobial properties could serve both purposes.

The use of food additives comes from ancient times, where people used simple substances to increase the shelf life and assign or highlight specific characteristics of some foods (Aun et. al., 2011). Nowadays, processed food has to be transported across large distances to reach consumers and, therefore, special requirements are needed to ensure products quality and safety, mainly in prevention of contamination and spoilage (Carocho et al., 2014). There are more than 25,000 additives used in food, from which some synthetic ones have been related with gastrointestinal, respiratory, dermatologic, and neurologic adverse reactions (Branen, Davidson, Salminen & Thorngate, 2001; Wilson & Bahna, 2005; Randhawa & Bahna, 2009). Due to those potential risks to consumer health, nowadays, there is a tendency to replace synthetic additives by natural ones (Carocho & Ferreira, 2013).

Natural ingredients with antioxidant properties could be used to replace synthetic additives, which might also have health benefits in the prevention of several diseases related to oxidative/nitrosative stress, such as cancer, cardiovascular diseases, atherosclerosis, neurological disorders, hypertension, or diabetes mellitus (Carocho & Ferreira, 2013). Likewise, antimicrobial activity of some natural ingredients could delay or inhibit the growth of pathogenic and/or toxin-producing microorganisms in food, as also minimize the incidence of foodborne diseases caused by food spoilage bacteria and fungi (Beuchat, 2001).

Foeniculum vulgare Mill. (fennel) is a biennial plant belonging to the family Apiaceae (Umbelliferaceae), distributed in central Europe and Mediterranean region, that showed both antioxidant (Sing, Maurya, Lampasona & Catalan, 2006; Barros, Heleno, Carvalho & Ferreira, 2009) and antimicrobial (Dadalioglu & Evrendilek, 2004; Lo Cantore, Iacobellis, De Marco, Capasso & Senatore, 2004; Soylu, Yigitbas, Soylu & Kurt, 2007; Barros et al., 2009) properties. Therefore, it seemed to us a promising matrix to incorporate in cottage cheese, which is highly appreciated regarding its organoleptic properties and nutritional value but has a very short shelf life (Díaz-Castro et al., 2012).

The present study aims to characterize and evaluate antioxidant/antimicrobial potential of *F. vulgare* decoction (phenolic-enriched extract) to be used as a natural ingredient in cottage cheese for two purposes: increase shelf life and provide bioactive properties.

Materials and methods

Standards and reagents

HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic and acetic acids were purchased from Prolabo (VWR International, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Phenolic standards were from Extrasynthèse (Genay, France). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also other individual fatty acid isomers, L-ascorbic acid, organic acid and sugar standards, and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Mueller-Hinton agar (MH) and malt agar (MA) were obtained from the Institute of Immunology and Virology, Torlak (Belgrade, Serbia). Water was treated in Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

Fennel-based natural ingredients: phenolic-enriched extracts

Preparation: Commercial samples of Foeniculum vulgare Mill. (fennel) were provided by Américo Duarte Paixão Lda. (Alcanede, Portugal). The dried samples were powdered (~20 mesh) and submitted to decoction in order to obtain phenolic-enriched extracts. Decoctions were performed by adding 1 g of plant material to 200 mL of distilled water, heated (heating plate, VELP scientific, Usmate, Italy), and boiled for 5 min. The mixtures were left to stand for 5 min and then filtered. The decoctions were frozen and lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA).

Chemical characterization: Phenolic compounds were determined in the decoctions, by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, CA, USA), as previously described by the authors (Barros et al., 2013). A Waters Spherisorb S3 ODS-2 C_{18}, 3 µm (4.6 mm × 150 mm) column thermostatted at 35 °C was used. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile, with a flow rate of 0.5 mL/min. Double online detection was carried out using diode array detector (DAD) using 280 and 370 nm as preferred wavelengths and a mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet. The phenolic compounds were identified by comparing their retention time, UV-vis and mass spectra with those obtained from standard solutions, when available. Otherwise, peaks were tentatively identified comparing the obtained information with available data reported in the literature. The results are expressed in mg/g of lyophilized decoction.

Evaluation of antioxidant properties

To obtain stock solutions of 5 mg/mL, the lyophilized decoctions were re-dissolved in water. The mentioned stock solutions were successively diluted until determination of EC_{50} values (sample concentration providing a value of 50% in the DPPH, β-carotene bleaching and TBARS assays or 0.5 absorbance in the reducing power assay).

DPPH radical-scavenging activity was evaluated using an ELX800 microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA), and calculated as a percentage of DPPH discolouration using the formula: $[(A_{DPPH} - A_S)/ A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution. Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader mentioned above. Inhibition of β-carotene bleaching was evaluated by the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, which is measured by the formula: (β-carotene absorbance after 2 h of assay/initial absorbance) x 100. Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS) was evaluated by the lipid peroxidation inhibition in porcine brain homogenates where the color intensity of the malondialdehyde–thiobarbituric acid (MDA–TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: [(A-B)/A] x 100%, where A and B were the absorbance of the control and the sample solution, respectively. Trolox was used as positive control in all the assays.

Evaluation of antimicrobial properties

Antibacterial activity was evaluated against Gram-negative bacteria: *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), *Enterobacter cloacae* (ATCC 35030), and 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 the authors (Sokovic et al., 2010). The minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations were determined following the same reference. Streptomycin and ampicillin were used as positive controls. Antifungal activity was evaluated against *Aspergillus fumigatus* (ATCC 1022), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus versicolor* (ATCC 11730), *Aspergillus niger* (ATCC 6275), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112), *Trichoderma viride* (IAM 5061), and *Penicillium verrucosum* var. *cyclopium* (food isolate), following the procedure previously described by the authors (Sokovic & van Griensven, 2006). The minimum inhibitory (MIC) and minimum fungicidal (MFC) concentrations were determined following the same reference. Bionazole and ketokonazole were used as positive controls.

Incorporation of the fennel-based natural ingredients in cottage cheese

Preparation of the cottage cheese samples: All the samples of cottage cheese were prepared by “Queijos Casa Matias Lda.” (one of the main producer companies of “Serra da Estrela” cheese, the most famous Portuguese cheese). Three groups of samples, each one with nine ewe’s cottage cheeses (250 g), were prepared: control sample (cottage cheese without the fennel-based natural ingredient); sample with the fennel decoction (it was incorporated in cottage cheese at the EC_{25} value previously determined by DPPH assay: 0.35 mg/mL, corresponding to 1.05 g for each 250 g cottage cheese sample); and sample with the fennel powder (it was incorporated at 1.75 mg/mL, considering the decoction yield of 20%, corresponding to 5.25 g for each 250 g cottage cheese sample). The samples (three different cottage cheeses for each storage time) were submitted to an evaluation of color, nutritional composition, and antioxidant activity, immediately after preparation and after seven and fourteen days of storage at 4 ºC.

Evaluation of color, nutritional composition and antioxidant activity of control and incorporated cottage cheese samples along storage time

The color of the samples was measured in a colorimeter (model CR-400, Konica Minolta Sensing, Inc., Japan), using the illuminant C and diaphragm aperture of 8 mm; the CIE *L*a*b** color space values were registered using the data software “Spectra Magic Nx” (version

CM-S100W 2.03.0006) (Fernandes et al., 2012). The color was measured with 3 readings on the top and bottom part, for each sample.

The samples were also analyzed for proximate composition (moisture, protein, fat, carbohydrates and ash) using the AOAC procedures. The crude protein content (N x 6.38) of the samples was estimated by 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 ºC. Total carbohydrates were calculated by difference. Total energy was calculated according to the following equations: Energy (kcal) = 4 x (g protein +g carbohydrate) + 9 x (g lipid).

Fatty acids were determined in the soxhlet extract by gas-chromatography coupled to flame ionization detector (GC-FID), according to the procedure previously described by the authors (Barros et al., 2013). The identification was made by comparing the relative retention times of fatty acid methyl esters from samples with standards. The results were expressed in relative percentage of each fatty acid.

Free sugars were determined in defatted samples by HPLC coupled to a refraction index (RI) detector, according to the procedure previously described by the authors (Barros et al., 2013). The compounds were identified by chromatographic comparisons with authentic standards, and quantification was performed using the internal standard (melezitose) method. Sugars content was expressed in g/100 g of cottage cheese.

Organic acids were determined in defatted samples by HPLC coupled to a photodiode array detector (PDA), according to the procedure previously described by the authors (Barros et al., 2013). Detection was carried out using 215 nm and 245 nm (for ascorbic acid) as preferred wavelengths. The organic acids found were quantified by comparison of the area of their peaks recorded at 215 nm with calibration curves obtained from commercial standards of each compound. The results were expressed in g/100 g of cottage cheese.

For evaluation of antioxidant activity, the samples were submitted to DPPH and reducing power assays, previously described.

Statistical analysis

In each group of study, three different samples were prepared that were analysed in triplicate from for each considered parameter. The results are expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This analysis was carried out using SPSS v. 22.0 program (IBM Corp., Armonk, NY: USA).

Results and discussion

Chemical characterization, antioxidant and antimicrobial properties of fennel phenolic-enriched extracts

The chromatographic profile of the fennel decoction can be observed in **Figure 6**. Up to seventeen phenolic compounds were identified, including twelve phenolic acids and derivatives, and five flavonoids (**Table 5**).

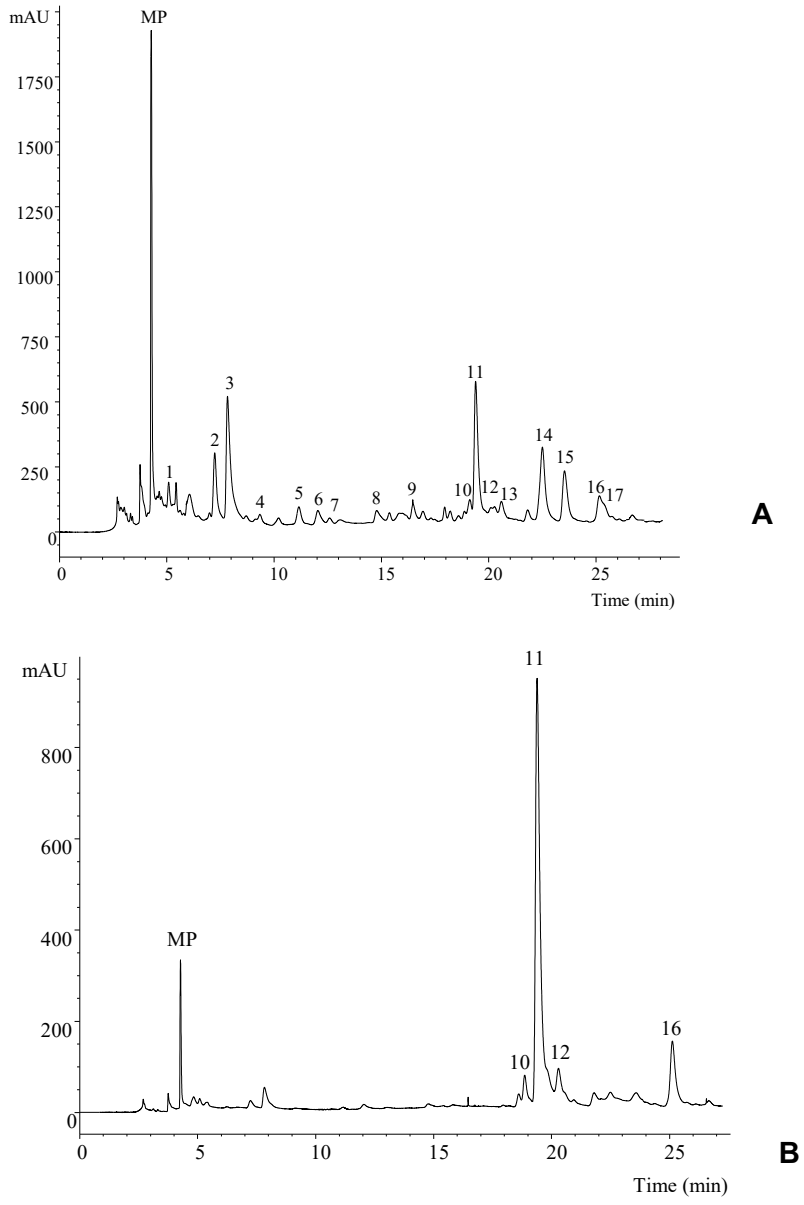


Figure 6. Profile of phenolic compounds in fennel deccotion, recorded at 280 nm (A) and 370 nm (B).

Table 5. Retention time (Rt), wavelengths of maximum absorption in the visible region (λmax), mass spectral data, identification and quantification of phenolic compounds in fennel decoction.

Compound	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*) (% base peak)	Tentative Identification	Quantification (mg/g)
1	5.1	328	353	191(100),179(62),173(5),161(5),135(45)	3-*O*-Caffeolyquinic acid	1.12 ± 0.02
2	7.2	326	353	191(60),179(89),173(100),161(7),135(31)	4-*O*-Caffeolyquinic acid	2.25 ± 0.09
3	7.8	326	353	191(100),179(20),173(14),161(5),135(14)	5-*O*-Caffeolyquinic acid	4.54 ± 0.15
4	9.5	256	137	109(100)	*p*-Hydroxybenzoic acid	0.04 ± 0.01
5	11.1	324	179	135(100)	Caffeic acid	0.31 ± 0.01
6	12.0	328	515	353(95),191(85),179(79),173(3),161(3),135(41)	1,3-Di-*O*-caffeoylquinic acid	0.76 ± 0.04
7	12.5	274	289	175(100),159(40),147(60),131(37),115(41)	Epicatechin	0.43 ± 0.01
8	14.8	324	367	193(11),191(100),173(12),134(5)	5-*O*-Feruloylquinic acid	0.32 ± 0.01
9	16.9	310	163	119(100)	*p*-Coumaric acid	tr
10	18.9	358	609	301(100)	Quercetin-3-*O*-rutinoside	0.28 ± 0.01
11	19.4	356	477	301(100)	Quercetin-3-*O*-glucuronide	8.81 ± 0.07
12	20.3	358	463	301(100)	Quercetin-3-*O*-glucoside	0.57 ± 0.02
13	20.6	326	515	353(87),191(20),179(62),173(100),161(4),135(18)	1,4-Di-*O*-caffeoylquinic acid	1.15 ± 0.07
14	22.5	328	515	353(77),191(100),179(24),173(4),161(15),135(7)	1,5-Di-*O*-caffeoylquinic acid	3.84 ± 0.08
15	23.5	330	601	557(20),515(13),353(12),233(100),191(8),179(9),173(21),161(4),135(3)	Malonyl di-*O*-caffeoylquinic acid	2.48 ± 0.14
16	25.1	354	491	315(100),300(42)	Isorhamnetin-3-*O*-glucuronide	1.43 ± 0.01
17	25.2	332	515	353(95),191(24),179(65),173(100),161(4),135(25)	3,4-Di-*O*-caffeoylquinic acid	1.43 ± 0.04
					Total phenolic acids	18.25 ± 0.62
					Total flavonoids	11.52 ± 0.11
					Total phenolic compounds	29.76 ± 0.73

p-Hydroxybenzoic acid (compound 4), 5-*O*-caffeolylquinic acid (compound 3), caffeic acid (compound 5), epicatechin (compound 7), *p*-coumaric acid (compound 9), quercetin-3-*O*-rutinoside (compound 10) and quecetin-3-*O*-glucoside (compound 12) were positively identified by comparison of their retention time, mass and UV-vis characteristics with commercial standards. Most of these compounds have been previously described in fennel samples by different authors (Parejo et al., 2004a; Parejo, Viladomat, Jaume & Codina, 2004b; Križman, Baričevič & Prošek, 2007; Faudale, Viladomat, Bastida, Poli & Codina, 2008; Rather, Dar, Sofi, Bhat & Qurishi, 2012; Roby, Sarhana, Selima & Khalel, 2013).

Compounds 1 and 2 showed the same pseudomolecular ion as compound 3 and were identified based on their fragmentation pattern according to the clues described by Clifford et al. (2003, 2005). Compound 1 was assigned as 3-*O*-caffeoylquinic acid based on the MS^2 base peak at *m/z* 191 (deprotonated quinic acid) and the second major ion at *m/z* 179 [caffeic acid-H]$^-$, with an intensity >60% of base peak, similar to those reported by Clifford and coworkers for 3-acylchlorogenic acids. Compound 2 showed a base peak at *m/z* 173 [quinic acid-H-H_2O]$^-$, accompanied by a secondary fragment ion at *m/z* 179 with approximately 89% abundance of base peak, consistent with 4-*O*-caffeoylquinic acid (Clifford et al., 2003, 2005). The presence of 3-, 4- and 5-*O*-caffeoylquinic acids in fennel samples was already reported by other authors (Parejo et al., 2004a,b; Križman et al., 2007; Faudale et al., 2008; Rather et al., 2012). Compound 8 was tentatively identified as 5-*O*-feruloylquinic acid taking into account its pseudomolecular ion and fragment ions with relative abundance similar to those of 5-*O*-caffeoylquinic acid. This compound was identified by Parejo et al. (2004a) in fennel wastes. Four compounds (peaks 6, 13, 14 and 17) showed the same pseudomolecular ion [M-H]$^-$ at *m/z* 515, corresponding to dicaffeoylquinic acids; they were also tentatively assigned based on their fragmentation patterns according to Clifford et al. (2005) as 1,3-; 1,4-; 1,5- and 3,4-*O*-dicaffeoylquinic acids, respectively. With the exception of 3,4-*O*-dicaffeoylquinic acid, all the other dicaffeoylquinic derivatives were previously reported in fennel (Parejo et al., 2004a,b; Križman et al., 2007; Faudale et al., 2008; Rather et al., 2012). Compound 15 presented a pseudomolecular ion [M-H]$^-$ at *m/z* 601, 86 mu higher than a di-*O*-caffeoylquinic acids and with similar MS^2 fragmentation pattern similar as those compounds; the mass difference can be related to a malonyl moiety, so that it was tentatively identified as malonyl di-*O*-caffeoylquinic acid. To the best of our knowledge such a compound has not been previously reported in fennel.

Compound 11 ([M-H]$^-$ at *m/z* 477) presented a UV spectrum with λ_{max} around 350 nm and a unique MS^2 product ion at *m/z* 301; it was identified as quercetin 3-*O*-glucuronide as confirmed by comparison with a standard obtained and characterized in our laboratory (Dueñas et al., 2008). This compound has been reported in fennel (Parejo et al., 2004a,b; Faudale et

al., 2008; Rather et al., 2012). Compound 16 showed a pseudomolecular ion $[M-H]^-$ at *m/z* 491, 14 mu higher than compound 11, and fragment ions at *m/z* 315 and 300, from the consecutive losses of 176 mu (glucuronide moiety) and 15 mu (methyl group), which allowed assigning it as a methylquercetin-*O*-glucuronide. It was tentatively identified isorhamnetin 3-*O*-glucuronide owing the previous identification of that compound in fennel by Parejo et al. (2004a). Quercetin-3-*O*-glucuronide was the most abundant compound in the studied fennel sample, whereas 5-*O*-caffeoylquinic acid was the most abundant phenolic acid. The quantitative results obtained in our study for fennel decoction (**Table 5**) cannot be compared with those given by other authors (Parejo et al., 2004b; Križman et al., 2007; Faudale et al., 2008; Roby et al., 2013) which expressed them regarding dry plant material.

Four different *in vitro* assays were applied to evaluate the antioxidant activity of the phenolic compounds-enriched extract of *F. vulgare* (fennel) prepared by decoction, and the results are shown in **Table 6**. The high antioxidant activity of fennel has been previously reported for methanol (Barros et al., 2009) and boiling aqueous (Mata et al., 2007) extracts. However, the decoction prepared in this study showed lower DPPH scavenging activity, reducing power and lipid peroxidation inhibition than the ones reported in the mentioned studies. A recent investigation described the evaluation of antioxidant properties of different fennel extracts (methanol, ethanol, diethyl ether and hexane), concluding that methanol and ethanol gave more efficient extracts than the other less polar solvents (Roby et al., 2013). Nevertheless, it is noted that water was not included in the mentioned study and that the mentioned solvents exhibit some toxicity to human, being the decoction used in the present study more suitable for incorporation in food matrices.

The antimicrobial and antifungal activity of the fennel extract was examined against a panel of eight microorganisms selected on the basis of their relevance to public health. The data were expressed as minimum inhibitory concentration (MIC), bactericidal concentration (MBC) and fungicidal concentration (MFC) and reported in **Table 6**. *Salmonella typhimurium* and *Bacillus cereus* were the most sensitive bacteria, while and *Aspergillus niger*, *Aspergillus versicolor* and *Penicillium funiculosum* were the most susceptible fungi, showing the lowest MIC values. Moreover, the fennel extract showed bactericide and fungicide effects against the tested microorganism. Some previous studies reported *F. vulgare* as a good source for the preparation of new therapeutic and antimicrobial agents (Lo Cantore et al., 2004; Singh et al., 2006). As for the antioxidant activity, Roby et al. (2013) described the antimicrobial activity of methanol and ethanol extracts prepared from fennel seeds showing much lower MICs (0.010-0.015 mg/mL) than those obtained in the present study.

Table 6. Antioxidant and antimicrobial activity of fennel phenolic-enriched extracts obtained by decoction.

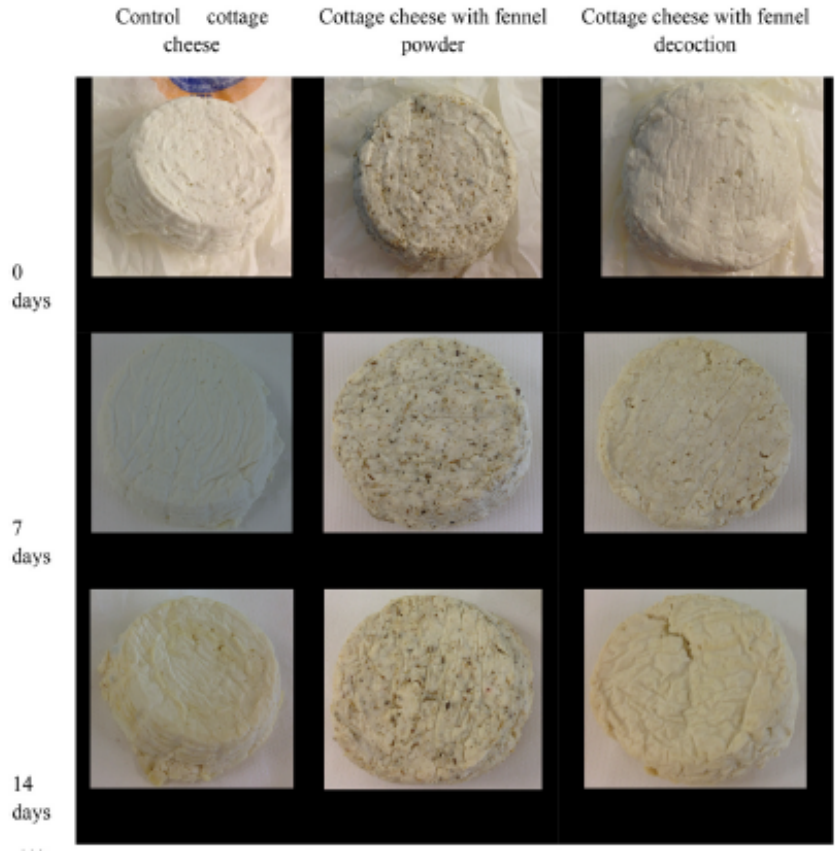
Antioxidant activity								
	DPPH scavenging activity		Reducing power		β-carotene bleaching inhibition		TBARS inhibition	
Fennel (EC_{50}, mg/mL)	0.75 ± 0.01		0.42 ± 0.06		0.17 ± 0.01		0.37 ± 0.01	
Trolox (EC_{50}, µg/mL)	41.43 ± 1.27		41.68 ± 0.28		18.21 ± 1.12		22.84 ± 0.74	
Antibacterial activity (mg/mL)								
	Staphylococcus aureus	*Bacillus cereus*	*Micrococcus flavus*	*Listeria monocytogenes*	*Pseudomonas aeroginosa*	*Escherichia coli*	*Enterobacter cloacae*	*Salmonella typhimurium*
Fennel								
MIC	0.20 ± 0.01	0.02 ± 0.01	1.00 ± 0.02	0.75 ± 0.1	0.20 ± 0.09	1.00 ± 0.02	0.75 ± 0.03	0.035 ± 0.009
MBC	0.75 ± 0.03	0.05 ± 0.005	1.50 ± 0.1	1.50 ± 0.1	0.75 ± 0.03	1.50 ± 0.2	3.0 ± 0.1	0.05 ± 0.01
Streptomycin								
MIC	0.04 ± 0.002	0.10 ± 0.003	0.20 ± 0.01	0.20± 0.001	0.20 ± 0.006	0.20 ± 0.003	0.20 ± 0.003	0.25 ± 0.007
MBC	0.10 ± 0.003	0.20 ± 0.06	0.30 ± 0.000	0.30 ± 0.02	0.30 ± 0.003	0.30 ± 0.03	0.30 ± 0.000	0.50 ± 0.003
Ampicillin								
MIC	0.25 ± 0.02	0.25 ± 0.01	0.25 ± 0.02	0.40 ± 0.03	0.75 ± 0.1	0.40 ± 0.02	0.25 ± 0.01	0.40 ± 0.01
MBC	0.40 ± 0.01	0.40 ± 0.00	0.50 ± 0.003	0.50 ± 0.003	1.20 ± 0.5	0.50 ± 0.05	0.50 ± 0.003	0.75 ± 0.1
Antifungal activity (mg/mL)								
	Aspergillus fumigatus	*Aspergillus versicolor*	*Aspergillus ochraceus*	*Aspergillus niger*	*Trichoderma viride*	*Penicillium funiculosum*	*Penicillium ochrochloron*	*Penicillium verrucosum*
Fennel								
MIC	3.00 ± 0.2	0.40 ± 0.09	0.75 ± 0.2	0.20 ± 0.03	0.75 ± 0.1	0.40 ± 0.09	1.50 ± 0.1	3.00 ± 0.1
MFC	6.00 ± 0.3	6.00 ± 0.6	6.00 ± 0.9	6.00 ± 0.75	1.50 ± 0.2	1.50 ± 0.1	3.00 ± 0.2	6.00 ± 0.3
Ketoconazole								
MIC	0.15 ± 0.01	0.10 ± 0.02	0.15 ± 0.002	0.15 ± 0.003	0.15 ± 0.002	0.20 ± 0.06	0.20 ± 0.02	0.10 ± 0.01
MFC	0.20 ± 0.02	0.20 ± 0.007	0.20 ± 0.000	0.20 ± 0.03	0.20 ± 0.000	0.25 ± 0.02	0.25 ± 0.03	0.20 ± 0.003
Bifonazole								
MIC	0.20 ± 0.000	0.20 ± 0.01	1.50 ± 0.1	0.20 ± 0.003	1.00 ± 0.07	0.20 ± 0.003	2.50 ± 0.3	0.20 ± 0.000
MBC	0.50 ± 0.01	0.50 ± 0.03	2.00 ± 0.02	0.50 ± 0.007	1.00 ± 0.01	0.50 ± 0.002	3.50 ± 0.2	0.30 ± 0.007

The antioxidant activity was expressed as EC_{50} values (Mean ± SD, n = 9), what means that higher values correspond to lower reducing power or antioxidant potential. EC_{50}: Extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. Minimum inhibitory concentration (MIC), bactericidal concentration (MBC) and fungicidal concentration (MFC).

Incorporation of fennel-based natural ingredients in cottage cheese

Effects on color and nutritional parameters

Considering the high antioxidant properties obtained in the present study for fennel decoction, this preparation was incorporated in cottage cheese samples. In addition to the incorporation of fennel decoction, fennel powder was also directly incorporated in other cottage cheese samples to compare effects and evaluate if the preparation of decoction would be worthy. Color parameters, nutritional value, fatty acids composition and antioxidant activity of the prepared cottage cheese samples were evaluated along shelf life (0 days, 7 days and 14 days). Pictures of the cheese throughout that period are shown in **Figure 7A**.



A

B

Figure 7. (A) Appearance of cottage cheese samples along shelf life. **(B)** Control sample of cottage cheese after 14 days of storage, showing signs of degradation.

The results of color evaluation are collected in **Table 7**. The *L** parameter indicates lightness so, higher values result in clearer objects; *a** value indicates the redness-greeness tendency and *b** value indicates the blueness-yellowness tendency. A statistical significant increase of *b** value (yellow component) of the control cottage cheese, was observed after 7 days of storage; after 14 days, all the parameters changed. The samples with fennel powder and decoction only showed increased *b** values after 14 days, maintaining *L** parameter (**Table 7; Figure 7A**). Some authors related the increase in these values with the occurrence of proteolysis and the Maillard reaction, which decreases the luminosity due to the production of browning compounds (Lucas, Rock, Agabriel, Chilliard & Coulon, 2008). The values obtained for the different parameters are in the same order of magnitude in all the sample groups. A study with ricotta cheese reported similar *L** (93.63-94.31) but lower *b** values (7.65-8.13) (Pizzilo, Claps, Cifuni, Fedele & Rubino, 2005) as those obtained in the present work. Another study with “coalho” cheese made from cow’s and goat’s milk and their mixture, found similar *L** and *b** values, as also an increase in *b** values along 28 days (Queiroga et al., 2013). No statistical significant differences were observed in *a** parameter; moreover, its value is close to zero and, therefore, the contribution to total color value is minimum.

Table 7. Color parameters, nutritional value and fatty acids composition of the cottage cheese samples along shelf life.

	Control cottage cheese			Cottage cheese with fennel powder			Cottage cheese with fennel decoction		
Storage days	**0**	**7**	**14**	**0**	**7**	**14**	**0**	**7**	**14**
Color parameters									
*L**	92.94±0.75^{a}	90.46±1.5^{a}	86.03±4.71^{b}	82.59±3.92^{a}	80.62±3.15^{ab}	76.47±5.49^{b}	88.14±1.49^{a}	84.38±5.22^{a}	84.59±2.32^{a}
*a**	-2.44±0.39^{a}	-2.34±0.25^{a}	-2.59±0.51^{a}	-1.47±0.36^{a}	-1.45±0.20^{a}	-1.22±0.48^{a}	-1.45±0.21^{a}	-1.55±0.40^{a}	-1.65±0.29^{a}
*b**	10.40 ±0.51^{c}	11.24 ±0.63^{b}	14.91±1.51^{a}	15.10±1.61^{b}	15.46±0.64^{b}	17.59±1.82^{a}	14.77±1.70^{b}	15.51±0.33^{b}	17.42±0.61^{a}
Nutritional value									
Moisture (g/100 g)	64.5±1.33^{a}	63.8±0.90^{a}	60.10±0.58^{b}	64.80±0.81^{a}	63.43±1.32^{b}	60.54±0.26^{c}	66.26±0.79^{a}	63.37±1.02^{a}	59.20±1.01^{b}
Protein (g/100 g)	11.01±0.02^{c}	12.08±0.09^{b}	13.25±0.07^{a}	11.70±0.11^{c}	12.30±0.08^{b}	13.34±0.24^{a}	11.30±0.36^{c}	12.09 ±0.04^{b}	13.62±0.12^{a}
Ash (g/100 g)	2.35±0.04^{a}	2.15±0.05^{c}	2.24±0.01^{b}	2.03±0.08^{b}	2.17±0.02^{a}	2.20±0.03^{a}	2.01±0.01^{c}	2.25 ±0.03^{b}	2.46±0.03^{a}
Fat (g/100 g)	18.98±0.33^{c}	19.97±0.09^{b}	22.33±0.20^{a}	19.45±0.44^{b}	19.38±0.04^{b}	20.40±0.02^{a}	18.06±0.51^{c}	19.10 ±0.12^{b}	22.55±0.01^{a}
Carbohydrates (g/100g)	3.16±1.00^{a}	2.00±0.14^{a}	2.08±0.21^{a}	2.02±1.07^{a}	2.72±0.07^{a}	3.52±0.05^{a}	2.38±0.35^{b}	3.17 ±0.09^{a}	2.18±0.02^{c}
Lactose (g/100 g)	2.00±0.06^{a}	1.95±0.03^{a}	1.77±0.04^{b}	1.71±0.01^{b}	1.83±0.04^{a}	1.75±0.07^{ab}	2.04±0.02^{a}	2.02 ±0.02^{a}	2.07±0.07^{a}
Energy (kcal/100 g)	227.47±6.98^{b}	236.08±0.24^{b}	262.30±0.98^{a}	229.96±0.73^{c}	234.48±0.13^{b}	251.07±0.01^{a}	220.03±0.95^{c}	233.00 ±0.71^{b}	266.09±0.19^{a}
Fatty acids									
C4:0	7.84±0.52^{a}	7.42±0.85^{ab}	6.57±0.55^{b}	6.86±0.75^{a}	5.12±0.16^{b}	6.72±0.06^{a}	6.74±0.32^{a}	6.27±0.01^{ab}	5.74±0.85^{b}
C6:0	7.09±0.68^{a}	6.79±0.63^{a}	6.28±0.30^{a}	6.41±0.03^{b}	5.52±0.49^{c}	6.70±0.37^{a}	6.22±0.31^{b}	5.97±0.35^{b}	6.93±0.15^{a}
C8:0	4.77±0.27^{a}	4.94±0.12^{a}	4.39±0.52^{a}	4.79±0.23^{b}	4.82±0.08^{b}	5.79±0.09^{a}	4.74±0.17^{b}	4.80±0.38^{ab}	5.39±0.47^{a}
C10:0	9.95±0.21^{a}	10.20±0.17^{a}	10.29±0.85^{a}	10.82±0.81^{b}	10.46±0.75^{b}	11.55±0.20^{a}	10.95±0.20^{b}	11.42±0.64^{b}	12.66±0.45^{a}
C12:0	4.48±0.02^{b}	4.49±0.01^{b}	5.02±0.32^{a}	5.20±0.38a^{b}	4.73±0.54^{b}	5.49±0.04^{a}	5.32±0.03^{a}	5.49±0.10^{a}	5.57±0.34^{a}
C14:0	8.74±0.30^{b}	8.79±0.07^{b}	9.85±0.12^{a}	9.95±0.50^{a}	9.29±0.46^{a}	9.48±0.07^{a}	10.11±0.11^{a}	10.34±0.11^{a}	10.18±0.37^{a}
C15:0	1.03±0.07^{b}	1.05±0.01^{b}	1.15±0.05^{a}	1.16±0.04^{a}	1.02±0.13^{b}	1.01±0.02^{b}	1.20±0.02^{a}	1.19±0.03^{a}	1.12±0.01^{b}
C16:0	20.70±0.56^{a}	19.96±0.27^{b}	20.59±0.90^{a}	20.66±0.24^{ab}	21.12±0.46^{a}	20.29±0.01^{b}	20.92±0.51^{ab}	21.28±0.54^{a}	20.47±0.26^{b}
C18:0	10.29±0.13^{a}	10.53±0.64^{a}	9.87±0.59^{a}	9.30±0.39^{b}	10.42±0.77^{a}	8.50±0.48^{b}	9.07±0.05^{a}	8.87±0.24^{ab}	8.47±0.42^{b}
C18:1n9	18.36±0.38^{a}	18.70±0.15^{a}	18.95±0.98^{a}	18.31±0.65^{b}	19.75±0.35^{a}	18.88±0.38^{b}	17.92±0.28^{a}	18.06±0.31^{a}	17.68±0.50^{a}
C18:2n6	2.80±0.05^{a}	2.71±0.16^{a}	2.70±0.30^{a}	2.49±0.14^{b}	2.94±0.25^{a}	2.20±0.01^{b}	2.54±0.02^{a}	2.44±0.08^{ab}	2.31±0.13^{b}
C18:3n3	1.58±0.13^{a}	1.61±0.04^{a}	1.52±0.21^{a}	1.43±0.12^{b}	1.71±0.13^{a}	1.21±0.02^{c}	1.47±0.04^{a}	1.37±0.08^{ab}	1.26±0.07^{b}

SFA (%)	76.23±0.60^{a}	75.70±0.15^{a}	75.41±1.62^{a}	76.56±0.97^{a}	74.11 ± 0.80^{b}	76.65±0.45^{a}	76.74±0.34^{b}	76.98±0.45^{ab}	77.72±0.75^{a}
MUFA (%)	19.09±0.42^{a}	19.40±0.19^{a}	19.80±1.06^{a}	19.05±0.69^{b}	20.64 ± 0.37^{a}	19.53±0.45^{b}	18.72±0.29^{a}	18.84±0.32^{a}	18.31±0.55^{a}
PUFA (%)	4.68±0.17^{a}	4.90±0.34^{a}	4.80±0.56^{a}	4.39±0.28^{b}	5.26 ± 0.43^{a}	3.81±0.01^{c}	4.54±0.04^{a}	4.18±0.14^{b}	3.96±0.21^{b}

The results are presented as mean±SD (n = 9). *L**, *a** and *b** (color parameters). Butiric acid (C4:0); Caproic acid (C6:0); Caprylic acid (C8:0); Capric acid (C10:0); Lauric acid (C12:0); Myristic acid (C14:0); Pentadecanoic acid (C15:0); Palmitic acid (C16:0); Stearic acid (C18:0); Oleic acid (C18:1n9); Linoleic acid (C18:2n6); α-Linolenic acid (C18:3n3); SFA- Saturated fatty acids; MUFA- Monounsaturated fatty acids; PUFA- Polyunsaturated fatty acids. The difference to 100% corresponds to other 17 less abundant fatty acids (data not shown). In each row and within each sample (Control cottage cheese, Cottage cheese with fennel powder and Cottage cheese with fennel decoction), different letters mean significant statistical differences along shelf life (p<0.05).

All the cottage cheese samples are characterized by high moisture, protein and fat contents (**Table 7**). The samples complied with the standard of identity for cottage cheese, which states that they should have a minimum fat free dry matter content of 18% (Codex standard, 2010). The moisture contents were similar to the ones reported by Queiroga et al. (2013), which also described a slight decrease in moisture along shelf life (28 days). Otherwise, Silva, Ramos, Moreno & Moraes (2010) found lower moisture contents (45.5-51.5 g/100 g) in "Coalho" cheese made from cow's milk. In the present study, as expected, a decrease in moisture levels was observed along the storage.

In our investigation, the amounts of protein and fat increased over time, however, it is necessary to take into account that moisture decreased and, therefore, a relative increase in nutrients concentration is expected. Nevertheless, the results obtained when expressed in dry matter basis showed maintenance of the protein content along shelf life, indicating that the total amount of protein does not change. A study with ripened cheeses described a decrease in protein content during storage (Pappa et al., 2006). Protein loss during ripening is related with protein hydrolysis and production of water-soluble nitrogen compounds, which are released in the brine (Pintado et al., 2008). This does not apply in our study, as cottage cheese do not suffer relevant ripening.

The energy values also increased along shelf life, being in the same magnitude in all the samples (**Table 7**); this variation is also due to the moisture decrease and increasing concentration of nutrients as previously explained. A study with fresh soft cheese described energetic values varying between 174 and 197 kcal/100 g (Krbavcic and Baric, 2004), lower than those obtained in our samples.

Lactose was the free sugar identified and quantified in the samples; fennel-based ingredients seem to protect lactose in the prepared cheeses, avoiding its decrease after 14 days of storage, which was observed in the control sample (**Table 7**).

Essential fatty acids and their derivatives represent an important nutritional role and have a high dietetic significance in dairy products. In all the samples, saturated fatty acid (SFA) predominated, followed by monounsaturated (MUFA) and then polyunsaturated (PUFA) fatty acids (**Table 7**). Studies with ricotta cheese showed similar fatty acid distribution as observed in the present study (Pizzillo et al., 2005). Palmitic acid (C16:0) and oleic acid (C18:1n9) were the predominant fatty acids in all the cheese samples (**Table 7**). The next most predominant fatty acid in the control cottage cheese was stearic acid (C18:0), while for the samples with fennel-based ingredients was capric acid (C10:0). Queiroga et al. (2013) reported the same predominant fatty acids in Coalho cheeses made from different types of milk. Otherwise, C6, C8, C10 and C12 fatty acids were described as the main fatty acids in cheeses made from goat's milk (Lucas et al., 2008; Ceballos et al., 2009).

Effects on antioxidant parameters

As expected, cottage cheese did not show relevant antioxidant properties; the reducing power observed after 7 and 14 days is probably related with the formation of reducing substances after lipid peroxidation process. The incorporation of fennel improved the antioxidant activity of cottage cheese (**Table 8**). Samples incorporated with plant powder revealed higher antioxidant properties than samples incorporated with decoction, either in 0 or 7 days of storage. A decrease in the antioxidant potential of the cottage cheese with both fennel preparations was observed along the shelf life. Nevertheless, it is important to highlight that the samples, after 14 days, still display antioxidant properties. At that time, cottage cheese incorporated with fennel decoction gave somewhat better DPPH scavenging activity than the one with fennel powder.

Food industry is focused on the development of novel functional foods containing health promoting natural ingredients. There are other available studies that also describe improvements in the nutritional characteristics, antioxidant and antimicrobial properties of cheese incorporated with different natural ingredients, such as *Agaricus bohusii* mushroom extract (Reis et al., 2012) or lupin milk (Elsamani, Habbani, Babiker & Ahmed, 2014).

Conclusions

Overall, the incorporation of fennel-based ingredients did not altered significantly the nutritional characteristics of control cottage cheese, but seems to avoid the increase in *b** color parameter (yellowness) after 7 days of storage (**Figure 7A**), and the decrease in lactose content observed after 14 days of storage in control samples. Furthermore, control samples of cottage cheese (without fennel-based ingredients) after 14 days of storage, were the only ones showing signs of degradation (**Figure 7B**). The incorporation of a fennel phenolic-enriched extract (decoction) improved the antioxidant properties of cottage cheese, up to 14 days of storage. To preserve the antioxidant activity along the shelf life, microencapsulation techniques could be applied to fennel decoction. The development of new functionalized dairy products is important for consumers that demand for valuable health effects while enjoying a highly appreciated product.

Table 8. Free radicals scavenging activity and reducing power (EC_{50} values, mg/mL) of cottage cheese samples along shelf life.

	Control cottage cheese			Cottage cheese with fennel powder			Cottage cheese with fennel decoction		
Storage days	**0**	**7**	**14**	**0**	**7**	**14**	**0**	**7**	**14**
DPPH assay	>200	>200	>200	30.05 ± 3.19^{b}	42.74 ± 0.35^{b}	49.42 ± 0.76^{a}	40.99 ± 0.12^{c}	44.20 ± 0.12^{b}	46.72 ± 0.09^{a}
Reducing power assay	>200	40.27 ± 0.52^{a}	14.85 ± 0.12^{b}	6.92 ± 0.13^{a}	5.75 ± 0.09^{b}	5.13 ± 0.03^{c}	8.46 ± 0.06^{a}	6.82 ± 0.10^{b}	5.50 ± 0.05^{c}

The results are presented as mean±SD (n = 9). In each row and within each sample (Control cottage cheese, Cottage cheese with fennel powder and Cottage cheese with fennel decoction), different letters mean significant statistical differences along shelf life ($p<0.05$).

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3.1.2. Desenvolvimento de um alimento lácteo funcional: exploração dos efeitos bioativos e de preservação da camomila (*Matricaria recutita* L.)

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Development of a functional dairy food: Exploring bioactive and preservation effects of chamomile (*Matricaria recutita* L.)

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Abstract

The antioxidant (free radical scavenging activity, reducing power and lipid peroxidation inhibition) and antimicrobial (against bacteria and fungi) potential of *Matricaria recutita* L. (chamomile) extracts obtained by decoction was demonstrated. The characterization of the extracts highlighted dicaffeoyl-2,7-anhydro-3-deoxy-2-octulopyranosonic acid (diCDOA) and luteolin-*O*-glucuronide as the main phenolic compounds. Extracts containing these natural ingredients were incorporated into cottage cheese, and the prepared products were compared with samples incorporated with chamomile powder and plain cottage cheese (control). Nutritional composition, colour and antioxidant activity of all samples were evaluated along storage time. Chamomile decoctions (natural bioactive ingredient) did not alter significantly the nutritional and fatty acids profiles of cottage cheese, but improved its antioxidant potential,

independent of the storage time. Moreover, it increased the shelf life since only the control samples showed signs of degradation after 14 days of storage. The development of this novel functional dairy product emphasizes the bioactive and preservation potential of chamomile.

Keywords: Functional food; Cottage cheese; *Matricaria recutita;* Antioxidant/antimicrobial properties; Natural preservative.

Introduction

Novel functional foods with health promoting natural ingredients instead of synthetic additives have been intensively developed and commercialized by food industry (Carocho, Barreiro, Morales & Ferreira, 2014; Caleja et al., 2015). Furthermore, there is an increasing concern among consumers to seek healthier and "natural" foods to avoid a series of food safety risks that have become a global problem (Sarig et al., 2003). Food additives are widely used for various purposes in order to improve the appearance, flavour, colour, texture or shelf life of the food, making this the most desirable form to the consumers' eyes. However, several studies have shown that consumption in excess can be harmful to health due to some toxicity effects (Randhawa & Bahna, 2009; Carocho et al., 2014).

There is a tendency for consumers to choose minimally processed foods instead of processed ones, or with minimum incorporation of synthetic additives, which are being passed over by natural ones. Natural additives include compounds or extracts from plants used to improve the qualities of food. One of the most studied effects of these additives is the antioxidant activity (Rasooli, 2007). Besides the importance of antioxidants as natural conservation ingredients, these compounds/extracts can also bring bioactive properties to food. Natural extracts rich in phenolic compounds arise as promissory alternatives to plant essential oils that, sometimes, could display toxic effects (Zapata & Smagghe, 2010; Assis, Gondim, Siqueira & Câmara, 2011). Furthermore, due to their antioxidant properties, these molecules have been related with the prevention of ageing and various diseases related with oxidative stress, such as coronary heart disease, cancer or neurodegenerative diseases (Procházková, Bousová & Wilhelmová, 2011; Weng & Yen, 2012).

Chamomile (*Matricaria recutita* L.) is a source of phenolic compounds, such as flavonoids, that are the main compounds contributing to its antioxidant properties (Mulinacci, Romani, Pinelli, Vincieri & Prucher, 2000; Nováková, Vildová, Mateus, Gonçalves & Solich, 2010; Lin & Harnly, 2012; Raal, Orav, Püssa, Valner, Malmiste & Arak, 2012; Guimarães et al., 2013; Matić, Juranić, Savikin, Zdunić, Nadvinski & Godevac, 2013; Roby, Sarhan, Selim & Khalel, 2013; Avula et al., 2014; Zielinski Haminiuk, Alberti, Nogueira, Demiate & Granato,

2014; Xie, Wang & Shi, 2014). Moreover, it has been used for its many beneficial health effects as antimicrobial, neuroprotective, anti-allergic, anti-inflammatory, and anticancer agent (Alanís, Calzada, Cervantes, Torres & Ceballos, 2005; Ranpariya, Parmar, Sheth & Chandrashekhar, 2011; Chandrashekhar et al., 2011; Bulgari et al., 2012; Silva, Barbosa, Seito & Fernandes, 2012; Matić et al., 2013).

Goat cheese is highly appreciated for its nutritional value, good digestibility and low allergenic properties. In addition, goat milk has been associated with certain therapeutic values in human nutrition (Haenlein, 2004; Díaz-Castro et al., 2012).

In the present work, the antioxidant and antimicrobial effects of chamomile (*M. recutita*) were explored, developing a functional dairy food with improved preservation and bioactive properties. Therefore, natural ingredients based in chamomile phenolic compounds extracts obtained by decoction were prepared, characterized and incorporated in cottage cheese. Besides these aqueous extracts, chamomile powder was also used to functionalize different samples. The effects on nutritional value, shelf life and bioactive properties of the control and the functionalized samples were comprehensively evaluated.

Materials and methods

Standards and reagents

HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic and acetic acids were purchased from Prolabo (VWR International, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Phenolic standards were from Extrasynthèse (Genay, France). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also other individual fatty acid isomers, L-ascorbic acid, organic acid and sugar standards, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and positive controls, streptomycin, ampicillin, bionazole and ketokonazole. Mueller-Hinton agar (MH) and malt agar (MA) were obtained from the Institute of Immunology and Virology, Torlak (Belgrade, Serbia). Water was treated in Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

Chamomile-based natural ingredients

Preparation

Commercial samples of *Matricaria recutita* L. (chamomile) were provided by Américo Duarte Paixão Lda. (Alcanede, Portugal). The dried samples were powdered (~20 mesh) and submitted to decoction in order to obtain phenolic-enriched extracts. The decoction was performed by adding 1 g of plant material to 200 mL of distilled water, heated (heating plate, VELP Scientific, Usmate, Italy) and boiled for 5 min. The mixture was left to stand for 5 min, filtered, and then frozen and lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA).

Characterization in phenolic compounds

Phenolic compounds were determined in the decoctions by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, CA, USA), as previously described by the authors (Barros et al., 2013). Double online detection was carried out in a diode array detector (DAD) using 280 at 370 nm as preferred wavelengths and a mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet. The phenolic compounds were identified by comparing their retention time, UV-vis and mass spectra with those obtained from standard solutions, when available. Otherwise, peaks were tentatively identified comparing the obtained information with available data reported in the literature. The results are expressed in mg/g of lyophilized decoction.

Antioxidant properties

The lyophilized decoctions were re-dissolved in water (5 mg/mL) and successively diluted until determination of EC_{50} values (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay).

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 and lipid peroxidation (thiobarbituric acid reactive substances, TBARS) inhibition were evaluated spectrophotometrically at 470 and 532 nm, respectively. The complete protocols were previously described by the authors (Barros et al., 2013). Trolox was used as positive control in all the assays.

Antimicrobial properties

Antibacterial activity was evaluated against Gram-negative bacteria: *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), *Enterobacter cloacae* (ATCC 35030), and 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 the authors (Sokovic, Glamočlija, Marin, Brkić & van Griensven, 2010). The minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations were determined following the same reference. Streptomycin and ampicillin were used as positive controls. Antifungal activity was evaluated against *Aspergillus fumigatus* (ATCC 1022), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus versicolor* (ATCC 11730), *Aspergillus niger* (ATCC 6275), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112), *Penicillium verrucosum* var. *cyclopium* (food isolate), and *Trichoderma viride* (IAM 5061), following the procedure previously described by the authors (Sokovic & van Griensven, 2006). The minimum inhibitory (MIC) and minimum fungicidal (MFC) concentrations were determined following the same reference. Bionazole and ketokonazole were used as positive controls.

Development of the functional food

Preparation of cottage cheese by incorporation with chamomile-based natural ingredients

All the samples of cottage cheese were prepared by "Queijos Casa Matias Lda." (one of the main producer companies of "Serra da Estrela" cheese; being the latter the most famous Portuguese cheese). Three groups of samples were prepared (cottage cheeses with 250 g): control samples (cottage cheese without the chamomile-based natural ingredient); samples with the chamomile decoction (it was incorporated in cottage cheese at EC_{25} value previously determined by DPPH assay: 0.165 mg/mL, corresponding to 0.495 g for each 250 g cottage cheese sample); and samples with the chamomile powder (it was incorporated at 0.644 mg/mL, considering the decoction yield of 25.6%, corresponding to 1.932 g for each 250 g cottage cheese sample). Samples corresponding to each group were pooled together and further divided in three subgroups, which were submitted to individual extraction procedures.

Evaluation of nutritional composition, colour and antioxidant activity along storage time

The samples (three different cottage cheeses for each storage time) were submitted to an evaluation of nutritional composition, colour and antioxidant activity, immediately after the incorporations and after 7 and 14 days of storage at 4 ºC.

The samples were analyzed for proximate composition (moisture, protein, fat, carbohydrates and ash) using the AOAC (2005) procedures. The crude protein content (N × 6.38) of the samples was estimated by 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 ºC. Total carbohydrates

were calculated by difference. Total energy was calculated according to the following equation: Energy (kcal) = 4 × (g proteins +g carbohydrates) + 9 × (g lipids).

Fatty acids, free sugars and organic acids were determined by gas-chromatography coupled to flame ionization detector (GC-FID), HPLC coupled to a refraction index (RI) detector, and HPLC coupled to a photodiode array detector (PDA) at 215 nm and 245 nm (for ascorbic acid), respectively, according to previous analytical validation and detailed description by the authors (Barros et al., 2013). Fatty acids were identified by comparison with standards and the results were expressed in relative percentage of each fatty acid. Free sugars were identified by comparison with standards, and further quantified (g/100 g of cottage cheese) by using the internal standard (melezitose). Organic acids were identified and quantified (g/100 g of cottage cheese) by using calibration curves from commercial standards.

The colour of the samples was measured in three different points, for each sample, by using a colourimeter (model CR-400, Konica Minolta Sensing Inc., Tokyo, Japan). Using the illuminant C and diaphragm aperture of 8 mm, the CIE *L*a*b** colour space values were registered using a data software "Spectra Magic Nx" (version CM-S100W 2.03.0006) (Fernandes, Antonio, Barreira, Oliveira, Martins & Ferreira, 2012).

For evaluation of antioxidant activity, the samples were submitted to DPPH and reducing power assays, previously mentioned.

Statistical analysis

All the experiments were performed using three independent samples, being three different extracts obtained from each sample (n=9) and all the assays were carried out in triplicate. In the assessment of differences among antimicrobial assays, results were evaluated using 1-way ANOVA. The homogeneity of variance, was tested by means of the Levene's tests. All dependent variables were compared using Tukey's honestly significant difference (HSD) or Tamhane's T2 multiple comparison tests, when homoscedasticity was verified or not, respectively.

Regarding the evaluation of the effects of functionalizing agent and storage time, an analysis of variance (ANOVA) with type III sums of squares was performed using the Repeated Measures Analysis procedure of the General Linear Model. Since the independence of variables could not be assumed, it was need to verify the sphericity criterion, which evaluates if the correlation between treatments is the same, assuming that variances in the differences among conditions are the same. Sphericity was evaluated trough the Mauchly's test; every time the sphericity assumption was violated, the Greenhouse-Geisser correction was applied. A 2-factor×3-levels matrix was studied.

All the statistical analysis were carried out using SPSS v. 22.0 program (IBM Corp., Armonk, NY, USA).

Results and discussion

Chemical characterization, antioxidant and antimicrobial properties of chamomile phenolic-enriched extracts

Up to nineteen phenolic compounds, including phenolic acids and flavonoids were detected in the *M. recutita* decoctions (Table 9).

Protocatechuic acid (compound 2), 5-*O*-caffeolylquinic acid (compound 3), caffeic acid (compound 5), myricetin-3-*O*-glucoside (compound 6), quercetin-3-*O*-glucoside (compound 8), luteolin-7-*O*-glucoside (compound 9), apigenin-7-*O*-glucoside (compound 15) and apigenin (compound 18) were positively identified by comparison of their retention time, mass and UV-vis characteristics with commercial standards. Most of these compounds have been previously described in *M. recutita* samples by different authors (Mulinacci et al., 2000; Nováková et al., 2010; Lin & Harnly, 2012; Raal et al., 2012; Guimarães et al., 2013; Matić et al., 2013; Avula et al., 2014; Zielinski et al., 2014; Xie et al., 2014).

Compounds 1 ($[M-H]^-$ at *m/z* 341) yielded a fragment at *m/z* 179 (caffeic acid) from the loss of 162 mu (hexosyl moiety), which allowed the tentative identification as caffeic acid hexoside. Compounds 11 and 12 were identified as *cis* and *trans* isomers of 3,5-*O*-dicaffeoylquinic acids, respectively. These compounds presented a similar fragmentation pattern to the ones previously reported by Guimarães et al. (2013). MS^2 base peak was at *m/z* 353, produced by the loss of one of the caffeoyl moieties $[M-H\text{-caffeoyl}]^-$, and subsequent fragmentation of this ion yielded the same fragments as 5-caffeoylquinic acid at *m/z* 191, 179 and 135, although in this case with a comparatively more intense signal at *m/z* 179 [caffeic acid-H]$^-$. Furthermore, *cis* hydroxycinnamoyl derivatives elute before the corresponding *trans* ones, as observed after UV irradiation (366 nm, 24 h) of hydroxycinnamic acids in our laboratory, justifying the elution order of these isomers. These and other dicaffeoylquinic derivatives have been previously reported in flowers of *M. recutita* (Nováková et al., 2010; Lin & Harnly, 2012; Raal et al., 2012; Guimarães et al., 2013).

Compounds 4, 13, 14 and 19 were tentatively assigned as caffeoyl-2,7-anhydro-3-deoxy-2-octulopyranosonic acids (CDOA) derivatives (Figure 8) owing to the previous identification of compounds with similar UV and mass spectra characteristics in other members of the Asteraceae familiy, such as *Erigeron breviscapus* (vant.) Hand. Mazz. (Zhang, Shi, Qu, & Cheng, 2007; Zhang, Zhao, Ma, Wu, & Zeng, 2010; Liao et al., 2010) and *Chamaemelum*

nobile (L.) (Zhao et al., 2014). To the best of our knowledge, such type of compounds has not been previously reported in *M. recutita* flowers.

Compound 4 showed a pseudomolecular ion $[M-H]^-$ *m/z* at 381 coherent with a mono-CDOA. Three different derivatives are possible, i.e., 3-CDOA, 4-CDOA and 9-CDOA (**Figure 8**), from which a tentative identity of 3-CDOA has been assigned taking into account its elution before the caffeic as observed by Zhang et al. (2007) and Liao et al. (2010). Compounds 13 and 14 possessed the same pseudomolecular ion ($[M-H]^-$ *m/z* at 543) and fragmentation patterns that allowed their identification as diCDOA. Two compounds with similar characteristics were also detected in *E. breviscapus* samples (Zhang et al., 2007, 2010; Liao et al., 2010), which were respectively identified as erigoster B (3,9-diCDOA) and 4,9-diCDOA (or 3,4 diCDOA), so that those identities were tentatively assumed for the compounds observed in our case. The pseudomolecular ion of compound 19 ($[M-H]^-$ *m/z* at 705) could be associated to 3,4,9-triCDOA (Zhang et al., 2007, 2010) or to a diCDOA glucoside, as reported by Liao et al. (2010). In our case, an identity as 3,4,9-triCDOA was assumed taking into account that for the diCDOA glucoside an elution earlier to the diCDOAs should be expected due to its greater polarity.

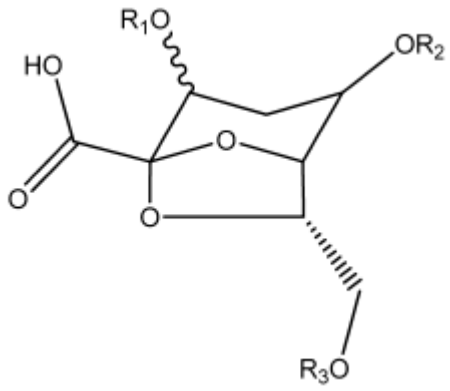


Figure 8. Structure of 2,7-anhydro-3-deoxy-2-octulopyranosonic acids; R_1-R_3 = possible locations for the caffeoyl moieties.

Table 9. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds in chamomile decoction. The results are presented as mean±standard deviation (n = 9).

Compound	Rt (min)	λ_{max} (nm)	Pseudomolecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (mg/g)
1	5.6	320	341	179(100)	Caffeic acid hexoside	0.25 ± 0.04
2	6.1	256/296	153	109(100)	Protocatechuic acid	0.42 ± 0.04
3	7.8	328	353	191(100),179(15),161(18),135(10)	5-*O*-caffeoylquinic acid	5.87 ± 0.03
4	8.5	328	381	293(5),251(8),219(18),203(11),179(70),161 (100),135(44)	3-CDOA	1.62 ± 0.06
5	11.1	324	179	135(100)	Caffeic acid	1.45 ± 0.03
6	16.1	358	479	317(100)	Myricetin-3-*O*-glucoside	4.57 ± 0.13
7	17.8	358	639	315(10),300(45)	Isorhmanetin-*O*-dihexoside	0.74 ± 0.09
8	19.2	358	463	301(100)	Quercetin-3-*O*-glucoside	0.70 ± 0.06
9	19.8	348	447	285(100)	Luteolin-7-*O*-glucoside	1.89 ± 0.18
10	20.2	348	461	285(100)	Luteolin-*O*-glucuronide	4.80 ± 0.54
11	21.9	328	515	353(100),191(73),179(64),161(4),135(23)	*cis* 3,5-*O*-Dicaffeoylquinic acid	1.71 ± 0.02
12	22.1	328	515	353(100),191(82),179(69),161(6),135(25)	*trans* 3,5-*O*-Dicaffeoylquinic acid	1.30 ± 0.06
13	22.6	328	543	381(62),363(21),319(10),251(6),221(24),203(16),179(83),161(82),135(42)	Erigoster B (3,9-diCDOA)	1.21 ± 0.02
14	23.0	328	543	381(65),363(18),319(7),251(3),221(18),203(7),179(59),161(50),135(25)	4,9-diCDOA (or 3,4 diCDOA)	6.83 ± 0.05
15	24.6	336	431	269(100)	Apigenin-7-*O*-glucoside	3.24 ± 0.11
16	27.2	356	519	315(100),300(30)	Isorhmanetin-*O*-acetylhexoside	0.14 ± 0.04
17	29.5	334	473	269(100)	Apigenin-7-*O*-acetylglucoside	1.08 ± 0.13
18	30.1	338	269	225(5),151(3),149(3),117(10)	Apigenin	0.73 ± 0.02
19	32.8	328	705	543(32),381(11),319(4),259(4),221(5),179(23),161(7),135(7)	3,4,9-triCDOA	3.00 ± 0.36
					Total phenolic acids	23.66 ± 0.27
					Total flavonoids	17.89 ± 0.91
					Total phenolic compounds	41.54 ± 1.18

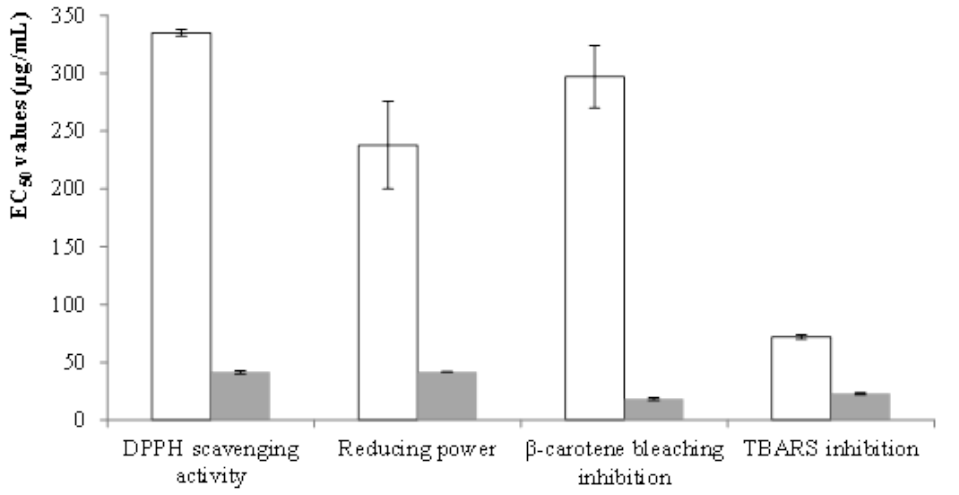
The four remaining compounds (7, 10, 16 and 17) corresponded to flavonoids. UV and mass spectra of compound 17 ($[M-H]^-$ at *m/z* 473) allowed its identification as apigenin 7-*O*-acetylglucoside, a compound consistently reported in *M. chamomilla* flowers (Kunde & Isaac, 1979; Pietta, Manera, & Ceva, 1987; Svehlikova et al., 2004; Weber, Herrmann, Hartmann, Joppe, Schmidt & Heinz-Jürgen, 2008; Lin & Harnly, 2012; Raal et al., 2012; Avula et al., 2014; Xie et al., 2014). Peak 10 presented a pseudo molecular ion $[M-H]^-$ at *m/z* 461, releasing a fragment at *m/z* 285 ($[M-176]^-$ (loss of a glucuronyl moiety). These characteristics and also its UV spectrum (λ_{max} at 348 nm) suggest that it could correspond to a luteolin *O*-glucuronide. As far as we know, that compound has not been cited in *M. recutita*, although luteolin glycosides are usually found in relevant amounts in chamomile flowers and infusions (Mulinacci et al., 2000; Atoui, Mansouri, Boskou & Kefalas, 2005; Nováková et al., 2010; Raal et al., 2012; Haghi, Hatami, Safaei & Mehran, 2014; Guimaraes et al., 2013; Avula et al., 2014). Compounds 7 and 16 presented pseudomolecular ions $[M-H]^-$ at *m/z* 639 and 519 releasing two common MS^2 fragments at *m/z* 315 ($[M-H-162-162]^-$ and $[M-H-42-162]^-$, loss of dihexosyl and acetylhexoside moieties, respectively) and 300 *m/z* ($[M-H-15]^-$, loss of a methyl moiety), pointing to they are methyl-quercetin glycosides. The presence of isorhamnetin (i.e., 3'-*O*-methylquercetin) derivatives in *M. recutita* flowers has been indicated by some authors (Kunde & Isaac, 1979; Nováková et al., 2010; Haghi et al., 2014), so that a tentative identity as isorhamnetin-*O*-dihexoside and isorhamnetin-*O*-acetylhexoside has been assumed for peaks 7 and 16, respectively. In our knowledge, such compounds have not been reported in *M. recutita*.

The decoctions of *M. recutita* presented a higher content in phenolic acids than flavonoids, being a diCDOA (tentatively identified as 4,9-diCDOA or 3,4 diCDOA) the most abundant compound present and luteolin-*O*-glucuronide the most abundant flavonoid.

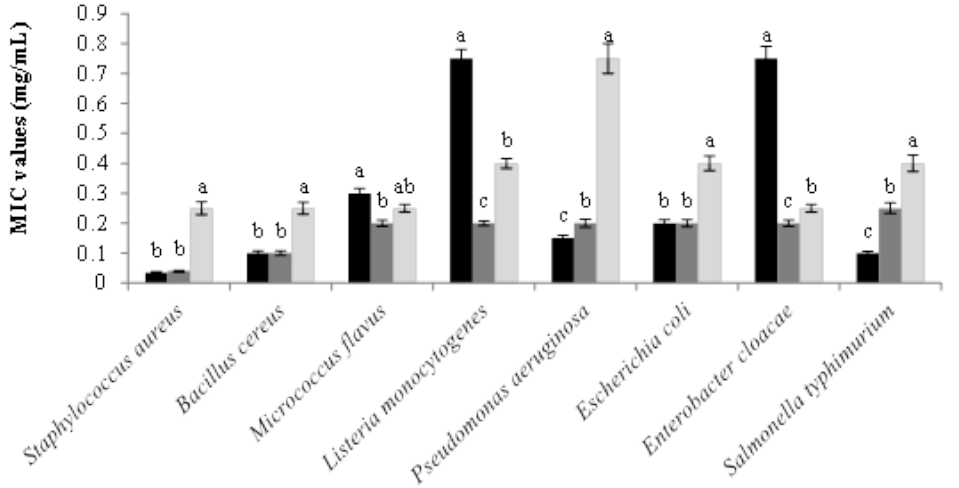
Matić et al. (2013) presented results for infusions and decoctions of *M. recutita*, in terms of area at 356 nm, therefore, it cannot be compared to our study, but nevertheless, two ferulic acid glucosides were the compounds with the highest area. Lin and Harnly (2012) and Mulinacci et al. (2000) did not present quantification data. The phenolic acid, 5-*O*-caffeolylquinic acid was the most abundant compound in *M. recutita* flowers studied by Zielinski et al. (2014) and Nováková et al. (2010). The latest authors also presented apigenin-7-*O*-glucoside as the most abundant flavonoid. Raal et al. (2012) presented quantification results for infusions of *Chamomilla recutita* of different commercial samples, packaged in different countries; these results were presented in mg/200 mL. Of the thirteen compounds identified, a ferulic acid glucoside was the major compound found. Avula et al. (2014) studied extracts of *Matricaria chamomilla* L. (Syn. *M. recutita* L.), identifying nine phenolic compounds and being *cis* GMCA [(Z)-2-β-D-glucopyranosyloxy-4-methoxycinnamic acid] the major molecule found.

Guimarães at al. (2013), also described and quantified the phenolic composition of the herbal flowers, infusion and decoction preparation of wild *M. recutita*, reporting a luteolin *O*-acylhexoside as the main phenolic compound found.

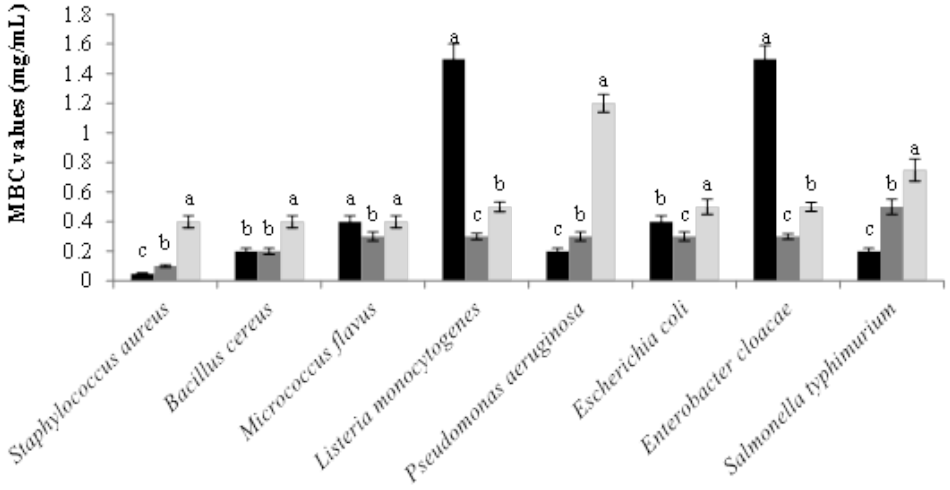
There are available in the literature several reports on the antioxidant activity of chamomile essential oils (e.g., Gawde, Cantrell, Zheljazkov, Astatkie & Schlegeld, 2014; Formisano, Delfine, Oliviero, Tenore, Rigano & Senatore, 2015), however, studies with other kind of extracts are not so frequent. Among the antioxidant activity assays chamomile extract showed to be more active against TBARS, which EC_{50} values were close to those obtained with the standard trolox (**Figure 9A**). DPPH scavenging activity and reducing power of the decoction was similar to the ones previously described for methanolic extracts (Guimarães at al., 2013), while the lipid peroxidation inhibition, measured by β-carotene bleaching and TBARS inhibition assays, was higher in the present study. Roby et al. (2013) reported a higher DPPH scavenging activity of chamomile methanolic and ethanolic extracts in comparison with diethyl ether and hexane ones.



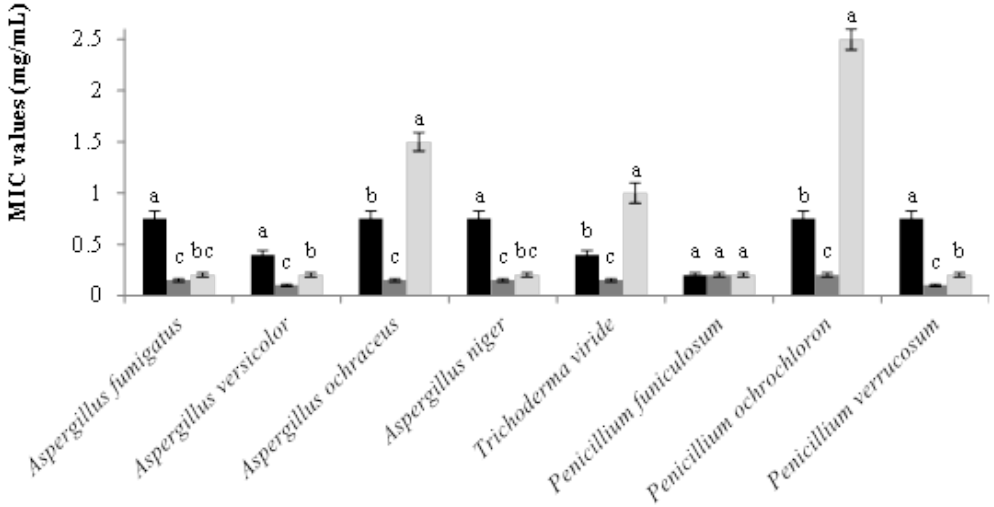
A



B


MBC values (mg/mL)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Staphylococcus aureus
Bacillus cereus
Micrococcus flavus
Listeria monocytogenes
Pseudomonas aeruginosa
Escherichia coli
Enterobacter cloacae
Salmonella typhimurium

C


MIC values (mg/mL)
2.5
2
1.5
1
0.5
0
Aspergillus fumigatus
Aspergillus versicolor
Aspergillus ochraceus
Aspergillus niger
Trichoderma viride
Penicillium funiculosum
Penicillium ochrochloron
Penicillium verrucosum

D

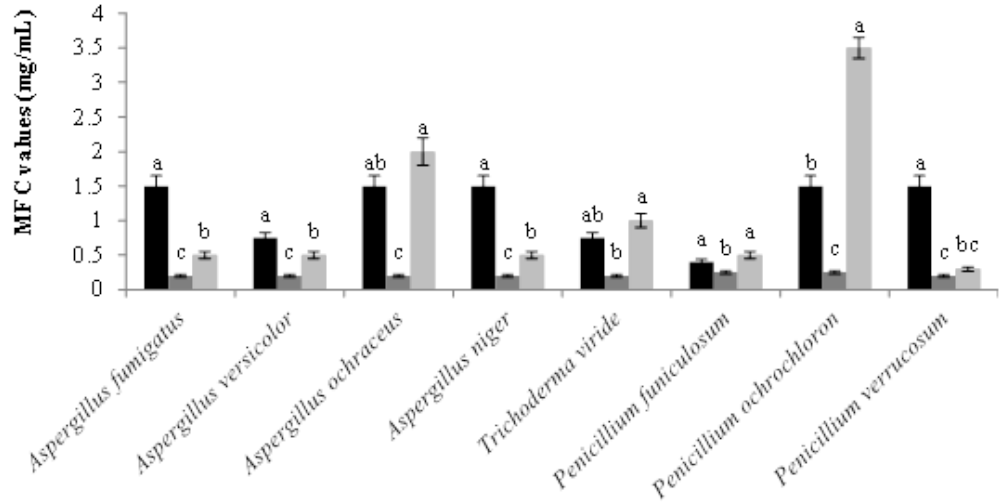

MFC values (mg/mL)
4
3.5
3
2.5
2
1.5
1
0.5
0
Aspergillus fumigatus
Aspergillus versicolor
Aspergillus ochraceus
Aspergillus niger
Trichoderma viride
Penicillium funiculosum
Penicillium ochrochloron
Penicillium verrucosum

Figure 9. (A) Antioxidant activity of chamomile phenolic compounds extract obtained by decoction (□) and of the standard trolox (■). The antioxidant activity was expressed as EC_{50} values (µg/mL) (Mean ± SD, n = 9), what means that higher values correspond to lower reducing power or antioxidant potential. EC_{50}: Extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. (B) Minimum inhibitory (MIC) and (C) bactericidal (MBC) concentrations for antibacterial activity of chamomile phenolic compounds extract obtained by decoction (■), and of the standards streptomycin (■) and ampicillin (■). (D) MIC and minimum fungicidal concentrations (E) (MFC) for antifungal activity of chamomile phenolic compounds extract obtained by decoction (■) and of the standards bifonazole (■) and ketoconazole (■). The antimicrobial activity results were expressed in mg/mL as Mean ± SD (n = 9).

The antibacterial and antifungal activities of the chamomile extract were tested against a panel of eight bacteria and fungi strains, respectively, selected on the basis of their relevance to public health. The most susceptible bacteria were *Staphylococcus aureus*, *Bacillus cereus* and *Salmonella typhimurium*, while *Penicillium funiculosum, Aspergillus versicolor* (**Figure 9B** and **C**) and *Trichoderma viride* were the most vulnerable fungi to the tested extract (**Figure 9D** and **E**). It should be highlighted that the chamomile extract showed higher activity (lower MIC values) than the standard ampicillin (**Figure 9B**) for five of the tested bacteria (*S. aureus*, *B. cereus, Pseudomonas aeruginosa, Escherichia coli* and *S. typhimurium*), and than ketoconazole (**Figure 9D**) for three of the fungi (*Aspergillus ochraceus, T. viride* and *Penicillium ochrochloron*). Roby et al. (2013) had previously reported *B. cereus* and *A. flavus* as the most sensitive microorganisms to chamomile extracts. Nevertheless, according to Petronilho, Maraschin, Coimbra and Rocha (2012), aqueous extracts were effective against molds and yeasts, while ethanolic extracts showed the highest activity against bacteria. Cvetanović, Svarc-Gajić, Masković, Savić and Nikolić (2014) concluded that chamomile extracts have antimicrobial activity independently on the solvent (water and ethanol 70%) and the extraction technique (Soxhlet, microwave-assisted, ultrasound-assisted and subcritical water extraction) used.

Incorporation of chamomile-based natural ingredients in cottage cheese

Three groups of cottage cheese were prepared: the control (samples without any chamomile-based ingredient), the samples with the characterized chamomile phenolic extract obtained by decoction and also the samples incorporated with chamomile powder (this group was prepared in order to evaluate if the decoction process would be necessary or if it would be enough to incorporate the all plant instead of an extract). All the samples were evaluated for nutritional characteristics, colour and antioxidant activity.

Effects on nutritional parameters

In all the evaluated parameters, it was intended to verify the effects of functionalizing agent, independently of the number of days kept in storage, as well as the specific effect of the storage time, irrespectively of having plain cottage cheese or one the prepared functionalized products. Thereby, results were compared through a 2-way ANOVA, following the generalized linear model coupled to the repeated measures analysis technique. In this analysis, it is important to check for the homogeneity of variances in the measures done for each assayed condition. Since the independence of variables cannot be assumed, the former requirement was evaluated by the Mauchly's sphericity test.

The results for moisture, protein, fat, carbohydrates and energy in the prepared cottage cheeses are presented in **Table 10**. The evaluated factors, functionalizing agent (FA) and storage time (ST) showed a significant interaction (FA×ST) in all cases, which hinders the possibility of identifying unequivocal effects of each individual factor. Nevertheless, some specific trends were observed: protein, ash, fat and carbohydrates contents reached maximal values after 14 days of storage (and consequently the highest energy values), while moisture and lactose had exactly the opposite behavior. The same tendency was described by Farahani, Ezzatpanah and Abbasi (2014) for protein and fat contents during ripening of Siahmazgi cheese (typical Iranian cheese). Nevertheless, other authors have reported a decrease in protein values along shelf life of ripe cheese (Pappa, Kandarakis, Anifantakis, Zerfiridis, Anifantakis and Sotirakoglou, 2006), and "coalho" cheese (Queiroga et al., 2013). The decrease in lactose is in agreement with the results obtained by Diezhandino, Fernández, González, McSweeney and Fresno (2015) during the ripening of Valdeón cheese (Spanish blue cheese).

Regarding the effect of FA, control samples tended to present higher contents in ash, fat and also the highest energy values. On the other hand, samples functionalized with chamomile decoctions presented the maximal values in protein and carbohydrates, while those prepared with chamomile powder gave higher moisture contents.

The significant interaction among factor was also observed for all studied fatty acids (**Table 10**), which were also significantly changed by each individual factor ($p < 0.05$). In all samples, the predominant fatty acids, independently of being added with chamomile-based ingredients, were palmitic (C16:0) and oleic (C18:1acids (**Table 10**), which is also supported by studies with other kinds of cheese such as Portuguese "coalho" cheese (Queiroga et al., 2013) and French goat milk cheeses (AFSSA, 2010). The order of abundance in fatty acids was saturated fatty acids (SFA), followed by monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA).

After a careful observation of the obtained data, and despite the detected significant differences, the effects caused by either FA or ST were very low in magnitude, indicating that the fatty acids profile of cottage cheese is not severely affected by both factors.

Table 10. Nutritional value (g/100 g), energy (kcal/100 g) and fatty acids composition (relative percentage) of the cottage cheese samples along shelf life. Results are presented as estimated marginal mean±standard error.

Storage days	Functionalizing agent (FA)			Mauchly's test of sphericity (*p*-value)	*p*-value[a]	Storage time (ST)			Mauchly's test of sphericity (*p*-value)	*p*-value[a]	FA×ST interaction	
	Control	Chamomile powder	Chamomile decoction			0 days	7 days	14 days			Mauchly's test (*p*-value)	*p*-value[a]
Nutritional value												
Moisture	62.5±0.2	64.0±0.2	62.3±0.2	0.965 (0.881)	<0.001	64.5±0.1	64.5±0.2	59.9±0.2	0.618 (0.185)	<0.001	0.617 (0.962)	<0.001
Protein	12.1±0.1	12.3±0.1	12.7±0.1	0.873 (0.621)	<0.001	11.6±0.1	12.2±0.1	13.3±0.1	0.429 (0.052)	<0.001	0.114 (0.137)	<0.001
Ash	2.26±0.04	2.09±0.05	2.24±0.05	0.498 (0.087)	0.005	2.23±0.05	2.05±0.05	2.32±0.04	0.538 (0.114)	<0.001	0.147 (0.211)	<0.001
Fat	20.4±0.4	19.3±0.2	19.3±0.2	0.656 (0.229)	<0.001	19.0±0.3	18.9±0.2	21.2±0.1	0.650 (0.222)	<0.001	0.016 (0.002)	<0.001
Carbohydrates	2.6±0.2	2.3±0.2	3.7±0.3	0.545 (0.119)	<0.001	2.7±0.2	2.5±0.3	3.3±0.3	0.894 (0.676)	0.002	0.026 (0.027)	<0.001
Lactose	1.91±0.04	1.72±0.04	2.03±0.04	0.270 (0.010)	<0.001	2.05±0.03	2.05±0.04	1.56±0.03	0.884 (0.651)	<0.001	0.362 (0.697)	<0.001
Energy	243±2	232±1	239±1	0.819 (0.497)	<0.001	228±2	228±2	257±2	0.877 (0.631)	<0.001	0.187 (0.309)	<0.001
Fatty acids												
C4:0	7.3±0.1	6.4±0.1	6.4±0.1	0.426 (0.051)	<0.001	7.3±0.1	6.3±0.1	6.5±0.1	0.629 (0.198)	<0.001	0.019 (0.003)	<0.001
C6:0	6.6±0.1	5.3±0.1	5.3±0.1	0.416 (0.047)	<0.001	5.9±0.1	5.5±0.1	5.8±0.1	0.410 (0.044)	0.001	0.006 (<0.001)	<0.001
C8:0	4.7±0.1	4.3±0.1	4.3±0.1	0.642 (0.212)	<0.001	4.4±0.1	4.3±0.1	4.6±0.1	0.432 (0.053)	<0.001	0.015 (0.002)	<0.001
C10:0	10.1±0.1	10.9±0.1	10.8±0.1	0.785 (0.428)	<0.001	10.5±0.1	10.1±0.1	11.1±0.1	0.442 (0.058)	<0.001	0.042 (0.019)	<0.001
C12:0	4.7±0.1	5.4±0.1	5.4±0.1	0.551 (0.124)	<0.001	5.1±0.1	4.9±0.1	5.4±0.1	0.625 (0.193)	<0.001	0.011 (0.001)	0.001
C14:0	9.1±0.1	10.7±0.1	10.6±0.1	0.486 (0.080)	<0.001	10.1±0.1	9.8±0.1	10.4±0.1	0.729 (0.331)	<0.001	0.026 (0.007)	<0.001
C15:0	1.08±0.02	1.27±0.02	1.25±0.01	0.239 (0.007)	<0.001	1.18±0.04	1.19±0.01	1.21±0.01	0.544 (0.118)	<0.001	0.003 (<0.001)	<0.001
C16:0	20.6±0.3	21.9±0.5	21.9±0.3	0.960 (0.868)	<0.001	21.5±0.1	21.6±0.2	21.2±0.2	0.894 (0.677)	<0.001	0.013 (0.001)	<0.001
C18:0	10.2±0.2	9.3±0.1	9.1±0.1	0.789 (0.437)	<0.001	9.6±0.1	9.9±0.1	9.1±0.2	0.810 (0.479)	<0.001	0.002 (<0.001)	0.001
C18:1	18.7±0.2	18.1±0.1	18.5±0.1	0.306 (0.016)	<0.001	18.0±0.1	19.3±0.2	18.1±0.1	0.589 (0.157)	<0.001	0.006 (<0.001)	<0.001

C18:2	2.8±0.1	2.5±0.1	2.5±0.1	0.698 (0.284)	<0.001	2.6±0.1	2.7±0.1	2.5±0.1	0.698 (0.284)	<0.001	0.061 (0.041)	0.035
C18:3	1.58±0.01	1.53±0.01	1.51±0.01	0.178 (0.002)	<0.001	1.54±0.01	1.59±0.01	1.48±0.02	0.452 (0.062)	<0.001	0.009 (0.001)	0.003
SFA	75.8±0.4	76.8±0.5	76.1±0.3	0.828 (0.516)	<0.001	76.9±0.4	75.0±0.2	76.9±0.5	0.536 (0.113)	<0.001	0.004 (<0.001)	<0.001
MUFA	19.3±0.1	19.0±0.1	19.5±0.1	0.347 (0.025)	0.001	18.8±0.2	20.2±0.1	18.8±0.3	0.267 (0.010)	<0.001	0.016 (0.002)	<0.001
PUFA	4.8±0.1	4.5±0.1	4.4±0.1	0.284 (0.012)	<0.001	4.5±0.1	4.7±0.1	4.4±0.1	0.524 (0.104)	<0.001	0.021 (0.004)	0.001

[a]Significance value for the tests of between subjects effects. When sphericity assumption was not met ($p<0.05$), the p-value was obtained from the Greenhouse-Geisser correction.

Butiric acid (C4:0); Caproic acid (C6:0); Caprylic acid (C8:0); Capric acid (C10:0); Lauric acid (C12:0); Myristic acid (C14:0); Pentadecanoic acid (C15:0); Palmitic acid (C16:0); Stearic acid (C18:0); Oleic acid (C18:1); Linoleic acid (C18:2); α-Linolenic acid (C18:3); SFA- Saturated fatty acids; MUFA- Monounsaturated fatty acids; PUFA- Polyunsaturated fatty acids. The difference to 100% corresponds to other 17 less abundant fatty acids (data not shown). The results of control cottage cheese samples were previously published by the authors (Caleja et al., 2015).

Effects on colour and antioxidant parameters

In the case of colour parameters, the interaction among factors was not significant for *L** and *b**, allowing to identify the exact way how each of the parameters is affected by the assayed conditions. Lightness (*L**) diminished along ST, being also lower in those samples prepared with chamomile powder. Similar *L** values were described in studies with ricotta cheeses (Pizzillo, Claps, Cifuni, Fedele & Rubino, 2005) and "coalho" cheeses (Queiroga et al., 2013). For *a** parameter, which is a direct indicator of redness, no statistical significant difference was found along ST, while samples prepared with chamomile powder presented slightly higher values. A study with ricotta cheeses presented also redness values close to zero (*a**=1.19-1.32) (Pizzillo et al., 2005). Other authors who studied the changes in colour characteristics during ripening of cheeses demonstrated that the *a** value did not show a defined trend (Buffa et al., 2001). Regarding the blueness (*b**) parameter, the highest values were obtained in samples functionalized with chamomile powder and those stored duting 14 days. On the other hand, the lowest values, i.e., the samples with less yellowness degree, were detected in plain cottage cheese and in non-stored samples. Ginzinger, Jaros, Lavanchy and Rohm (1999) confirmed that yellowness index increased with cheese aging. Previous research related with cheese colour as a function of ripening time reported a decrease in *L** value and an increase in *b** value (Rohm and Jaros,1996; Buffa, Trujillo, Pavia & Guamis, 2001).

The appearance of the different cottage cheese samples after 14 days of storage is shown in **Figure 10**, where it can be observed that only the control sample showed signs of degradation.

Figure 10. Cottage cheese after 14 days of storage: control sample with signs of degradation (A); sample with chamomile decoction (B) and sample with chamomile powder (C).

The incorporation of chamomile-based ingredients improved the antioxidant activity (DPPH scavenging activity and reducing power) of plain cottage cheese samples (**Table 11**), without great differences among chamomile powder and decoction. Along the shelf life, DPPH scavenging activity showed a slight decrease, while reducing power showed a high increase, which might be related with the decrease in lactose (**Table 10**) and the consequent release of their reducing monomers. Another study conducted in our laboratory (Caleja et al., 2015) showed that the incorporation of a fennel (*Foenniculum vulgare* Mill.) phenolic-enriched extract, obtained by decoction, improved the antioxidant properties of cottage cheese, but the EC_{50} values reported demonstrate that the improvement antioxidant capacity of fennel was lower than the one showed by chamomile in the present work.

Conclusions

The characterization of *M. recutita* (chamomile) extracts obtained by decoction revealed the presence of phenolic compounds, which can be related with their antioxidant and antimicrobial activities. The incorporation of those extracts (chamomile based ingredients) to goat cottage cheese improved its antioxidant properties without significantly modifying the nutritional characteristics or fatty acids profiles. Moreover, only the control samples not added with the chamomile extracts presented signs of degradation after 14 days of storage. In view of the growing interest of consumers for functional foods without chemical additives and taking into account that the studied dairy product is highly appreciated, it would be interesting to go further in the development of this novel formulation in order to preserve the antioxidant activity along the shelf life of cottage cheese by using stabilization/microencapsulation techniques.

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Table 11. Colour parameters, free radicals scavenging activity and reducing power of the cottage cheese samples along shelf life. Results are presented as estimated marginal mean±standard error.

		Colour parameters			**Antioxidant activity** (EC_{50} values, mg/mL)	
		*L**	*a**	*b**	DPPH scavenging activity	Reducing power
Functionalizing agent	Control	90±1	-2.5±0.1	12.2±0.2	200[b]	85±1
	Chamomile powder	80±1	-1.8±0.1	18.6±0.4	41±1	5.9±0.1
	Chamomile decoction	88.6±0.3	-2.3±0.1	16.3±0.3	44±1	6.9±0.2
Mauchly's test of sphericity (*p*-value)		0.443 (0.017)	0.490 (0.028)	0.685 (0.151)	0.271 (0.010)	0.140 (0.001)
p-value[a]		<0.001	<0.001	<0.001	<0.001	<0.001
Storage time (ST)	0 days	88±1	-2.2±0.1	13.8±0.3	90±1	72±1
	7 days	87±1	-2.1±0.1	14.8±0.2	96±1	17.6±0.1
	14 days	83±1	-2.2±0.1	18.6±0.3	99±1	8.5±0.1
Mauchly's test of sphericity (*p*-value)		0.715 (0.186)	0.682 (0.148)	0.854 (0.455)	0.350 (0.025)	0.101 (<0.001)
p-value[a]		<0.001	0.439	<0.001	<0.001	<0.001
FA×ST interaction						
Mauchly's test of sphericity (*p*-value)		0.050 (0.001)	0.097 (0.010)	0.105 (0.013)	<0.001 (<0.001)	0.001 (<0.001)
p-value[a]		0.318	0.023	0.130	0.001	<0.001

[a]Significance value for the tests of between subjects effects. When sphericity assumption was not met ($p<0.05$), the *p*-value was obtained from the Greenhouse-Geisser correction. [b]The presented value corresponds to the maximum assayed concentration, since it was not possible to reach 50% of activity up to that concentration. The results of control cottage cheese samples were previously published by the authors (Caleja et al., 2015).

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3.1.3. Requeijões funcionalizados com extratos de funcho e camomila: desempenho comparativo entre formas livres e microencapsuladas

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Cottage cheeses functionalized with fennel and chamomile extracts: Comparative performance between free and microencapsulated forms

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Abstract

Globally, there is a trend for healthy food products, preferably incorporating natural bioactive ingredients, replacing synthetic additives. From previous screening studies, extracts of *Foeniculum vulgare* Mill. (fennel) and *Matricaria recutita* L. (chamomile) maintained the nutritional properties and improve the antioxidant activity of cottage cheese. Nevertheless, this effect was limited to 7 days. Accordingly, aqueous extracts of these plants were microencapsulated in alginate and incorporated in cottage cheese to achieve an extended bioactivity. Plain cottage cheese and cheese functionalized with direct addition of free decoctions were also prepared and compared. Independently of plant species, "functionalization type" factor did not show a significant effect on the nutritional parameters, as also confirmed in the linear discriminant analysis, where these parameters were not selected

as discriminating variables. Furthermore, samples functionalized with microencapsulated extracts showed higher antioxidant activity after the 7th day, thereby demonstrating that the main purpose of this experimental work was achieved.

Keywords: *Foeniculum vulgare*; *Matricaria recutita*; cottage cheese; functional foods; microencapsulation.

Introduction

Plant-derived bioactive extracts and compounds are interesting ingredients to functionalize foods (Carocho, Barreiro, Morales & Ferreira, 2014). Aqueous extracts of *Foeniculum vulgare* Mill. (fennel) and *Matricaria recutita* L. (chamomile) are good sources of phenolic compounds, exhibiting different biological activities such as antioxidant and antimicrobial properties, as previously reported by our research group (Caleja et al., 2015a; Caleja et al., 2015b). In these previous works, the preserving potential of fennel and chamomile extracts obtained from decoction was explored through their direct use as natural preservers. Their incorporation in cottage cheese maintained the nutritional characteristics and improved the antioxidant properties, namely the free radical's scavenging activity. However, after 7 days under storage the cheese samples shown an antioxidant capacity decrease which was associated with extracts degradation (Caleja et al., 2015a; Caleja et al., 2015b).

In fact, the use of natural bioactive extracts/compounds as food additives presents limitations because after extraction they can become susceptible to degradation. Therefore, microencapsulation may be considered as an appropriate process to overcome these limitations once this technique can provide protection against the action of several environmental agents like oxygen, light, moisture or heat, ensuring an increase of their stability (Betz & Kulozik, 2011; Dias, Ferreira, & Barreiro, 2015a). This process will preserve the bioactive compound by means of a surrounding coating shell around it (reservoir type particles) or by embedding it, homogeneously or heterogeneously, in a matrix (matrix type particles) (Çam, Içyer, & Erdogan, 2014). The controlled release along time or oriented to a specific site, can be achieved by means of different mechanisms, which depend on the used encapsulation materials, production process, and microcapsules' morphology and desired application (Martins et al., 2014). Alginate, a natural polymer obtained from bacteria and algae, is widely used for microencapsulation in several fields, namely in food industry (Goh, Heng & Chan, 2012). This polymer is classified as non-toxic for oral administration being usually commercialized in its salt form (e.g. sodium alginate). In the presence of bivalent cations (e.g. Ca^{2+}) it gels giving rise to a material that resists acidic pH and dissolves at basic medium

(disruption of the ionic network). In this way the release of the encapsulated compounds will occur in the intestinal tract (George & Abraham, 2006). Besides, it presents good stability, biocompatibility, exudate-retaining ability and moderate antimicrobial activity (Goh, Heng & Chan, 2012). Its use in the food industry is allowed by the FDA – Food and Drug Administration (USA) and EFSA – European Food Safety Agency.

There are some documented examples dealing with the application of microencapsulation to natural extracts for use in functional foods (Dias, Ferreira, & Barreiro, 2015a). Our research group had successfully encapsulated *Fragaria vesca* L. (Dias et al., 2015b) and *Rubus ulmifolius* Schott (Martins et al., 2014) extracts that were further incorporated in κ-carrageenan gelatin and yogurts, respectively.

In the present study, aqueous extracts of *F. vulgare* and *M. recutita* were prepared by decoction. Then, these extracts were used to functionalize cottage cheeses following two main strategies: i) direct use (extracts in its free form), and ii) use after stabilization through microencapsulation with alginate (extracts in its microencapsulated form). Microencapsulation was achieved by using an atomization/coagulation technique following a procedure developed in our research group (Dias et al., 2015b; Martins et al., 2014). The incorporation of *F. vulgare* and *M. recutita* extracts in cottage cheeses was compared with samples without free or encapsulated extracts (control). Moreover the gain derived from the use of the microencapsulated form over the use of the free form was also evaluated (specifically colour, nutritional value and antioxidant activity of the functionalized cottage cheese as a function of storage time). Besides studying individual changes induced by each of the defined factors (storage time and functionalization type) through a 2-way ANOVA, data were also analyzed by a linear discriminant analysis to determine which of the assayed independent variables (studied parameters) defined mostly the differences in the average score profiles of the prepared cheese samples.

Materials and methods

Standards and reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic and acetic acids were purchased from Prolabo (VWR International, France). Sodium alginate was provided by Fluka Chemie (USA). All other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

Preparation of the bioactive extracts

Commercial samples of *Foeniculum vulgare* Mill. (fennel) and *Matricaria recutita* L. (chamomile) were provided by Américo Duarte Paixão Lda. (Alcanede, Portugal). The dried samples were powdered (~20 mesh) and submitted to decoction. Decoctions were performed by adding 5 g of plant material to 200 mL of distilled water, heated (heating plate, VELP scientific, Usmate, Italy), and allowed to boil during 5 min. The mixtures were left to stand for 5 min and filtered through Whatman No. 4 paper. The decoctions were then frozen and lyophilized in order to obtain the final extracts (FreeZone 4.5, Labconco, Kansas City, MO, USA).

Microencapsulation of the plant extracts and characterization

Microencapsulation

Microspheres containing extracts of *F. vulgare* or *M. recutita* were prepared by using an atomization/coagulation technique as previously described by the authors (Dias et al., 2015b; Martins et al., 2014).

Calcium alginate (matrix material) was obtained by combining sodium alginate with calcium chloride ($CaCl_2$) (coagulation agent). Briefly, the atomization solution was prepared by dissolving firstly, 100 mg of the extract with 20 mL of distilled water under stirring at 250 rpm and room temperature, followed by filtration to remove eventual non-soluble trace residues. In a second step, 800 mg of sodium alginate were added and the solution was kept under stirring in the same conditions until complete dissolution was achieved. The obtained alginate solution containing the extract was then atomized using a NISCO Var J30 system (Zurich, Switzerland) at a feed rate of 0.2 mL/min and a nitrogen pressure of 0.1 bar to produce the microspheres. The atomized microspheres undergo coagulation upon contacting with a $CaCl_2$ aqueous solution (500 mL at a concentration of 4% (w/v)) over a period of 4 hours. The resulting microspheres were collected by filtration under reduced pressure, washed twice with distilled water, and further lyophilized and stored under dark conditions at 4 °C.

Microcapsules characterization

Microspheres were analyzed by optical microscopy (OM) using a Nikon Eclipse 50i microscope (Tokyo, Japan) equipped with a Nikon Digital Sight camera and NIS Elements software for data acquisition.OM analysis was applied to assess the size and morphology of the microspheres after the atomization and coagulation stages. It was also possible to infer the presence/absence of extract inside the microspheres.

The effective extract incorporation into the alginate matrix was investigated by FTIR analysis. For that purpose, spectra of pure alginate, free extracts of *F. vulgare* or *M. recutita*,

and the corresponding microspheres were collected on a FTIR Bomen (model MB 104) by preparing KBr pellets at a sample concentration of 1% (w/w). The spectra were recorded at a resolution of 4 cm^{-1} in the spectral range between 650 and 4000 cm^{-1} and by co-adding 48 scans. The encapsulation efficiency (EE) was also evaluated through the quantification of the non-encapsulated extract. For that purpose, the remaining extract in the coagulation and in the first washing solution were quantified by HPLC and added. The second washing solution presented no extract. The encapsulation efficiency was calculated according to the following expression:

$$EE = [(M_{e\text{-}t} - M_{e\text{-}ne})\ (M_{e\text{-}t})] \times 100$$

in which $M_{e\text{-}t}$ represents the theoretical amount of extract (the amount of extract used in the microencapsulation process), $M_{e\text{-}ne}$ corresponds to the non-encapsulated extract remaining after the encapsulation process.

Since the extracts are complex mixtures, the major phenolic compounds present in the extracts of fennel (quercetin-3-*O*-glucoside; Caleja et al., 2015a) and chamomile (luteolin-*O*-glucuronide; Caleja et al., 2015b) were selected for EE evaluation.

Functionalization of cottage cheese with the plant extracts

Preparation of the cottage cheese samples

All the cottage cheese samples were prepared by “Queijos Casa Matias Lda.” (Seia, Portugal), by using the milk serum obtained after the production of cheese. The remaining serum (liquid component) was pumped into a vat where it was mixed and heated to a temperature that can range between 83-85°C. After a few minutes at that temperature, the serum started to flocculate and rose to the surface where it was scooped into individual forms, left for a few minutes and packed with parchment paper. The incorporation of the extracts was carried out immediately before packaging, individually into each one of the forms mentioned above, in order to guarantee a better distribution of the extract by the cottage cheese mass.

Five groups, each one with nine ewe’s cottage cheeses (250 g), were prepared: (i) cottage cheeses without plant extracts; (ii) cottage cheeses with free fennel extract; (iii) cottage cheeses with free chamomile extract; (iv) cottage cheeses with microencapsulated fennel extract and (v) cottage cheeses with chamomile microencapsulated extract. For the samples prepared with free extracts, 100 mg of extract per each cottage cheese (250 g) were used. In the case of microencapsulated extract, 900 mg of microspheres (quantity containing 100 mg of free extract) per each cottage cheese were used. The samples were analyzed in what concerns colour, nutritional composition (protein, fat, carbohydrates, ash and energy), and free radical’s scavenging activity, immediately after preparation and after seven and fourteen days

of storage in the original package (parchment paper) at 4 ºC in the refrigerator. Assays were done in triplicate.

Effects of storage time on colour, nutritional and antioxidant activity of cottage cheese samples

The colour of the sampled cottage cheeses was evaluated in the top and bottom of the cheese, by using readings in three different points for each determination. by using a colourimeter (model CR-400, Konica Minolta Sensing Inc., Tokyo, Japan). Using the illuminant C and diaphragm aperture of 8 mm, the CIE *L*a*b** colour space values were registered using a data software "Spectra Magic Nx" (version CM-S100W 2.03.0006) (Fernandes, Antonio, Barreira, Oliveira, Martins & Ferreira, 2012).

The proximate composition (protein, fat and ash) of the samples was analyzed according to the AOAC (2005) procedures.

The crude protein content (N × 6.38) was estimated by the Kjeldahl method (AOAC, 991.02); the crude fat (AOAC, 989.05) was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content (AOAC, 935.42) was determined by incineration at 600±15 ºC. Total carbohydrates were calculated by difference. Total energy was calculated according to the following equation: Energy (kcal) = 4 × (g proteins +g carbohydrates) + 9 × (g lipids). Free sugars were determined in defatted samples by HPLC coupled to a refraction index (RI) detector, according to previous analytical validation and detailed description by the authors (Barros et al., 2013). Fatty acids were identified by comparison with standards and the results were expressed in relative percentage of each fatty acid. Free sugars were identified by comparison with standards, and further quantified (g/100 g of cottage cheese) by using an internal standard (melezitose).

For the antioxidant activity evaluation, the samples were submitted to DPPH radical's scavenging activity, which was performed in a ELX800 microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) at 515 nm. The complete protocol was previously described by the authors (Caleja et al., 2015a).

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 standard deviation. An analysis of variance (ANOVA) with type III sums of squares was performed using the general linear model (GLM) procedure. The dependent variables were analyzed using 2-way ANOVA with the factors "storage time" (ST)

and “functionalization type” (FT). When a statistically significant interaction was detected for these two factors, they were evaluated simultaneously by the estimated marginal means plots for all levels of each factor. Furthermore, if no statistical significant interaction is 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.

In addition, a linear discriminant analysis (LDA) was used to compare the effect of ST and FT over all the assayed parameters. 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. This procedure uses a combination of forward selection and backward elimination steps, where the inclusion of a new variable is preceded by verifying the significance of all previously selected variables (Zielinsky et al., 2014). The basic purpose of the discriminant analysis was estimating the relationship between a single categorical dependent variable (cheese formulation) and a set of quantitative independent variables (the values obtained in all the assays). Through this method, it is possible to determine which of the independent variables contributed more for the differences in the average score profiles of the different cheese samples. To verify the significance of the canonical discriminating functions, Wilk’s Λ test was used. A leaving-one-out cross validation procedure was carried out to assess the model performance.

Results and discussion

Characterization of the microencapsulated plant extracts

The OM analysis showed an efficient incorporation of the extracts, confirming a homogeneous distribution of the extract within the microspheres, recognized as brown droplets well distributed in the alginate matrix. It was also observed that the microspheres had different shapes and sizes; the larger showed round shape while the smaller had a pear-like shape. Their final size at magnification of 100x varied between 68.1 and 306.5 μm. Figure 11 shows the microcapsules morphology at different preparation stages, as well as, after being lyophilized. The EE, determined by quantification of the major compounds identified in the extracts (quercetin-3-*O*-glucoside for microspheres with fennel and 5-*O*-caffeoylquinic acid for microspheres with chamomile), was estimated as approaching 100% for both samples. Only traces of these compounds were detected in the coagulation and washing solutions. The presence of the extract inside the microspheres was also confirmed by FTIR analysis (data not shown).

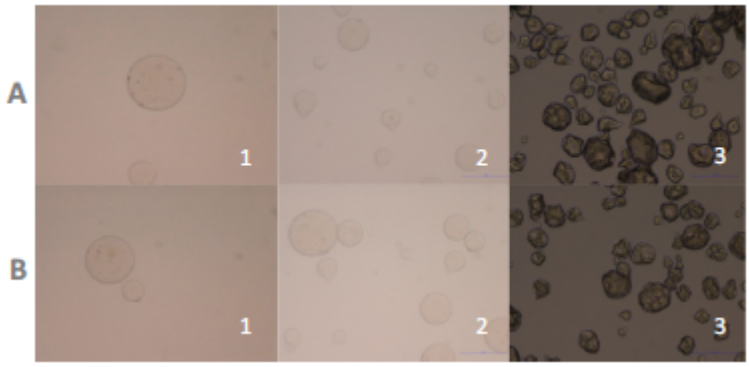

Figure 11. Morphology of fennel (A) and chamomile (B) microspheres by OM analysis under magnifications of 100X of the microspheres immediately after atomization (1) and after 4 hours coagulation period (2) and lyophilized microspheres (3).

Effects of incorporating plant-based natural extracts in cottage cheese

Five groups of cottage cheese were prepared: i) control (samples without plant extracts); ii) samples with free fennel extract; iii) samples with free chamomile extract; vi) samples with microencapsulated fennel extract; and v) samples with microencapsulated chamomile extract.

For all the evaluated parameters, it was aimed to assess the effects of each functionalization type (i.e. used functionalization agent: free or microencapsulated extracts), independently of the number of days kept in storage, as well as the influence of the storage time, regardless of having plain cottage cheese or functionalized samples. Therefore, the standard deviations should not be regarded as a measure of accuracy of the applied methodologies, since they reflect results obtained from samples prepared in different conditions. The interaction among factors (ST×FT) was also evaluated. When a significant interaction was found ($p<0.050$), no multiple comparisons can be performed. In those cases, the influence of each factor was assessed by interpreting the estimated marginal means (EMM) plots. The statistical analysis was performed separately for each one of the selected plant species.

The achieved results for water, fat, protein, ash, carbohydrates, lactose and energy in the prepared cottage cheeses are presented in **Table 12**. The evaluated factors showed a significant interaction (ST×FT) in all cases, indicating that the quantitative variations in these parameters evolved dissimilarly throughout ST, either using fennel or chamomile. Considering each individual factor, it is obvious that ST had a higher effect on these parameters than FT.

In fact, while the variations induced by ST were significant in all cases, FT produced a significant effect only in ash (for fennel) and carbohydrates (for both plant species). Except for water, ash and lactose, which tended to decrease along ST, the assayed parameters tended

to present higher values for longer storage periods (especially after 14 days). A similar behavior has been previously reported in cheeses of Portuguese (Caleja et al., 2015b), Spanish (Diezhandino, Fernández, González, McSweeney & Fresno, 2015) and Iranian (Farahani, Ezzatpanah & Abbasi, 2014) origin.

The interaction among factors had a less marked effect on the colour parameters, since it was only significant, in both plants, for *b** parameter, thereby allowing to perform some multiple comparisons (**Table 13**). Lightness (*L**) diminished along ST, showing also to be tendentiously lower in functionalized samples (particularly those with direct incorporation of the extracts). Despite the observed differences the *L** values were generally in the range of those obtained in similar cheese types (Caleja et al., 2015b; Pizzillo, Claps, Cifuni, Fedele & Rubino, 2005; Queiroga et al., 2013). Regarding the *b** parameter, the highest values were obtained in samples stored during 14 days, , a yellowing tendency independently of FT. The increase in this particular parameter is a common feature in stored cheeses (Buffa, Trujillo, Pavia & Guamis, 2001; Ginzinger, Jaros, Lavanchy & Rohm, 1999; Rohm & Jaros,1996), which seem to be maintained in functionalized formulations, as those prepared herein. For *a** parameter, which might be interpreted as a direct indicator of greenness-redness tendency, it was possible to observe a slight increase along ST, while control and functionalized samples resulted to be statistically indistinguishable. However, the magnitude of the values obtained for this parameter is in the expected range for this type of products (Caleja et al., 2015a,b; Pizzillo et al., 2005).

Table 12. Nutritional parameters, lactose (g/100 g fw) and energy (kcal/100 g) in the cottage cheese samples along shelf life. Results are presented as estimated mean±standard deviation.

		Water	Fat	Protein	Ash	Carbohydrates	Lactose	Energy
Foeniculum vulgare								
ST	0 days	70±1	14±1	12.0±0.3	1.3±0.1	2.5±0.4	1.9±0.1	186±7
	7 days	66±1	17±1	12.4±0.2	1.1±0.1	3.6±0.4	1.1±0.4	215±3
	14 days	54±2	24±1	18.0±0.5	1.0±0.1	3.7±0.5	0.2±0.1	301±9
	p-value (n=27)	<0.001	<0.001	<0.001	<0.001	0.007	<0.001	<0.001
FT	None	63±7	18±4	15±3	1.1±0.1	3.2±0.5	1.3±0.4	238±49
	Microspheres	64±6	18±4	14±2	1.2±0.1	2.9±0.5	1.0±0.5	229±46
	Extract	63±8	18±4	14±3	1.1±0.1	3.6±0.5	0.9±0.5	235±55
	p-value (n=27)	0.443	0.532	0.546	<0.001	<0.001	0.906	0.459
ST×FT	*p*-value (n=81)	<0.001	<0.001	<0.001	0.013	<0.001	<0.001	<0.001
Matricaria recutita								
ST	0 days	69±1	15±1	12±1	1.2±0.1	3±1	1.8±0.1	193±4
	7 days	66±1	17±1	12±1	1.1±0.1	4±1	1.6±0.1	215±6
	14 days	53±2	24±1	18±1	1.1±0.1	4±1	0.2±0.1	304±13
	p-value (n=27)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
FT	None	63±7	19±4	15±3	1.1±0.1	3±1	1.3±0.5	238±49
	Microspheres	64±6	18±4	14±3	1.2±0.1	3±1	1.2±0.5	228±44
	Extract	62±8	19±4	14±3	1.1±0.1	5±1	1.2±0.5	245±54
	p-value (n=27)	0.753	0.863	0.681	0.056	0.004	0.244	0.793
ST×FT	*p*-value (n=81)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Table 13. Colour parameters and free radicals scavenging activity in the cottage cheese samples along shelf life. Results are presented as estimated marginal mean±standard error.

		DPPH scavenging activity	*L**	*b**	*a**
Foeniculum vulgare					
ST	0 days	152±70	91±3 a	10±1	-2.3±0.2 b
	7 days	152±69	90±4 ab	11±1	-2.2±0.2 b
	14 days	144±44	89±2 b	16±2	-0.9±0.3 a
	p-value (n=27)	0.885	0.001	<0.001	<0.001
FT	None	200*	92±2 a	12±3	-2.0±0.4
	Microspheres	178±31	91±3 a	13±3	-1.8±0.5
	Extract	69±20	88±3 b	12±2	-1.7±0.5
	p-value (n=27)	<0.001	0.003	0.054	0.256
ST×FT	*p*-value (n=81)	<0.001	0.052	0.005	0.263
Matricaria recutita					
ST	0 days	145±80	92±2 a	11±1	-2.3±0.2
	7 days	151±70	91±2 a	11±1	-2.3±0.4
	14 days	142±60	89±2 b	17±2	-0.8±0.2
	p-value (n=27)	0.856	0.009	<0.001	<0.001
FT	None	200*	92±2 a	12±3	-2.0±0.5
	Microspheres	188±19	90±2 b	13±3	-1.6±0.5
	Extract	50±12	90±2 b	14±4	-1.8±0.5
	p-value (n=27)	<0.001	<0.001	0.460	0.292
ST×FT	*p*-value (n=81)	<0.001	0.076	<0.001	0.034

*Corresponds to the maximum assayed concentration. Different letters mean significant statistical differences.

Fennel and chamomile were previously reported for their high antioxidant activity, as assayed in alcoholic and aqueous extracts (Barros et al., 2009; Guimarães et al., 2013; Mata et al., 2007). In previous studies, it was reported that the incorporation of chamomile or fennel improved the antioxidant activity of cottage cheese (Caleja et al., 2015 a,b). Herein, the incorporation of chamomile- and fennel-based ingredients improved the DPPH scavenging activity of plain cottage cheese samples, especially in the case of extracts, either using chamomile or fennel (**Table 13**). Since the compounds present in the extract were readily available when these were incorporated in the free form, it was somehow predictable that the antioxidant activity would be higher in these cases. However, it was previously verified that the DPPH scavenging activity tended to decrease throughout the shelf-life of the functionalized cheese (Caleja et al., 2015b). Thereby, the encapsulation in microspheres represented a way to tentatively preserve the scavenging activity throughout the shelf-life of the developed product.

As it can be seen in Figure 12, samples functionalized with free extracts tended to lose some of the initial activity; on the other hand, it is obvious (particularly after the 7th day of storage) that the microencapsulation of the extracts allows achieving increasing levels of scavenging activity throughout ST, which represents a good indicator of the protective effect intended with the application of the microencapsulation technique.

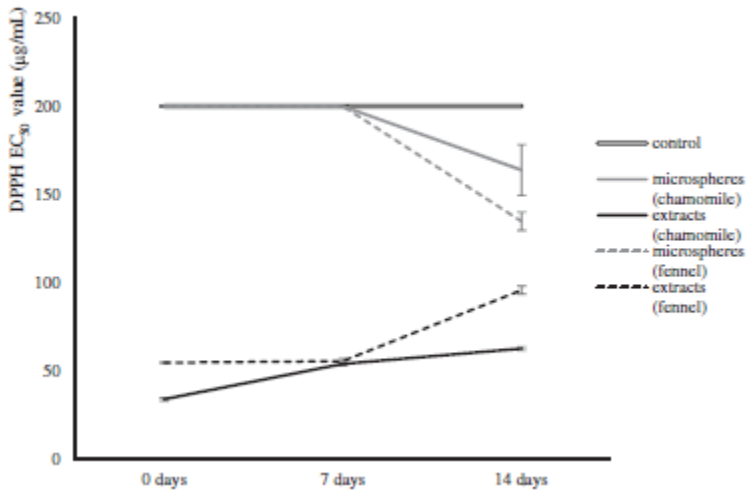

Figure 12. Effects of storage time and functionalizing agent on the DPPH scavenging activity of cottage cheese functionalized with chamomile- or fennel-based extracts.

The significant interaction among factors was also observed for all studied fatty acids (FA), with most of them being also significantly changed by each individual factor (**Table 14**). Nevertheless, given the number of detected significant changes, chamomile extracts seemed

to have higher capacity to maintain the FA profiles of plain cottage cheese. Besides the tabled fatty acids, C11:0, C13:0, C14:1, C15:0, C16:1, C17:0, C17:1, C18:2, C18:3, C20:1, C20:4, C21:0 and C20:5 were also detected, but in trace amounts (<0.2%). As verified in previous studies, saturated fatty acids (SFA) were the predominant forms, followed by monounsaturated (MUFA) and small amounts of (PUFA), which is in agreement with the FA profiles typically detected in this type of lactic products (Caleja et al., 2015a,b; Pizzillo et al., 2005; Queiroga et al., 2013).

Linear Discriminant Analysis

As discussed in section Characterization of the microencapsulated plant extracts, it was possible to identify different statistically significant effects (either induced by ST or FT) in most of the evaluated parameters. However, to characterize better each level ("0 days", "7 days" and "14 days" in one case, "none", "microspheres" and "extracts", in the other) of the assayed factors, it is essential knowing which of the parameters contributed more decisively to define those levels. Accordingly, different linear discriminant analysis (LDA) were performed with the basic objective of evaluating the linkage between the ST or FT levels (categorical dependent variables) and the matrix of obtained results (quantitative independent variables). The significant independent variables were selected following the stepwise method of the LDA, according to the Wilks' λ test. Only variables with a statistically significant classification performance ($p < 0.050$) were maintained by the statistical model.

Initially, the results obtained for fennel were compared considering the effects induced by ST (**Figure 13a** and **Figure 13c**). The two defined discriminant functions included 100% of the observed variance by selecting fat, protein, lactose, energy, DPPH, *b**, C4:0, C12:0, C14:0, C18:1 and PUFA as the variables with the strongest discriminant effect. Function 1, which was mostly correlated (data not shown) to fat, protein and energy (higher in "14 days" samples) projected the markers corresponding to the 14 days period far from the remaining samples, proving their significant differences. On the other hand, the separation of non-stored samples and those stored for 7 days was dictated by function 2, which was highly correlated to lactose (higher in non-stored samples), C12:0 and C14:0 (lower in non-stored samples).

Regarding the effect of FT, function 1, which separated markers corresponding to the extracts, was more correlated to DPPH (higher scavenging activity in "extracts") and lactose (higher in non-functionalized samples). Markers from non-functionalized samples and those incorporated with microencapsulated extracts scored differently regarding function 2, which correlated more to SFA, and, particularly, with C18:0 (higher in non-functionalized samples). The other selected variables were water, fat, carbohydrates, *b**, C6:0, C10:0, C16:0 and PUFA.

A similar study was applied to the results obtained from chamomile-based functionalized cheese. The 2 defined functions regarding ST effect included 100.0% of the observed variance, selecting the variables water, protein, lactose, DPPH, C4:0, C10:0, C12:0, C16:0 and C18:0 as those with significant discriminant ability. The graph representation (Figure 13c) indicates clearly that samples stored during 14 days are those with highest differences. The correlation among variables and discriminant functions specifies that lactose, water (clearly lower in “14 days” samples) and protein (higher in “14 days” samples) were the variables which contributed most to this separation. On the other hand, non-stored samples and those submitted to 7 days of storage were nearly equal regarding the variables correlated with function 1; their main differences were related to C12:0, C10:0 (higher in “7 days” samples), which resulted to be the variables with the highest correlation with function 2.

Regarding the effect of FT, the separation among markers (**Figure 13d**) reveals that the most marked differences among plain cottage cheese and samples functionalized by directly adding decocted extracts are related to function 1, which was verified to have the strongest correlations with DPPH scavenging activity and carbohydrates. On the other hand, samples functionalized with microencapsulated extracts distinguish from plain cottage cheese through function 2, which was more highly correlated with SFA, and, particularly, C18:0. The other selected variables as having discriminant ability were fat, protein, ash, lactose, *L**, C4:0, C6:0 and C16:0.

In all the performed LDA, the classification performance was 100% accurate, either for original grouped cases, as well as for the cross-validated grouped cases.

Table 14. Fatty acids profile (%) of the cottage cheese samples along shelf life. Results are presented as estimated mean±standard deviation.

		C4:0	C6:0	C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	SFA	MUFA	PUFA
Foeniculum vulgare													
ST	0 days	4.0±0.1	3.5±0.1	3.3±0.1	8.1±0.2	4.7±0.1	10.7±0.2	23.7±0.3	12±2	22±2	72±2	24±2	3.8±0.1
	7 days	3.7±0.3	3.8±0.1	3.6±0.1	8.8±0.2	5.0±0.1	11.1±0.3	24.1±0.5	12±2	20±1	74±1	22±1	3.6±0.2
	14 days	2.4±0.5	3.2±0.4	3.4±0.2	8.5±0.3	5.0±0.2	11.1±0.2	24.1±0.3	13±2	21±2	73±2	23±2	3.9±0.3
	p-value (n=27)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	0.053	0.002	<0.001	<0.001	<0.001
FT	None	3.5±0.4	3.5±0.1	3.3±0.1	8.3±0.3	4.9±0.2	10.9±0.3	24.0±0.4	14±1	19±1	75±1	21±1	3.8±0.1
	Microspheres	3.7±0.5	3.7±0.2	3.5±0.2	8.6±0.4	4.9±0.1	11.1±0.2	24.3±0.2	10±1	22±1	72±2	25±2	3.8±0.1
	Extract	3.0±0.5	3.3±0.4	3.4±0.2	8.5±0.3	4.9±0.2	10.8±0.2	23.7±0.4	13±1	21±1	73±1	23±1	3.8±0.4
	p-value (n=27)	0.015	<0.001	<0.001	0.005	0.328	0.006	<0.001	<0.001	<0.001	<0.001	<0.001	0.443
ST×FT	*p*-value (n=81)	<0.001	0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Matricaria recutita													
ST	0 days	3.9±0.1	3.5±0.1	3.3±0.1	8.2±0.2	4.7±0.1	10.7±0.2	23.7±0.3	12±2	22±1	73±1	24±1	3.7±0.1
	7 days	3.8±0.3	3.7±0.1	3.5±0.2	8.8±0.4	5.1±0.2	11.0±0.2	23.8±0.5	11±2	21±2	73±2	23±2	3.6±0.2
	14 days	3.0±0.2	3.4±0.1	3.3±0.1	8.3±0.2	4.9±0.1	11.0±0.2	24.2±0.4	13±2	21±2	73±2	23±2	3.7±0.1
	p-value (n=27)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.005	0.234	0.155	0.201	0.004
FT	None	3.5±0.4	3.5±0.1	3.3±0.1	8.3±0.3	4.9±0.2	10.9±0.3	24.0±0.4	14±1	19±1	75±1	21±1	3.8±0.1
	Microspheres	3.6±0.3	3.5±0.1	3.3±0.1	8.4±0.3	4.8±0.1	10.9±0.2	23.9±0.5	11±1	23±1	72±1	25±1	3.6±0.1
	Extract	3.6±0.5	3.5±0.2	3.5±0.2	8.6±0.4	5.0±0.2	10.9±0.3	23.8±0.3	12±2	21±1	73±1	24±1	3.6±0.2
	p-value (n=27)	0.951	0.452	0.001	0.006	0.062	0.553	0.196	<0.001	<0.001	<0.001	<0.001	<0.001
ST×FT	*p*-value (n=81)	<0.001	<0.001	0.001	0.022	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.005

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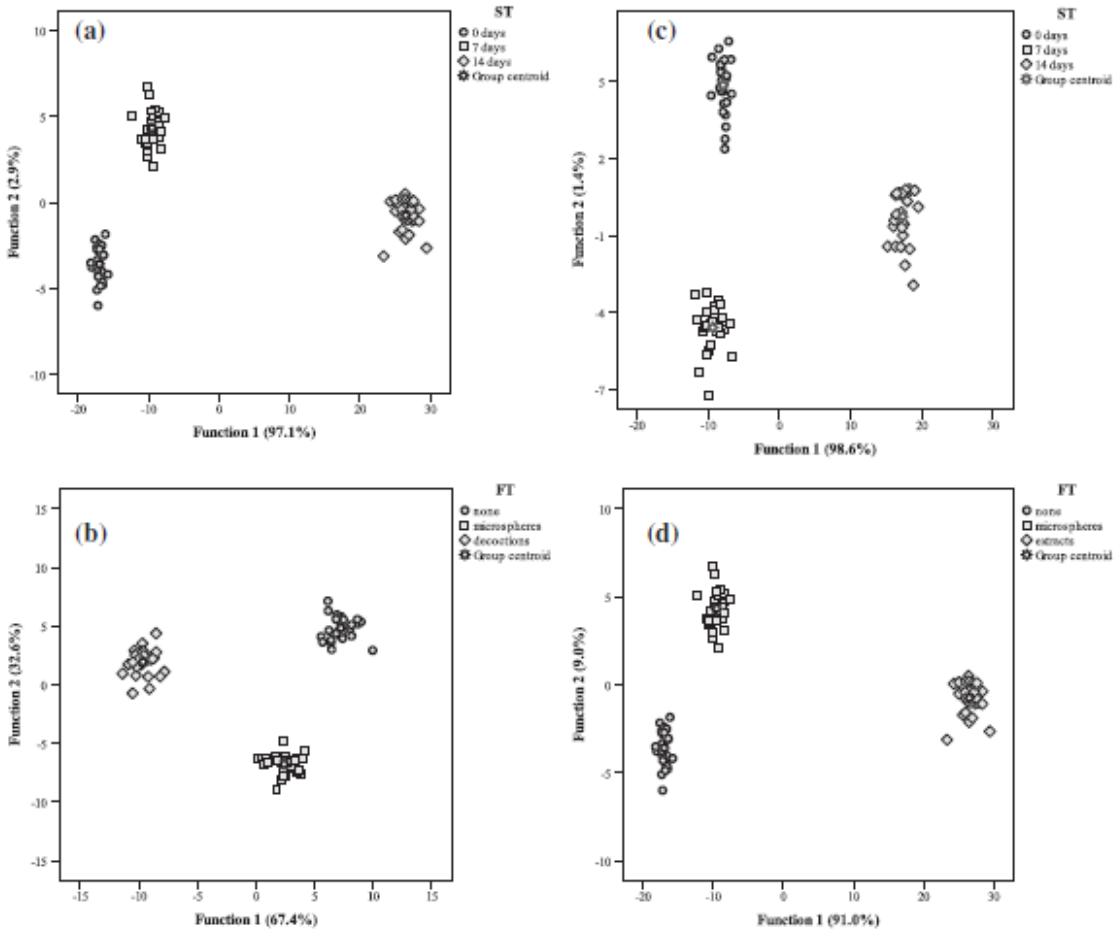

Figure 13. Canonical discriminant functions coefficients defined from the evaluated parameters. **(a)** Effect of storage time in samples incorporated with fennel-based extracts. **(b)** Effect of functionalization type in samples incorporated with fennel-based extracts. **(c)** Effect of storage time in samples incorporated with chamomile-based extracts. **(d)** Effect of functionalization type in samples incorporated with chamomile-based extracts.

Conclusions

Considering all the results at once, it might be concluded that ST exerted its main effects on the nutritional composition of cottage cheese, as the variables more related with function 1 (that include most of the variance in both cases) are related to the nutritional parameters. Likewise, it is possible to identify DPPH scavenging activity as the main variable in discriminating non-functionalized and functionalized samples, since it was the variable most correlated with function 1. In view of these results, it is possible to conclude that cottage cheese was effectively functionalized without causing significant changes in nutritional and colour parameters and in fatty acids profiles. Furthermore, it is possible to conclude that the microsphere encapsulation might be applied as a feasible technique to preserve the

antioxidant activity throughout longer storage times (**Figure 12**). In fact, the microencapsulated compounds seemed to be released after the 7th day, granting an increase in the antioxidant activity, while this bioactivity indicator started to increase for cheese samples incorporated with free extracts after the same period.

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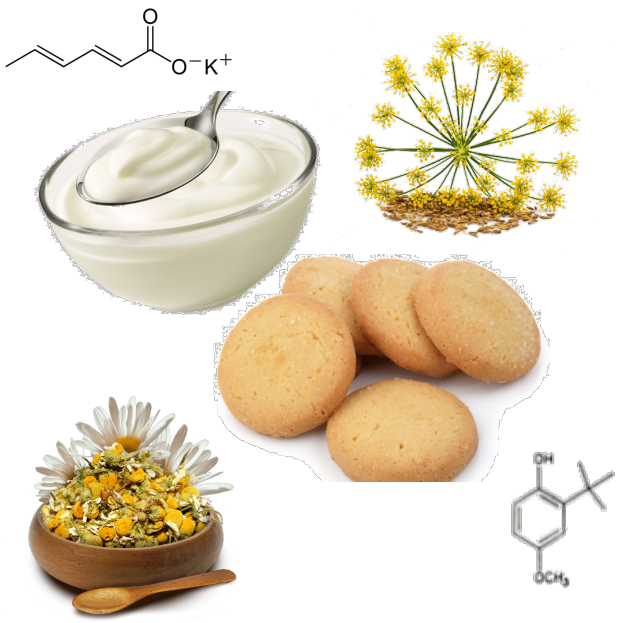
3.2. **Estudos comparativos dos efeitos conservantes de funcho e camomila comparativamente com aditivos artificiais**



Neste sub-capítulo são apresentados dois estudos onde se avaliaram os efeitos da incorporação das decocções de funcho e camomila comparativamente com aditivos artificiais normalmente utilizados pela indústria alimentar em produtos lácteos e de pastelaria. São também apresentados os resultados de um estudo sensorial sobre as preferências dos consumidores de biscoitos.

3.2.1. Fortificação de iogurtes com diferentes conservantes antioxidantes: estudo comparativo entre aditivos naturais e sintéticos

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Fortification of yogurts with different antioxidant preservatives: A comparative study between natural and synthetic additives

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Abstract

Yogurt is a highly appreciated product all over the world; so, the industry has been focused on the development of a wide range of yogurts with different characteristics and functionalities. However, consumers demand more and more so-called "natural" products and, therefore, the aim of this work was to compare the effects of natural versus synthetic antioxidant preservatives in yogurts. *Matricaria recutita* L. (chamomile) and *Foeniculum vulgare* Mill. (fennel) decoctions were tested as natural additives, while potassium sorbate (E202) was used as a synthetic additive. The fortification of yogurts with natural and synthetic antioxidants did not cause significant changes in the yoghurt pH and nutritional value, in comparison with control samples (yogurt without any additive). However, the fortified yogurts showed higher antioxidant activity, mainly the yogurts with natural additives (and among these, the ones with chamomile decoction). Overall, it can be concluded that plant decoctions can be used to develop novel yogurts, by replacing synthetic preservatives and improving the antioxidant properties of the final product, without changing the nutritional profile.

Keywords: Yogurt; *Matricaria recutita*; *Foeniculum vulgare*; potassium sorbate (E202); antioxidant preservatives.

Introduction

Yogurt is a fermented dairy product obtained by lactic acid fermentation through the action of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermo-philus*. The resulting lactic acid reacts with milk protein, promoting the characteristic texture and sensorial properties of this product (Serafeimidou, Zlatanos, Kritikos, & Tourianis, 2013). Yogurts are highly appreciated, and since they are regarded as important in human diet, they are produced and consumed massively in many countries (O´Connel & Fox, 2001; Serafeimidou et al., 2013; Shori & Baba, 2014). However the literature points out that dairy food products have a limited content of bioactive compounds, which can remove some value from these products. Therefore, to overcome this limitation some authors suggest the incorporation of plant or fruits based additives to fortify the yogurt (Karaaslan, Ozden, Vardin, & Turkoglu, 2011; Martins et al., 2014; Bertolino et al., 2015).

In the food industry, synthetic additives are used to improve the characteristics and properties of processed foods, and include preservatives (antimicrobials, antioxidants and antibrowning), nutritional additives and coloring, flavoring, texturizing and miscellaneous agents (Branen, Davidson, Salminen & Thorngate, 2002; Dickson-Spillmann, Siegrist & Keller, 2011; Carocho, Barreiro, Morales & Ferreira, 2014). However, many studies have confirmed that the excessive consumption of synthetic food additives is related with gastrointestinal, respiratory, dermatological, and neurological adverse reactions (Wilson & Bahna, 2005; Randhawa & Bahna, 2009; Carocho et al., 2014).

Potassium sorbate is one of the main preservatives used in food industry, being also extensively used as an antimicrobial agent since it can effectively inhibit the growth of fungi, aerobic bacteria and yeasts (Karabulut, Lurie & Droby, 2001; Fandos & Dominguez, 2007; Liu, Wang & Young, 2014). Despite being considered as safe, effective, and presenting a lower toxicity than other preservatives (Karabulut et al., 2001; Karabulut, Romanazzi, Smilanick & Lichter, 2005; Fandos and Dominguez, 2007), some authors consider that the use of this preservative has adverse effects on human health (Kamankesh, Mohammadi, Tehrani, Ferdowsi & Hosseini, 2013). In particular, some cases of allergic effects have been described, such as urticaria and asthma (Code of Federal Regulations, 1999, Tfouni & Toledo, 2002; Goren et al., 2015), and also cases of intolerance (Code of Federal Regulations, 1999; Hannuksela & Haahtela, 1987; Goren et al., 2015).

Antioxidants present in plants, algae and mushrooms are excellent natural additives and have been presented as alternatives to synthetic additives. Vitamins, polyphenols and

carotenoids are considered the most natural antioxidant molecules (Baines & Seal, 2012; Carocho & Ferreira, 2013a; Carocho, Morales & Ferreira, 2015). Due to their high antioxidant power, the polyphenols are considered among the most interesting and relevant natural compounds to be used as food preservatives and bioactive ingredients (Caleja et al., 2015a; Caleja et al., 2015b; Carocho et al., 2015).

The antioxidant and antimicrobial potential of *Matricaria recutita* L. (chamomile) and *Foeniculum vulgare* Mill. (fennel) decoctions (rich in phenolic compounds such as quercetin-3-*O*-glucoside and 5-*O*-caffeolylquinic acid, or di-caffeoyl-2,7-anhydro-3-deoxy-2-octulopyranosonic acid and luteolin-*O*-glucuronide, respectively) was previously demonstrated by our research group, as well as their efficient use as preservatives in cottage cheese (Caleja et al., 2015a; Caleja et al., 2015b). The aim of the present study was to propose the use of these polyphenol rich extracts to yogurts, and to compare their performance with a synthetic additive commonly used in dairy products.

Materials and methods

Standards and reagents

Acetonitrile was of HPLC grade from Fisher Scientific (Lisbon, Portugal). Fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA), as also sugar standards. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, Massuchets, USA). Water was treated in Milli-Q water purification system (Millipore Corporation, Billerica, Massachusetts, USA).

Natural and synthetic additives

Dried samples of *Matricaria recutita* L. (chamomile) and *Foeniculum vulgare* Mill. (fennel) were provided by Américo Duarte Paixão Lda. (Alcanede, Portugal). In order to prepare the decoction extracts, the samples were reduced to powder (~20 mesh) and added (5 g) to 200 mL of distilled water. The mixture was heated (VELP Scientific plate, Usmate, Italy), allowed to boil for 5 minutes and then left to stand for 5 min, filtered, frozen and lyophilized (FreeZone 4.5, Labconco, Kansas City, Missouri, USA). The lyophilized extracts obtained by the described decoction procedure were used as natural additives. The characterisation of the extracts in terms of individual phenolic compounds was previously carried out by HPLC-DAD-ESI/MS and described by the authors (Caleja et al., 2015a; Caleja et al., 2015b). Five flavonoids (mainly quercetin-3-*O*-glucoside) and twelve phenolic acids (mainly 5-*O*-caffeolylquinic acid) were found in fennel extract (Caleja et al., 2015a), while di-caffeoyl-2,7-anhydro-3-deoxy-2-octulopyranosonic acid and luteolin-*O*-glucuronide were

identified as the main phenolic compounds present in the chamomile extract (Caleja et al., 2015b).

The synthetic additive used was potassium sorbate 99% (E202) and it was supplied by Acros organics (Geel, Belgium).

Fortification of yogurts with the natural and synthetic additives

Four groups of samples were prepared (yogurts with 100 g each): control samples (yogurts without additives); samples with potassium sorbate (E202); samples with the fennel decoction; and samples with the chamomile decoction. For each portion of 100 g of yogurt, 40 mg of the additive (natural or synthetic) were incorporated. All the yogurts were prepared in duplicate.

Yogurts were prepared from UHT (ultra high temperature) milk (fat 3.6%; protein 3.3% and carbohydrates 5.0%) mixed with natural yogurt purchased at the local market. After fortification with the different additives, each corresponding sample was left for overnight incubation at 44 ºC for yogurt production.

Nutritional composition, physico-chemical analyses and evaluation of the antioxidant activity of the samples along shelf life

The samples were analyzed immediately after preparation and after seven and fourteen days of storage at 4 ºC. All the analyses were performed in triplicate.

Nutritional parameters

The samples were also analyzed for proximate composition (moisture, protein, fat, ash and carbohydrates) using the AOAC (2005) procedures. The crude protein content (N × 6.38) of the samples was estimated by 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 ºC and total carbohydrates were calculated by difference. Total energy was calculated according to the following equation: Energy (kcal) = 4 × (g proteins +g carbohydrates) + 9 × (g lipids). Free sugars were detected by HPLC coupled to refraction index detector and the identification was performed by comparison with standards, and further quantified (g/100 g of yogurt) by using an internal standard (melezitose). Fatty acids were analyzed by GC coupled to a FID detector and the identification was performed by comparison with commercial standards. Results were expressed in relative percentage of each fatty acid.

Physico-chemical parameters

The color of the samples was measured in six different points (three on the top and three on bottom), for each sample, by using a colorimeter (model CR-400, Konica Minolta Sensing Inc., Tokyo, Japan). Using the illuminant C and diaphragm aperture of 8 mm, the CIE *L*a*b** color space values were registered using a data software "Spectra Magic Nx" (version CM-S100W 2.03.0006) (Fernandes, Antonio, Barreira, Oliveira, Martins & Ferreira, 2012).

The pH of the samples was measured directly in the samples with a HI 99161 pH-meter (Hanna Instruments, Woonsocket, Rhode Island, USA).

Antioxidant activity

The lyophilized samples (3 g) were extracted with methanol/water (80:20) at room temperature during 1h under stirring. The extract was filtered with Whatman paper filter Nº 4 (Sigma-Aldrich, Missouri, USA) and the remaining solid residue subjected to an additional extraction at the same conditions. The resulted extracts were evaporated under reduced pressure in a rotatory evaporator until complete removal of methanol. Finally, the evaporated extract was dissolved in methanol at a concentration of 200 mg/mL for the antioxidant activity evaluation. DPPH radical-scavenging activity and reducing power were evaluated at 515 and 690 nm, respectively, using ELX800 microplate Reader (Bio-Tek Instruments, Inc., Winooski, Vermont, USA) (Caleja et al., 2015a).

Statistical analysis

The data were expressed as means±standard deviation. An analysis of variance (ANOVA) with type III sums of squares was carried out in the SPSS software. By using a multivariate general linear model, the dependent variables were analyzed through a 2-way ANOVA with the factors "incorporation" (I) and "storage time" (ST). When a significant interaction was detected for both factors (I and ST), they were evaluated simultaneously by the estimated marginal means. If no statistical significant interaction was detected, the means were compared using Tukey's multiple comparison test, relying on a previous assessment of the equality of variances through the Levene's test. All statistical tests were performed at a 5% significance level and using the SPSS software, version 22 (IBM Corp., USA).

Results and discussion

The four types of yogurts; control, fortified with E202, fennel and chamomile were labeled, and analyzed after three storage periods (0, 7 and 14 days). The results are presented in **Table 15** to**Table 18**. The results are presented as the mean value of each storage time

(ST) regardless of the fortification treatment, and also the mean value of each fortification (F) regardless of the storage time. With this type of statistical analysis and representation, the best fortification type (control, E202, fennel decoction and chamomile decoction) could be determined independently of the storage time, but also the influence of the storage time irrespectively of the incorporation type. Thus, the standard deviations should not be regarded as a measure of accuracy of the methodologies, given that they encompass the results of the non-fixed factor (F or ST). Furthermore, the interaction among both factors (ST × F) could also be determined through this study. Every time a significant interaction was detected ($p<0.05$), no multiple comparisons could be carried out, therefore, the influence of each factor could be evaluated from the Whisker Box Plots representation, first for all samples then for all storage times. **Table 15** shows the nutritional parameters, the energy value and the individual sugars, detected through HPLC-RI, expressed in g/100 g of fresh weight. Overall, and as expected, moisture and fat were the most abundant nutrients in the yoghurts. For the majority of the parameters, the interaction between ST and F was significant, with only moisture not showing significant interaction. In terms of the individual factors, their effect was significant in most cases, except moisture, proteins and carbohydrates, concerning the effect of F. Thus, the fortification type has lower influence than the storage time on these parameters. Overall, none of the incorporations had a significant influence on the nutritional profile of the yogurts.

Moisture and protein values are in agreement with the results reported by Serafeimidou et al. (2013), in yogurts prepared with cow and sheep milk; however, ash values were higher than the ones described by these authors. Two free sugars were quantified in the studied samples: lactose and galactose (**Table 15**). The changes in these two sugars are not drastic, regardless of the fortification applied. Previous studies focused on the influence of the addition of different halzenut skins to yogurts have described the presence of these two sugars as well as glucose, although it was detectedin very low amounts (Bertolino et al., 2015).

Table 15. Macronutrients, free sugars composition (g/100 g) and energy value (kcal/100 g) of the yogurts along shelf life and with different fortifications.

		Moisture	Fat	Protein	Ash	Carbohydrates	Energy	Galactose	Lactose
Storage time (ST)	0 days	87.6±0.2b	2.58±0.18	3.92±0.12	0.67±0.02	5.25±0.4	60±1	0.49±0.10	3.28±0.13
	7 days	87.5±0.3a	2.42±0.23	3.77±0.10	0.70±0.02	5.62±0.33	59±2	0.56±0.07	2.96±0.28
	14 days	87.3±0.8ab	2.32±0.13	3.84±0.05	0.70±0.02	5.79±0.81	60±3	0.62±0.09	2.78±0.32
p-value (n=24)	Tukey's HSD test	0.036	<0.001	<0.001	0.001	0.005	<0.001	<0.001	<0.001
Fortification (F)	Control	87.6±0.5	2.55±0.22	3.81±0.04	0.70±0.03	5.39±0.66	60±2	0.54±0.10	2.98±0.20
	E202	87.4±0.7	2.38±0.22	3.87±0.08	0.68±0.02	5.64±0.73	59±3	0.44±0.05	2.74±0.4
	Fennel decoction	87.3±0.5	2.54±0.16	3.91±0.12	0.70±0.02	5.57±0.52	61±2	0.62±0.09	3.13±0.25
	Chamomile decoction	87.6±0.3	2.30±0.13	3.79±0.14	0.67±0.02	5.62±0.31	58±2	0.63±0.02	3.18±0.01
p-value (n=18)	Tukey's HSD test	0.058	<0.001	0.098	<0.001	0.606	<0.001	<0.001	<0.001
ST×F (n=72)	*p*-value	0.086	<0.001	<0.001	<0.001	0.016	<0.001	<0.001	<0.001

In each row and within each storage period, different letters mean significant statistical differences between control yogurts, yogurts with E202, yogurts with fennel decoction and yogurts with chamomile decoction ($p<0.05$).

Table 16. Fatty acids composition of the yogurts (expressed in relative percentage of each fatty acid) along shelf life and with different fortifications.

		C4:0	C6:0	C8:0	C10:0	C12:0	C14:0	C15:0	C16:0	C16:1	C18:0	C18:1n9	C18 :2n9	SFA	MUFA	PUFA
Storage time (ST)	0 days	3.4±0.5	2.8±0.3	1.5±0.2	2.7±0.2	3.4±0.2	10.7±0.3	1.05±0.02	32.9±0.5	1.60±0.02	11.1±0.2	24±2	2.7±0.2	70±2	26±2	3.1±0.4
	7 days	2.5±0.3	2.4±0.3	1.4±0.2	2.7±0.2	3.4±0.1	10.9±0.2	1.06±0.01	33.5±0.7	1.60±0.07	11.2±0.3	24±1	2.4±0.3	70±1	27±1	2.8±0.4
	14 days	3.1±0.8	2.4±0.1	1.3±0.2	2.6±0.3	3.4±0.3	10.9±0.4	1.07±0.03	33.8±0.5	1.61±0.06	11.3±0.2	23.5±0.8	2.4±0.4	71±1	26±1	2.8±0.5
p-value (n=24)	Tukey's HSD test	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.051	<0.001	<0.001	0.025	<0.001
Fortification (F)	Control	2.9±0.6	2.6±0.3	1.5±0.1	2.8±0.2	3.6±0.2	11.1±0.3	1.08±0.02	33.2±0.3	1.58±0.04	11.0±0.1	24±1	2.3±0.4	71±1	27±1	2.6±0.4
	E202	3.4±0.7	2.6±0.5	1.5±0.4	2.7±0.3	3.4±0.2	10.8±0.1	1.05±0.02	33.2±0.7	1.59±0.08	11.1±0.3	24±1	2.5±0.2	71±1	26±1	3.0±0.2
	Fennel decoction	3.2±0.6	2.4±0.2	1.3±0.1	2.6±0.2	3.3±0.2	10.7±0.3	1.05±0.02	33.7±0.4	1.63±0.02	11.4±0.2	23±1	2.6±0.3	71±1	26±1	3.1±0.4
	Chamomile decoction	2.6±0.5	2.6±0.2	1.3±0.1	2.6±0.1	3.3±0.1	10.8±0.4	1.06±0.03	33.6±1.0	1.61±0.04	11.3±0.2	24±1.	2.6±0.4	70±2	27±1	3.1±0.4
p-value (n=18)	Tukey's HSD test	<0.001	0.006	<0.001	<0.001	<0.001	<0.001	0.329	<0.001	0.205	<0.001	<0.001	<0.001	<0.001	<0.001	0.001
ST×F (n=72)	*p*-value	<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

The results are presented as mean±SD. Butiric acid (C4:0); Caproic acid (C6:0); Caprylic acid (C8:0); Capric acid (C10:0); Lauric acid (C12:0); Myristic acid (C14:0); Pentadecanoic acid (C15:0); Palmitic acid (C16:0); Palmitoleic acid (C16:1); Stearic acid (C18:0); Oleic acid (C18:1n9); Linoleic acid (C18:2n6); SFA- Saturated fatty acids; MUFA- Monounsaturated fatty acids; PUFA- Polyunsaturated fatty acids.

The twelve most abundant fatty acids detected in the yogurt samples are presented in **Table 16**. It was possible to identify twenty-three fatty acids in the studied samples, the remaining eleven were detected in trace amounts (data no shown). The most abundant ones were oleic acid (C18:1n9), followed by palmitic acid (C16:0), stearic acid (C18:0) and myristic acid (C14:0). As expected, saturated fatty acids prevailed over unsaturated ones, with the monounsaturated showing higher amounts than polyunsaturated. The variation among SFA and PUFA was very slight, both along storage time and with the different fortification types. Among the presented fatty acids, a significant interaction among ST and F was found for all of them. In terms of C15:0 and C16:1, storage time had a higher impact than the fortification type and, inversely, for C18:1n9 (the most abundant fatty acid), the highest contributing factor for its variation was the fortification type, expressed by the respective p-values for each factor (Table 2). In 2001, Serafeimidou et al. (2013) described the same trends in terms of abundancies (SFA>MUFA>PUFA), but identified a higher number of fatty acids.

The results of two physico-chemical parameters (color and pH) of the yogurts are displayed in **Table 17**. For all parameters, the interaction was significant ($p<0.05$), but some general tendencies could be extracted from the Whisker Box Plot representation. The incorporations did not alter the color parameters of the yogurts to the naked eye, as can be confirmed in **Figure 14**. For color parameters it was not observed a significant change in L^* (lightness) in the different samples and along storage time. The a^* value has a minor contribution for total color but has the same tendency as L^* value. The samples with no added extract, with E202 and with Fennel were not significantly different regarding color parameters (graphs not shown). A slight distinctive mark was observed only for yogurts with chamomile fortification in b^* parameter (**Figure 15A**). In food industry, color control is used to produce another product for the market and could also be used as a control quality parameter along expected storage time. Its change along storage is not desired, however different color products, as is current in food industry, namely in yogurts, are a distinctive mark.

In a similar study with yoghurts fortified with grape (*Vitis vinifera*) seed extracts, the same tendency was observed, as the supplementation with seed extracts did not affect the pH values of the yogurts however, the color of different seeds (Agiorgitiko and Moschofilero seed extracts were red and yellow, respectively) affected the color of the yogurts, which was not visually detected (Chouchouli et al., 2013). Another study with yogurts fortified with bioactive compounds obtained from different grape varieties, presented positive values of a^* and displayed light red color (Karaaslan et al., 2011).

Table 17. Color parameters and pH of the yogurts along shelf life and with different fortifications.

		*L**	*a**	*b**	pH
Storage time (ST)	0 days	92±1	-2.7±0.8	10±1	4.4±0.1
	7 days	93±1	-2.7±0.4	10±1	4.5±0.1
	14 days	92±1	-3.0±0.4	10±1	4.5±0.1
p-value (n=24)	Tukey's HSD test	<0.001	<0.001	<0.001	0.001
Fortification (F)	Control	92±1	-3.0±0.3	8.8±0.5	4.5±0.4
	E202	92±1	-3±1	9.7±0.7	4.60±0.06
	Fennel decoction	92±1	-2.6±0.3	10.5±0.7	4.3±0.1
	Chamomile decoction	91±2	-2.6±0.5	11.5±0.7	4.53±0.07
p-value (n=18)	Tukey's HSD test	<0.001	<0.001	<0.001	<0.001
ST×F (n=72)	*p*-value	<0.001	<0.001	<0.001	<0.001

The results are presented as mean±SD. *L**: Lightness, *a**: red-green, *b**: yellow-blue (color parameters).

Figure 14. Visual appearance of different yogurt samples **(A)** Control yogurt, **(B)** yogurt with E202, **(C)** yogurt with fennel decoction and **(D)** yogurt with chamomile decoction.

In terms of the pH values, none of the fortifications changed the parameters, which is quite important to maintain the yogurts' stability during the storage time. The values are also within the acceptable range and in accordance with previously published studies with the same foodstuffs (Ersöz, Kınık, Yerlikaya & Açu, 2011; Serafeimidou et al., 2013; Bertolino et al., 2015). According to Gohil, Ahmed, Davies and Robinson (1995), the analysis of this parameter in yogurts is very important with respect to public safety. The same authors report that pathogens such as *Listeria monocytogenes* in yogurts die out rapidly at pH values between 4.2 and 4.5. Another study reported the survival of *Salmonella* and *Escherichia coli* for up to

10 and 7 days respectively, at high pH values in yoghurts (Massa, Altieri & Quaranta de Pace, 1997).

Table 18. Antioxidant activity of the yogurts (expressed in EC_{50} values, mg/mL) along shelf life and with different fortifications.

		Reducing Power	DPPH scavenging activity
Storage time (ST)	0 days	25±6	105±54
	7 days	26±6	107±56
	14 days	27±6	121±57
p-value (n=27)	Tukey's HSD test	<0.001	<0.001
Fortification (F)	Control	32.4±0.4	195±5
	E202	29±2	111±20
	Fennel decoction	27±1	94±4
	Chamomile decoction	16.4±0.8	45±3
p-value (n=36)	Tukey's HSD test	<0.001	<0.001
ST×F (n=108)	*p*-value	<0.001	<0.001

The results are presented as mean ± SD.

The results of the reducing power and DPPH radical scavenging activity of the yogurt samples along shelf life are given in **Table 18**. Once again, the interaction among the storage time and fortification type was significant, therefore, tendencies were extracted from the Whisker Box Plots representation of all data regardless storage time (**Figure 15B**). Similar analysis was performed for all samples at different storage times without significant differences being observed. Still, by interpreting the table, the best assay was the reducing power, given the lower EC_{50} values. Yogurts fortified with fennel and chamomile decoctions seem to have conferred a higher antioxidant capacity to the yogurts than the synthetic additive, as can be confirmed on **Figure 15B and Figure 15D**, although it was more apparent for the reducing power assay. **Figure 15B** represents the box-plot representation for the reducing power for each incorporation for all the storage times. It is clear that, for the results expressed in EC_{50} values, yogurts incorporated with chamomile displayed the highest activity, lower EC_{50} values, followed by fennel incorporations. As expected, the control samples showed the lower reducing power capacity. Interestingly, the antioxidant capacity of the yogurts fortified with the chemical additive, lost their antioxidant capacity more rapidly than the plant fortified yogurts, mainly from the seventh day of storage onwards, proving the excellent capacity of the plants, especially over longer periods of storage, as could be seen from the graphic bars for all fortifications at each storage time (**Figure 15C**). **Figure 15D** represents the box-plot graphs for each fortification for all the storage times, except for the DPPH assay. Once again, chamomile

decoction was the best antioxidant, although the chemical additive showed a very similar activity as fennel incorporated yogurts. Still, after the seventh day, the antioxidant capacity of the yogurts fortified with the chemical additive lost considerable antioxidant capacity, while the fortified with natural products, although they lost some antioxidant capacity, this reduction was very slight (**Figure 15E**).

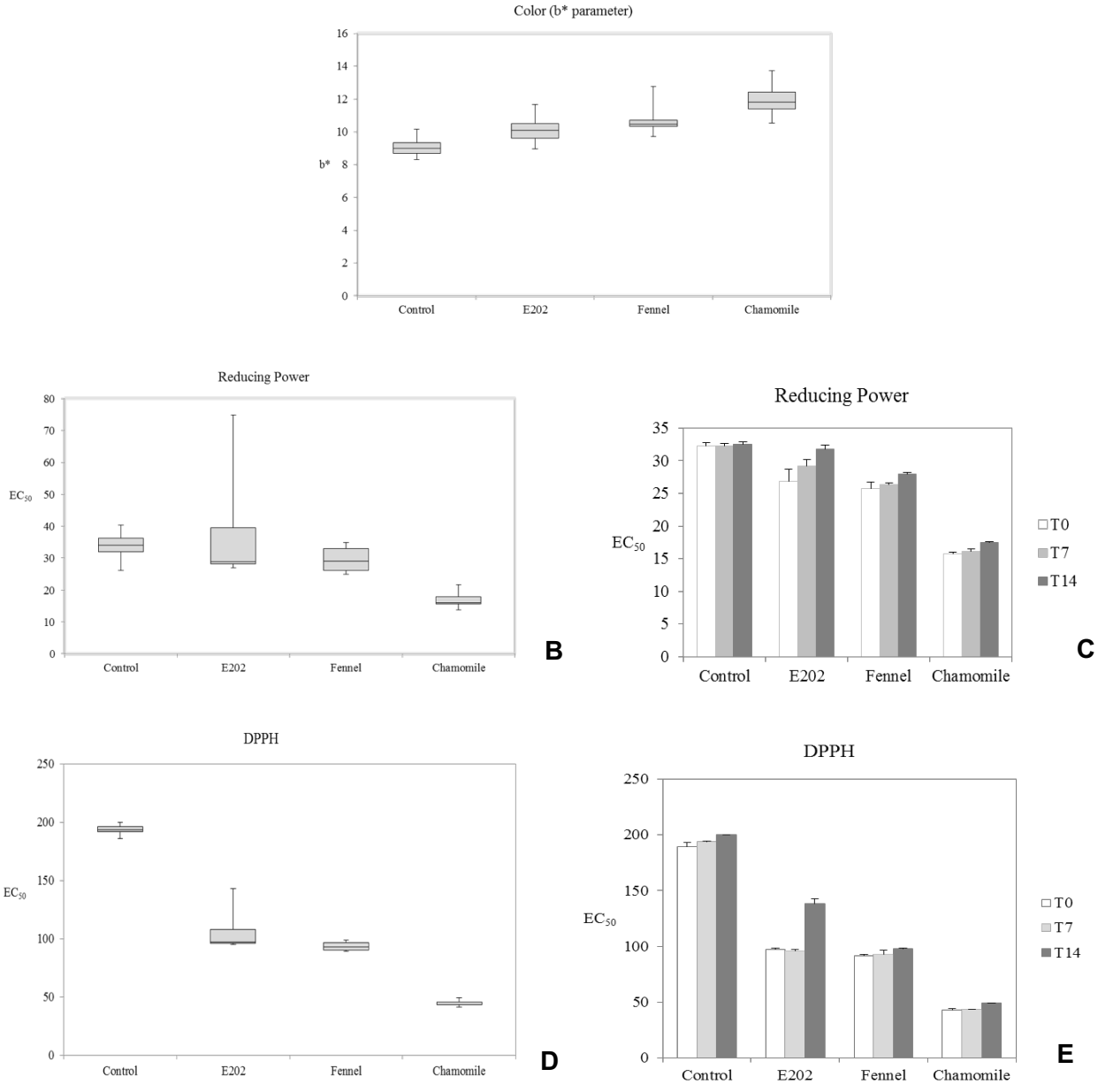

Figure 15. A- Color parameter (*b** value); **B-** Reducing Power assays whisker box plots; **C-** Reducing Power EC_{50} values for different samples at different storage times; **D-** DPPH assays whisker box plots; **E-** DPPH EC_{50} values for the different samples at different storage times.

Similar studies describe that the antioxidant activity of yogurts was enhanced by the presence of natural extracts, for example, in studies with yogurts fortified with white and red dragon fruit (Zainoldin and Baba, 2009), callus (Karaaslan et al, 2011), grape seed (Chouchouli et al., 2013) or with wild blackberry (Martins et al, 2014) extracts.

Conclusions

Yogurt is a highly appreciated product all over the world and, concomitantly, dairy industry is in constant research for innovative products. So, yogurts fortified with antioxidants from natural origin are an interesting food to satisfy consumer demands for healthy products. This study revealed that the introduction of aqueous extracts prepared from plants improves the antioxidant activity of yoghurts, showing higher capacity than the synthetic additive, potassium sorbate, used as antioxidant preservative by the food industry, including the dairy sector. Furthermore, the incorporation of these decoctions did not significantly alter the nutritional profile, external appearance, pH and individual fatty acids, proving that natural plant extracts are beneficial for consumers and do not pose nutritional changes in the yogurts during their normal storage period.

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3.2.2. Estudo comparativo entre antioxidantes naturais e sintéticos: avaliação do desempenho após incorporação em biscoitos

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A comparative study between natural and synthetic antioxidants: Evaluation of their performance after incorporation into biscuits

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Abstract

Currently, the food industry is focused in replacing the use of synthetic by natural antioxidants. The present study focused on the use of fennel and chamomile extracts, rich in phenolic compounds, as natural antioxidants in biscuits and compared their performance with a synthetic antioxidant widely used, the butylated hydroxyl anisole (BHA). The complete nutritional profile, free sugars, fatty acids and antioxidant activity were determined immediately after baking and also after 15, 30, 45 and 60 days of storage. The results showed that the incorporation of natural and synthetic additives did not cause significant changes in colour or in nutritional value of biscuits when compared with control samples. Both natural and synthetic additives conferred similar antioxidant activity to the biscuits. Therefore, natural additives are a more convenient solution for consumers who prefer foods “free” from synthetic additives. Additionally, natural additives were obtained by aqueous extraction, an environment friendly and safe process.

Keywords: Biscuits; *Matricaria recutita* L.; *Foeniculum vulgare* Mill.; Butylated Hydroxyl Anisole (BHA); antioxidant.

Introduction

The affordable cost together with the good nutritional quality, availability in different tastes and long shelf life are some of the reasons which turn the biscuits into the most popularly consumed bakery items all around the world (Gandhi, Kotawaliwale, Kawalkar, Srivastava, Parihar, & Raghu Nadh, 2001). To maintain its high consumption, the biscuit texture, colour, and sensory parameters should be in line with consumer's expectations (Bajaj, Urooj, & Prabhasankar, 2006), which increasingly demand minimally processed foods and avoid the presence of synthetic additives (Carocho, Morales, & Ferreira, 2015).

Crackers, cookies and biscuits are widely consumed and stored for extended periods of time before consumption, thus, keeping quality of these baked foods is of great economic importance (Reddy, Urooj, & Kumar, 2005). Antimicrobials, antioxidants and antibrowning agents are among the additives mostly used by the food industry to preserve products for longer periods (Carocho, Barreiro, Morales, & Ferreira, 2014). In the last century, butylated hydroxyl anisole (BHA) has been used as antioxidant in foods (Freitas, & Fatibello-Filho, 2010; EFSA, 2011). However, the use of this synthetic molecule has been associated with a possible toxicity, and it has been reported that it has some side effects such as carcinogenesis, which has led to some restraint in its use (Branen, 1975; Ito, Fukushima, Hassegawa, Shibata, & Ogiso, 1983; Reddy et al., 2005).

Some authors have developed studies in biscuits where they intended to compare the use of natural antioxidants from plant or fruit extracts with synthetic BHA. For example, the incorporation of fresh mango peel extracts in biscuits improved their antioxidant properties, in comparison with BHA (Ajila, Naidu, Bhat, & Prasada Rao, 2007; Ajila, Leelavathi, & Prasada Rao, 2008). The same tendency was demonstrated by Reddy et al. (2005) who used ethanolic extracts from three plant foods as sources of natural antioxidants: amla (*Emblica officianalis* Gaertn), drumstick leaves (*Moringa oleifera* Lam.) and raisins (*Vitis vinifera* L.) for application in biscuits. The addition of these extracts gave an excellent antioxidant effect to the biscuits compared with the effect of BHA. Bajaj et al. (2006) have also studied the effects of different forms of mint (*Mentha spicata* L.), namely powder and ethanolic extracts, in biscuits.

Natural extracts from plant origin could provide alternatives to synthetic preservers, namely antioxidants, also providing bioactive properties and bringing additional value to the final products (Rasooli, 2007; Ye, Shen, Xu, Lin, Yuan, & Jones, 2013; Pasqualone et al. 2015). Some bakery, dairy and meat products have already been developed incorporating natural extracts from aromatic plants, spices and fruit powder, for antioxidant purposes (Reddy et al.,

2005; Bajaj et al., 2006; Shah, Don Bosco, & Mir, 2014; Caleja et al., 2015a; Caleja et al., 2015b).

In particular, aqueous extracts prepared from *Foeniculum vulgare* Mill. (fennel) and *Matricaria recutita* L. (chamomile) were successively incorporated as natural antioxidants and antimicrobials for cottage-cheese (Caleja et al., 2015a; Caleja et al., 2015b; Caleja et al., 2016a) and yogurts (Caleja, Barros, Antonio, Carocho, Oliveira, & Ferreira, 2016b), being those properties attributed to phenolic compounds namely di-caffeoyl-2,7-anhydro-3-deoxy-2-octulopyranosonic acid and luteolin-*O*-glucuronide in fennel (Caleja et al., 2015a) and quercetin-3-*O*-glucoside and 5-*O*-caffeolylquinic acid in chamomile (Caleja et al., 2015b). Furthermore, the infusions of the abovementioned plants have been traditionally used for the treatment of hypertension, neurological diseases, and allergies (Ranpariya, Parmar, Sheth, & Chandrashekhar, 2011; Rather, Dar, Sofi, Bhat, & Qurishi, 2012; Matić, Juranić, Savikin, Zdunić, Nadvinski, & Godevac, 2013).

In order to generalize the use of fennel and chamomile aqueous extracts (prepared by decoction) as natural antioxidants, the present work evaluated their performance, in two different doses, in a novel food matrix (biscuits), and compared the results with a widely used synthetic antioxidant (BHA), in relation to the storage time.

Materials and methods

Preparation of the natural antioxidants from plant origin and synthetic antioxidant

Commercial samples of *Matricaria recutita* L. (chamomile flowers) and *Foeniculum vulgare* Mill. (fennel aerial parts) were provided by Américo Duarte Paixão Lda. (Vale da Trave, Santarém, Portugal). The dried samples were powdered (~20 mesh; Ultra Centrifugal Mill ZM 200, Porto, Portugal) and decoctions were prepared by adding 5 g of plant material to 200 mL of distilled water (Milli-Q water purification system, TGI Pure Water Systems, Greenville, SC, USA), heated (heating plate, VELP Scientific, Usmate, Italy) and boiled for 5 min. The mixture was left to stand for 5 min, filtered, and then frozen and lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA). The prepared ingredients were previously characterized in terms of antioxidant compounds; fennel extract was rich in quercetin-3-*O*-glucuronide, 5-*O*-caffeolylquinic acid and 1,5-Di-*O*-caffeolylquinic acid (Caleja et al., 2015a), while chamomile extract presented di-caffeoyl-2,7-anhydro-3-deoxy-2-octulopyranosonic acid, 5-*O*-caffeolylquinic acid, luteolin-*O*-glucuronide and myricetin-3-*O*-glucoside as main phenolic compounds (Caleja et al., 2015b).

Butylated hydroxyl anisole (E320-BHA) was used as synthetic additive being supplied by Merck-Schuchardt (Darmstadt, Germany).

Preparation of the biscuits by incorporation of natural and synthetic antioxidants

To prepare the biscuits, a traditional recipe was followed: one egg was thoroughly mixed with 125 g of sugar. The antioxidants were dissolved in 60 mL of water and added to the mixture. Then, 300 g of wheat flour were sequentially added to the mixture while mixing vigorously with a hand mixer at 450W during 8 minutes (Bosch, Munich, Germany). After 15 minutes of rest the dough with the intended consistency was reduced to 5 mm thickness and cut by a round biscuit cutter with 50 mm internal diameter. Six lots of biscuits (30 per lot, 6 biscuits for each storage time) were prepared: i) control biscuits- without any antioxidant, designated by C; ii) two lots of biscuits with fennel extract (80 mg and 800 mg, designated by Fen and Fen10, respectively); iii) two lots of biscuits with chamomile extract (80 mg and 800 mg, designated by Cham and Cham10, respectively); iv) biscuits with synthetic additive, BHA (80 mg). The biscuits were baked in an electric oven for 10 min at 180 ºC. All samples were lyophilized, finely crushed and analyzed, in triplicate, immediately after preparation and after fifteen, thirty, forty-five and sixty days of storage (at room temperature and packed in a sealed plastic bag covered with aluminum paper).

Evaluation of the colour parameters of the biscuit samples along storage time

The colour of the samples was measured in three different points on the top, for each sample, 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) (Fernandes, Antonio, Barreira, Oliveira, Martins, & Ferreira, 2012).

Evaluation of the nutritional properties

Proximate composition with reference to the contents of protein (N×5.70, AOAC 978.04), fat (AOAC 920.85) and ash (AOAC 923.03), was determined following AOAC methods (AOAC, 2005). Total energy was calculated following the equation: Energy (kcal) = 4 × (g proteins + g carbohydrates) + 9 × (g lipids). Fatty acids were determined, after Soxhlet extraction, 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 (Barros et al., 2013). Free sugars were determined in defatted samples by HPLC coupled to a refraction index (RI) detector (Barros et al., 2013), identified by comparison with standards, and further quantified (g/100 g of biscuit) considering the internal standard (melezitose).

Evaluation of the antioxidant activity of the biscuit samples

All lyophilized samples (3 g) were extracted for 1 h, using a procedure previously described by Caleja et al. (2016b). After recovering, the extracts were dissolved in methanol in a final concentration of 200 mg/mL. The antioxidant activity evaluation was performed using two *in vitro* assays: 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity and reducing power (RP), following the experimental methodologies adopted by the authors (Caleja et al., 2016a,b).

Statistical analysis

The experimental data was checked for normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test) assumptions. When it was not possible to apply an analysis of variance (ANOVA) and for data that did not follow a normal distribution, non-parametric tests were performed to evaluate significant differences at a level of 5, using the EXCEL software, Microsoft Office Professional Plus 2010, version 14.0.7159.5000, with the add-in Analysis ToolPak (Microsoft Corp., USA).

Results and discussion

The different types of biscuits (control, incorporated with BHA, fennel, fennel_10, chamomile, and chamomile_10) were analyzed in five storage times (0, 15, 30, 45 and 60 days) and the results are presented as the mean value of each storage time (ST) regardless of the incorporated additive, and also the mean of each additive (A) regardless of the storage time (**Table 19-Table 22**). In this way it is possible to determine the best additive regardless of cookie storage time and vice versa. So, the standard deviations presented in the tables should not be regarded as a measure of accuracy of the methodologies, since they include the results for one of the factors (ST or A).

Colour is an important quality attribute for consumer acceptance of bakery products. In bakery, colour can give indication when cooking is complete, besides that colour changes during storage provide information about its freshness (Mamat, Abu Hardan, & Hill, 2010). Table 19 presents the results for the different colour parameters of the biscuits (L^*, a^*, b^*). The total instrumental colour is the contribute of L^*, a^* and b^* values (Fernandes et al., 2012), where for this particular case the contribute of a^* value is less relevant. With storage time, it was observed a slight diminish of yellow colour (b^*-value) and no statistical significant changes of lightness (L^*-value). For different additives, the lower values of L^* are presented by the biscuits with Fennel (Fen10) and Chamomile (Cham10). And the higher values for b^* parameter are presented by the samples Control (C), Fennel (Fen) and Chamomile (Cham10). Some authors affirm that the colour of biscuits may result from Maillard reactions between

reducing sugars and amino acids (Mundt, & Wedzicha, 2007; Köksel, & Gökmen, 2008; Pasqualone, Bianco, Paradiso, Summo, Gambacorta, & Caponio, 2014). The colour of the additive may influence the biscuits colour. For example, incorporation of bee pollen caused a darkening in the surface of the biscuit (Krystyjan, Gumul, Ziobro, & Korus, 2015), but in this study it was not possible to observe any difference between samples (with or without additives), what could be considered a positive aspect, if it is intended to maintain the traditional aspect of the biscuits.

Table 19. Colour parameters of the biscuits in relation to storage time (ST) and food additive (A) for top side.

		*L**	*a**	*b**
Storage time (ST)	0 days	75.59 ± 2.59 a	5.24 ± 2.37 a	29.73 ± 3.09 a
	15 days	74.91 ± 2.44 a, b	5.08 ± 1.50 a	28.32 ± 2.10 b
	30 days	76.15 ± 1.58 a	4.69 ± 1.44 a	29.13 ± 2.45 a
	45 days	76.60 ± 2.24 a	4.50 ± 1.81 a	28.16 ± 2.25 b
	60 days	76.01 ± 2.16 a	4.45 ± 1.75 a	28.29 ± 1.74 b
Additive (A)	C	75.35 ± 2.15 a, b	5.81 ± 2.54 a	30.61 ± 3.10 a
	BHA	76.48 ± 2.11 a	3.80 ± 1.14 b	28.21 ± 1.69 b
	Fen	76.53 ± 2.04 a	5.07 ± 1.31 a	29.54 ± 2.07 a
	Fen10	74.71 ± 2.64 b	5.61 ± 1.39 a	27.42 ± 1.92 b
	Cham	76.78 ± 2.01 a	4.22 ± 1.71 b	27.13 ± 1.95 b
	Cham10	75.26 ± 1.98 b	4.24 ± 1.57 b	29.46 ± 1.52 a

The results are presented as mean ± SD. *L**: Lightness, *a**: red-green, *b**: yellow-blue. Control (C); Butylated hydroxyl anisole (BHA); Fennel in the same amount of BHA (Fen) or Fennel ten times more (Fen10); Chamomile in the same amount of BHA (Cham) or Chamomile ten times more (Cham10).
In each column, within each storage period and within each additive, different letters mean statistical significant differences ($p < 0.05$).

Table 20 shows the content of macronutrients, sucrose and energy of the biscuits during storage and with different additives. It is possible to verify that the carbohydrates are the most abundant macronutrients in biscuits. The results are presented as the mean value of each storage time (ST) regardless of the additive incorporated, and also the mean of each additive (A) regardless of the storage time. With this type of statistical analysis and representation, the best additive (control, BHA, fennel decoction and chamomile decoction) could be determined independently of the storage time, but also the influence of the storage

time irrespectively of the additive type. When it was observed a variation in both factors the samples were not classified. Regarding carbohydrates, samples with additives, synthetic or natural, were similar along storage time, except immediately after cooking. For proteins, the samples with chamomile additive were similar to control samples, but the higher protein content observed in samples with fennel additive highlights its higher protective role regarding this parameter. Carbohydrates did not vary with additive type. Only some differences were observed with storage time, with lower values registered after 15 days of storage. One free sugar was detected in the studied samples: sucrose, which was also one of the ingredients used in biscuits preparation.

Table 21 shows the six most abundant fatty acids detected in biscuits samples. Although twenty-three fatty acids were identified in the studied samples, seventeen were detected only in trace amounts (data not shown). The most abundant fatty acids were linoleic acid (C18:2n6) and oleic acid (C18:1n9). The prevalence of these fatty acids would be expected since the literature confirming the predominance of oleic and linoleic acid in the composition of hen eggs (Belitz, Grosh, & Schieberle, 2009) and flour (Nikolić, Sakač, & Mastilović, 2011). Unsaturated fatty acids (polyunsaturated followed by monounsaturated) were found in higher amounts than saturated fatty acids.

In the present study, the samples with natural additives revealed higher values of PUFA, and lower values of SFA (probably related with a higher inhibition of lipid peroxidation). As can be observed in **Table 21** PUFA with storage time and biscuits additive, its variation is dominated by the sample type or additive, and not by the storage time.

Table 20. Macronutrients, free sugars composition (g/100 g) and energy value (kcal/100 g) of the biscuits in relation to storage time (ST) and food additive (A).

		Moisture	Fat	Protein	Ash	Carbohydrates	Energy	Sucrose
Storage time (ST)	0 days	4.08 ± 0.55 a	1.45 ± 0.07 b	8.16 ± 0.53 a	1.03 ± 0.11 b	87.58 ± 2.22 a	386.81 ± 2.22 a	30.85 ± 0.62 b
	15 days	3.99 ± 0.46 a	1.51 ± 0.05 b	8.46 ± 0.44 a	1.04 ± 0.14 b	85.00 ± 0.50 b	387.43 ± 1.76 a	32.96 ± 1.07 a
	30 days	4.33 ± 0.50 a	1.31 ± 0.10 c	8.35 ± 0.57 a	1.37 ± 0.15 a	84.64 ± 0.50 b,c	383.74 ± 2.46 b	31.68 ± 1.76 b
	45 days	4.49 ± 0.98 a	1.45 ± 0.27 b,c	8.50 ± 0.49 a	1.40 ± 0.12 a	84.17 ± 0.90 c	383.68 ± 4.00 b	30.93 ± 0.86 b
	60 days	4.21 ± 1.63 a	1.74 ± 0.17 a	8.49 ± 0.56 a	1.06 ± 0.12 b	84.50 ± 1.11 b,c	387.64 ± 6.83 a,b	31.02 ± 0.63 b
Additive (A)	C	3.91 ± 0.54 a	1.63 ± 0.20 a	8.28 ± 0.29 b	1.15 ± 0.21 a	85.02 ± 0.65 a	387.91 ± 2.58 a	31.92 ± 0.93 a
	BHA	4.86 ± 1.28 a	1.55 ± 0.16 a	8.10 ± 0.35 b	1.29 ± 0.26 a	84.68 ± 2.07 a	383.15 ± 4.68 b	30.30 ± 1.08 b
	Fen	3.64 ± 0.63 a	1.54 ± 0.16 a	8.99 ± 0.60 a	1.12 ± 0.22 a	85.53 ± 1.95 a	388.67 ± 2.83 a	31.78 ± 1.22 a
	Fen10	3.51 ± 0.70 a	1.48 ± 0.22 a,b	8.78 ± 0.31 a	1.14 ± 0.15 a	85.56 ± 1.41 a	388.77 ± 3.91 a	31.45 ± 0.38 a
	Cham	4.56 ± 0.37 a	1.41 ± 0.24 a,b	8.15 ± 0.36 b	1.19 ± 0.20 a	85.31 ± 1.86 a	384.05 ± 2.91 b	31.41 ± 2.00 a
	Cham10	4.83 ± 0.69 a	1.35 ± 0.14 a	8.04 ± 0.36 b	1.20 ± 0.19 a	85.16 ± 2.09 a	382.62 ± 2.91 b	32.07 ± 1.08 a,b

In each column, within each storage period and within each additive, different letters mean statistical significant differences ($p < 0.05$). Control (C); Butylated hydroxyl anisole (BHA); Fennel in the same amount of BHA (Fen) or Fennel ten times more (Fen10); Chamomile in the same amount of BHA (Cham) or Chamomile ten times more (Cham10).

Table 21. Fatty acids composition of the biscuits (expressed as relative percentage of each fatty acid) in relation to storage time (ST) and food additive (A).

		C16:0	C16:1	C18:0	C18:1n9	C18 :2n6	C18:3n3	SFA	MUFA	PUFA
Storage time (ST)	0 days	20.51 ± 0.93 a	1.91 ± 0.19 a	3.89 ± 0.42 a	28.63 ± 1.42 a	39.72 ± 2.27 a	2.57 ± 0.37 a	25.33 ± 1.25 a	31.15 ± 1.54 a	43.52 ± 2.52 a
	15 days	20.57 ± 0.82 a	1.97 ± 0.18 a	3.82 ± 0.40 a	28.76 ± 1.63 a	39.65 ± 2.43 a	2.54 ± 0.37 a	25.32 ± 1.16 a	31.32 ± 1.72 a	43.35 ± 2.68 a
	30 days	20.63 ± 0.98 a	1.99 ± 0.20 a	3.77 ± 0.40 a	28.61 ± 1.56 a	39.73 ± 2.42 a	2.56 ± 0.36 a	25.27 ± 1.27 a	31.20 ± 1.64 a	43.54 ± 2.67 a
	45 days	20.94 ± 0.94 a	1.93 ± 0.16 a	3.92 ± 0.45 a	28.51 ± 1.62 a	39.46 ± 2.47 a	2.44 ± 0.36 a	25.27 ± 1.26 a	31.07 ± 1.73 a	43.17 ± 2.61 a
	60 days	20.85 ± 0.84a	1.86 ± 0.29 a	4.10 ± 0.35 a	27.51 ± 2.20 a	40.09 ± 2.41 a	2.52 ± 0.33 a	25.76 ± 1.36 a	29.97 ± 2.47 a	44.18 ± 2.99 a
Additive (A)	C	21.65 ± 0.18 b	2.01 ± 0.04 b	4.32 ± 0.10 a	30.46 ± 0.41 a	36.73 ± 0.19 e	2.07 ± 0.03 e	26.88 ± 0.15 a	33.07 ± 0.39 a	40.05 ± 0.31 a
	BHA	20.45 ± 0.63 d	2.10 ± 0.07 a	3.81 ± 0.29 c	29.36 ± 0.59 b	38.88 ± 1.03 c	2.63 ± 0.28 b	25.13 ± 0.90 d	32.10 ± 0.56 b	42.77 ± 1.21 c
	Fen	21.91 ± 0.23 a	2.08 ± 0.05 a	3.88 ± 0.11 b	28.01 ± 0.73 c	39.15 ± 0.42 c	2.15 ± 0.04 d	26.72 ± 0.32 b	30.67 ± 0.78 c	42.61 ± 0.57 c
	Fen10	19.81 ± 0.34 e	1.55 ± 0.08 d	3.76 ± 0.34 c	26.86 ± 1.20 d	42.51 ± 0.51 b	2.64 ± 0.03 c	24.51 ± 0.65 e	29.01 ± 1.31 d	46.48 ± 0.82 b
	Cham	19.73 ± 0.18 e	1.85 ± 0.14 c	3.37 ± 0.36 d	26.23 ± 0.58 e	42.97 ± 0.34 a	3.03 ± 0.08 a	24.00 ± 0.43 f	28.71 ± 0.73 e	47.29 ± 0.45 a
	Cham10	20.64 ± 0.18 c	2.00 ± 0.12 b	4.28 ± 0.22 a	29.49 ± 1.28 a,b	38.12 ± 0.68 d	2.65 ± 0.07 c	25.80 ± 0.44 c	32.09 ± 1.36 a,b,c	42.11 ± 1.02 c

The results are presented as mean±SD. Palmitic acid (C16:0); Palmitoleic acid (C16:1); Stearic acid (C18:0); Oleic acid (C18:1n9); Linoleic acid (C18:2n6); α-linolenic acid (C18:3n3); SFA- Saturated fatty acids; MUFA- Monounsaturated fatty acids; PUFA- Polyunsaturated fatty acids. Control (C); Butylated hydroxyl anisole (BHA); Fennel in the same amount of BHA (Fen) or Fennel ten times more (Fen10); Chamomile in the same amount of BHA (Cham) or Chamomile ten times more (Cham10).

In each column, within each storage period and within each additive, different letters mean significant statistical differences ($p < 0.05$).

The results of reducing power (RP) and DPPH radical scavenging activity of the biscuits samples during storage are presented in **Table 22**. As expected, the control sample showed the lower reducing power capacity and the EC_{50} value of samples fortified with plant extracts decreased with the increase of the quantity of the extract. Natural additives with higher concentration revealed a strong RP (i.e., lower EC_{50} values). It was also possible to verify that storage time had no influence in RP and just have influence after 60 days in DPPH scavenging activity. Along storage time, after 60 days, there was a slight tendency for antioxidant activity reduction (higher EC_{50} values). However, relevant variations could be observed within a longer period of time. The antioxidant activity was higher (lower EC_{50} value) when the concentration of natural additive was higher (Fen10 and Cham10) for both *in vitro* assays, DPPH and Reducing Power, but in the same order of magnitude as the synthetic additive BHA. However, the presence of the natural extracts can bring some additional benefits (Caleja et al., 2015a; Caleja et al., 2015b) and without deleterious effects.

Table 22. Antioxidant activitity (EC_{50} values expressed in mg/mL) in relation to storage time (ST) and food additive (A).

		DPPH	Reducing Power
Storage time (ST)	0 days	128.55 ± 62.28 b	83.41 ± 32.15 a
	30 days	149.24 ± 47.91 b	88.38 ± 35.56 a
	60 days	171.40 ± 31.68 a	99.04 ± 36.66 a
Additive (A)	C	> 200 a	135.13 ± 3.69 a
	BHA	88.32 ± 29.38 e	53.54 ± 9.91 d
	Fen	191.73 ± 9.68 b	121.43 ± 16.64 b
	Fen10	120.73 ± 35.00 c,d	62.60 ± 3.52 c
	Cham	185.15 ± 8.78 b	113.39 ± 11.07 b
	Cham10	104.77 ± 31.41 d	55.57 ± 2.42d

Control (C); Butylated hydroxyl anisole (BHA); Fennel in the same amount of BHA (Fen) or Fennel ten times more (Fen10); Chamomile in the same amount of BHA (Cham) or Chamomile ten times more (Cham10).
The results are presented as mean±SD. In each column, within each storage period and within each additive, different letters mean statistical significant differences ($p < 0.05$).

A similar study incorporating fresh peel extracts of two different varieties of mango in biscuits demonstrated higher antioxidant properties, when compared to BHA (Ajila et al., 2007; Ajila et al., 2008). Alternative and promising natural antioxidants have been used by other authors, but obtained by methanolic extraction (Reddy et al., 2005; Bajaj et al., 2006). These natural additives were obtained by aqueous extraction, a more environment friendly and safer process to obtain food additives (Reddy et al., 2005). A recent study described the high antioxidant activity of functional biscuits prepared with wheat purple, due to its high levels of bioactive compounds (Pasqualone et al., 2015). Other previous studies have proven the

capacity of fennel and chamomile extracts to bring antioxidant activity to cottage cheese and yogurt (Caleja et al., 2015a; Caleja et al., 2015b; Caleja et al., 2016b).

Conclusions

Bakery industry is in constant innovation and biscuits are products worldwide appreciated and consumed by different categories of consumers. Therefore, the production of this type of product enriched with natural sources of antioxidants may be attractive for consumers who are increasingly concerned about the choice of healthy foods. This study revealed that the addition of fennel and chamomile decoctions improves the antioxidant activity of biscuits, bringing beneficial advantages without adverse effects, contrary to what has been reported for the synthetic additive BHA. It should be noted that the incorporation of these aqueous extracts caused no significant changes in appearance or nutritional profile of biscuits thus proving that the use of such extracts can be important for the release of new healthier pastry products on the market.

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3.2.3. Efeitos da incorporação de extratos de funcho e camomila na palatabilidade dos biscoitos: avaliação da aceitabilidade por análise sensorial

Submitted: *Food Quality and Preference*

Effects of fennel and chamomile extracts incorporation in the palatability of biscuits: acceptability evaluation by sensory analysis

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Abstrat

Current consumers' trends point towards food products incorporating natural bioactive agents, preferably absent from synthetic additives. Using plant-based ingredients is generally well-accepted, since these compounds provide pleasant properties in addition to their functional effect. However, when developing new foods, it is important to compare different recipes, aiming obtaining suitable products. Herein, lyophilized decoctions from fennel or chamomile were used (in different concentrations) to functionalize biscuits. Owing to the potential preserving effect of these natural extracts additional formulations were prepared with BHA. All prepared samples (including blank formulations) were evaluated by a panel of 120 tasters, who answered a check-all-that-apply questionnaire. Despite the similarity of prepared biscuits, those containing the highest concentration of chamomile or fennel were generally preferred. Besides their better palatability, these biscuits might be considered new functional foods, owing to the health-promoting properties of bioactive compounds naturally present in both decoctions. In addition, all assayed sensory parameters were evaluated as predictor variables to assess the "predisposition to buy" each biscuit, resulting that "global quality" and "taste" were classified as the best predictors, as indicated by their higher relevance in tasters'

intention to buy the tested biscuits. The present findings might offer a new production strategy for health-promoting biscuits with validated acceptability.

Keywords: biscuit formulation; synthetic *vs*. natural additives; chamomile; fennel; consumers' acceptability.

Introduction

The diet-related health status represents and increasing awareness among consumers. Some of the current trends demand safe and health-promoting natural ingredients, mostly obtained from plant species (Krishnaiah, Sarbatly, & Nithyanandam, 2011; Patil, Rudra, Varghese, & Kaur, 2016). In fact, using aqueous plant extracts as a substitute for synthetic additives may represent an alternative to produce healthier foods, meeting the consumers' expectations (Baltsavias, Jurgens, & van Vliet, 1999). Some of these expectations are exemplified by reducing fat levels, increasing protein content, substituting sugars by natural sweeteners, using natural dyes or natural preservatives, thereby decreasing the need for synthetic ingredients (Baltsavias et al., 1999). Furthermore, product labeling has a high impact on consumers, who progressively seek products with the least number of synthetic ingredients (Vázquez, Curia, & Hough, 2009). Although the evolution in biscuit producers concerns, mainly oriented to improve the nutritional composition by reducing fat and sugar content, some authors describe that this type of biscuits are usually less appreciated by consumers, although the growing demand for healthier foods (Biguzzi, Schlich, & Lange, 2014; Biguzzi, Lange, & Schlich, 2015). In fact, there are several factors that lead consumers to have higher acceptability for functional products: i) greater knowledge related to health and well-being; ii) the labeling of this type of food is associated with more natural and healthier products; iii) familiarity and confidence in a brand (Bimbo et al., 2017).

Usually, products enriched with vegetable extracts are very well-accepted, because the potential changes induced in the sensory quality of the product tend to please consumers (Krystyjan, Gumul, Ziobro, & Korus, 2015; Pasqualone, Bianco, Paradiso, Summo, Gambacorta, & Caponio, 2014). In previous studies (Caleja et al., 2015a,b), the aqueous extracts of fennel and chamomile revealed high antioxidant and antimicrobial activities, most likely due to the presence of phenolic compounds such as di-caffeoyl-2,7-anhydro-3-deoxy-2-octulopyranosonic acid and luteolin-*O*-glucuronide in fennel and quercetin-3-*O*-glucoside and 5-*O*-caffeolylquinic acid in chamomile. Therefore, incorporating fennel or chamomile decoctions in biscuit' formulations might be considered as a feasible way of attaining a new functional food.

However, changing the traditional formulation of a determined food product can modify its properties, which, in turn, might affect the acceptability of the newly developed product (Baltsavias et al., 1999). Actually, changes in food composition might cause significant effects in texture, mouthfeel and palatability (Ambigaipalan & Shahidi, 2015; Tarancón, Salvador, Sanz, Fiszman, & Tárrega, 2015), driving food companies to concern about how consumers appreciate the sensory characteristics of the product and which attributes define their acceptability, with the general purpose of designing food products that entirely fulfil consumers' expectations (Guinard, Uotani, & Schlich, 2001; Ten Kleij & Musters, 2003). Therefore, sensory panel studies are mandatory in the process of developing any new food formulation (Ten Kleij, & Musters, 2003; Tarancón et al., 2015).

Accordingly, this work was planned to evaluate the sensory properties of traditional biscuits incorporating natural ingredients obtained from *Foeniculum vulgare* Mill. (fennel) and *Matricaria recutita* L. (chamomile), and their further comparison with formulations including the synthetic additive butylated hydroxyl anisole (BHA), labeled as E320. In addition, the main purpose of the work developed herein was achieving a formulation with improved health effects, while maintaining, or ideally enhancing, its organoleptic quality. The sensory analysis of all prepared formulations was performed by a group of 120 tasters that answered a 10 parameter questionnaire (mostly based in a 9-level hedonic scale).

Materials and methods

Preparation of chamomile and fennel extracts

Américo Duarte Paixão Lda. (Vale da Trave, Santarém, Portugal) provided the commercial samples of *Matricaria recutita* L. (chamomile) and *Foeniculum vulgare* Mill. (fennel) aerial parts. To prepare the decoctions, 5 g of dry and crushed (~20 mesh; Ultra Centrifugal Mill ZM 200, Porto, Portugal) vegetal material were added to 200 mL of distilled water (Milli-Q water purification system, TGI Pure Water Systems, Greenville, SC, USA) and put in a heating plate (VELP Scientific, Usmate, Italy) until reaching boiling point. Samples were then boiled for 5 min and left to stand until cooling down to room temperature. Afterwards, the solutions were filtered, frozen and lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA).

Preparation of biscuits incorporating natural extracts or synthetic preservatives

The base recipe of biscuits included 4 traditional ingredients: egg, sugar, wheat flour and water. The batter was prepared by sequentially mixing (MFQ 3540, Bosch, Munich, Germany) 1 egg, sugar (125 g/egg), plain water (60 mL/egg) or antioxidant solution (60 mL/egg

of an aqueous solution containing: ii) 80 mg of lyophilized fennel decoction iii) 800 mg of lyophilized fennel decoction, iv) 80 mg of lyophilized chamomile decoction, v) 800 mg of lyophilized chamomile decoction, or vi) 80 mg of BHA) and flour (300 g/egg). The batter was then left to stand for 15 minutes and further stretched until having a uniform thickness of 5 mm. A circular stainless steel cutter (50 mm) was used to shape the biscuits. A total of 750 biscuits (30 extra biscuits, 5 for each formulation, were produced to prevent any possible loss, guaranteeing samples for 120 tasters) were prepared: i) control biscuits (C); ii) biscuits with 80 mg of fennel extract (F80); iii) biscuits with 800 mg of fennel extract (F800); iv) biscuits with 80 mg of chamomile extract (Ch80); v) biscuits with 800 mg of chamomile extract (Ch800); vi) biscuits with 80 mg of BHA (BHA). Biscuits were placed in trays and baked at 180 °C for 10 minutes (HE-635, Teka, Haiger, Germany). The sensory analysis was conducted in the same day of biscuit preparation.

Sensory analysis

A total of 120 (three of which were excluded) consumers (87 women and 31 men) from 6 to 75 years old participated in the study. All participants accepted voluntarily to do the evaluation and also to be involved in the study. The preparation and presentation of samples were appropriate for the product, as well as the proposed questionnaire, according to ISO 16820:2004 and ISO 6658:2005. Each cookie was identified with a three-digit number randomly generated by a computer program. The six biscuit samples were served in two different plastic dishes (3/dish) and presented monadically, following a Williams design. A glass of water (neutral, tasteless and odorless) at room temperature was available for tasters, who were asked to swallow samples and to rinse their mouths with water to cleanse their palate between samples (Tarancón et al., 2015). For each sample, consumers had to score the acceptability using a nine-point hedonic scale to evaluate “color” (average intensity of color), “appearance” (uniformity of the surface of biscuit), “odor” (intensity of aroma), “chewiness” (force required to chew), “crunchiness” (energy with which the biscuit makes crack-crunch-bang during the first two or three bites), “taste”(total intensity of taste that persists in the mouth after swallowing the sample), “sweetness” (sweet flavor intensity) and “overall quality” (assessment taking into account all the characteristics analyzed). Additionally, consumers classified the presence or absence of noticeable defects and express their “buying predisposition” as “yes”, “maybe” or “no”. The values were recorded on the evaluation sheet for further data compilation and analysis.

Statistical analysis

Wherever possible, data were expressed as mean±standard deviation. All statistical tests were performed at a 5% significance level using IBM SPSS Statistics for Windows, version 22.0. (IBM Corp., USA).

An analysis of variance (ANOVA), followed by Tukey's test (homoscedastic distributions) or Tamhane's T2 test (heteroscedastic distributions) was used to classify the statistical differences among the mean scores obtained for each biscuit formulation. The fulfillment of the one-way ANOVA requirements, specifically the normal distribution of the residuals and the homogeneity of variance, was tested by means of the Shapiro Wilk's and the Levine's tests, respectively.

Different consumers' acceptability maps were obtained by applying principal components analysis (PCA). In this approach, the affinity (correlation) of each studied variable with different mathematical functions (principal components) is evaluated. The number of dimensions kept for data analysis was assessed by the respective eigenvalues (which should be greater than one), by the Cronbach's alpha value (that must be positive) and also by the total percentage of variance (that should be as high as possible) explained by the selected components. The principal components were plotted considering different grouping factors (biscuit consumption habits, age group and gender) to verify possible associations among preference results and any level of those factors. The number of plotted dimensions (three) was chosen for being the maximum number still allowing a straightforward interpretation.

Additionally, a linear discriminant analysis (LDA) was used to evaluate the parameters included in the sensory analysis ("color", "appearance", "odor", "chewiness", "crunchiness", "taste", "sweetness" and "overall quality") as potential predictors for the outcome variable "buying predisposition". The independent variables (predictors) were entered together and the usual assumptions of LDA were fully verified. To validate the significance of the canonical discriminating functions, Wilk's Λ test was used. A leaving-one-out cross validation procedure was carried out to assess the model performance.

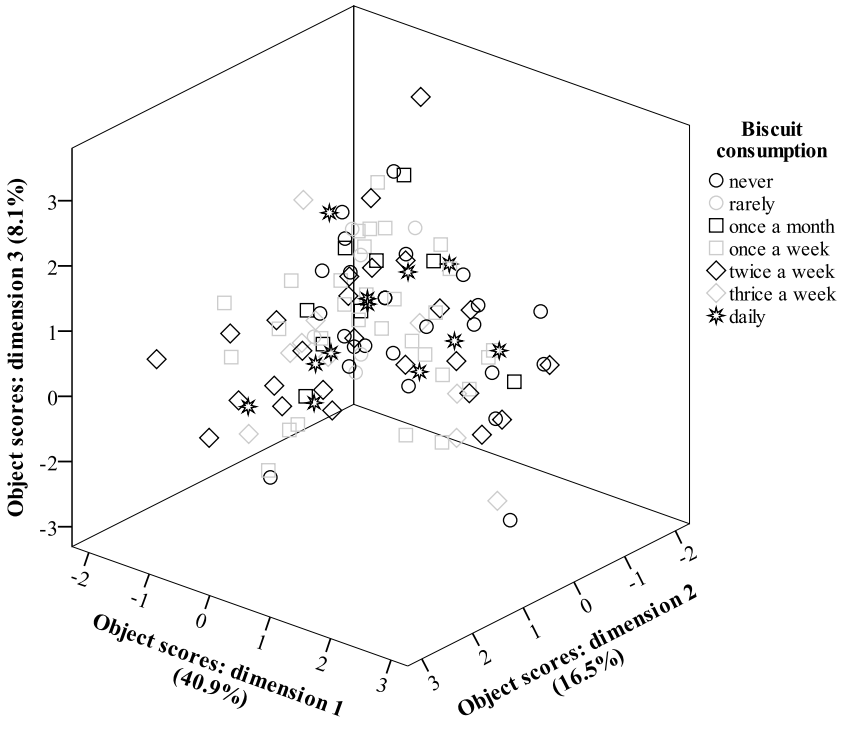
Results and discussion

A total of 117 valid questionnaires were obtained, answered by a group of individuals with ages varying from 6 to 75 years old, with high predominance of the age group between 26 and 55 years (74 individuals). Besides the nine evaluated sensory qualities ("color", "appearance", "presence of defects", "odor", "chewiness", "crunchiness", "taste", "sweetness" and "global quality"), the frequency of consumption of similar products and the future "predisposition to buy" each prepared biscuit formulations were also characterized.

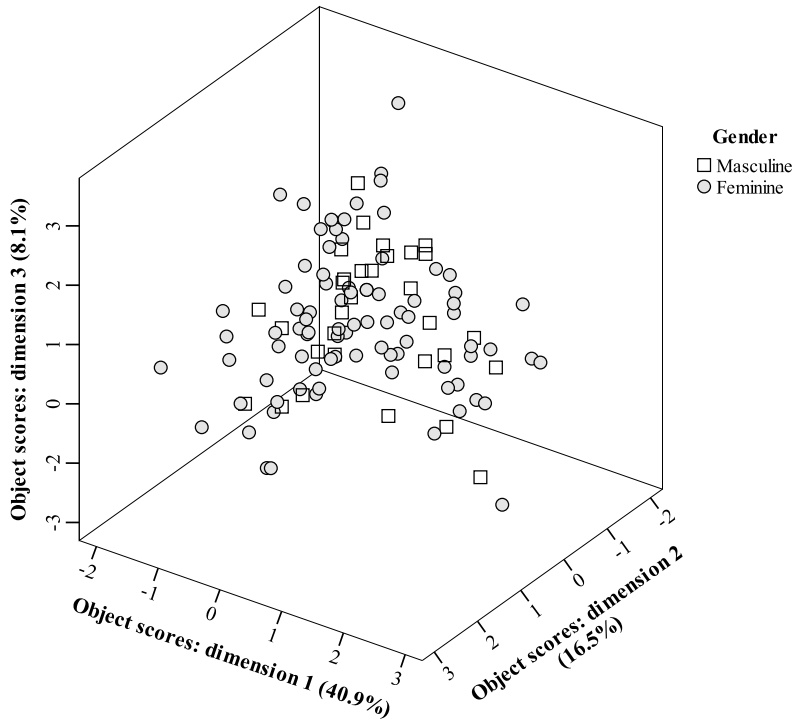
A high number of research studies has been dedicated to the analysis of the interaction among sensory and extrinsic product attributes and its effect in consumer preferences. In fact, this research field has been identified as one of the keystones to improve likelihood of food products' market success (Asioli et al., 2017). Likewise, when evaluating sensory characteristics in between-subjects experimental designs, the check-all-that-apply (CATA) questions are commonly used (Jaeger et al., 2017).

Accordingly, the present study was designed to study the interaction among sensory attributes and consumer preferences, in order to raise the potential acceptability of prepared biscuit formulations.

Different preference maps (**Figure 16**), explaining 65.5% of the overall variance, were obtained by principal component analysis. The presented maps resulted from plotting the three most significant functions (first: Cronbach's α = 0.962, eigenvalue = 18.545, explained variance = 40.9%; second: Cronbach's α = 0.870, eigenvalue = 6.908, explained variance = 16.5%, third: Cronbach's α = 0.714, eigenvalue = 3.355, explained variance = 8.1%). In order to verify if the preference was somehow linked to the frequency of biscuit consumption (**Figure 16A**), age class (**Figure 16B**) or gender (**Figure 16C**), the results were sequentially plotted after considering each of those three factors as labelling variables. As it might be observed, preference results were not influenced in any case, since no individual clusters corresponding to the levels of each factor were formed; *i.e.*, consumers were scattered randomly, thereby revealing a considerable homogeneity in their preferences. Since no visible separation was achieved, it would be meaningless to analyze correlations among studied factors and produced principal components.


Biscuit consumption
never
rarely
once a month
once a week
twice a week
thrice a week
daily
Object scores: dimension 3 (8.1%)
Object scores: dimension 1 (40.9%)
Object scores: dimension 2 (16.5%)

a)


Gender
Masculine
Feminine
Object scores: dimension 3 (8.1%)
Object scores: dimension 1 (40.9%)
Object scores: dimension 2 (16.5%)

b)

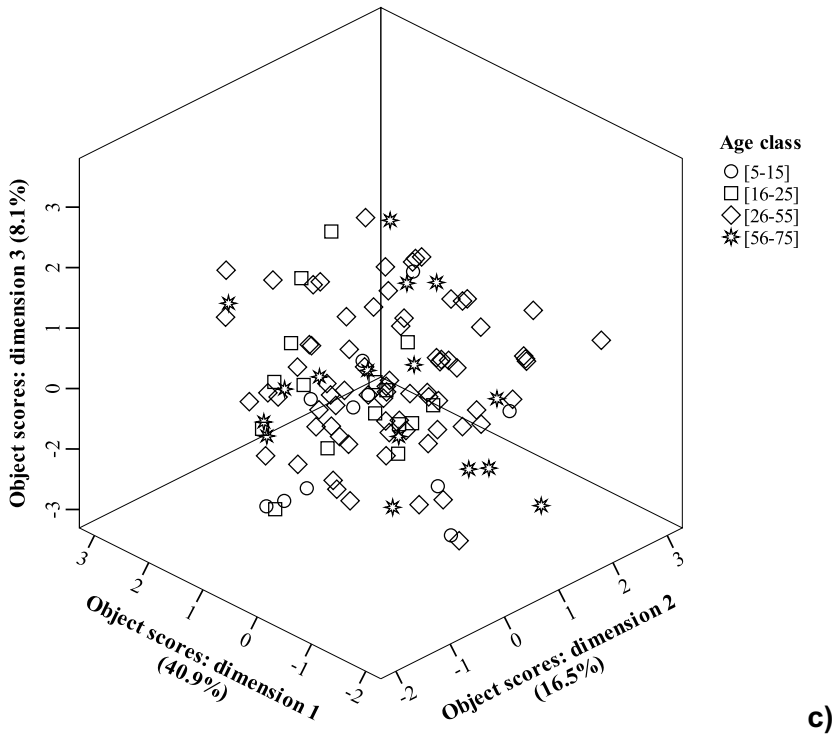


Figure 16. Consumers' preference maps considering "biscuit consumption frequency" **(a)**, "age group" **(b)** or "gender" as labelling variables **(c)**.

Besides the apparent similarity among tasters, the assayed formulations were also classified with close scores (**Table 23**). Nevertheless, some individual significant ($p < 0.05$) differences were found, specifically for "appearance" (F80, Ch80 and BHA had the highest scores), "crunchiness" (BHA and Ch80 had the best result, while C was the least appreciated) and "taste" (Ch800 was the preferred formulation, while C was classified with the worst mark). For the remaining parameters ("color", "odor", "chewiness", "sweetness" and "global quality"), biscuits were generally scored with half-scale values, indicating that tasters liked these biscuits, but did not became exceptionally pleased (or unpleased) with their characteristics. These average scores obtained in quality descriptors, could somehow be explained by the simple recipe that was employed, especially when compared to the plethora of similar products available in market.

However, the option for a plain recipe is justified by the purpose of verifying if the incorporation of natural preservatives could provide any type of unpleasant characteristics to a specific type of biscuit. Actually, preparing formulations with a low number of ingredients is probably the most reasonable approach to ensure the maximum reliability of results. Combining a higher number of ingredients could somehow mask potential changes induced by the incorporation of the selected extracts. In turn, the prepared formulations were kept as

simple as possible, so that any potential organoleptic change could be easily detected by tasters' panel.

The fact of using a simple formulation might also justify the similar results obtained in evaluations performed by tasters with different characteristics (*e.g.*, their age or biscuit consumption habits). Even so, some significant differences were found among biscuits incorporating any kind of preservative and the corresponding blank formulation (**Table 23**). In those cases, the most noteworthy result is the higher score that biscuits including chamomile extract at the highest concentration got in "taste" parameter. In a similar study (Bajaj *et al.* 2006), where different biscuits were added with powdered mint, the main difference among blank and functionalized formulations was linked to the improved texture of biscuits incorporated with mint powder. Nevertheless, those biscuits presented a pale green color, which, despite not having caused a great reduction in their acceptability, might represent an important limitation in terms of market implementation. This particular issue was not a problem in the case of biscuits prepared in the current study, since chlorophyll pigments are not extracted with water.

Table 23. Average scores for sensory qualities and buying predisposition of each biscuit formulation. Except for the "presence of defects" and "buying predisposition", the results are presented as mean±SD.[1]

		Color	Appearance	Odor	Chewiness	Crunchiness	Taste	Sweetness	Global quality
Formulation	Control	4±2	4±2 b	4±2	4±2	4±2 b	4±2 b	5±2	6±2
	Ch80	4±2	6±2 a	4±2	5±2	5±2 a	5±2 ab	5±2	6±2
	Ch800	4±2	4±2 b	4±2	5±2	5±2 ab	5±2 a	5±2	6±2
	F80	4±2	6±2 a	4±2	5±2	4±2 ab	4±2 ab	5±2	6±2
	F800	4±2	4±2 b	4±2	5±2	4±2 ab	5±2 ab	5±2	6±2
	BHA	4±2	5±2 a	4±2	5±2	5±2 a	5±2 ab	5±2	6±2
Homoscedasticity[2]	*p*-value (n = 36)	0.707	0.152	0.531	0.488	0.834	0.703	0.811	0.685
One-way ANOVA[3]	*p*-value (n = 36)	0.156	<0.001	0.624	0.562	0.012	0.011	0.335	0.060

[1]Differences among means were evaluated using the Tukey's HSD (homoscedastic distribution) or the Taman's T2 (heteroscedastic distribution) multiple comparison tests.

[2]Homoscedasticity among formulations was tested by means of the Levene test: homoscedasticity, p-value>0.05; heteroscedasticity, p-value<0.05.

[3]p<0.05 meaning that the mean value of the evaluated parameter of at least one formulation differs from the others (in this case multiple comparison tests were performed).

In general, the comparison among formulations was made more difficult by the high standard deviations obtained in each parameter. From a mathematical point of view, the mean value is limited by the magnitude of standard deviation. If, for instance, the standard deviation is in the units range, there is no sense in presenting decimal places in the mean value. Therefore, we could not present decimal places in mean values described in **Table 23**. Accordingly, to attain a more accurate understanding of values obtained for each parameter, as also to obtain a global overview of all studied descriptors, collected results were presented simultaneously in **Figure 17**. As it might be observed, and despite the statistical difference in ANOVA results, biscuits prepared with the highest concentrations of chamomile and fennel stood out for their top scores in "taste", "sweetness" and, most of all, "overall quality", which indicates that "taste" and "sweetness" are key factors to define the "overall quality" of biscuits. This conclusion is reinforced by the lower scores obtained for Ch800 and F800 in other parameters such as "appearance", which did not cause a decrease in the scores obtained for "overall quality".

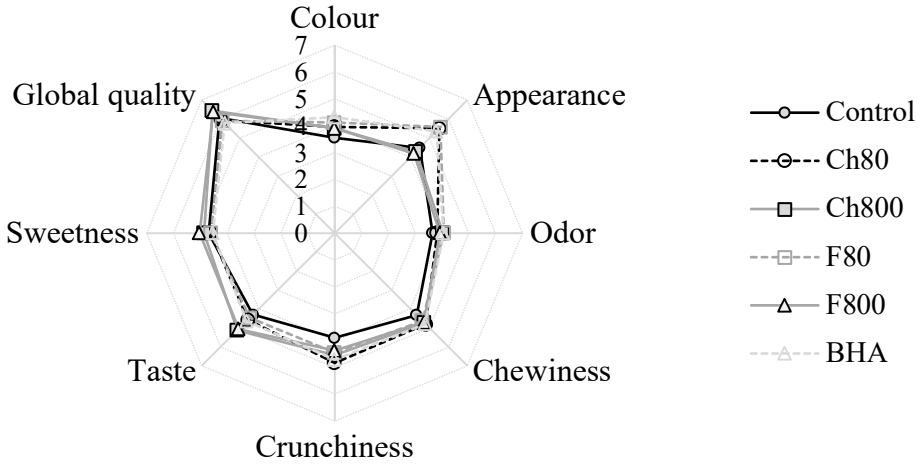


Figure 17. Overall scores for all evaluated sensorial parameters.

In either case, these results should be considered together with those obtained regarding the presence of visible defects in biscuits, as well as the ones gathered in the question aiming to evaluate the "predisposition to buy" each tested biscuit formulations. The C formulation registered the highest number (35) of biscuits classified as having some kind of visual defect, followed by Ch800 (31), Ch80 (25), BHA (24), F80 (23) and F800 (20). Concerning buying attitude, BHA formulation got the worst results, considering that 32 tasters answered as not wanting to buy those particular biscuits (37 answered "yes" and 48 "maybe");

C formulation was the second worst ("no": 24; maybe: 49; yes: 44), followed by F80 ("no": 23; maybe: 49; yes: 45), Ch80 ("no": 22; maybe: 54; yes: 41), Ch800 ("no": 18; maybe: 42; yes: 57) and F800 ("no": 12; maybe: 57; yes: 48).

In addition, and in order to find the sensory parameters with the highest usefulness as predictor variables for results obtained in "predisposition to buy" (which was used as outcome variable), a linear discriminant analysis was performed.

As a first remark, it should be highlighted that the number of observations (117 tasters × 6 formulations = 702) fulfill the rule of thumb indicating that the number of observations must be at least as high as five times the number of predictor variables (8 in this case). Before the analysis, the typical LDA assumptions were verified. The predictor variables ("color", "appearance", "odor", "chewiness", "crunchiness", "taste", "sweetness" and "overall quality") were independent, normally distributed (according to Shapiro-Wilks test) and did not show multicollinearity (high correlation with each other). In this particular indicator, the correlations among variables ranged from 0.020 (among "chewiness" and "global quality") to 0.622 (among "sweetness" and "taste"). Likewise, and as indicated by Box's test of equality of covariance matrices, variances were equal among groups. No outliers were detected in the box-plots. Finally, the mutual exclusivity of group membership in the outcome variable ("buying predisposition") was guaranteed by the answer possibilities ("yes", "maybe" or "no").

In total, 131 "no" answers, 299 "maybe" answers and 272 "yes" answers were obtained. The two defined discriminant functions plotted in **Figure 18** explained 100% of observed variance (first: 98.2%, eigenvalue: 0.538; second: 1.8%, eigenvalue: 0.010). Considering the variance explained by each function, eigenvalues and Wilk's λ test significance (p<0.001), only function 1 was considered in the obtained model, since function 2 was not useful to justify the variability among scores obtained in CATA questionnaires.

Function 1 was mainly correlated with "global quality" (0.956) and "taste" (0.353), thereby indicating that these two parameters were the most important to predict the "buying predisposition" of participating tasters. The classification performance was not as accurate as it was intended, reaching 56.4% for originally grouped cases and 55.7% for cross-validated cases. Nevertheless, it might be considered that "global quality" and "taste" were in fact the most relevant variables in deciding whether buying (markers trendily distributed in the positive side of horizontal axis) or not (markers mainly located in the negative side of horizontal axis) the biscuits assayed in this study.

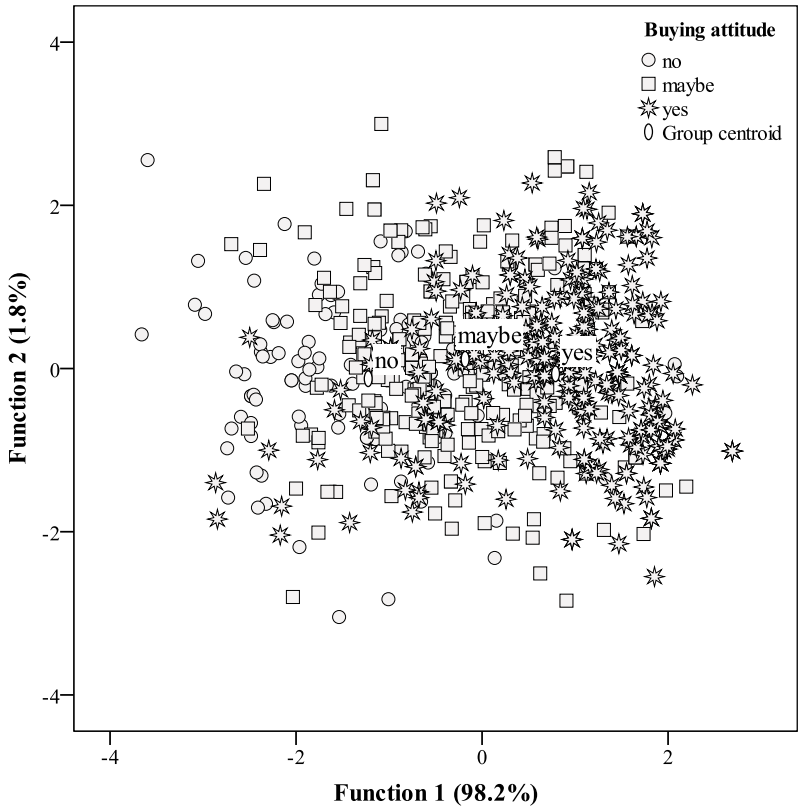


Figure 18. Mean scores of "predisposition to buy" answers distributed according to the discriminant functions defined from the evaluated sensorial parameters scores, which were used as potential predictor variables.

Conclusions

Despite the relatively high number of individuals in the consumer panel, which represents a vital factor to achieve a successful market product, the obtained conclusions are limited by the range of studied population sample, especially considering that sensory sensitiveness can be influenced by geographical conditions. Nevertheless, the preference maps obtained in this survey were not influenced by biscuit consumption frequency, age group or gender of tasters, which is a good indicator of the overall acceptance of results. Regarding the potential buy of the developed biscuits, the most important predictor variables were "taste" and "overall quality".

Overall, sensory analysis highlighted Ch800 as the best formulation, independently of tasters' gender, age or biscuit consumption frequency. Owing to the antioxidant and antimicrobial properties of the main compounds (quercetin-3-*O*-glucoside and 5-*O*-caffeolylquinic acid) in chamomile decoctions (but also in fennel decoctions), this natural ingredient might be considered as an advantageous alternative to synthetic additives such as

BHA, rendering interesting benefits from consumers' acceptability point of view, as well as considering its potential health-promoting properties. Therefore, the present findings could offer a new approach in biscuits production, especially by providing a product with higher healthiness resulting from using natural plant extracts as functionalizing agents.

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4.

Extratos ricos em compostos fenólicos para aplicação como aditivos naturais na indústria de pastelaria

Neste capítulo descreve-se a obtenção de extratos ricos em determinados compostos a partir de folhas *Melissa officinalis* L. (cidreira) e flores de *Castanea sativa* Mill. (castanheiro). Após a descrição detalhada de todo processo de otimização para a obtenção dos extratos é apresenta a caracterização química e avaliação das suas propriedades antioxidantes, antimicrobianas e de hepatotoxicidade para cada um. Neste capítulo descreve-se ainda o processo de incorporação destes extratos em produtos de pastelaria bem como, a avaliação dos efeitos dessa incorporação em comparação com aditivos artificiais.

4.1. **Desenvolvimento de um extrato rico em ácido rosmarínico obtido apartir de cidreira com aplicação como conservante natural**

Neste capítulo descreve-se a otimização da extração de ácido rosmarínico obtido a partir de *Melissa officinalis* L. (cidreira) e apresenta-se a sua caracterização química e avaliação das suas propriedades antioxidantes, antimicrobianas e hepatotoxicidade. A incorporação deste extrato em *cupcakes* e a avaliação do efeito da sua incorporação comparativamente com um aditivo artificial é também apresentada neste capítulo.

4.1.1. Extração de ácido rosmarínico a partir de *Melissa officinalis* L. por técnicas de extração assistida por calor, micro-ondas e ultrassons: um estudo comparativo através de análise de superfície de resposta

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Extraction of rosmarinic acid from *Melissa officinalis* L. by heat-, microwave- and ultrasound-assisted extraction techniques: A comparative study through response surface analysis

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Abstrast

The goal of this study was to compare the extraction of rosmarinic acid from *Melissa officinalis* L. using three techniques (heat-, microwave- and ultrasound- assisted extraction). In order to obtain the conditions that maximize the rosmarinic acid extraction, a response surface methodology was applied using the circumscribed central composite design of three variables with five levels. The relevant independent variables used for the process optimization were time, temperature and ethanol-water proportion for heat-assisted- and microwave-extration, whereas for the ultrasound method the ultrasonic power was variable. The responses used as criteria were the amount of rosmarinic acid was determined by HPLC-DAD

and the extraction yield of the obtained residue. Ultrasound extraction proved to be the most effective method, capable of yielding 86.3 ± 4.1 mg rosmarinic acid/g plant per dry weight (dw) at the optimal extraction conditions (33.0 ± 3.2 min, 371.7 ± 19.3 W and 39.9 ± 1.4% of ethanol). According to the content of rosmarinic acid, microwave- and heat-assisted extractions techniques were less effective, producing 49.4 ± 2.3 (at 26.5 ± 2.1 min, 108.6 ± 10.2 ºC and 25.5 ± 0.9 % of ethanol) and 59.4 ± 2.2 (at 106.2 ± 5.1 min, 88.0 ± 2.9 ºC and 34.5 ± 1.6 % of ethanol), respectively. Additionally, the solid/liquid ratio effect at the optimal values in a dose-response format was studied in view of its plausible transference at industrial level, showing a decreasing non-linear pattern from 5 to 120 g/L. In brief, the obtained results highlight the potential applications of using the leaves from *M. officinalis* as a source of rosmarinic acid.

Keywords: Heat- /microwave-/ultrasound- assisted extraction; *Melissa officinalis* L.; response surface methodology; rosmarinic acid.

Introduction

Melissa officinalis L. (lemon balm) is a plant of the Lamiaceae family that has been consumed since several decades in form of decoction, infusion or directly in food [1,2]. It is a millenarian plant popularly known for presenting multiple benefits to consumer's well-being. This has aroused great interest among researchers to prove these same properties scientifically [3]. In addition to the recognized effects in assisting digestion problems, rheumatism or headaches, several studies have demonstrated their antioxidant, hypoglycemic, hypolipidemic, antimicrobial, anticancer, antidepressant, anxiolytic, anti-inflammatory and spasmolytic capabilities [4–8].

Such beneficial effects of *M. officinalis* extracts may be primarily attributed to the presence of phenolic compounds [1]. Scientific research revealed that this plant contains various phytochemicals including terpenes (mono-terpenes, sesquiterpenes and triterpenes) and phenolic compounds (phenolic acids, flavonoids and tannins) [3,9]. Argyropoulos and Müller [10] have revealed that the main active compounds of lemon balm are volatiles (*e.g.* geranial, neral, citronellal and geraniol), triterpenes (*e.g.* ursolic acid and oleanolic acid), and phenolic compounds (*e.g. cis* and *trans* rosmarinic acid isomers, caffeic acid derivatives, luteolin, naringin and hesperidin). However, rosmarinic acid (RA) has been identified by many authors as the most abundant phenolic compound [1,11,12] and, recently, a direct relationship has been established between the presence of phenolic acids (mainly hydroxycinnamic acid derivatives such as RA) and the *in vitro* bioactivities demonstrated by *M. officinalis* extracts [1,13,14].

Due to consumers pressure because of the issues that are involved with several undesirable effects of synthetic compounds on human health, the food industry is being forced to seek for alternatives [12]. Natural-derived alternatives from plant extracts with proven benefits are being incorporated into food matrices in order to partially or totally replace those synthetic additives. As a consequence, the food industry has invested in solutions based on plants, mushrooms and algae, to be used as natural ingredients, which can act as food additives (*e.g.* by increasing shelf-life) and, simultaneously, bring health benefits, since they retain the original natural bioactivity of the natural sources [15].

The production of natural ingredients needs preliminary studies concerning the isolation of these compounds and the establishment of the best extraction methodology and conditions [16,17]. Although a wide variety of solid-liquid extraction procedures is available to obtain natural ingredients, the use of extended time periods of extraction, the need of large amounts of solvents and the partial loss of natural molecules (such as phenolic compounds) was some of the disadvantages identified by some authors , like as [16,18].

Heat-assisted extraction (HAE) is the conventional method mostly used for the extraction of natural compounds. Its advantage is the simplicity of its procedure, whereas its disadvantage is related with the extended periods of extraction and the high temperatures required [19]. Ultrasound-assisted extraction (UAE) is an alternative extraction method that has been pointed out by several authors as fast and clean, since it does not leave any residues in the extract, improves extract quality, productivity, yield and process selectivity [20,21]. Currently, this method is applied to prepare extracts for the phyto-pharmaceutical, cosmetic, and liqueur industries [21]. Another green technique with growing relevance is the microwave-assisted extraction (MAE). It presents short extraction cycles, uses reduced solvent amounts, provides high extraction rates, combined with lower costs [22,23]. UAE and MAE are currently amongst the foremost green techniques for accelerating extraction processes [21].

The effectiveness of these techniques depends on several variables and operating conditions and therefore, should not be generalized to any matrix due to the existing variability in composition. Consequently, it is necessary to select and optimize the extraction conditions depending on the used matrix, with the aim to ensure maximum yields with minimal time, solvent and energy [23,24]. One way to accomplish the optimization of any system is by measuring, independently, the influence of each variable when all the other ones are fixed. However, this type of approach does not provide the optimal operating conditions neither the interactions between variables. In this context, the application of mathematical models such as the response surface methodology (RSM) are gaining importance among the scientific community [23]. Based on an experimental design, the RSM analysis allows the simultaneous

optimization of the variables taking into account complex interactions between them, supporting the prediction of the responses and their maximization [25,26].

In this study, the responses of the extraction performance were expressed in terms of RA extraction yield per g of dry weight (mg RA/g of plant dw), purity of RA in the extracted residue - R (mg RA/g R) and extracted residue quantity (g of R/g of plant dw). Therefore, the aim of the present study was to optimize the RA extraction yield from *M. officinalis*, in order to be considered its use in food, pharmaceutical and cosmetic industries. With this purpose, different extraction techniques (HAE, UAE and MAE) were studied and compared. From this study, it was expected to achieve the following targets: 1) optimize the primary variable conditions for RA extraction from *M. officinalis* maximizing the response criteria used; and 2) develop a consistent process in a pre-industrial form for contributing the understanding the potential of RA for industrial applications.

Material and methods

Samples

Melissa officinalis L. dry leaves were provided by the company Pragmático Aroma Lda. ("Mais Ervas") based in Trás-os-Montes, Portugal. The samples were reduced to powder (~20 mesh). The obtained powder was mixed to guarantee the sample homogeneity and stored in a desiccator at room temperature (~25 ºC), protected from light, until further analysis.

Standards and reagents

Formic acid, HPLC grade ethanol and acetonitrile were from Fisher Scientific (Lisbon, Portugal). Rosmarinic acid standard was purchased from Extrasynthèse (Genay, France). Water was treated in Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

Response format values for the results presentation

The results were expressed in two response (*Y*) format values: Y_1, in mg of RA per g of plant dry weight material (mg RA/g plant dw), which was specifically used to analyse the RA extraction yields; and Y_2, in mg of RA obtained in the extracted residue (mg RA/g R), which was specifically used to evaluate the RA purity in the extracts. Both responses were equally analysed, but more considerations regarding the first one (mg RA/g plant dw) were taken in the results presentation because, it would be the guiding response in terms of optimization or industrial transference. Note, that by dividing those responses Y_1/Y_2, it was obtained obtain g of R/g plant dw that provides the information regarding the extracted residue quantity.

Description of the extraction techniques and used variables

The relevant variables, and the selection of appropriate tested ranges, for each one of the studied extraction techniques, were obtained based on the combination of single variable preliminary experiments, on previous extractions studies performed at our laboratory and on bibliographic surveys. A detailed description of the study ranges for the selected variables (their definition can be consulted below in the respective points), used in the RSM design, is displayed in **Table 24**. The solid/solvent ratio was kept constant (30 g/L) for all techniques. The used solvent was an ethanol/water mixture characterized in terms of ethanol content (%, w/w).

Table 24. Experimental domain and codification of independent variables in the CCCD factorial design with 5 range levels.

CODED VALUES	NATURAL VALUES								
	MACERATION			*ULTRASOUND*			*MICROWAVE*		
	t (min)	*T (ºC)*	*S (%)*	*t (min)*	*P (W)*	*S (%)*	*t (min)*	*T (ºC)*	*S (%)*
-1.68	30	30	0	3	100	0	3	60	0
-1	54.3	42.2	20.3	11.5	180	20.3	11.5	85	20.3
0	90	60	50	24	300	50	24	120	50
+1	125.7	77.9	79.8	36.5	420	79.8	36.5	155	79.8
+1.68	150	90	100	45	500	100	45	180	100

Heat-assisted extraction (HAE)

The samples of dry powdered leaves (600 mg) were placed in a beaker with 20 mL of solvent. The beaker was then placed in a thermostatic water bath under continuous electro-magnetic stirring for the required time. The variables and ranges tested were: time (t or X_1, 30 to 150 min), temperature (T or X_2, 30 to 90 ºC) and ethanol solvent proportion (S or X_3, 0 to 100 %).

Microwave-assisted extraction (MAE)

MAE process was performed using a Biotage Initiator Microwave (Biotage® Initiator+, Uppsala, Sweden) using closed vessels. The samples of dried powdered leaves (300 mg) were extracted with 10 mL of solvent. In enclosed microwave systems, the pressure and T are correlated and the applied power is linked to the needed t to reach the selected T or pressure. In consequence, T was selected as the main variable and the microwave power was set to 400 W. Under the selected conditions, the needed t to reach the selected T was always less than 20 s thus guaranteeing a fast heating process (this time interval can be neglected

considering the studied extraction time range). Therefore, the final variables and ranges tested were t (X_1, 3 to 45 min), T (X_2, 60 to 180 ºC) and S (X_3, 0 to 100 %).

Ultrasound-assisted extraction (UAE)

The UAE was carried out using an ultrasonic device (QSonica sonicators, model CL-334, Newtown, CT, USA). The samples of dried powdered leaves (1.5 g) were extracted with 50 mL using different times (t or X_1, 3 to 45 min), ultrasound power ranges (P or X_2, 100 to 500 W) and ethanol content (S or X_3, 0 to 100 %), while temperature was monitored in order to be below 30-35 ºC.

Chemical characterization

The extracts were filtered through a 0.22 mm nylon filter and submitted to LC-DAD analysis. The chromatographic data were acquired from Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA), as previously described [26]. Detection was car- ried out using a DAD detector (280 and 370 nm, as preferred wave- lengths) and quantification was performed using a calibration curve with rosmarinic acid, constructed based on its UV signal. The results were expressed in mg RA/g plant dw and in mg RA/g R.

Experimental design

The study of the impact of all the defined independent variables was carried out using one-factor-at-a-time, to pick the most influential one, and to determine the initial range of the processing variables. Through the analysis of these experimental results (data not shown), X1 (time in min), X2 (temperature in ºC) and X3 (ethanol proportion in %) were chosen as variables for the RSM design. Therefore, the combined effect of these three variables were studied using a circumscribed central composite design (CCCD) using five levels for each one [27]. Thus, this design produces 20 response combinations, six of which are replicas at central point of the experiment and the others are independent experimental points built around the centre as a sphere. According to Box & Hunter (2005) the centre point is presumed to be close to the optimum position for the response, so it is repeated to maximize the prediction. In order to minimize the unpredictable effects in the observed responses, experimental runs were random. The mathematical expressions used to calculate the design distribution, code and decode the tested variables can be found in detail in the supplemental section and in Table 24. Once the optimal conditions (X1, X2 and X3) were found, the following natural optimization step is to describe the pattern of the solid/liquid ratio (S/L or X4, expressed in g/L) in view of an industrial process application.

Mathematical model

The response surface models were fitted by means of least-squares calculation using the following second-order polynomial equation:

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{ii} X_i^2 \quad (1)$$

where Y is the dependent variable (response variable) 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} the coefficients of quadratic effect and n is the number of variables. As responses, the three format values, Y_1, mg RA/g plant dw; Y_2, mg RA/g R; and Y_1/Y_2, g R/g plant dw, were used.

Procedure to optimize the variables to a maximum response

For optimization of RA extraction, a maximized process of the model produced responses was achieved, using a simple method tool to solve non-linear problems [28,29]. Limitations were made to the variable coded values to avoid unnatural conditions (*i.e.*, times lower than 0).

Dose-response analysis of the solid to liquid ratio

At the optimized best conditions of time (X_1, *t*), temperature or ultrasound power (X_2, *T or P*) and ethanol solvent proportion (X_3, *S*) a dose-response analysis of the solid to liquid ratio (X_4, *S/L ratio*) was performed to describe its behaviour. To depict the response effect as function of the variation of the *S/L ratio* the Weibull (W) equation [30] was used with some modifications to fit the purposes as follow:

$$W\left(S/L\right) = K\left\{1 - \exp\left[-\ln 2\left(S/L/m\right)^a\right]\right\} \quad (2)$$

where K is the maximum extraction value (response criteria units, *i.e.* if Y_1 the units would be mg RA/g plant dw), m (*S/L* units, in this case g plant dw/L) the dose required for 50% of the maximum extraction value (K) and a shape parameter related to the maximum slope of the response. The rate of the process parameter (v, *i.e.* if assessing the Y_1 response criterion the units would be mg RA/g plant dw per g plant dw/L) can be obtained by using the parametric values of Eq. (2) as follows:

$$v = \frac{K\alpha}{m}\left(\ln 2\right)^{1/a} G^G \exp\left(-G\right) \text{ ; where } G = \frac{\alpha - 1}{\alpha} \quad (3)$$

The parameter v provides the information related to the decreasing average value of the extraction as function of the increase of the *S/L* variable. Therefore, the three parametric values of *K, m* and *v* can be used to assess the *S/L* trends.

Numerical methods, statistical analysis and graphical illustrations

The statistical analysis and fitting of the experimental results were carried out according to the equations for the responses obtained using a Microsoft Excel spreadsheet in three phases:

1) Coefficients measurement was achieved using the nonlinear least-square (quasi-Newton) method provided by the macro Solver in Microsoft Excel, by minimization of the sum of quadratic differences between observed and model-predicted values [31].

2) Coefficients significance was obtained via 'SolverAid' [31] to determine the parametric confidence intervals. The terms that were not statistically significant ($p > 0.05$) were dropped to simplify the model.

3) Model reliability was confirmed by applying the following standards: a) the Fisher *F*-test ($\alpha = 0.05$) to determine the consistency of the constructed models to describe the obtained data (Shi & Tsai, 2002); b) the 'SolverStat' macro to make assessment of parameter and model prediction uncertainties [32]; c) R^2 to explain the proportion variability of the dependent variable obtained by the model.

Results and discussion

Preliminary experiments to select the relevant variables and instrumental parameters to centre their experimental domain before the RSM application

Although there are scientific documents dealing with RA extraction from natural matrices [11,18,33], no complete studies could be found describing the conditions of RA extraction from *M. officinalis* leaves and the effects of the main process variables of conventional and alternative extraction techniques, as well as the comparison between them. In general, the leaves of *Rosmarinus officinalis* are the most studied plant material for the extraction of RA. However, due to composition variability associated to natural sources, the conditions of *R. officinalis* cannot be directly extrapolated to be used with *M. officinalis*. Consequently, to find the optimal conditions and choose a suitable technique able to maximize RA extraction from *M. officinalis*, independent studies are necessary.

To define the conditions that maximize RA extraction from *M. officinalis*, it is necessary to understand how the relevant process variables affect the performance of different techniques (HAE, UAE and MAE). These variables can be divided into non-intrinsic factors (such as *S* and *S/L*) and intrinsic factors (*t* and *T* for the HAE and MAE systems, and *t* and *P* for the UAE system). Preliminary tests to evaluate these variables, and to determine their experimental domain for a RSM design, were conducted using one-factor-at-the-time (keeping

others at a constant value). Additionally, a literature survey was carried out focusing on studies involving the extraction of RA from *M. officinalis* using similar processes.

Regarding the intrinsic factors of HAE, UAE and MAE, although worthy conclusions can be derived from the consulted bibliographic material [11,18,33], results may be highly dependent on variations not directly foreseen in these studies. Thus, to optimize the response criteria the following ranges for the intrinsic factors were used: for HAE *t* (30-150 min) and *T* (30-90 °C); for MAE *t* (3-45 min) and *T* (60-180 °C); and for UAE *t* (3-45 min) and *P* (100-500 W).

Therefore, in all extracting systems, the non-intrinsic variables and ranges were selected as follows:

1) The solvent type and composition are key factors for the successful extraction of the desired compounds. Due to the RA chemical structure, the use of different water-based solvent mixtures was found in the literature. Generally, authors combine water with different contents of methanol, ethanol or acetone [11,33,34]. Considering the principles of green chemistry, binary mixtures of ethanol with water were selected as the extraction solvent. In all systems, the ethanol content was tested from 0 to 100 % and confirmed as impacting significantly the RA extraction yield and, therefore, selected in the appropriate range.

2) Regarding the *S/L* factor, a broad range was tested, finding that lower values lead to an enhanced extraction yield, but also contribute to a significant waste of solvent. A higher *S/L* will result in lower extraction yields but in a better rationalization of raw materials consumption. Although the differences were significant, it was initially discarded as a variable to be optimized at the RSM study, and the value of 30 g/L was selected to be used in this study with all the tested extraction techniques. Once the optimal conditions for each technique were defined in terms of the other studied variables, the study proceeded with the analysis of the *S/L* condition by a dose-response analysis.

In conclusion, the efficiency of the HAE, UAE and MAE processes for RA extraction from *M. officinalis* was performed by the application of a RSM of three variables in a *CCCD* (five level values of each factor). The optimization of RA extraction yield in HAE, UAE and MAE with the RSM provides a strong solution that minimizes the errors with a short number of experimental trials. As stated before [35], the multivariable fitting decreases the number of parameters needed to analyse the response leading to better estimations and reducing their interval of confidence. A detailed description of all the tested values for each one of the used techniques can be found in **Table 24**. In **Figure 19**, a comprehensive summary of the different steps carried out for optimizing is presented.

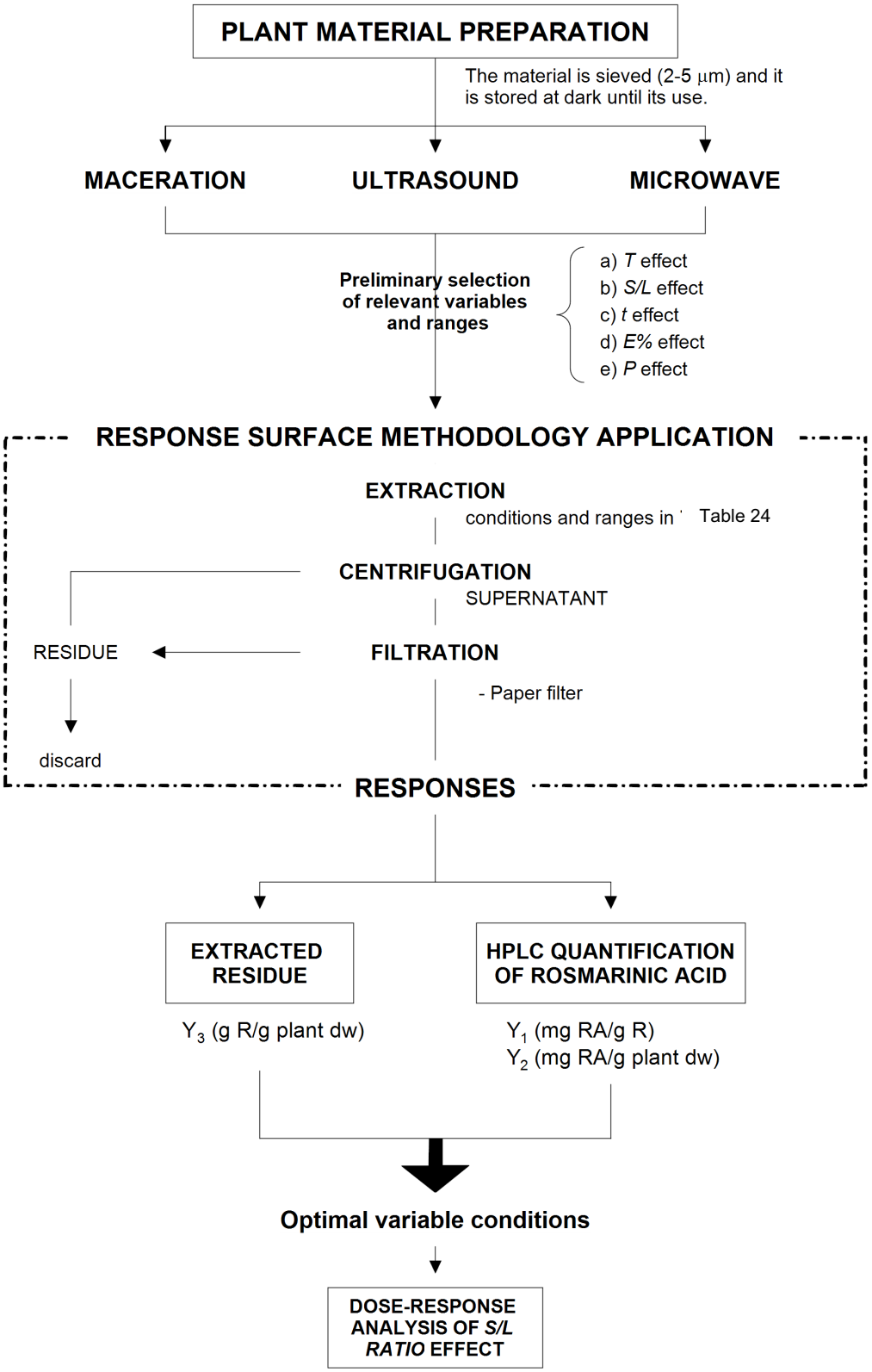

Figure 19. Diagram of the different steps carried out for optimizing the conditions that maximize the extraction responses Y_1 (mg RA/g plant dw), Y_2 (mg RA/g R) and Y_1/Y_2 (g R/g plant dw) of rosmarinic acid (RA) and the total extracted residue (R).

Mathematical models derived from the RSM for a CCCD with three variables and statistical assessment

The results obtained by the statistical CCCD are shown in the first part of **Table 25** for each one of the employed extraction techniques. By fitting the second-order polynomial model of Eq. (1) to the obtained responses using nonlinear least-squares estimations, the parametric values are obtained and presented in **Table 26**. Therefore, the resulting models for each assessed extraction technique are the following:

For the response format Y_1 (mg RA/g plant dw):

for HAE:
$$Y_{ME}^{Y_1} = 55.5 + 0.57t + 3.2T - 9.6S - 0.62t^2 - 1.47T^2 - 14.3S^2 - 3.1TS \quad (4)$$

for UAE:
$$Y_{UAE}^{Y_1} = 141.7 + 4.4t + 1.8T - 18.4S - 40.8S^2 - 8.9TS \quad (5)$$

for MAE:
$$Y_{MAE}^{Y_1} = 0.39 + 0.02t + 0.02T - 0.08S - 0.04S^2 + 0.01TS \quad (6)$$

For the response format Y_2 (mg RA/g R):

for HAE:
$$Y_{ME}^{Y_2} = 79.3 + 8.7t + 10.1T - 6.9S - 3.1t^2 - 3.4T^2 - 19.8S^2 + 7.6tT + 3.9t \quad (7)$$

for UAE:
$$Y_{UAE}^{Y_2} = 145.8 + 17.1t + 17.2T + 9.4t^2 - 25.1S^2 + 23.5tT + 8.5tS \quad (8)$$

for MAE:
$$Y_{MAE}^{Y_2} = 0.55 - 0.10S - 0.05t^2 - 0.04T^2 - 0.07S^2 - 0.04tT - 0.02tS \quad (9)$$

For the response format Y_1/Y_2 (g R/g plant dw):

for HAE:
$$Y_{ME}^{Y_1/Y_2} = 44.9 + 0.92t - 3.6T - 9.1S - 1.1t^2 - 5.3T^2 - 5.5S^2 \quad (10)$$

for UAE:
$$Y_{UAE}^{Y_1/Y_2} = 192.2 - 27.0T - 10.7S - 9.4t^2 - 26.6T^2 - 6.7S^2 \quad (11)$$

for MAE:
$$Y_{MAE}^{Y_1/Y_2} = 0.23 + 0.01t + 0.02T - 0.04S - 0.01T^2 - 0.02S^2 \quad (12)$$

Those coefficients, which showed confidence interval values (α= 0.05) higher than the parameter value, were consider as non-significant (*ns*) and were not used for the model development. Equations from (4) to (12) translate the response patterns for the three response criteria formats (Y_1 in mg RA/g plant dw, Y_2 in mg RA/g R and Y_1/Y_2 in g R/g plant dw) showing a relatively high complexity of the possible sceneries.

For the HAE, UAE and MAE techniques and for each response criteria, the linear and quadratic effects are found to play an important and significant role, while regarding the interactive effects, no significant effects were found for the MAE system.

Table 25. Experimental RSM results of the CCCD for the optimization of the three main variables involved (X_1, X_2 and X_3) in the ME, UAE and MAE for the three response value formats assessed (Y_1, mg RA/g plant dw; Y_2, mg RA/g R; and Y_1/Y_2 g R/g plant dw). Variables, natural values and ranges in **Table 24**. Three replicates were performed for each condition for each technique.

VARIABLE CODED VALUES			EXPERIMENTAL RESPONSES								
			MACERATION (ME)			*ULTRASOUND (UAE)*			*MICROWAVE (MAE)*		
X_1	X_2	X_3	Y_1	Y_2	Y_1 / Y_2	Y_1	Y_2	Y_1 / Y_2	Y_1	Y_2	Y_1 / Y_2
-1	-1	-1	42.70	102.07	0.418	48.90	112.41	0.435	40.06	161.54	0.248
1	-1	-1	42.81	109.49	0.391	44.24	88.13	0.502	43.49	181.73	0.239
-1	1	-1	54.65	128.50	0.425	56.90	120.38	0.473	41.47	151.73	0.273
1	1	-1	58.54	136.13	0.430	74.34	167.43	0.444	33.94	124.17	0.273
-1	-1	1	23.31	81.79	0.285	28.08	97.51	0.288	21.51	124.58	0.173
1	-1	1	25.53	92.38	0.276	30.75	84.78	0.363	19.83	107.78	0.184
-1	1	1	25.44	78.44	0.324	37.76	92.55	0.408	22.84	105.10	0.217
1	1	1	25.86	77.27	0.335	79.15	196.51	0.231	22.43	98.11	0.229
-1.68	0	0	55.81	142.01	0.393	56.57	151.26	0.374	39.27	173.75	0.226
1.68	0	0	56.50	140.09	0.403	93.64	222.23	0.421	50.45	207.31	0.243
0	-1.68	0	50.46	149.74	0.337	61.74	150.34	0.411	46.43	222.52	0.209
0	1.68	0	57.07	143.52	0.398	86.96	174.80	0.432	19.63	60.97	0.322
0	0	-1.68	27.24	57.59	0.473	41.46	73.17	0.567	47.57	186.79	0.255
0	0	1.68	7.67	94.69	0.081	14.35	105.52	0.136	17.02	209.24	0.081
0	0	0	52.45	133.12	0.394	78.88	146.15	0.579	42.59	178.46	0.239
0	0	0	56.62	143.70	0.374	81.31	150.20	0.541	47.67	203.13	0.235
0	0	0	56.41	143.16	0.345	77.50	144.86	0.575	48.01	202.85	0.237
0	0	0	57.16	145.07	0.384	83.22	150.03	0.555	44.50	191.33	0.228
0	0	0	53.86	136.69	0.376	75.93	144.54	0.525	49.33	212.03	0.233
0	0	0	55.64	141.22	0.391	77.91	149.63	0.521	46.74	196.94	0.237

Figure 20, **Figure 21** and **Figure 22** show the extraction results for the three response criteria formats (Y_1 in mg RA/g plant dw, Y_2 in mg RA/g R and Y_1/Y_2 in g R/g plant dw), respectively. The figures are divided in three columns, each one shows the results for each of the tested techniques. Additionally, each column is divided into two sections (A and B). The section A shows the 3D surface plots for the three possible variable combinations produced by Eqs. (4) to (12). The binary action between variables is presented when the excluded variable is positioned at the centre of the experimental domain (see **Table 24**). Subsection B illustrates the capability to predict the obtained results and the residual distribution as a function of each one of the considered variables.

Table 26. Parametric results of the second-order polynomial equation of Eq. (1 for the ME, UAE and MAE extracting techniques assessed and in terms of the extraction behaviour of the three response value formats (Y_1, mg RA/g plant dw; Y_2, mg RA/g R; and Y_1/Y_2 g R/g plant dw), according to the CCCD with 5 range levels (**Table 24**). 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. Analysis of significance of the parameters (α=0.05) are presented in coded values. Additionally, the statistical information of the fitting procedure to the model is presented.

COEFFICIENTS		**PARAMETRIC RESPONSES TO THE CENTRAL COMPOSITE DESIGNS FOR EACH TECHNIQUE**								
		MACERATION (ME)			***ULTRASOUND (UAE)***			***MICROWAVE (MAE)***		
		Y_1	Y_1	Y_1 / Y_2	Y_1	Y_1	Y_1 / Y_2	Y_1	Y_1	Y_1 / Y_2
Fitting coefficients obtained										
Intercept	b_0	55.49±1.73	141.74±1.72	0.393±0.01	79.378±2.33	145.85±6.40	0.549±0.02	44.986±2.61	192.21±14.68	0.236±0.01
	b_1	0.571±0.25	4.373±1.63	0.023±0.01	8.725±1.55	17.087±5.00	*ns*	0.923±0.77	*ns*	0.003±0.01
Linear effect	b_2	3.022±1.15	1.769±1.63	0.018±0.01	10.149±1.55	17.221±5.00	*ns*	-3.608±1.73	-26.96±9.74	0.025±0.01
	b_3	-9.627±1.15	-18.38±1.63	-0.081±0.01	-6.901±1.55	*ns*	-0.094±0.01	-9.060±1.73	-10.68±9.74	-0.038±0.01
	b_{11}	-0.629±0.31	*ns*	*ns*	-3.087±1.51	9.374±4.84	-0.051±0.01	-1.151±0.97	-9.390±8.17	*ns*
Quadratic effect	b_{22}	-1.473±1.12	*ns*	*ns*	-3.353±1.51	*ns*	-0.043±0.01	-5.332±1.69	-26.64±9.48	0.012±0.01
	b_{33}	-14.31±1.12	-40.84±1.57	-0.038±0.01	-19.77±1.51	-25.06±4.84	-0.067±0.01	-5.593±1.69	-6.746±5.47	-0.022±0.01
	b_{12}	*ns*	*ns*	*ns*	7.602±2.02	23.501±6.53	-0.043±0.01	*ns*	*ns*	*ns*
Interactive effect	b_{13}	*ns*	*ns*	*ns*	3.908±2.02	8.557±6.53	-0.017±0.01	*ns*	*ns*	*ns*
	b_{23}	-3.152±1.50	-8.940±2.13	0.006±0.00	2.498±2.02	*ns*	*ns*	*ns*	*ns*	*ns*
Statistical information of the fitting analysis										
Obs		40	40	40	40	40	40	40	40	40
df		31	34	34	30	33	33	33	34	34
R^2		0.9500	0.9685	0.9350	0.9540	0.9034	0.9120	0.8947	0.8644	0.9271
R^2adj		0.9326	0.9533	0.9222	0.9401	0.8947	0.9065	0.8956	0.8532	0.9173
MEC		280.1	960.3	0.008	602.7	1363.9	0.016	158.0	2397.5	0.003
RMSE		16.73	30.99	0.088	24.55	36.93	0.126	12.57	48.96	0.050
MAPE		12.15	11.46	8.32	12.78	11.05	6.55	12.41	15.23	5.07
DW		2.228	2.071	2.101	1.321	2.043	2.047	2.179	1.659	2.093

ns: non-significant coefficient; *Obs*: Number of observations; *df*: Number of degrees of freedom; R^2: Correlation coefficient; R^2adj: The adjusted determination coefficient for the model; *MSE*: The Mean Square of the Error; *RMSE*: The Root Mean Square of the Errors; *MAPE*: The Mean Absolute Percentage Error; and *DW*: The Durbin-Watson statistic.

In almost all combinatory 3D responses of **Figure 20**, **Figure 21** and **Figure 22**, the amount of extracted material increases to an optimum value and then decreases as a function of each of the assessed independent variables. Therefore, in almost all combinations, an absolute optimum can be found at one single point along with the response, allowing computing the conditions that lead to the absolute maximum.

In statistical terms, the tests used to assess the competence of the models showed that the non-significant parameters of both RSM approaches (**Table 26**) did not improve the reached solution and, in contrast, all significant parameters were highly consistent ($p < 0.01$). This was also verified by the achieved high R^2 and R^2_{adj} values, indicating the percentage of variability explained by the model. The distribution of the residuals presented in **Figure 20**, **Figure 21** and **Figure 22** was arbitrarily around zero and no group of values or autocorrelations were observed. Additionally, the agreement between the experimental and predicted values implies an acceptable explanation of the results obtained by the independent variables used. Therefore, the models developed in Eqs. (4) to (12) are completely functional and adequate to be used for prediction and process optimization.

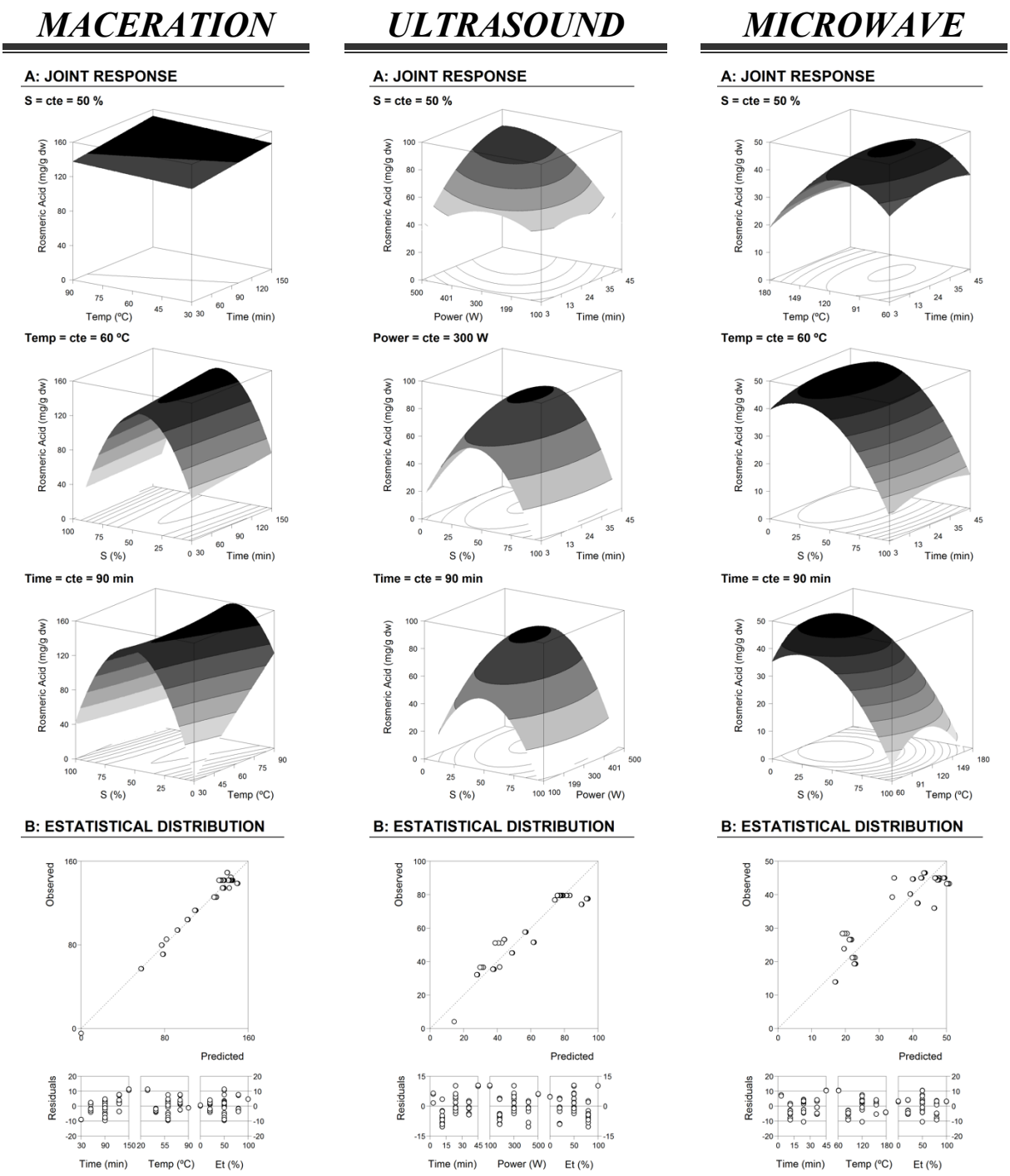

Figure 20.*Part A:* Shows the joint graphical 3D analysis in terms of the extraction behaviour for the Y1 (mg RA/g plant dw) responses for the optimization of the three main variables involved (X1, X2 and X3) in the ME, UAE and MAE. Each of the net surfaces represents the theoretical three-dimensional response surface predicted with the second order polynomial of Eq. (1. The binary actions between variables are presented when the excluded variable is positioned at the center of the experimental domain (**Table 24**). The statistical design and results are described in **Table 25**. Estimated parametric values are shown in **Table 26**. *Part B:* To illustrate the goodness of fit, two basic graphical statistic criteria are used. The first one, the ability to simulate the changes of the response between the predicted and observed data; and the second one, the residual distribution as a function of each of the variables. Additionally, for both parts (A and B), note all the differences in the axes scales.

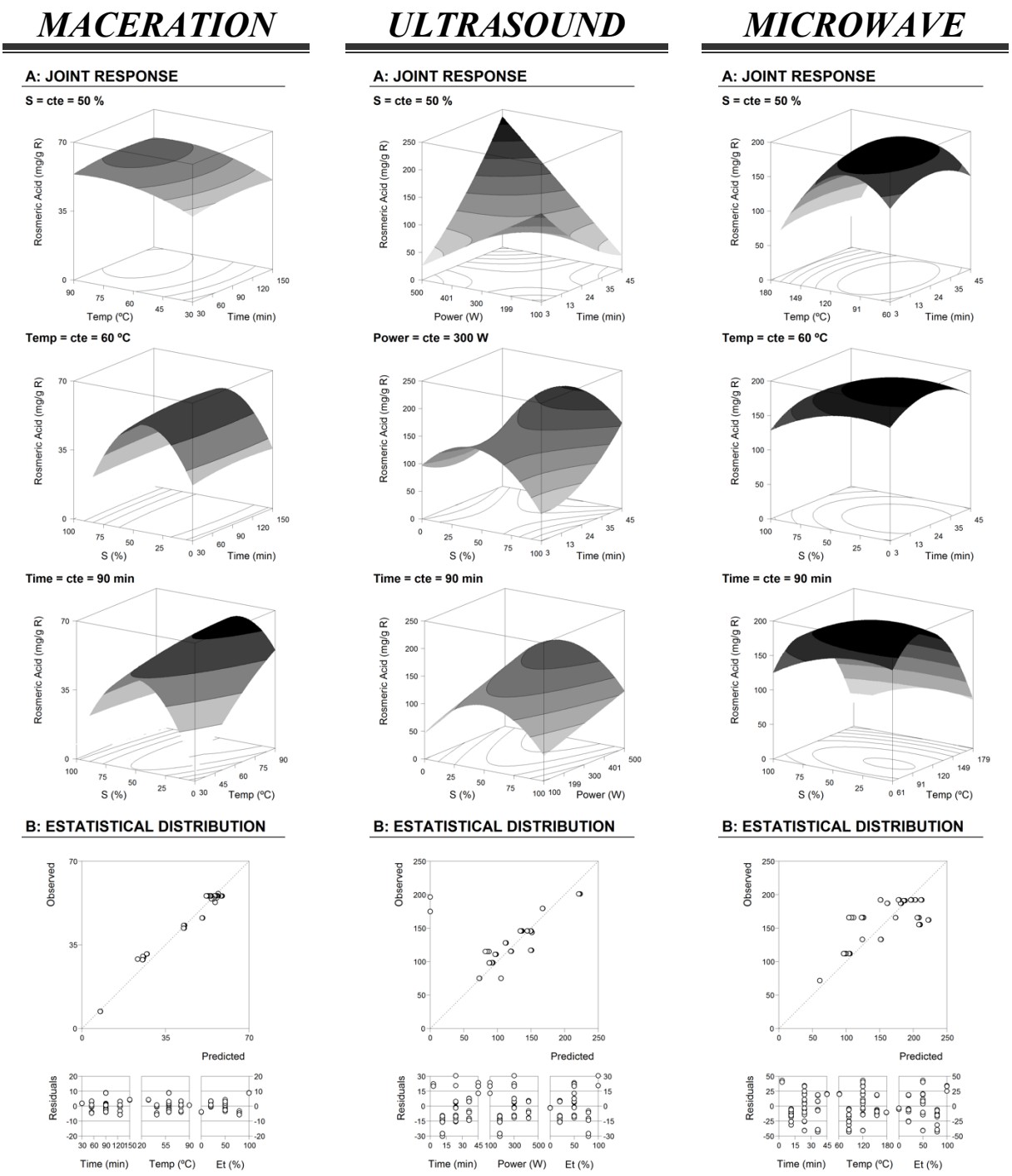

Figure 21. *Part A:* Shows the joint graphical 3D analysis in terms of the extraction behaviour for the Y2 (mg RA/g R) responses for the optimization of the three main variables involved (X1, X2 and X3) in the ME, UAE and MAE. Each of the net surfaces represents the theoretical three-dimensional response surface predicted with the second order polynomial of Eq. (1. The binary actions between variables are presented when the excluded variable is positioned at the centre of the experimental domain (**Table 24**). The statistical design and results are described in **Table 25**. Estimated parametric values are shown in **Table 26**. *Part B:* To illustrate the goodness of fit, two basic graphical statistic criteria are used. The first one, the ability to simulate the changes of the response between the predicted and observed data; and the second one, the residual distribution as a function of each of the variables. Additionally, for both parts (A and B), note all the differences in the axes scales.

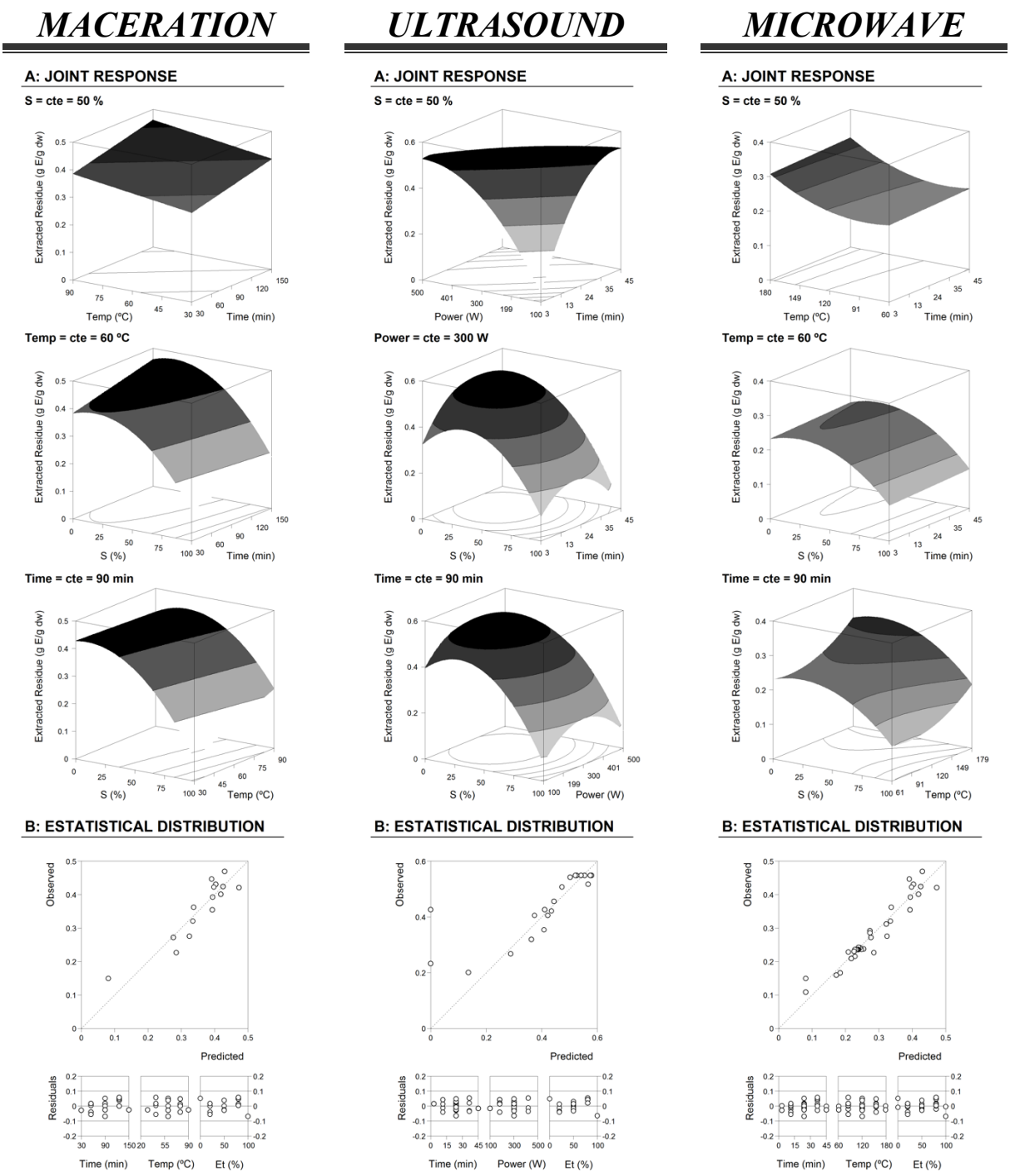

Figure 22. <u>*Part A:*</u> Shows the joint graphical 3D analysis in terms of the extraction behaviour for the Y_1/Y_2 (g R/g plant dw) responses for the optimization of the three main variables involved (X_1, X_2 and X_3) in the ME, UAE and MAE. Each of the net surfaces represents the theoretical three-dimensional response surface predicted with the second order polynomial of Eq. (1. The binary actions between variables are presented when the excluded variable is positioned at the centre of the experimental domain (**Table 24**). The statistical design and results are described in **Table 25**. Estimated parametric values are shown in **Table 26**. <u>Part B:</u> To illustrate the goodness of fit, two basic graphical statistic criteria are used. The first one, the ability to simulate the changes of the response between the predicted and observed data; and the second one, the residual distribution as a function of each of the variables. Additionally, for both parts (*A* and *B*), note all the differences in the axes scales.

Table 27: Variable conditions in natural values that lead to optimal response values for RSM using a CCCD for each of the extracting techniques assessed (ME, UAE and MAE), for the three individual response value formats (Y_1, mg RA/g plant dw; Y_2, mg RA/g R; and Y_1/Y_2 g R/g plant dw) and for the global optimal conditions.

CRITERIA		OPTIMAL VARIABLE CONDITIONS			OPTIMUM RESPONSE	
		X_1: t (min)	X_2: T (ºC) or P(W)	X_3: S (%)		
A) Individual optimal variable conditions:						
Maceration (ME)	Y_1	106.2±5.1	88.0±6.1	34.8±6.1	60.4±2.7	*mg RA/g plant dw*
	Y_2	90.0±9.1	90.0±6.1	37.8±3.7	161.6±7.2	*mg RA/g R*
	Y_1/Y_2	150.0±8.4	90.0±6.2	22.5±2.1	0.493±0.06	*g R/g plant dw*
Ultrasound (UAE)	Y_1	45.0±3.6	500.0±5.5	52.9±2.2	97.6±8.4	*mg RA/g plant dw*
	Y_2	45.0±3.2	500.0±4.1	58.5±5.1	298.3±9.3	*mg RA/g R*
	Y_1/Y_2	26.0±2.1	290.7±11.7	28.5±3.2	0.583±0.11	*g R/g plant dw*
Microwave (MAE)	Y_1	29.0±1.9	108.2±4.9	25.9±1.1	49.5±2.6	*mg RA/g plant dw*
	Y_2	24.0±2.2	102.3±6.2	26.4±1.1	203.3±1.2	*mg RA/g R*
	Y_1/Y_2	45.0±4.5	178.8±9.1	24.3±7.1	0.334±0.09	*g R/g plant dw*
B) Global optimal variable conditions:						
Maceration (ME)	Y_1				59.4±2.2	*mg RA/g plant dw*
	Y_2	106.2±5.1	88.0±2.9	34.5±1.6	158.3±12.1	*mg RA/g R*
	Y_1/Y_2				0.487±0.04	*g R/g plant dw*
Ultrasound (UAE)	Y_1				86.3±4.1	*mg RA/g plant dw*
	Y_2	33.0±3.2	371.7±19.3	39.9±1.4	178.5±13.2	*mg RA/g R*
	Y_1/Y_2				0.517±0.07	*g R/g plant dw*
Microwave (MAE)	Y_1				49.4±2.3	*mg RA/g plant dw*
	Y_2	26.5±2.1	108.6±10.2	25.5±0.9	202.1±13.1	*mg RA/g R*
	Y_1/Y_2				0.246±0.03	*g R/g plant dw*

Numerical optimal conditions that maximize the extraction and experimental verification of predictive models

By applying a simple procedure inserting restrictions to the experimental ranges, the optimal absolute or relative (marked as (*) when the optimal value may be outside of the experimental range studied) conditions that maximize the responses criteria are achieved (**Table 27**).

- For the HAE system: for response criteria Y_1 (mg RA/g plant dw) that correspond to the RA extraction yield, the optimal conditions were found to be 106.2 ± 5.1 min, 88.0 ± 6.1 ºC and 34.8 ± 6.1% of ethanol, producing a maximum response value of 60.4 ± 2.7 mg RA/g plant dw; for response criteria Y_2 (mg RA/g R) that analysed the RA purity in the extracted residue the optimal conditions were found to be 90.0 ± 9.1 min, (*) 90.0 ± 6.1 ºC and 37.8 ± 3.7% of ethanol, producing a maximum response value of 161.6 ± 7.2 mg RA/g R; and for response criteria Y_1/Y_2 (g R/g plant dw) that analysed the yield of the extracted residue the optimal conditions were found to be (*) 150.0 ± 8.4 min, (*) 90.0 ± 6.2 ºC and 22.5 ± 2.1% of ethanol, producing a maximum response value of 0.493 ± 0.06 g R/g plant dw.

- For UAE response: for Y_1, the optimal conditions were found to be (*) 45.0 ± 3.6 min, (*) 500.0 ± 5.5 W and 52.9 ± 2.2% of ethanol, producing 97.6 ± 8.4 mg RA/g plant dw; for Y_2, conditions were at (*) 45.0 ± 3.2 min, (*) 500.0 ± 4.1 W and 58.5 ± 5.1% of ethanol, producing 298.3 ± 9.3 mg RA/g R; and for Y_1/Y_2, the optimal conditions were found to be 26.0 ± 2.1 min, 290.7 ± 11.7 W and 28.5 ± 3.2% of ethanol, producing 0.583 ± 0.11 g R/g plant dw.
- For MAE response: for Y_1, the optimal conditions were found to be 29.0 ± 1.9 min, 108.2 ± 4.9 ºC and 25.9 ± 1.1% of ethanol, producing 49.5 ± 2.6 mg RA/g plant dw; for Y_2 the optimal conditions were found to be 24.0 ± 2.2 min, 102.3 ± 6.2 ºC and 26.4 ± 1.1% of ethanol, producing 203.3 ± 1.2 mg RA/g R; and for Y_1/Y_2 the optimal conditions were found to be (*) 45.0 ± 4.5 min, (*) 178.8 ± 9.1 ºC and 24.3 ± 7.1% of ethanol, producing 0.334 ± 0.09 g R/g plant dw.

Figure 23 shows the optimized isolines projections for the combination of the three main variables involved (X_1, X_2 and X_3) in the HAE, UAE and MAE of the three response value formats (Y_1, mg RA/g plant dw; Y_2, mg RA/g R; and Y_1/Y_2, g R/g plant dw) to describe visually the tendencies of each response and guide the selection of the most favourable conditions, taken into account simultaneously all responses. Each of the contour graphs represents the projection in XY plane of the theoretical three-dimensional response surface predicted with the second order polynomial of Eq. (1). The binary actions between variables are presented when the excluded variable is positioned at the individual optimum of the experimental domain (**Table 27**).

When combining, the information produced by the three responses criteria (Y_1, Y_2 and Y_1/Y_2), the complete behaviour of each relevant variable influencing the responses is defined in global terms. The global optimizing results are presented in **Table 27** and summarized below:

- For the HAE system: the optimal global conditions were at 106.2 ± 5.1 min, 88.0 ± 2.9 ºC and 34.5 ± 1.6% of ethanol, producing 59.4 ± 2.2 mg RA/g plant dw (Y_1), 158.3 ± 12.1 mg RA/g R (Y_2) and 0.487 ± 0.04 g R/g plant dw (Y_1/Y_2).
- For UAE response: the optimal global conditions were at 33.0 ± 3.2 min, 371.7 ± 19.3 W and 39.9 ± 1.4% of ethanol, producing 86.3 ± 4.1 mg RA/g plant dw (Y_1), 178.5 ± 13.2 mg RA/g R (Y_2) and 0.517 ± 0.07 g R/g plant dw (Y_1/Y_2).
- For MAE response: the optimal global conditions were at 26.5 ± 2.1 min, 108.6 ± 10.2 ºC and 25.5 ± 0.9% of ethanol, producing 49.4 ± 2.3 mg RA/g plant dw (Y_1), 202.1 ± 13.1 mg RA/g R (Y_2) and 0.246 ± 0.03 g R/g plant dw (Y_1/Y_2).

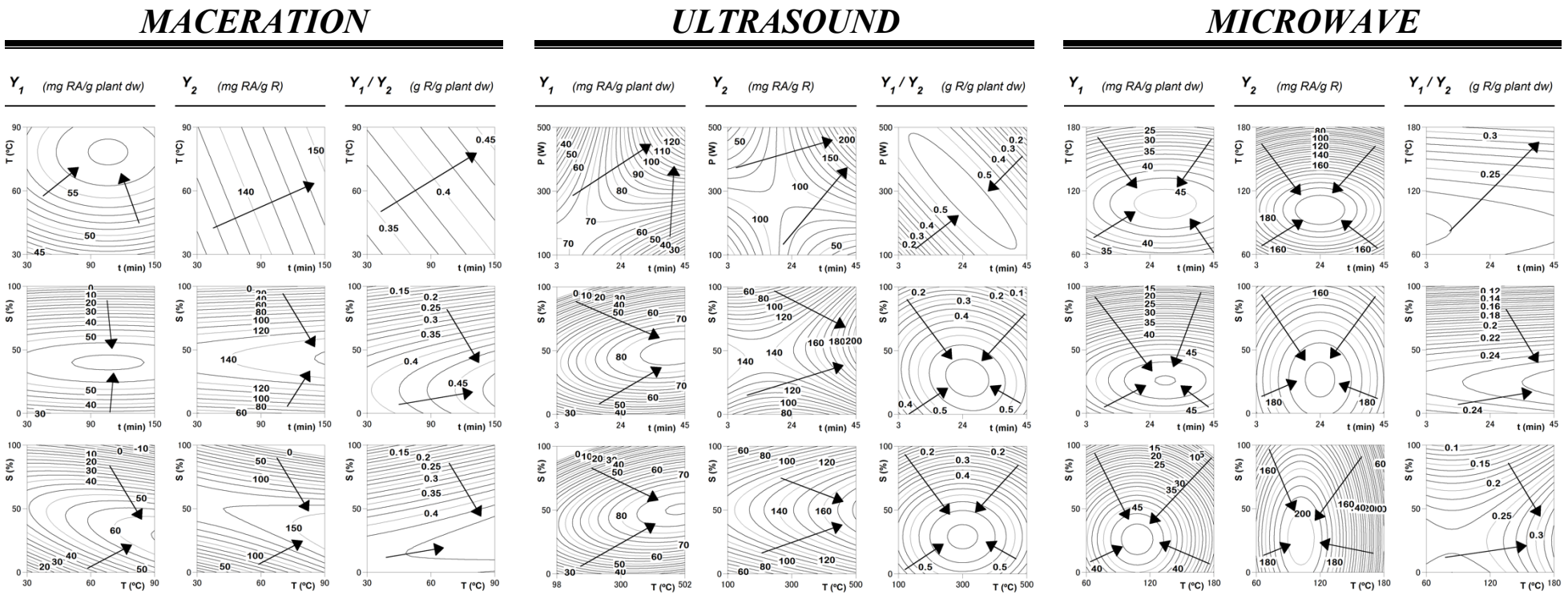

Figure 23. Shows the optimized isolines projections for the combination of the three main variables involved (X_1, X_2 and X_3) in the ME, MAE and UAE of the three response value formats (Y_1, mg RA/g plant dw; Y_2, mg RA/g R; and Y_1/Y_2 g R/g plant dw) to describe visually the tendencies of each response and guide the selection of the most favourable conditions, taken into account simultaneously all responses. Each of the contour graphs represents the projection in XY plane of the theoretical three-dimensional response surface predicted with the second order polynomial of Eq. (1. The binary actions between variables are presented when the excluded variable is positioned at the individual optimum of the experimental domain (**Table 27**). The statistical design and experimental results are described in **Table 25**. Estimated parametric values are shown in **Table 26**.

For all techniques, the conditions that lead to the optimal values were experimentally tested in order to ensure the accuracy of the presented results. **Figure 24A**, shows the summarized individual 2D responses as a function of the defined variables for HAE, UAE and MAE extraction techniques to guide the selection of the most favourable conditions. The line represents the variable response pattern when the others are located at the optimal values presented in the third part of **Table 27**. The dots (⊙) presented alongside the line highlight the location of the optimal value. Comparing the results of extraction efficiencies among the techniques, UAE gave significantly higher values, while HAE and MAE extraction generated lower values. Regarding the extraction time, MAE was the fastest extraction method while HAE was the longest.

Dose-response analysis of the solid-to-liquid effect at the optimum conditions

The studies on *S/L* were performed at the global optimal conditions predicted by the polynomial models obtained for each extraction technique. Preliminary results indicated that the experimental limit value was proximal to 120 g/L. Therefore, the dose-response process of extraction was designed to verify the *S/L* patterns between 5 g/L until 100 g/L. The *S/L* effect can be described by a simple non-linear relationship using Eq. (2). The effects of the *S/L* dose-response for the HAE, UAE and MAE techniques are presented in **Figure 24B**. Only the response criterion Y_1 (mg RA/g plant dw) is presented because the other responses (Y_2 and Y_3) showed similar trends. In all cases, the responses obtained through the HAE, UAE and MAE systems are consistent with the previous results. **Figure 24B1** shows the experimental results (dots) and model predictions of Eq. (2) (lines) obtained for each technique. The *S/L* increase leads to a decrease in the extraction ability of the solvent; consequently, the extraction responses reach a maximum value at minimum values of *S/L* and a minimum at higher values of *S/L* (in our case 120 g/L was the experimental limit achieved) and the respective losses are justified by parametric results derived from Eq. (2) and (3) in terms of the response criterion Y_1 (mg RA/g plant dw) as follows.

- For the HAE system: the value of *K* that accounts for the maximum yield extraction of RA was found to be 118.6 ± 12.2 mg RA/g plant dw; the value of *m* that accounts for the *S/L* value that provides 50% of the parameter value *K* (IC_{50}) was found to be 34.4 ± 4.7 g plant dw/L; the value of *v* that provides the decreasing average rate of process was found to be 0.680 ± 0.09 mg RA/g plant dw per g plant dw/L; the coefficient of determination R^2 achieved was 0.9834; and the dimensionless shape parameter *a* was found to be 0.6 ± 0.1.

- For UAE response: K = 153.8 ± 17.8 mg RA/g plant dw; m = 48.1 ± 6.3 g plant dw/L; v = 1.523 ± 0.32 mg RA/g plant dw per g plant dw/L; R^2 = 0.9886; and a = 1.4 ± 0.1.
- For MAE response: K = 132.6 ± 11.2 mg RA/g plant dw; m = 57.5 ± 7.1 g plant dw/L; v = 0.797 ± 0.32 mg RA/g plant dw per g plant dw/L; R^2 = 0.9929; and a = 1.0 ± 0.1.

In statistical terms, the mathematical model of Eq. (2) used to assess non-linear *S/L* trends showed highly significant parameters for all techniques, a fact that was also verified by the achieved high R^2 values, indicating the good agreement of the model with the produced experimental results. From this parametric analysis, the different trends of the *S/L* effect can be determined at the optimal extraction conditions achieved for each one of the assessed techniques. By means of the most relevant parameters (*K, m* and *v*), the following outcomes can be derived:

- For the parametric value of *K*: the technique which provided the higher RA extraction yield was UAE followed by MAE and HAE.
- For the parametric value of *m*: the technique that needed the lower *S/L* values at 50% of the response was HAE followed by UAE and MAE.
- For the parametric value of *v*: the technique which provided the lower average extraction rate values was HAE followed by MAE and UAE.

Consequently, the dose-response in terms of the response criterion Y_1 (mg RA/g plant dw) can be explained by the parametric results derived from Eq. (2) and (3), and this trend was visually interpreted in **Figure 24B2**, for comparison purposes, in which the modelling predictions obtained for each technique are represented jointly up to the determined experimental limit value of 120 g/L. It can be observed that UAE is the technique were higher RA yields are achieved in the S/L interval range of 0-50 g/L, as corroborated by the higher values of parameter *K*. However, the decrease observed for the UAE system are slightly higher than for the MAE and HAE process, as noted by the parameters *m* and *v*. In fact, the HAE system is more efficient to extract RA at *S/L* ranges of 50 to 100 g/L. The higher loss induced by the increase of S/L for the UAE system causes a higher dose-response (> 100 g/L) and the extraction yields are better with the HAE and MAE systems. A similar behaviour was found for the other response criteria (Y_2 and Y_3, data not showed), considering the same variation magnitude for both.

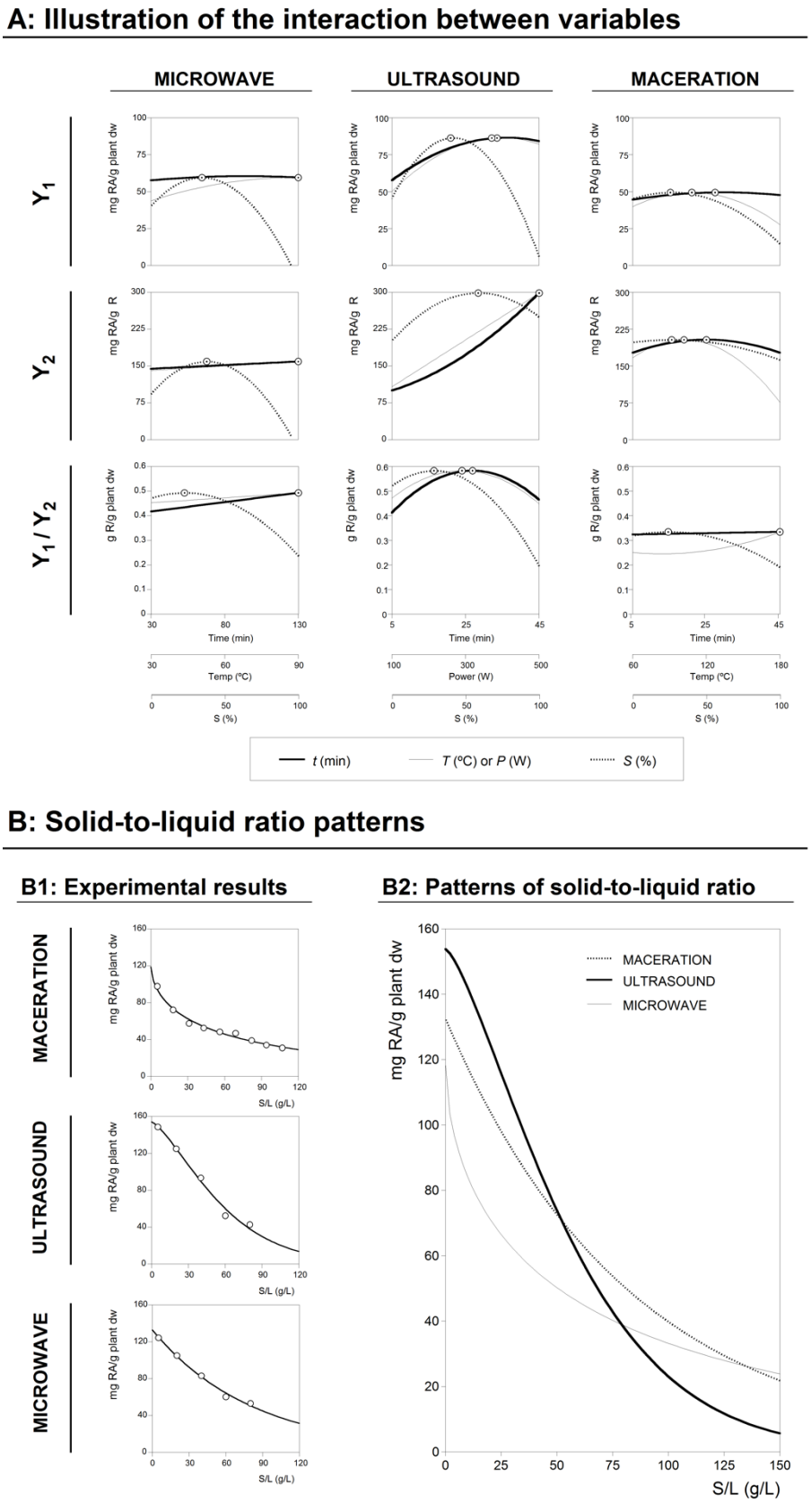

Figure 24. Final summary of the effects of all variables assessed for ME, UAE and MAE systems. *Part A*: Shows the individual 2D responses of all studied responses as a function of

all the variables assessed. The variables in each of the 2D graphs were positioned at the individual optimal values of the others (**Table 27**). The dots (⊙) presented alongside each line highlight the location of the optimum value. Lines and dots are generated by the theoretical second order polynomial models of Eqs. (4) to (6) for ME, Eqs. (7) to (9) for UAE and Eqs. (10) to (12 for MAE systems. Part B: Shows the dose response of *S/L ratio* at the global optimal values of the other three variables (**Table 27**). Only the response criterion Y_1 (mg RA/g plant dw) is presented because the other responses (Y_2 and Y_3) showed similar trends. First part (B1) shows the experimental results (○), meanwhile the lines are the predicted pattern by Eq. (2). The limit value (~120 g/L) shows the maximum achievable experimental concentration until the sample cannot be physically stirred at laboratory scale. Second part (B2) shows the dose-response trends for ME, UAE and MAE jointly for comparison purposes.

Comparison with other studies involving the extraction of rosmarinic acid

In the literature, there are several techniques that also describe the extraction of RA from *M. officinalis*. The most relevant are supercritical carbon dioxide extraction, liquid CO_2 extraction, UAE, MAE and HAE [36–38]. There are few works that compare the conventional and alternative extraction techniques [11,18,33]. In a study developed by Ince et al. (2013) the extraction of RA from the leaves of lemon balm was studied through three techniques (HAE UAE and MAE) using water as solvent. This study revealed a higher yield for MAE followed by UAE and HAE. These results are in opposition to the results described in this manuscript where UAE was more efficient. This may be because in the herein work the proportion of the hydro-alcoholic mixtures was included as variable. This fact is corroborated by the results found by other authors dealing with the extraction of RA: a) Kim et al. (2010) found better RA extraction yields with hydro-alcoholic mixtures (methanol-based); b) Bellumori et al. (2016) reported that MAE and UAE using water:ethanol mixtures or water:acetone mixtures considerably increase the archived yields (using *R. officina*lis L.) comparatively with more traditional used solid-liquid extraction processes; c) Bernatoniene et al. (2016) described that UAE with 90% ethanol would increase the RA extraction yield from rosemary leaves; and d) even when other techniques, such as supercritical CO_2 extraction, were used to extract RA, hydro-alcoholic mixtures with 70% ethanol were reported to produce the better results [40].

M. officinalis leaf is standardized to contain not less than 4% of total hydroxycinnamic acid derivatives expressed as RA [14]. However, several studies in literature reporting the total amount of RA extracted from different species, shows some variability. *In vitro* cultured and in commercial samples of *M. officinalis* infusions, Barros et al. (2013) found that RA was the most abundant compound, being higher in commercial samples, especially in tea bag samples (55.68 mg/g dw) and lower in *in vitro* culture samples (15.46 mg/g dw). Other authors using a variety of *M. officinalis* grown in Slovakia found 17.03 mg/g dw of RA [13]. In a study analysing commercial herbal teas containing *M. officinalis* leaves, authors indicated that, depending on

the supplied source, variations from 5.2 to 32.6 mg/g dw of RA were found[14]. Ince et al. (2013), using MAE and UAE extraction processes with *M. officinalis* leaves, found 39.8 mg RA/g dw. To the best of our knowledge, the highest content reported in literature, from leaf material of *M. officinalis* extracted in aqueous ethanol using medium pressure liquid–solid extraction, was 96.41 mg RA/g dw [41].

For comparison purposes, some authors have studied the possibility of obtaining RA from six different plants using UAE, concluding that *Hypericum perforatum* was the plant providing the highest RA yield (0.99 mg/g dw), followed by *M. officinalis* (0.62 mg/g dw) and *R. officinalis* (0.51 mg/g dw) [33]. Bellumori et al. (2016) using *R. officinalis* reported the value of 67.7 mg/g dw (UAE), and a much lower one with MAE (32.9 mg/g dw).

Several factors may have contributed to the achieved variability. The most relevant one could be the lack of optimization approaches, specifically in what concerns RA extraction optimization. Authors report the extracting values under conditions not properly optimized, or by studying variables not adequately selected. Other reasons could be ascribed to the use of different techniques and solvents, to the use of different plant species (*i.e. M. officinalis* and *R. officinalis*), regional varieties and gathering conditions. This last point was studied in some works by using a large time-period collection, i.e. samples of *M. officinalis* collected over several years and over different seasons [10,42].

Conclusions

RA extraction from *M. officinalis* leaves was optimized using three different extraction techniques (HAE, UAE and MAE) and the obtained results were compared. The combined effects of three independent variables (*t*, *T* and *S* for HAE and MAE, and for the UAE the *P* was used instead of *T*) were studied to maximize the three response value formats (Y_1, mg RA/g plant dw; Y_2, mg RA/g R; and Y_1/Y_2, g R/g plant dw). RSM methodology was applied using a CCCD of three factors with five-levels and second-order polynomial models, which were successfully designed and experimentally verified, showing that the studied variables have significant effects on the RA extraction yield. UAE was found to be the most effective method, capable of yielding 86.3 ± 4.1 mg RA/g plant dw and 0.517 ± 0.07 g R/g plant dw, extract comprising a content of 178.5 mg RA/g R. These results were achieved at the following optimal extraction conditions: 33.0 ± 3.2 min, 371.7 ± 19.3 W and 39.9 ± 1.4% of ethanol. Comparatively, MAE and HAE lead to results generally inferior, for all the assessed responses. Although MAE was found to be a much faster technique, considerably faster than HAE and slightly faster than UAE, lower RA extraction yields and extracts with lower content of RA were obtained.

Considering the study of the solid/liquid ratio effect, an important variable from an industrial point of view, the optimal values in the dose-response format have shown a decreasing non-linear correlation from 5 to 120 g/L. In terms of response criterion Y_1 (mg RA/g plant dw), it was observed that the UAE was the technique conducting to higher RA extraction yields in the S/L range of 0-50 g/L. Nevertheless, for the medium *S/L* range, HAE extraction was more efficient and for S/L values higher than 100 g/L, HAE and MAE systems revealed both to be more efficient.

In all cases, the used mathematical models (RSM and dose-response models) can be assumed as adequate models to optimize the process of RA extraction from *M. officinalis*, resulting in extracts enriched in RA. These extracts constitute interesting natural ingredients, able to substitute synthetic-derived counterparts, and with application in a wide range of industrial fields (food, pharmaceutical and cosmetic applications).

This work offers an overview through the use of environmentally compatible extraction processes, and the obtained results indicate the viability of using *M. officinalis* leaves as a productive source to obtain RA-enriched extracts, which may constitute interesting approaches to be transposed to a large productive scale.

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4.1.2. Extrato de cidreira (*Melissa officinalis* L.) rico em ácido rosmarínico como potenciador de propiedades funcionais em *cupcakes*

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Suitability of lemon balm (*Melissa officinalis* L.) extract rich in rosmarinic acid as a potential enhancer of functional properties in cupcakes

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Abstract

Melissa officinalis (lemon balm) and its extracts have been reported profusely for possessing bioactive properties, offering the possibility to be used to develop/enrich food products with additional functional capabilities, providing health benefits to consumers. The antioxidant, antibacterial and antifungal activity of lemon balm extract, as well as it potential hepatotoxicity were thoroughly evaluated. Then the extracts were incorporated in cupcakes and their preserving effect, chemical composition, colour parameters and antioxidant activity was compared with that provided by potassium sorbate. In general, the variables with highest differences among different storage times were energy level, sucrose, glucose, palmitic acid (C16:0) and oleic acid (C18:1n9). On the other hand, *L** (top), *a** (top), *b** (top), pH, capric acid (C10:0) and lauric acid (C12:0) showed the highest variation according to cupcake formulation.

The results observed indicate that the lemon balm extract rich in rosmarinic acid can provide advantageous functional properties to pastry products.

Keywords: Cupcake, bioactivity, lemon balm, potassium sorbate (E202), proximate composition, colour parameters, fatty acids.

Introduction

Cakes, biscuits and cookies are pastry products appreciated worldwide and consumed by individuals from different age groups and social ranks. Some of the reasons underlying this success include their affordable cost, great availability of flavours and textures and average shelf-life (Caleja, Barros, Antonio, Oliveira, & Ferreira, 2017; Gandhi et al., 2001; Jeddou et al., 2017). Owing to the generally high fat content of pastry products, these are particularly prone to oxidative deterioration, demanding the employment of effective antioxidants to maintain their integral quality (Bera, Lahiri, & Nag, 2006). However, the commonly used artificial antioxidants, especially if consumed excessively, were associated to several adverse effects on consumers' health (Lennerz et al., 2015). This scenario boosts the search for antioxidants obtained from natural species, which often present high levels of polyphenolic compounds, tocopherols, ascorbic acid or carotenoids, all representing good alternative to artificial antioxidants, such as BHA or BHT (Maqsood, Benjakul, & Shahidi, 2013), which is also in line with the current consumers' demand for healthier, safer and more convenient food (Shim, Seo, Lee, Moon, Kim, & Park, 2011).

Furthermore, in addition to the preserving ability of different natural extracts, they contain compounds with excellent bioactivity, which might represent a way to improve the overall effect of pastry products consumption. In fact, several studies have proven the excellent preservative capacity of different plant extracts in different food matrices. *Foeniculum vulgare* Mill. And *Matricaria recutita* L., for instance, are two examples of medicinal plants with various beneficial physiological properties, namely in the prevention and treatment of several diseases (Roby, Sarhan, Selim, & Khalel, 2013). Furthermore, extracts from both plants, most likely due to their antioxidant and antimicrobial properties, showed to be highly effective in preserving different food products, such as cottage cheese (Caleja et al., 2015 a,b), yoghurts (Caleja, Barros, Antonio, Carocho, Oliveira, & Ferreira, 2016) and biscuits (Caleja et al., 2017a). Obviously, in addition to the achieved preservation effect, plant extracts can improve the bioactivity levels in the functionalized food products, without changing their appearance or nutritional value. This double preserving-functionalizing effect was also observed in a "Serra da Estrela" cheese (a Portuguese specialty cheese), when incorporated with aqueous extracts

Castanea sativa Mill. and *Ocimum basilicum* L. (Carocho, Barreira, Bento, Fernández-Ruiz, Morales, & Ferreira, 2016; Carocho et al., 2016b).

The purpose of the present study is obtaining lemon balm extract rich in rosmarinic acid (ERA), validating their antioxidant and antimicrobial properties and assessing their lack of toxicity, and further incorporating these extracts in cupcakes to verify their suitability as an alternative to the commonly used artificial additive (potassium sorbate).

Material and methods

Standards and reagents

Fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA), as also sugar standards (L-rhamnose, L-(+)-arabinose, D-(-)-fructose, D-(+)-glucose, D-(+)-sucrose, D-(-)-mannitol, D-(+)-trehalose, D-(+)-maltose, D-(+)-raffinose and D-(+)-melezitose) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Acetonitrile (HPLC grade) was purchased from Fisher Scientific (Lisbon, Portugal). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, Massachusetts, USA). The standard, rosmarinic acid was from Extrasynthese, Genay Cedex, France. All other solvents and reagents were obtained from common sources and water was treated in Milli-Q water purification system (Millipore Corporation, Billerica, Massachusetts, USA).

Natural ingredients

Preparation of lemon balm extract rich in rosmarinic acid

Commercial samples of *Melissa officinalis* L. (lemon balm) were provided by Pragmático Aroma Lda. (“Mais Ervas”) based in Trás-os-Montes, Portugal (three bags containing ~300 g each). The lemon balm extract rich in rosmarinic acid (ERA) were obtained using an ultrasonic device (QSonica sonicators, model CL-334, Newtown, CT, USA) comprising an ultrasound power in the range between 100 and 500 W at a frequency of 20 kHz, equipped with a digital timer. The dried powdered leaves samples (1.5 g) were extracted with 50 mL of a mixture of ethanol:water (30:70), for 33 minutes using 371W ultrasound power, the temperature was monitored in order to be below 30-35 ºC (Caleja et al., 2017b). After evaporating the ethanol, the samples were lyophilized and stored in a desiccator at room temperature (average 25 ºC), protected from light, until further analysis.

Potassium sorbate 99% (E202) was used as an artificial additive (Acros Organics, Geel, Belgium).

Chemical characterization

The dry lyophilized extract obtained from lemon balm was re-dissolved in the extraction solvent (mixture of ethanol:water (30:70), filtered through a 0.22 µm nylon filter and submitted to LC-DAD-ESI/MSn analysis. The chromatographic data were acquired from Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA), as previously described by Bessada et al. (2016). Chromatographic separation was achieved with a Waters Spherisorb S3 ODS-2 C18 (3 µm, 4.6 × 150 mm, Waters, Milford, MA, USA) column thermostatted at 35 °C. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was isocratic 15% B (5 min), 15% B to 20% B (5 min), 20-25% B (10 min), 25-35% B (10 min), 35-50% B (10 min), and re-equilibration of the column, using a flow rate of 0.5 mL/min and an injection volume of 10 µL. Double online detection was carried out in the DAD using 280 and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet.

MS detection was performed in negative mode, using a Linear Ion Trap LTQ XL mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an ESI source. Nitrogen served as the sheath gas (50 psi); the system was operated with a spray voltage of 5 kV, a source temperature of 325 °C, a capillary voltage of -20 V. The tube lens offset was kept at a voltage of -66 V. The full scan covered the mass range from *m*/*z* 100 to 1500. The collision energy used was 35 (arbitrary units). Data acquisition was carried out with Xcalibur® data system (ThermoFinnigan, San Jose, CA, USA).

Rosmarinic acid was identified by comparing its retention times, UV-vis and mass spectra with the standard compound. For quantitative analysis, a calibration curve of rosmarinic acid was constructed based on the UV signal (y = 191291x – 652903, R^2=0.999). The results were expressed as mg/g of extract.

Evaluation of antioxidant properties

The lyophilized samples were re-dissolved in ethanol (5 mg/mL) and successively diluted to allow determining the corresponding EC_{50} values (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay).

Following the previously described protocols (Barros et al., 2013a), 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. Additionally, β-carotene bleaching inhibition and lipid peroxidation inhibition, were respectively evaluated by measuring the colour decay of a β-carotene solution as induced by a linoleate solution (at 470

nm) and by using the thiobarbituric acid reactive substances (TBARS) assay (at 532 nm). Trolox was used as positive control in all assays.

Evaluation of toxicity

The hepatotoxicity was evaluated following in a cell line obtained from porcine liver acquired from certified abattoirs (Guimarães et al., 2013). A phase-contrast microscope was used to monitor the growth of the cell cultures, which 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, supplemented with FBS (10%), penicillin (100 U/mL) and streptomycin (100 µg/mL). Ellipticine was used as a positive control. The results were expressed as GI_{50} values in µg/mL (sample concentration that inhibited 50% of the net cell growth).

Evaluation of antimicrobial properties

Antibacterial activity was evaluated according to a previously described methodology (Soković, Glamoćlija, Marin, Brkić, & van Griensven 2010) using four Gram-negative bacteria: *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), *Enterobacter cloacae* (ATCC 35030), and four Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240), and *Listeria monocytogenes* (NCTC 7973). The minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations were determined and streptomycin and ampicillin were used as positive controls.

Furthermore, the antifungal activity was evaluated using eight reference species: *Aspergillus fumigatus* (ATCC 1022), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus versicolor* (ATCC 11730), *Aspergillus niger* (ATCC 6275), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112), *Trichoderma viride* (IAM 5061) and *Penicillium verrucosum* var. cyclopium (food isolate) (Soković & van Griensven, 2006). The MIC and minimum fungicidal concentration (MFC) were determined. Bifonazole and ketokonazole were used as positive controls.

Preparation of cupcakes

To prepare the cupcakes, a traditional base recipe was followed: four eggs were thoroughly mixed with 100 g of sugar and lemon zest. Then, 100 g of melted butter were added, intercalated with 125 g of flour and 2 g of yeast powder, and further mixed with a hand mixer at 450W (MFQ 3540, Bosch, Munich, Germany). The ERA (lyophilized) or the potassium sorbate powder were mixed in the dry ingredients. The dough was distributed in silicone baking cups and placed in the oven (HE-635, Teka, Haiger, Germany) for 15 minutes at 180 ºC. Three

different lots (12 per lot, further divided in 3 groups of 4 cupcakes for each storage time) of cupcakes were prepared: i) control cupcakes without any additives; ii) cupcakes with 1.1 g of ERA; iii) cupcakes with 1.1 g of potassium sorbate (E202). All samples were lyophilized, finely crushed and analysed (in triplicate), immediately after preparation, after three days of storage or after five days of storage (cupcakes were stored at room temperature and packed in a sealed plastic bag covered with aluminium paper).

Evaluation of the colour parameters and pH of cupcakes

Colour was measured on the top and on the bottom (in three different points in each case) of all cupcakes using a colorimeter (model CR-400, Konica Minolta Sensing Inc., Tokyo, Japan). The illuminate C was used, with a diaphragm aperture of 8 mm, after being calibrated against a standard white tile. The CIE L^* (lightness), a^* (greenness/redness), b^* (blueness/yellowness) colour space values were registered using "Spectra Magic Nx" (version CM-S100W 2.03.0006) (Fernandes, Antonio, Barreira, Oliveira, Martins, & Ferreira, 2012).

The pH of the samples was measured in three different points directly in the samples with a HI 99161 pH-meter (Hanna Instruments, Woonsocket, Rhode Island, USA).

Evaluation of the nutritional properties

Following the AOAC methods (AOAC, 2016), the contents of protein, fat and ash, was determined. Total energy was calculated following the equation: Energy (kcal) = 4 × (g proteins + g carbohydrates) + 9 × (g lipids).

In addition, fatty acids and free sugars were also determined. Fatty acids were analysed in hexane extracts (obtained in a Soxhlet apparatus) by gas-chromatography coupled to flame ionization detector (GC-FID). Fatty acids were identified by comparison with standards (standard 47885, Sigma-Aldrich, St. Louis, Missouri, USA) and expressed as relative percentages of each fatty acid (Barros et al., 2013a). Free sugars were determined in hydro-ethanolic extracts obtained from defatted samples by HPLC coupled to a refraction index (RI) detector (Barros et al., 2013a), identified by comparison with standards, and further quantified (g/100 g of cupcake) based on the internal standard (melezitose).

Antioxidant activity

Three independent samples (≈3 g) of each cupcake formulation and storage time were with a mixture of ethanol:water (30:70) during 1 h, using a procedure previously described by Caleja et al. (2016). After removing the solvent, the extracts were dissolved to a final concentration of 200 mg/mL. The antioxidant activity evaluation was performed using two *in vitro* assays: 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity and reducing

power (RP), following the experimental methodologies adopted by the authors (Caleja et al., 2016).

Statistical analysis

The statistical tests were done considering a 5% significance level using IBM SPSS Statistics for Windows, version 22.0. (IBM Corp., Armonk, NY, USA). Except for antimicrobial assays results, data were expressed as mean±standard deviation; differences in the number of decimal places are justified by the magnitude of standard deviations.

An analysis of variance (ANOVA) with type III sums of squares was performed using the general linear model (GLM) procedure, to compare the prepared cupcakes. The dependent variables were analysed using 2-way ANOVA with the factors “cupcake formulation” (CF) and “storage time” (ST). In parameters showing results with a statistically significant interaction among factors, those were evaluated simultaneously by the estimated marginal means plots for all levels of each factor. In cases with no statistical significant interaction, means were compared using Tukey’s multiple comparison test, after checking the equality of variances through a Levene’s test.

In addition, a linear discriminant analysis (LDA) was used to compare the effects of CF and ST on the differences observed in the evaluated parameters. 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. The main purpose was estimating the relationship between single categorical dependent variables (cupcake formulations or storage times) and the quantitative independent variables (evaluated parameters), thereby determining which independent variables contributed more to the differences in the average score profiles of both factors. To verify the significance of the canonical discriminating functions, Wilk’s Λ test was used. A leaving-one-out cross validation procedure was carried out to assess the model performance.

Results and discussion

Rosemary Extract (E392) is the only allowed food preservative extract within the European Union (Carocho, Morales, & Ferreira, 2015). This additive is extracted from rosemary (*Rosmarinus officinalis* L.) leaves and stems and Rosmarinic acid is one of the main constituents. Nowadays, in food industry thousands of plants have been screened for suitability to be used as preservatives.

In the present work, the aqueous extract of *M. officinalis* (lemon balm), previously reported as containing high levels of rosmarinic acid (Barros et al., 2013a; Caleja et al., 2017b;

Döring et al., 2014), was used as a natural preserver/functionalizing agent in cupcake formulations. Before the extracts incorporation, their bioactivity was characterized, specifically by evaluated antioxidant activity, antibacterial activity, antifungal activity and potential hepatotoxicity, which are among the acknowledged effects of *M. officinalis* (Shakeri et al., 2016).

Considering the results obtained in four distinct assays (**Table 28**), the strong antioxidant activity of ERA is obvious, especially concerning TBARS formation inhibition (EC_{50} = 25±2 µg/mL extract), reducing power (EC_{50} = 49±1 µg/mL extract) and DPPH scavenging activity (EC_{50} = 79±2 µg/mL extract), which is in line with previously obtained results (Barros et al., 2013b; Dias et al., 2012; Kamdem et al., 2013; Pereira et al., 2015; Pereira et al., 2017).

The antibacterial (**Table 28**) is noteworthy since the ERA gave lower MIC and MBC than streptomycin and ampicillin for all bacterial species, Gram positive or Gram negative, except for *Staphylococcus aureus.* Among Gram positive bacteria, the most sensitive were *Enterobacter cloacae* (MIC = 0.075 mg/mL extract; MFC = 0.15 mg/mL extract). The MIC and MBC values are slightly better, despite comparable, to those obtained with the decoctions of the same species (Carocho et al., 2015b; Shakeri et al., 2016). Nevertheless, the antifungal activity was even stronger, especially in comparison to ketoconazole, as it might be confirmed by the tenfold differences in MIC among some fungal species such as *Aspergillus ochraceus*. Actually, and except for the MFC values measured in *Aspergillus fumigatus*, *Penicillium funiculosum* and *Penicillium ochrochloron*, the *M. officinalis* extract showed higher activity than ketoconazole and bifonazole against all fungal species, either considering MIC or MFC results. A good level of antifungal activity was also observed when evaluating *M. officinalis* activity against related fungal species (Shakeri et al., 2016) as well as against phytopathogenic fungi (El Ouadi et al., 2017).

Interestingly, the ERA did not show acute cytotoxicity, as revealed by maintenance of the PLP cell culture (the GI_{50} value could not be determined, since cells maintain their growth when exposed to the maximum tested extract concentration: 400 µg/mL).

Furthermore, the quantification of rosmarinic acid (181 ± 3 mg of rosmarinic acid/g of extract) in the optimized extract obtained from lemon balm was also determined.

According to the former advantageous properties, the ERA were incorporated in cupcakes (standard formulation) aiming to add some beneficial effects to their consumption. Besides comparing the newly formulated cupcakes with the traditional recipe (no additives included), a set of cupcakes containing the commonly used preservative, potassium sorbate (E202), was also prepared and evaluated. Furthermore, all performed assays were done at three different periods (baking day, after 3 days of storage and after 5 days of storage).

Characterization of different cupcake formulations

Owing to their high consumption level, cupcakes might be considered as a suitable product to test different functionalization approaches. In fact, this particular food product was previously functionalized with xanthophylls (Xavier et al., 2017) or anthocyanin-enriched extracts (Abdel-Moemin, 2016), besides being formulated with different flours (Kim & Shin, 2014), trying to fulfil the current consumers' preference towards food products containing natural additives instead of synthetic compounds.

In the present work, three different cupcake formulations were prepared: i) control (cupcakes without any incorporated extract); ii) cupcakes with ERA; iii) cupcakes added with potassium sorbate. Furthermore, the potential differences among formulations were also evaluated throughout time, by analysing samples from cupcakes submitted to different storage times: preparation day, 3 days after preparation and 5 days after preparation. In this sense, two different factors (CF and ST) might contribute to the variability among different analysed parameters. Therefore, it was also mandatory to verify their interaction (CF×ST), in order to check if the effect of one factor depended on the variation of the other. Concerning the parameters where a significant interaction was found ($p<0.050$), the statistical classification was not possible, and the results were discussed based on the estimated marginal means (EMM) plots (data might be provided upon request).

The proximate analysis results (**Table 29**) showed significant differences, either among different formulations, as well as in result of ST, for almost all parameters, except glucose ($p = 0.533$) and sucrose ($p = 0.962$) regarding CF, thereby indicating a generally more significant effect of ST in comparison to CF. However, the interaction was also significant in all cases, except protein content ($p = 0.352$), allowing to classify the results only in this case. As indicated, protein content was statistically higher in control samples (7.3±0.5 g/100 g), among different CF, and in samples stored during 3 days (7.3±0.4 g/100 g), in what concerns ST. Considering the EMM plots, additional overall conclusions could also be obtained for the remaining parameters: moisture (as expected) tended to decrease along time (36±3 g/100 g at preparation day, 30±4 g/100 g after 5 days of storage), showing also lower values (30±4 g/100 g) in control samples. These differences are linked to the variation in carbohydrates (since these are calculated by difference), which increased along time (38±1 g/100 g at preparation day to 43±2 g/100 g after 5 days of storage), and registered maximum values in control samples. The most noticeable difference regarding fat content resulted from ST, as indicated by the higher contents (20±1 g/100 g) quantified in samples stored for 5 days. Energy values increased along ST (345±12 kcal to 378±21 kcal) and showed highest values in control samples, most likely due to carbohydrate content.

Despite the relevance of nutritional parameters, the visual appearance is also highly important, as it represents the primary consumers' acceptability criterion. In **Figure 25**, the photographs of each of the cupcakes samples are shown where visually the results described can be verified and the similarities between the control cupcake (**Figure 25A**) and cupcake with potassium sorbate (**Figure 25C**) distinguished from cupcake with lemon balm extract (**Figure 25B**) which presents a more "integral aspect".

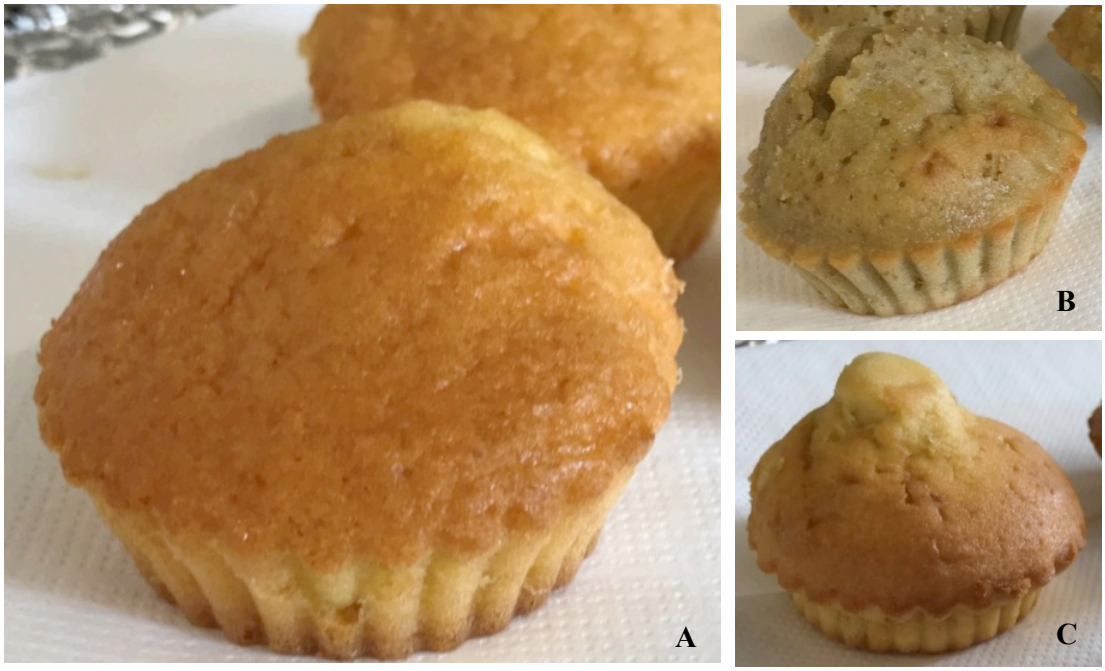

Figure 25. Appearance of cupcakes samples along shelf life (A) cupcake control, (B) cupcake with lemon balm extract rich in rosmarinic acid and (C) cupcake with potassium sorbate.

Table 28. Antioxidant activity (EC_{50} values, μg/mL extract) of lemon balm extract rich in rosmarinic acid (ERA). Values are given as mean±standard deviation.

	Antioxidant activity (EC_{50} values, μg/mL extract)			
	DPPH scavenging activity	Reducing power	β-carotene bleaching inhibition	TBARS formation inhibition
ERA	79±2	49±1	407±9	25±2

	Antibacterial activity (MIC and MBC values, mg/mL extract)							
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
	Gram positive bacteria							
	Escherichia coli		*Pseudomonas aeruginosa*		*Salmonella typhimurium*		*Enterobacter cloacae*	
ERA	0.10	0.15	0.15	0.30	0.15	0.30	0.075	0.15
Streptomycin	0.20	0.30	0.20	0.30	0.25	0.50	0.20	0.30
Ampicillin	0.40	0.50	0.75	1.20	0.40	0.75	0.25	0.50
	Gram negative bacteria							
	Staphylococcus aureus		*Bacillus cereus*		*Micrococcus flavus*		*Listeria monocytogenes*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
ERA	0.15	0.30	0.075	0.15	0.10	0.15	0.15	0.30
Streptomycin	0.04	0.10	0.10	0.20	0.20	0.30	0.20	0.30
Ampicillin	0.25	0.45	0.25	0.40	0.25	0.40	0.40	0.50

	Antifungal activity (MIC and MFC values, mg/mL extract)															
	Aspergillus fumigatus		*Aspergillus ochraceus*		*Aspergillus versicolor*		*Aspergillus Niger*		*Penicillium funiculosum*		*Penicillium ochrochloron*		*Trichoderma viride*		*Penicillium verrucosum*	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
ERA	0.15	0.30	0.10	0.20	0.075	0.15	0.04	0.075	0.15	0.30	0.15	0.30	0.075	0.15	0.05	0.075
Ketoconazole	0.25	0.50	1.50	2.00	0.20	0.50	0.20	0.50	0.20	0.50	2.50	3.50	1.00	1.00	0.20	0.30
Bifonazole	0.15	0.20	0.15	0.20	0.10	0.20	0.15	0.20	0.20	0.25	0.20	0.25	0.15	0.20	0.10	0.20

Table 29. Nutritional composition (g/100 g) and energy (kcal) values for different cupcake formulations (CF) and storage times (ST). Results are presented as mean±standard deviation.[1]

		Moisture	**Fat**	**Protein**	**Ash**	**Carbohydrates**	**Glucose**	**Sucrose**	**Energy**
CF	Control	30±4	20±1	7.3±0.5 a	0.70±0.04	42±3	0.17±0.02	37±4	375±23
	Lemon balm extract rich in rosmarinic acid (ERA)	35±2	19±1	6.8±0.4 b	0.67±0.05	39±1	0.19±0.05	37±4	350±11
	Potassium sorbate (E202)	33±4	19±1	6.9±0.4 b	0.75±0.05	40±3	0.18±0.05	37±4	356±15
	ANOVA *p*-value (n = 27)[2]	<0.001	0.002	<0.001	<0.001	<0.001	0.533	0.962	<0.001
ST	0 days	36±3	18±1	6.8±0.4 b	0.74±0.05	38±1	0.20±0.05	34±2	345±12
	3 days	32±2	19±1	7.3±0.4 a	0.70±0.05	40±2	0.21±0.04	39±3	358±8
	5 days	30±4	20±1	6.9±0.5 b	0.69±0.05	43±2	0.13±0.04	39±4	378±21
	ANOVA *p*-value (n = 27)[3]	<0.001	<0.001	<0.001	0.004	<0.001	<0.001	<0.001	<0.001
CF×ST	*p*-value (n = 81)[4]	<0.001	<0.001	0.358	<0.001	<0.001	<0.001	<0.001	<0.001

[1]The results presented for each CF included the contribution of values measured in all ST; likewise, results for each ST were obtained considering the values measured for all CF. [2]If $p<0.050$, the corresponding parameter presented a significantly different value for at least one CF. [3]If $p<0.050$, the corresponding parameter presented a significantly different value for at least one ST. [4]$p<0.050$ indicates a significant interaction among factors, thereby precluding performing multiple comparison tests.

Accordingly, the colour parameters (*L**: lightness, *a**: redness and *b**: yellowness) were evaluated in the top and bottom crusts of the prepared cupcakes (**Table 30**). All measured parameters showed significant differences among different CF, while no statistical differences at all were detected for cupcake samples stored during different periods. The interaction among factors was again significant in most cases, with *b** (top) as the only parameter that was not affected cooperatively by both factors ($p = 0.809$). This parameter showed statistically higher values (50±5) in cupcakes added with potassium sorbate. According to the EMM plots, it was possible to conclude that cupcakes incorporating ERA presented lower *L** (top: 56±3), *a** (top: 6±2 and bottom: 11±4) and *b** (top: 33±2 and bottom: 34±2) than the remaining formulations, thereby indicating that this particular type of cupcakes might result less visually attractive (especially for younger consumers).

Despite a certain tendency for higher values in cupcakes added with potassium sorbate and lower values in control cupcakes, differences in pH values were barely noticeable.

Concerning fatty acids profile, the main molecules (**Table 31**) were also affected cooperatively by CF and ST, except in the case of myristic acid (C14:0) and palmitic acid (C16:0). Regarding the individual effect of each factor, caproic acid (C6:0), C14:0 and stearic acid (C18:0) did not show significant variation among different CF, in line with the verified for lauric acid (C12:0) and C14:0 concerning the ST effect. Accordingly, it was only possible to present the statistical classification in the case of C16:0, which showed the highest values in cupcakes functionalized with ERA and in non-stored cupcakes, for each respective factor. In the remaining cases, it was possible to conclude that butyric acid (C4:0) presented lower values (4±1%) in cupcakes with ERA and in control cupcakes (4±1%), C6:0 (2.3±0.4%) and capric acid (C10:0) (3.2±0.5%) presented lower percentages at the preparation day, C10:0 (4.3±0.5%) and C12:0 (4.5±0.4%) showed higher levels in cupcakes without additives (control), C18:0 tended to lower values (9.5±0.4%) after 5 days of storage and oleic acid (C18:1n9) presented lower percentages in control (23±1%) samples and in non-stored (28±1%) samples. The same tendencies were reflected in grouped fatty acids (SFA, MUFA and PUFA), mostly showing the higher tendency of saturated fatty acids along storage, when compared to the more sensitive unsaturated forms.

Table 30. Colour parameters evaluated in cupcake formulations (CF) and storage time (ST). Results are presented as mean±standard deviation.[1]

		L(top)*	***a***(top)*	***b***(top)*	***L***(bottom)*	***a***(bottom)*	***b***(bottom)*	**pH**
CF	Control	64±5	12±4	46±2 b	56±3	15±2	37±3	7.1±0.2
	Lemon balm extract rich in rosmarinic acid (ERA)	56±3	6±2	33±2 c	54±3	11±4	34±2	7.3±0.2
	Potassium sorbate (E202)	66±6	13±4	50±4 a	50±5	18±3	38±4	7.5±0.2
	ANOVA *p*-value (n = 27)[2]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
ST	0 days	63±7	9±4	43±8	53±5	14±5	36±3	7.3±0.4
	3 days	60±6	12±6	42±8	53±3	15±5	36±3	7.3±0.2
	5 days	63±6	11±4	44±7	53±3	15±3	37±3	7.3±0.2
	ANOVA *p*-value (n = 27)[3]	0.215	0.100	0.596	0.806	0.797	0.768	0.797
CF×ST	*p*-value (n = 81)[4]	0.006	0.001	0.809	<0.001	0.001	<0.001	<0.001

[1]The results presented for each CF included the contribution of values measured in all ST; likewise, results for each ST were obtained considering the values measured for all CF. [2]If $p<0.050$, the corresponding parameter presented a significantly different value for at least one CF. [3]If $p<0.050$, the corresponding parameter presented a significantly different value for at least one ST. [4]$p<0.050$ indicates a significant interaction among factors, thereby precluding performing multiple comparison tests.

Considering the purpose of providing a new food product with specific bioactivity, the improvement in the antioxidant activity achieved in cupcakes incorporated with ERA was evident. The average EC_{50} values for DPPH scavenging activity (obtained for all storage times) were 68±12 mg/mL extract for control cupcakes, 41±12 mg/mL extract for cupcakes incorporated with ERA and 44±18 mg/mL extract for cupcakes added with potassium sorbate. However, the antioxidant activity increase was more noticeable in the reducing power assay: EC_{50} values = 70±12 mg/mL extract for control cupcakes, 9±1 mg/mL extract for cupcakes incorporated with ERA and 37±13 mg/mL extract for cupcakes added with potassium sorbate, clearly highlighting cupcakes incorporated with ERA as the ones having highest bioactive potential.

In either case, a significant percentage of the detected antioxidant activity was lost throughout storage, as indicated by the EC_{50} values for DPPH scavenging activity (0 days: 44±18 mg/mL extract; 3 days: 54±7 mg/mL extract; 5 days: 65±14 mg/mL extract) and reducing power (0 days: 29±15 mg/mL extract; 3 days: 39±19 mg/mL extract; 5 days: 48±21 mg/mL extract).

Linear Discriminant Analysis

After evaluating several individual changes among the studied parameters, either due to CF or ST effects, the next step was identifying the parameters with the most significant changes for each factor, as well as the ones more highly correlated to each factor level (0, 3 or 5 days in one case and control, cupcakes functionalized with lemon balm extract or added with potassium sorbate in the other). With that purpose in mind, two linear discriminant analysis (LDA) were performed, intended to respectively evaluate the association between CF or ST (categorical dependent variables) and the matrix of obtained results (quantitative independent variables). The significant independent variables were selected according to the stepwise method of LDA, considering the Wilks' λ test. Only variables with a statistically significant classification performance ($p < 0.050$) were selected in the obtained statistical models.

Table 31. Major (detected above 1%) fatty acids (relative percentage) in cupcake formulations (CF) and storage time (ST). Results are presented as mean±standard deviation.[1]

		C4:0	C6:0	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1n9	SFA	MUFA	PUFA
CF	Control	5±1	3±1	4.3±0.3	4.5±0.4	10.2±0.5	32±1 ab	9.8±0.5	23±1	74±2	25±1	0.7±0.2
	Lemon balm extract rich in rosmarinic acid (ERA)	4±1	3±1	3.1±0.4	3.5±0.3	10.1±0.4	33±1 a	9.8±0.3	27±3	70±3	29±3	0.6±0.2
	Potassium sorbate (E202)	5±1	3±1	3.3±0.5	3.6±0.4	10.2±0.4	32±1 b	9.8±0.4	26±2	71±2	28±2	0.6±0.2
	ANOVA *p*-value (n = 27)[2]	<0.001	0.235	<0.001	<0.001	0.560	0.001	0.846	<0.001	<0.001	<0.001	0.210
ST	0 days	4±1	2.3±0.4	3.2±0.5	3.7±0.5	10.0±0.4	33±1 a	9.9±0.5	28±3	69±3	30±3	0.5±0.2
	3 days	5±1	3.2±0.5	3.7±0.5	4.0±0.5	10.3±0.4	32±1 ab	9.9±0.4	24±1	73±1	27±1	0.8±0.1
	5 days	6±1	4±1	3.9±0.5	3.8±0.4	10.2±0.5	32±1 b	9.5±0.4	23±2	74±2	25±2	0.7±0.1
	ANOVA *p*-value (n = 27)[3]	<0.001	<0.001	0.001	0.160	0.054	0.037	0.002	<0.001	<0.001	<0.001	<0.001
CF×ST	*p*-value (n = 81)[4]	<0.001	<0.001	0.003	0.010	0.228	0.103	<0.001	<0.001	<0.001	<0.001	<0.001

The results are presented as mean ± SD. Butyric acid (C4:0); Caproic acid (C6:0); Capric acid (C10:0); Lauric acid (C12:0); Myristic acid (C14:0); Palmitic acid (C16:0); Stearic acid (C18:0); Oleic acid (C18:1n9); SFA-Saturated fatty acids; MUFA-Monounsaturated fatty acids; PUFA-Polyunsaturated fatty acids.[1]The results presented for each CF included the contribution of values measured in all ST; likewise, results for each ST were obtained considering the values measured for all CF. [2]If $p<0.050$, the corresponding parameter presented a significantly different value for at least one CF. [3]If $p<0.050$, the corresponding parameter presented a significantly different value for at least one ST. [4]$p<0.050$ indicates a significant interaction among factors, thereby precluding performing multiple comparison tests.

Regarding ST effect, the two defined discriminant functions included 100.0% (first function: 85.8%; second function: 14.2%) of the observed variance (**Figure 26A**). From the initial 28 variables, only 10 (carbohydrates, fat, ash, glucose, sucrose, energy, *b** (bottom), PUFA, DPPH scavenging activity and reducing power) were selected as having discriminant ability to separate samples corresponding to each ST. The first function was highly correlated to carbohydrates, energy and glucose, and its main contribution was the separation of markers corresponding to samples stored 5 days, which might be characterized as having particularly high energy and carbohydrates levels in one side, and the lowest glucose contents, on the other. Function 2, in contrast, allowed separating samples corresponding to non-stored samples from those stored for 3 days, mainly due to the differences in sucrose and PUFA (both lower in non-stored samples) and DPPH scavenging activity (higher in non-stored samples) the variables with highest correlation with function 2. Overall, non-stored cupcakes showed higher similarity with those stored for 3 days, as also indicated by the classification performance of the discriminant model: 96.3% for the original grouped cases (3 of the 27 non-stored cupcakes were classified as having been stored for 3 days) and 93.8% (3 of the 27 non-stored cupcakes were classified as having been stored for 3 days; 2 of the 27 cupcakes stored for 3 days were classified as not having been stored; all cupcakes stored during 5 days were correctly classified) for the cross validated ones.

In what concerns CF effect, the two defined discriminant functions included 100.0% (first function: 81.6%; second function: 18.4%) of the observed variance (**Figure 26B**). The model selected 12 variables (*L** (top), *a** (top and bottom), *b** (top and bottom), pH, C6:0, C10:0, SFA, MUFA, DPPH scavenging activity and reducing power) as having significant capacity to discriminate samples corresponding to each CF. As observable in **Figure 26B**, function 1 separated cupcakes functionalized with ERA from the remaining samples, mostly due to the values of *L**, *a** and *b** (all with lower values in cupcakes prepared with ERA) at the top part of cupcakes. The three variables with highest correlation with function 2 were reducing power and DPPH scavenging activity (higher EC_{50} values in control cupcakes) C10:0 and 12:0 (higher in control cupcakes) and pH (lower in control cupcakes), thereby being effective in separating control samples from those added with potassium sorbate. In this case, the classification performance was 100% accurate for originally grouped cases and 98.8% for cross-validated ones.

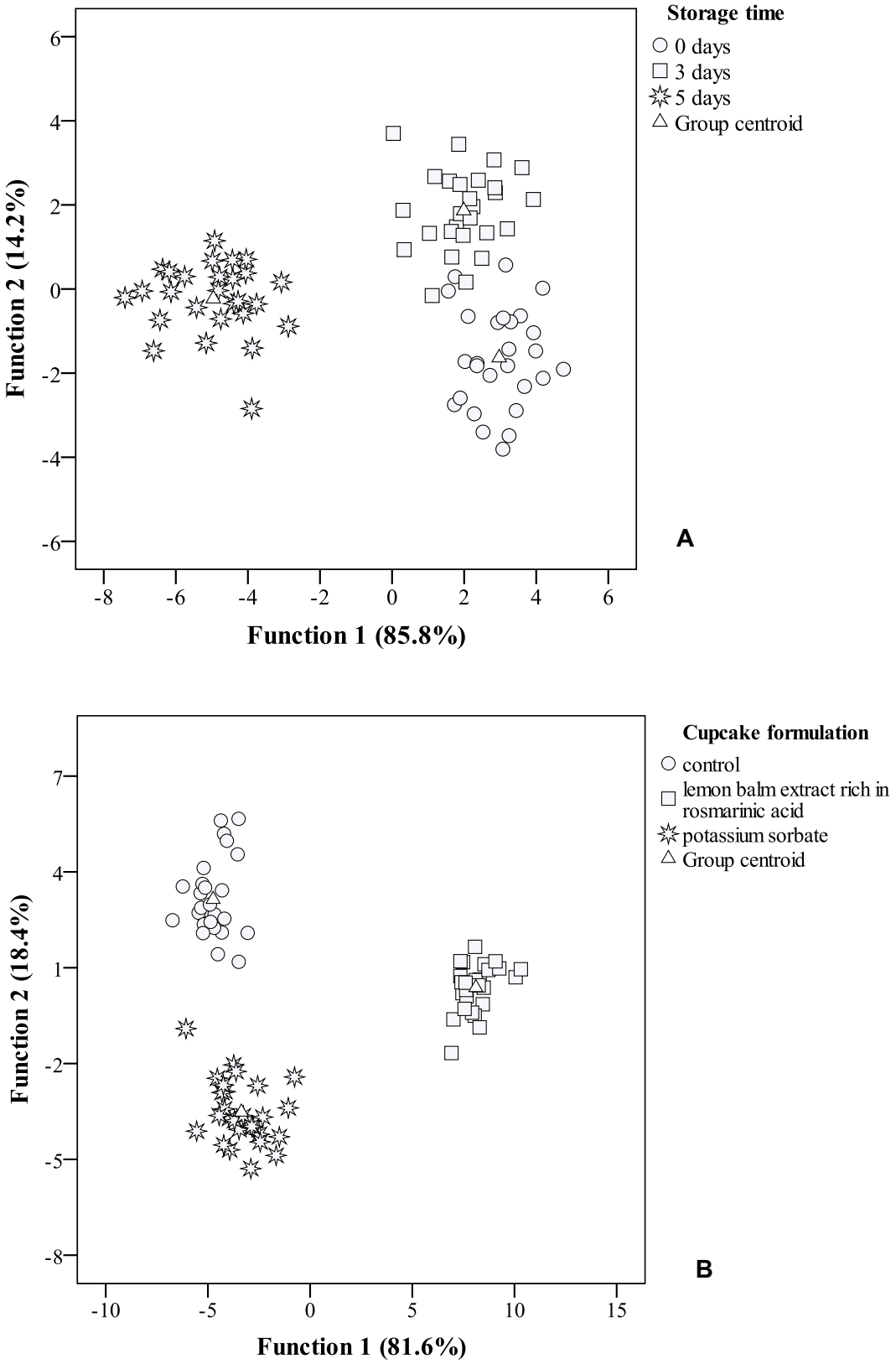

Figure 26. Canonical discriminant functions coefficients defined from the evaluated parameters. **A:** Storage time effect. **B:** Cupcake formulation effect.

Conclusions

Overall, CF exerted more pronounced effects than ST over the parameters evaluated in these cupcakes. Energy level, carbohydrates, sucrose, glucose and PUFA were the variables with highest differences among different ST, while *L** (top), *a** (top), *b** (top), reducing power, DPPH scavenging activity, C10:0, 12:0 and pH presented the most significant changes according to CF effect. In general, and in view of the strong bioactivity (antioxidant, antibacterial and antifungal activities) exhibited by ERA, the obtained results reinforce its suitability to be used as natural additive to provide advantageous properties to pastry products, potentially benefiting consumers' health.

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4.2. **Flor de castanheiro apresentada como conservante natural**

Neste capítulo são apresentados dois artigos onde de descreve a otimização da técnica de maceração para a obtenção de um extrato rico em compostos fenólicos obtido a partir de flores de castanheiro. É apresentado também todo o estudo antioxidante, antimicrobiano e de hepatotoxicidade para a posterior aplicação num produto de pastelaria tipicamente português. Os efeitos da sua incorporação no pastel de nata são comparados com um aditivo artificial.

4.2.1. Desenvolvimento de um preservante natural a partir de flores masculinas de castanheiro: Otimização da técnia assistida por calor

Submitted: *Food Chemistry*

Development of a natural preservative obtained from male chestnut flowers: Optimization of heat- assisted extraction technique

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Abstract

The aim of this work was to optimize the extraction conditions of phenolic compounds from male chestnut flowers using heat-assisted extraction with the purpose of developing extracts rich in phenolic compounds for its potential application in the food industry as a natural ingredient. The study conditions of time (t), temperature (T) and water-ethanol solvent mixture (S) that favours the extraction of active phenolic compounds was optimized by response surface methodology using a 5-level experimental design. The responses used as criteria were the quantification of the individual phenolic compounds identified by HPLC-DAD-ESI/MS and the extraction yield of the obtained residue. Based on their chromatographic, UV-vis and mass spectra characteristics, fourteen compounds were identified of which seven were hydrolysable tannins and seven were flavonoids. The results revealed that the hydrolysable tannins were recovered in higher quantities in relation to the flavonoids, being trigalloyl-HHDP-glucoside the major compound found. The models developed were successfully fitted to the data and used to determine optimal extraction conditions for maximization purposes of the responses assessed. In this regard, the conditions that maximized the total phenolic content was found at t= 20.0±37.7 min, T= 25.0±5.7 ºC S= 0.0±8.7% ethanol and 82.8 g/L of solid-to-liquid ratio producing an extract with 86.5 mg of phenolic compounds per g of extract. The results highlight the potential of valorising chestnut flowers agro-residues as a productive source of active

phenolic compounds for the development of bio-based ingredients for food, pharmaceutical and cosmeceutical industrial applications that may be able to compete with synthetic compounds.

Keywords: Heat-assisted extraction; *Castanea sativa*; Male chestnut flowers; Natural ingredients; Hydrolysable tannins; Flavonoids; Extraction optimization.

Introduction

The chestnut tree (*Castanea sativa* Mill.) fruit represent one of the most economically important agro-food material in the northeaster region of Portugal, in which the fruit represents the most exported plant part to Europe [1]. Despite the importance of the fruit for the region, previous scientific work can be mentioned to illustrate how almost all parts of the chestnut tree have been studied in order to find potential industrial applications [2,3]. Among them, the most relevant are: 1) chestnut wood, which is used in the production of furniture and it is considered of high quality [4]; 2) chestnut leaves and flowers, which have been used since ancient times in the preparation of infusions due to the high concentration of active phenolic compounds (PC) beneficial to the human health, especially in treatments of colds, cough or diarrhoea [5]; and 3) chestnut honey, although it is not a standard by-product from the production of chestnut fruits, it is highly appreciated and its production is totally attached to the chestnut tree agro-industry. Outside of those uses, tones of agro-residues are generated annually (branches, leaves, flowers, etc) and used, in the better cases, as natural fertilizers or, in a less environmental friendly cases, incinerated. Reduction of environmental impacts of by-products from industrial processes have been continually emphasized in the last two decades, in which scientists have emphasized the transformation of industries using advance sustainable process of agro-industrial activities [6]. As a consequence, typically discarded by-products generated, have been valorized [7].

Recent research has shown that male chestnut flowers (CF) or extracts possess high abundance of PC that can be used in the preservation of foods due to their capacity to inhibit lipid peroxidation and microbial proliferation [4,8,9], and used as a natural ingredient while enhancing the health of consumers [10]. These properties as well as the medicinal effects referred above have been related to their PC [4,8,9]. In this regard, recently studies have incorporated CF into different Portuguese products such as "Serra da Estrela" cheese and "económicos" dried cakes, and results have confirmed the potential of these natural matrices in the development of new food products which can meet consumers expectations [11,12].

Differently, recent scientific evidences have related the consumption of synthetic compounds in foods with undesirable effects in human health. Such results are pushing the food-industry to look for alternatives that meet consumers' needs towards a more natural market [13]. In this way, the food industry has been searching in the substitution of this type of synthetic additives by natural ingredients obtained from plants, mushrooms or algae, with already proven human health benefits [14].

In order to bring natural additives into a real and efficient alternative to the widely used artificial analogues, it is necessary to find promising sources and develop sustainable and efficient recovery processes for these compounds. However, the efficiency of these processes are affected by considered variables (e.g., time, temperature, ultrasonic power and solvent) [15–17]. The production of natural ingredients is more complex than it seems, since it always requires a preliminary study on the type of compounds as well as the best conditions and methodologies to be applied in there extraction [18–20]. Therefore, it is necessary to use appropriate experimental designs and optimization tools to determine optimal extraction conditions that led to the best response values. Moreover, different extraction parameters such as the solvent used, time and energy, as well as the possible loss of natural compounds, should be also taken into consideration [21]. To guarantee a maximum yield with the minimum of time, solvent and energy used, it is essential to select and optimize the best extraction conditions [22]. Through response surface methodology (RSM) it is possible to optimize the relevant variables simultaneously, obtaining mathematical solutions capable of describing, within the tested experimental interval, the ideal conditions that maximize the used response criteria [23].

Therefore, this study intends to optimize the conditions for the recovery of phenolic compounds from CF using one of the most known techniques for the extraction of natural compounds, the heat-assisted extraction (HAE), in order to be used in the food industry. The three most relevant independent variables for each process were combined in a RSM system for the extraction process optimization.

Material and methods

Samples

Male chestnut (*Castanea sativa* Mill.) flowers (CF) were collected near Bragança (Samil) in the northeastern region of Portugal in June of 2017 (41º46´52´´N, 6º45´54´´W). The samples were lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA) and reduced to a fine powder (~20 mesh). The obtained powder was mixed to guarantee the sample

homogeneity and stored in a desiccator at room temperature (~25 ºC), protected from light, until further analysis.

Heat-assisted extraction (HAE) technique

The relevant variables, and the selection of appropriate tested ranges, were obtained based on the combination of single variable preliminary experiments, in previous extractions studies performed in our laboratory and using bibliographic surveys. The solid/solvent ratio was kept constant (30 g/L). The used solvent was an ethanol/water mixture characterized in terms of ethanol content (%, w/w).

The dried powdered CF (600 mg) were placed in a beaker with 20 mL of solvent. The beaker was then placed in a thermostatic water bath under continuous electro-magnetic stirring (CIMAREC i Magnetic Stirrer with a fixed agitation speed 500 rpm, Thermo Scientific, San Jose, CA, USA) for the required time. The variables and ranges tested were: time (t or X1, 20 to 120 min), temperature (T or X2, 25 to 85 ºC) and ethanol solvent proportion (S or X3, 0 to 100 %).

Identification and quantification of phenolic compounds 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/MS (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA) system. Detection was carried out by DAD, using 280, 330, and 370 nm as the preferred wavelength, coupled to a Linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an ESI source and working in negative mode. Data acquisition was carried out with Xcalibur® data system (Thermo Finnigan, San Jose, CA, USA). The phenolic compounds were characterized according to their UV, mass spectra, retention times in comparison with authentic standards when available, and with literature. For quantification, calibration curves were generated by injection of known concentration (2.5–100 µg/mL) of standard compounds: ellagic acid (y = 26719x - 317255; R2 = 0.999); gallic acid (y = 131538x + 292163; R2 = 0.997); quercetin 3-*O*-glucoside (y = 34843x – 160173; R2 = 0.999).

Determination of extraction yield

The residue (R) resulting from each extraction was determined gravimetrically in crucibles, first by partial evaporation of the solvent at 60 ºC and then by a heat treatment at 100 ºC for 24 h. The results were expressed in percentage (%).

Response value formats for results presentation

The quantified phenolic compounds were grouped in two forms: a) by groups, as hydrolysable tannins (Hta), flavonoids (Fla) and total phenolic compounds (Phe, including all quantified phenolics); b) major compounds (P5, P7, P8, P9, P11, P13 and P14) and minor compounds (P1, P2, P3, P4, P6, P10 and P12). Therefore, the response criteria to optimize the extraction conditions of Hta, Fla and Phe from CF using RSM were: extraction yield (in %, which provides information regarding the quantity of extracted residue) and the compounds content in the individual and grouped terms (mg/g R, which was specifically used to evaluate the compounds purity in the extracts).

Experimental design, modelling and optimization

Experimental design

A five-level Central Composite Circumscribed Design (CCCD) coupled with RSM was implemented to optimize the HAE and UAE conditions for the extraction of phenolic compounds from CF. The coded and natural values of the independent variables X1 (processing time, t in min), X2 (temperature, T in ºC) and X3 (solvent, S in % of ethanol, v/v) are presented in **Table 32**. The CCCD includes 6 replicated centre points and a group of axial points to allow rotatability, which ensures that the variance of the model prediction is constant at all points equidistant from the design centre. The experimental runs were randomized to minimize the effects of unexpected variability in the observed responses.

Table 32. Experimental domain and codification of independent variables in the *CCCD* factorial design with 5 range levels.

CODED VALUES	**NATURAL VALUES**		
	t (min)	*T (ºC)*	*S (%)*
-1.68	20	25	0
-1	40,3	37,2	20.3
0	70	55	50
+1	99,7	72,8	79.7
+1.68	120	85	100

Mathematical modelling

The response surface models were fitted by means of least-squares calculation using the following second order polynomial model with interactions:

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{ii} X_i^2 \quad ((13)$$

In this equation, Y represents the dependent variable (response variable) to be modelled, Xi and Xj are the independent variables, b0 is the constant coefficient, bi is the coefficient of linear effect, bij is the coefficient of interaction effect, bii is the coefficient of quadratic effect, and n is the number of variables. The extraction yield of the residue (R) obtained (in %) and the individual and grouped phenolic compounds (14 compounds of 2 major groups, in mg/g R) were used as dependent variables.

Procedure to optimize the variables to a maximum response

A simplex method was used to optimize the predictive model by solving nonlinear problems in order to maximize the extraction yield and the recovery of phenolic compounds [24]. Certain limitations were imposed (i.e., times lower than 0) to avoid variables with unnatural conditions.

Dose-response analysis of the solid to liquid ratio

The analysis of the solid-to-liquid ratio (S/L or X4, expressed in g/L) was performed by a dose-response at the optimal conditions of the variables found by the RSM (X1, X2, and X3). The aim was to achieve the S/L conditions that leads to a more productive processes for industrial applications. As described previously [25,26], to depict the response effect as function of the S/L, the Weibull (W) equation for increasing (↑) and decreasing (↓) responses was used (with some parametric modifications to fit the searched purposes):

$$\uparrow W(X_4) = K \exp\left[\ln\left(1 - {}^{n}\!/_{100}\right)\left(\frac{X_4}{m_n}\right)^a \right] \quad \text{or} \quad \downarrow W(X_4) = K - K \exp\left[\ln\left(1 - {}^{n}\!/_{100}\right)\left(\frac{X_4}{m_n}\right)^a \right] \quad (2)$$

where K is the maximum extraction value (the units would be in mg/g R for all the responses except for the extraction yield that would be in %), a is a shape parameter related to the maximum slope of the response, n is any desired level between 0 to 100% of the responses that would be achieved and mn would be the S/L value (X4) for the selected n response level (m10, m25, m75, m95, etc.). For example, if the n value is selected as 99%, the mn parameter will display de S/L needed to achieve the 99% of the assessed response (m99%). When the

response shows increasing patterns (↑), the Weibull equation that is used to describe the response will present a mn parameter of n=99%. When the response shows decreasing patterns (↓), a mn 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 (m99%). However, when the response decreases, the value of m99% 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 m50%). If other mn 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.

Numerical methods, statistical analysis and graphical illustrations

Fitting procedures, coefficient estimates and statistical calculations were achieved as previously described by other authors [27]. In brief, a) the parameters determination was accomplished using the quasi-Newton algorithm (least-square) by running the integrated macro '*Solver*' in Microsoft Excel minimizing the differences between observed and predicted values; b) the coefficient significance was evaluated using the '*SolverAid*' macro to determine their intervals (α=0.05); and c) the model consistency was proved by means of several statistical criteria: i) the Fisher F-test (α=0.05) used for the assessment the adequacy of the models to describe the observed data; ii) the '*SolverStat*' macro was used for the assessment of parameter and model prediction uncertainties [28]; iii) the R^2 was interpreted as the proportion of variability of the dependent variable explained by the model.

Results and discussion

Experimental data and response criteria for RSM optimization

The HPLC phenolic profile (for hydrolysable tannins identification recorded at 280 nm and for flavonoid identification recorded at 370 nm) of the CF extract obtained can be seem in **Figure 27**. **Table 33** shows the retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data and identification of phenolic compounds of the peaks displayed in **Figure 27**. Identification of phenolic compounds was carried out and cross checked through their chromatographic characteristics, such as retention time, mass spectrum, UV absorption. In total, fourteen different phenolic compounds were detected of which seven were hydrolysable tannins and the other seven were flavonoids. In the present work the trigaloyl-HHDP-glucoside and quercetin-3-*O*-glucuronide were the major compounds

within the two classes found (hydrolysable tannin and flavonoid, respectively). All the detected compounds have been previously identified [8,29]. Of the fourteen compounds identified, seven (P1, P2, P3, P4, P6, P10 and P12) were considered minor, because they were found in very low amounts of which only two were classified as flavonoids.

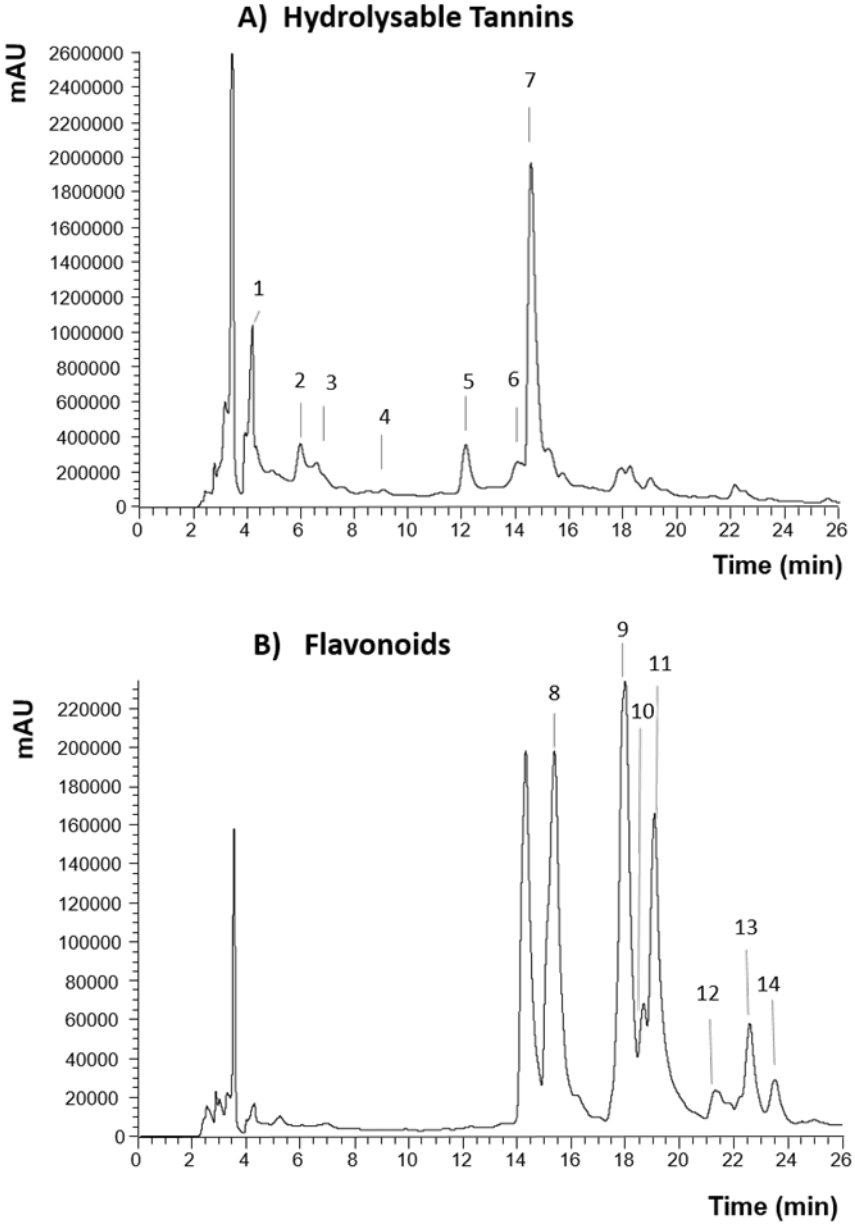

Figure 27. Example of a HPLC profile of phenolic compounds of the chestnut flower extract obtained.

Table 33. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data and identification of phenolic compounds.

Peak ID	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*) (% base peak)	Tentative identification	Phenolic compound classification
1	4.7	280	783	481(30),301(100)	Bis-HHDP-glucoside[A]	hydrolysable tannin
2	6.1	277	633	463(20),301(100)	Galloyl-HHDP-glucoside[A]	hydrolysable tannin
3	6.6	275	937	767(3),637(21),467(100),301(5)	Trigalloyl-HHDP-glucoside[A]	hydrolysable tannin
4	9.0	272	637	593(100),469(19),169(5)	Galloyl derivative[B]	hydrolysable tannin
5	12.1	275	939	631(23),469(100),169(5)	Pentagalloyl-glucoside[B]	hydrolysable tannin
6	13.9	275	937	767(3),637(19),467(100),301(5)	Trigalloyl-HHDP-glucoside[A]	hydrolysable tannin
7	14.5	273	937	767(3),637(20),467(100),301(3)	Trigalloyl-HHDP-glucoside[A]	hydrolysable tannin
8	15.2	355	479	317(100)	Myricetin-3-*O*-glucoside[C]	flavonoid
9	17.7	353	477	301(100)	Quercetin-3-*O*-glucuronide[C]	flavonoid
10	18.5	353	477	301(100)	Quercetin-3-*O*-glucuronide[C]	flavonoid
11	18.8	354	463	301(100)	Quercetin-3-*O*-glucoside[C]	flavonoid
12	21.1	343	593	285(100)	Kaempferol-3-*O*-rutinoside[C]	flavonoid
13	22.4	347	447	285(100)	Kaempferol-3-*O*-glucoside[C]	flavonoid
14	23.3	350	477	315(100)	Isorhamnetin-3-*O*-glucoside[C]	flavonoid

Phenolic compounds used for quantification: compounds [A]- ellagiic acid ($y = 26719x - 317255$; $R^2 = 0.999$); compounds [B]- gallic acid ($y = 131538x + 292163$; $R^2 = 0.997$); compounds [C]- quercetin 3-*O*-glucoside ($y = 34843x – 160173$; $R^2 = 0.999$).

The content in the final residue produced and the compounds distribution are strongly influenced by the extraction conditions. As already described, trying to understand the effects of each of the variables involved in an extraction system individually, while other variables are fixed, it is not as efficient as analysing all the effects in conjunction. Therefore, the first approach to optimize the efficiency of the HAE system for the recovery of PC in CF was to perform a simple independent test of each variable (data not showed) to set the convenient ranges for an optimization study under RSM system. Once it was performed, the application of a RSM was conducted for three variables in a CCCD with five levels, being the final rages selected as: t (20-120 min), T (25-85ºC), and S (0-100 %). A detailed description of the coded and natural values of the selected variables for each technique in the CCCD with tree variables is presented in the first part of **Table 34**, and in the second part are presented the experimental values of the 28 experimental runs of the CCCD design. For optimization purposes, the quantified phenolic compounds (**Table 34**) were grouped in two forms: a) by group of compounds, as hydrolysable tannins (Hta), flavonoids (Fla) and total phenolic compounds (Phe, including all quantified phenolics); b) major compounds (P5, P7, P8, P9, P11, P13 and P14) and minor compounds (P1, P2, P3, P4, P6, P10 and P12). Therefore, the yield of the extraction (in %) and the compounds content in the individual and grouped terms were used as response criteria to optimize the conditions for their extraction from CF using RSM.

The values of the extraction yield ranged from 8.02 to 42.83% (or g R/100 g CF dw) with the experimental runs nº 16 and 3, respectively (**Table 34**). The highest group of compounds detected were Hta and ranged from 4.23 to 43.62 mg/g R, corresponding to the experimental runs nº 6 and 15, respectively. The Fla group ranged from 4.62 to 17.49 mg/g R (experimental runs nº 6 and 18, respectively). Regarding the individual content of the identified compounds (P1 to P14, more details in **Table 33**), in which compound P7 (35.41 mg/g R, experimental run 15) showed the highest content followed by P5 (6.81 mg/g R, run 18), P8 (6.34 mg/g R, run 19), P9 (4.51 mg/g R, run 15), P11 (2.21 mg/g R, run 15), P13 (1.56 mg/g R, run 18), and P14 (1.32 mg/g R, run 18). All of them comprised the response criteria used for optimizing the conditions that favours their maximization.

Table 34. Variables, natural values, ranges and experimental RSM results of the *CCCD* for the optimization of the three main variables involved (X_1, X_2 and X_3) in the HAE for all the response values assessed: *Extraction yield* (%), T. hydrolysable tannins (*Hta*), T. Flavonoids (*Fla*), T. Phenolics (*Phe*) and major (*P5*, *P7*, *P8*, *P9*, *P11*, *P13* and *P14*) and minor (*P1*, *P2*, *P3*, *P4*, *P6*, *P10* and *P12*) compounds. Three replicates were performed for each condition for each technique.

CODED VALUES			YIELD	CLASS			COMPOUNDS IDENTIFIED BY HPLC							
							MAJOR							MINOR
X_1	X_2	X_3		*Hta*	*Fla*	*Phe*								
t (min)	*T (ºC)*	*S (%)*	*(%)*	*(mg/g R)*	*(mg/g R)*	*(mg/g R)*	P5 *(mg/g R)*	P7 *(mg/g R)*	P8 *(mg/g R)*	P9 *(mg/g R)*	P11 *(mg/g R)*	P13 *(mg/g R)*	P14 *(mg/g R)*	P1,2,3,4,6,10,12 *(mg/g R)*
-1(40,3)	-1(37,2)	-1(20,3)	38.12	26.11	10.07	36.18	1.62	21.64	2.24	3.03	1.40	0.93	0.69	4.64
-1(40,3)	-1(37,2)	1(79,7)	26.73	6.10	4.95	11.05	0.45	3.15	1.15	1.50	0.96	0.90	0.45	2.49
-1(40,3)	1(72,8)	-1(20,3)	42.83	28.00	8.67	36.66	1.46	23.44	2.48	1.78	1.69	0.79	0.57	4.46
-1(40,3)	1(72,8)	1(79,7)	35.94	15.49	6.60	22.09	2.29	4.02	2.88	1.31	0.79	0.77	0.85	9.17
1(99,7)	-1(37,2)	-1(20,3)	32.77	25.34	10.21	35.55	2.59	13.45	3.06	1.79	1.75	0.85	0.69	11.37
1(99,7)	-1(37,2)	1(79,7)	32.99	4.23	4.62	8.85	0.80	3.43	0.98	1.14	0.84	0.68	0.38	0.60
1(99,7)	1(72,8)	-1(20,3)	42.55	21.98	7.62	29.61	2.00	17.71	2.01	1.18	1.54	0.71	0.55	3.91
1(99,7)	1(72,8)	1(79,7)	35.52	6.48	4.62	11.10	0.76	4.25	0.66	1.96	0.74	0.64	0.62	1.47
1.68(120)	0(55)	0(50)	42.41	7.95	6.61	14.56	0.97	6.54	2.95	1.44	1.16	0.58	0.48	0.44
-1.68(20)	0(55)	0(50)	35.45	18.41	5.67	24.08	1.14	12.37	1.23	1.74	1.12	0.81	0.77	4.90
0(70)	-1.68(25)	0(50)	38.82	7.51	5.13	12.64	0.86	5.31	1.30	1.58	1.03	0.73	0.49	1.34
0(70)	1.68(85)	0(50)	42.06	12.48	4.94	17.41	1.27	7.21	1.28	1.35	1.12	0.59	0.61	3.99
0(70)	0(55)	-1.68(0)	35.24	23.99	10.59	34.58	1.86	17.03	2.58	2.58	2.12	0.91	0.61	6.89
0(70)	0(55)	1.68(100)	15.61	6.06	5.96	12.01	0.45	2.10	1.31	1.52	1.22	1.14	0.77	3.51
-1.68(20)	-1.68(25)	-1.68(0)	22.30	43.62	15.94	59.56	3.98	35.41	4.14	4.51	2.21	1.23	0.96	7.13
-1.68(20)	-1.68(25)	1.68(100)	8.02	5.93	9.64	15.57	4.72	1.20	2.02	1.84	1.33	1.55	0.92	1.97
-1.68(20)	1.68(85)	-1.68(0)	34.81	34.23	8.26	42.49	2.73	31.50	1.29	1.41	2.20	0.86	0.60	1.90
-1.68(20)	1.68(85)	1.68(100)	18.71	33.44	17.49	50.93	6.81	11.82	4.82	1.93	1.43	1.56	1.32	21.25
1.68(120)	-1.68(25)	-1.68(0)	31.44	26.23	14.59	40.82	4.13	11.75	6.34	1.50	1.79	0.93	0.76	13.61
1.68(120)	-1.68(25)	1.68(100)	15.33	3.31	5.47	8.79	2.28	1.04	1.14	0.89	1.14	1.00	0.27	1.04
1.68(120)	1.68(85)	-1.68(0)	34.96	35.27	10.33	45.61	3.65	30.64	2.59	1.07	2.02	0.87	0.67	4.10
1.68(120)	1.68(85)	1.68(100)	32.70	15.55	6.35	21.89	1.46	14.09	0.85	2.45	0.77	0.70	0.87	0.71
0(70)	0(55)	0(50)	41.99	7.48	6.38	13.86	0.62	6.86	1.16	1.54	1.16	0.61	0.51	1.40
0(70)	0(55)	0(50)	41.71	8.43	6.84	15.27	0.59	7.26	1.23	1.45	1.33	0.61	0.51	2.29
0(70)	0(55)	0(50)	38.68	9.62	6.68	16.30	0.66	8.30	1.22	1.61	1.19	0.63	0.50	2.20
0(70)	0(55)	0(50)	40.58	8.42	5.95	14.37	0.57	7.22	1.01	1.68	0.98	0.60	0.48	1.84
0(70)	0(55)	0(50)	42.20	7.73	5.67	13.39	0.67	6.56	1.14	1.53	0.82	0.60	0.49	1.57
0(70)	0(55)	0(50)	41.00	8.67	5.88	14.55	0.70	7.30	1.01	1.56	0.95	0.63	0.48	1.93

Theoretical response surface models

As in many research fields, when trying to develop theoretical models to predict and comprehend the effects of independent variables on certain response variables, it is necessary to evaluate its precision by fitting these models to the experimental values [26]. The 12 response values (yield, Hta, Fla and Phe, P5, P7, P8, P9, P11, P13, P14 and the total sum of the minor compounds) presented in **Table 34** were fitted to a second-order polynomial model, with interactions of Eq. (1) using a nonlinear algorithm (least-squares estimations), to develop mathematical models for each response criteria proposed.

Part A of **Table 35** shows the estimated coefficient values obtained from the polynomial model of Eq. (1) and the coefficient of correlation (R2) for each parametric response of the extraction process. These parametric values translate the response patterns and show the complexity of the possible interactions between variables. However, not all the parameters of Eq. (1) were used in the model, since some coefficients showed values that their effect can be neglected due to their lack of significance (non-significative, ns). The significant ones were assessed at a 95% confidence level ($\alpha = 0.05$) and statistic tests were used to analyse the models adequacy, validating that no substantial improvement could be produce by inserting the statistically ns parametric values [24]. The resulting models for each of the 12 assessed responses (Eqs. 3 to 14) are presented in **Table 36**. In all cases, R2 coefficients higher than 0.85 were obtained, which indicates that the percentage of variability of each response can be explained by the model. Indeed, it suggests that more than 85% of variability was successfully explained by the model, thus indicating the suitability and a high level of correlation between the observed 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 35**, which validates the models of Eqs. (3) to (14). These workable models were applied in the subsequent prediction and optimization steps, which allows the determination of the optimal conditions that will maximize the responses.

The obtained model coefficients (part A of **Table 35**) are empirical and cannot be associated with physical or chemical significance. However, they are useful for predicting the results of untested extraction conditions [30] and their numerical values can be used for direct comparisons. In fact, the higher the absolute value of the coefficients is, the more important will be the weight of the corresponding variables. Correspondingly, when a factor has a positive effect, the response increases as the value of the involved variable increase, and when the factor has a negative effect, the response decreases.

Table 35. First part of the table shows the fitting coefficients and R^2 determined for the models obtained for of all the response values assessed: *Extraction yield* (%), T. hydrolysable tannins (*Hta*), T. Flavonoids (*Fla*), T. Phenolics (*Phe*) and major (*P5*, *P7*, *P8*, *P9*, *P11*, *P13* and *P14*) and minor (*P1*, *P2*, *P3*, *P4*, *P6*, *P10* and *P12*) compounds. The second part of the table shows the optimal processing conditions of extraction in the HAE and the maximal response values produced.

Response variables	A: Fitting coefficients obtained after applying the RSM equation											B: Optimal conditions and response values			
	Intercept	*Linear effect*			*Quadratic effect*			*Interactive effect*			R^2	*t (min)*	*T (ºC)*	*S (%)*	*Optimum*
	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)					
Extraction yield	40.83±1.02	1.75±0.21	2.92±0.61	-3.86±0.31	ns	ns	-5.56±0.58	ns	0.63±0.13	0.43±0.03	0.9324	120.0±12.4	85.0±6.7	44.5±9.7	48.87±2.99
Hydrolysable tannins (Hta)	9.34±1.67	-2.68±0.32	2.34±0.22	-6.50±0.92	2.38±0.48	ns	3.04±0.28	ns	ns	1.76±0.18	0.9102	20.0±3.3	25.0±3.7	0.0±6.7	41.14±0.96
Flavonoids (Fla)	5.56±0.59	-0.72±0.13	ns	-1.12±0.13	0.53±0.19	ns	1.28±0.39	ns	-0.65±0.23	0.89±0.23	0.8468	20.0±1.7	85.0±14.7	100.0±17.7	14.38±0.33
Total Phenolics (Phe)	14.90±1.09	-3.40±0.16	2.11±0.16	-7.62±1.06	2.91±1.26	ns	4.32±1.26	ns	-0.87±0.76	2.65±0.76	0.9110	20.0±3.7	25.0±5.7	0.0±8.7	55.37±2.20
Compound P5	0.37±0.26	-0.31±0.14	ns	ns	0.37±0.18	0.37±0.18	0.41±0.18	-0.12±0.10	-0.39±0.10	0.15±0.10	0.8671	20.0±5.2	85.0±6.7	100.0±9.7	6.00±0.85
Compound P7	7.88±1.53	-1.68±0.32	2.09±0.92	-6.15±0.92	2.81±0.86	ns	ns	1.02±0.65	1.25±0.35	ns	0.8921	20.0±3.6	85.0±7.7	0.0±1.7	33.14±0.10
Compound P8	1.22±0.19	ns	-0.17±0.11	-0.43±0.11	0.33±0.12	ns	0.28±0.12	-0.20±0.07	-0.37±0.07	0.39±0.07	0.9009	120.0±22.4	25.0±2.7	0.0±1.7	6.65±0.83
Compound P9	1.55±0.15	-0.23±0.09	-0.13±0.09	-0.17±0.09	ns	ns	0.15±0.09	0.19±0.07	0.14±0.07	0.24±0.07	0.9328	20.0±3.1	25.0±1.7	0.0±2.7	4.47±0.73
Compound P11	1.06±0.08	-0.06±0.01	ns	-0.29±0.05	ns	ns	0.19±0.05	ns	ns	ns	0.8850	20.0±1.6	25.0±1.7	0.0±3.7	2.21±0.56
Compound P13	0.64±0.07	-0.10±0.02	-0.05±0.04	0.05±0.04	ns	ns	0.15±0.04	ns	-0.05±0.03	ns	0.8889	20.0±1.3	25.0±1.7	100.0±4.7	1.55±0.60
Compound P14	0.51±0.02	-0.08±0.01	0.04±0.01	0.02±0.01	0.04±0.02	ns	0.06±0.02	0.02±0.01	-0.04±0.01	0.07±0.01	0.9357	20.0±0.5	85.0±12.7	100.0±5.7	1.31±0.57
Compound P1,2,3,4,6,10,12	2.61±0.25	-0.89±0.29	ns	-0.53±0.19	ns	ns	1.35±0.37	-1.12±0.28	-1.40±0.28	1.53±0.28	0.8855	20.0±3.0	85.0±17.7	100.0±2.7	18.53±1.33

ns: non-significant coefficient; R^2: Correlation coefficient.
Optimum values of the optimized conditions are all presented in mg/g R except for the extraction yield that is expressed in %.
Parametric values of part are codified following the experimental design of Table 2.
Optimal conditions and response values of part B are decoded. Note that the values for the variable S, when the result is close to 0 the confidence intervals may seem ns, however all presented results are fully significant.

Table 36. Mathematical models of the extraction process derived from the second-order polynomial model with interactions of Eq. (1).

Extraction yield	$Y_{Yield} = 40.8 + 1.75t + 2.92T - 3.86S - 5.56S^2 + 0.63tS + 0.43TS$	Eq. (3)
Hydrolysable tannins (Hta)	$Y_{Elg} = 9.34 - 2.68t + 2.34T - 6.5S + 2.38t^2 - 3.04S^2 + 1.76TS$	Eq. (4)
Flavonoids (Fla)	$Y_{Fla} = 5.56 - 0.72t - 1.12S + 0.53t^2 + 1.28S^2 - 0.65tS + 0.89TS$	Eq. (5)
Total Phenolics (Phe)	$Y_{Phe} = 14.9 - 3.4t + 2.11T - 7.62S + 2.91t^2 + 4.32S^2 - 0.87tS + 2.65TS$	Eq. (6)
Compound P5	$Y_{P5} = 0.37 - 0.31t + 0.37t^2 + 0.37T^2 + 0.412S^2 - 0.12tT - 0.39tS + 0.15TS$	Eq. (7)
Compound P7	$Y_{P7} = 7.88 - 1.68t + 2.09t^2 - 6.15S + 2.81t^2 + 1.02tT + 1.25tS$	Eq. (8)
Compound P8	$Y_{P8} = 1.22 - 0.17T - 0.43S + 0.33t^2 + 0.28S^2 - 0.2tT - 0.37tS + 0.39TS$	Eq. (9)
Compound P9	$Y_{P9} = 1.55 - 0.23t - 0.13T - 0.17S + 0.15S^2 + 0.19tT + 0.14tS + 0.24TS$	Eq. (10)
Compound P11	$Y_{P11} = 1.06 - 0.06t - 0.29S + 0.19S^2$	Eq. (11)
Compound P13	$Y_{P13} = 0.64 - 0.1t - 0.05T + 0.05S + 0.15S^2 - 0.05tS$	Eq. (12)
Compound P14	$Y_{P14} = 0.51 - 0.08t + 0.04T + 0.02S + 0.04t^2 + 0.06S^2 + 0.02tT - 0.04tS + 0.07TS$	Eq. (13)
Compound P1,2,3,4,6,10,12	$Y_{Minor} = 2.61 - 0.89t - 0.53S + 1.35S^2 - 1.12tT - 1.40tS + 1.53TS$	Eq. (14)

Based on the mathematical expressions, no associations were found between the response variables of Hta, Fla and Phe. However, certain features regarding the general effects of the variables are displayed. 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. Alexandre et al. [31] also found S as the most relevant variable in the extraction of bioactive compounds. Regarding the linear, quadratic, and interactive parametric effects of the developed equations, it was found that they play an important and significant role in all evaluated responses. For the linear effect, the variables T and S had strong values; meanwhile, the effect of t was negligible in almost all cases. All independent variables had moderate quadratic or nonlinear effects. Regarding the interactive effects, the interactions of the variable t with the other variables (tT and tS) were of minor relevance; meanwhile, the TS interaction had a strong significance in describing the behaviour of almost all responses. To make the combined effects more explicit and to visually describe the extraction trends, the results were presented in the response surface plots discussed below.

Effect of the independent variables on the target responses and optimal extraction conditions

Although the parametric coefficients translate the responses, for a better understanding of response patterns, graphical representations in 2D and 3D are provided in **Figure 28** and **Figure 29** (part A).

The **Figure 28** illustrates the 3D response surface plots of extraction yield and grouped phenolic compounds as Hta, Fla, Phe and sum of minor compounds (P1, P2, P3, P4, P6, P10 and P12). Each of the responses presented in Figure 2 are described by two main parts (A and B). Part A that shows the 3D analysis as a function of each independent variable. The grid surfaces were built using the theoretical values (**Table 35**) predicted with Eq. (1). Meanwhile, part B of **Figure 28** illustrates the goodness of fit through two graphical representations that can be used as a statistical criteria: 1) the ability to simulate response changes between the observed and predicted values; and 2) the residual distribution as a function of each variable. Observing the response surface plots of the extraction yield (**Figure 28**), it is possible to verify that the optimum value can be found as being a single point in almost all combinations, which allows computing the extraction conditions that lead to an absolute maximum. The responses are affected differently by the tested variables. For all response criteria, all experimental points were found to be close to the line of perfect fit, suggesting an accurate correlation between the predicted and experimental values, what further confirms the suitability of the employed model. Additionally, the residuals distribution presented was in all cases arbitrarily distributed around zero, and no group of values or autocorrelations were observed.

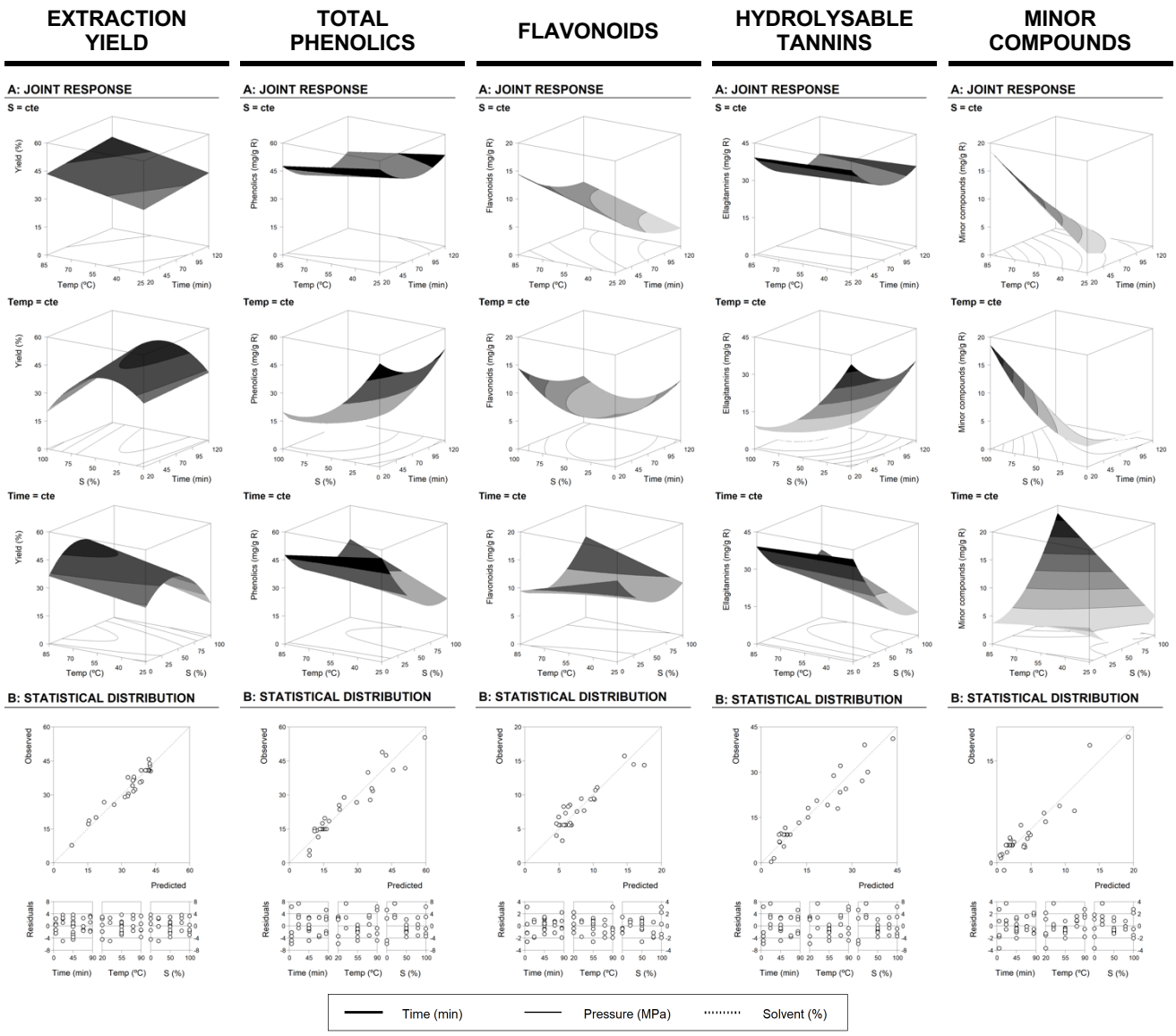

Figure 28. Response surface plots of extraction yield and grouped phenolic compounds: T. hydrolysable tannins (Hta), T. Flavonoids (Fla), T. Phenolics (Phe) and minor compounds (P1, P2, P3, P4, P6, P10 and P12). Part A: 3D analysis as a function of each independent variable. The grid surfaces were built using the theoretical values (**Table 35**) predicted with Eq. (1). For representation purposes, the excluded variable was positioned at the optimum of their experimental domain (**Table 35**). Part B: illustration of the goodness of fit through two graphical statistical criteria, namely the ability to simulate response changes between observed and predicted values and the residual distribution as a function of each variable.

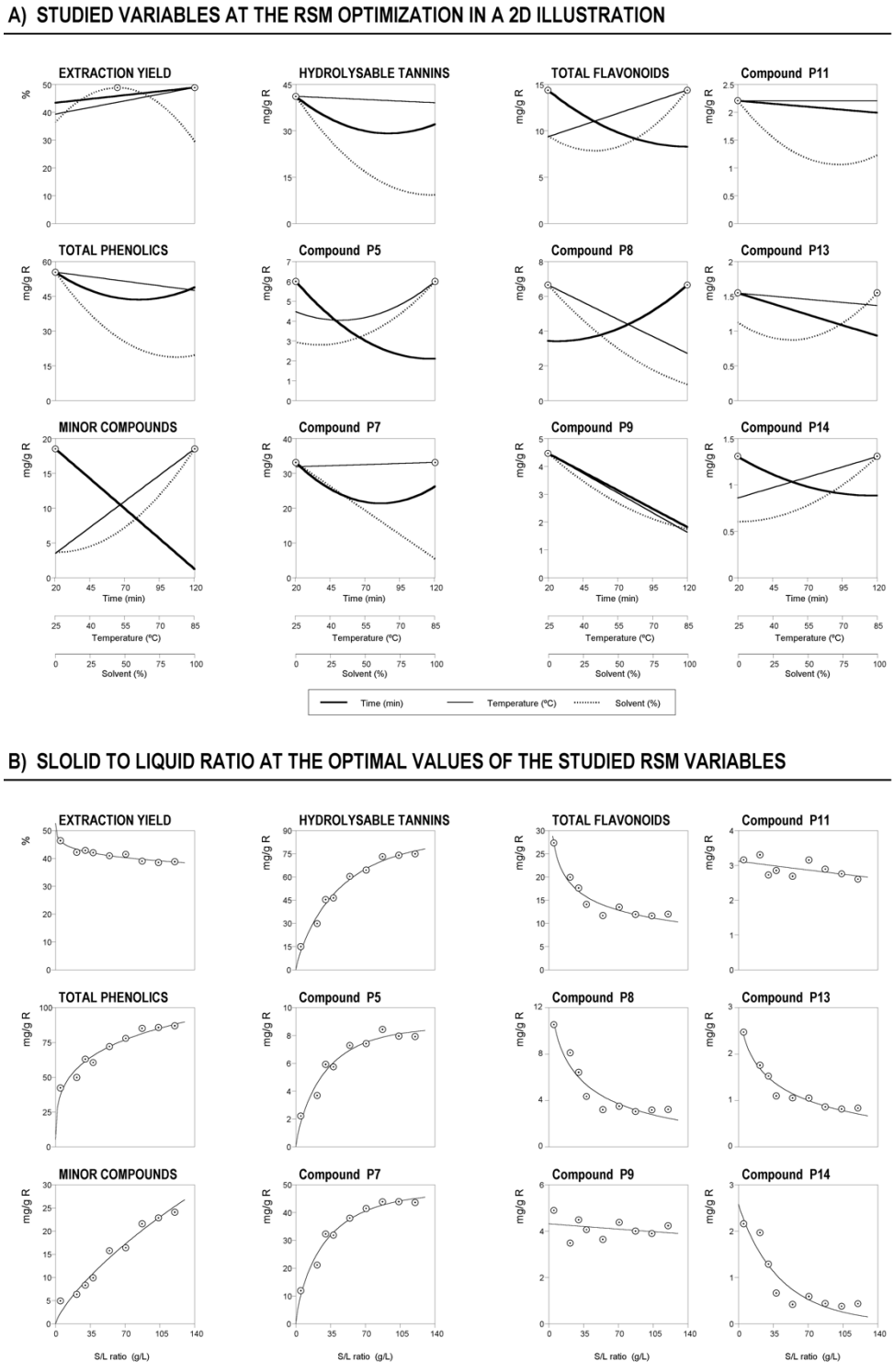

Figure 29. 2D graphical response of the effects of the independent variables for all the response values assessed: *Extraction yield* (%), T. hydrolysable tannins (*Hta*), T. Flavonoids (*Fla*), T. Phenolics (*Phe*) and major (*P5*, *P7*, *P8*, *P9*, *P11*, *P13* and *P14*) and minor (*P1*, *P2*, *P3*, *P4*, *P6*, *P10* and *P12*) compounds. Dots (⊙) represent the optimal values. In each plot, each independent variable was positioned at the optimal value of the other two variables (**Table 35**).

The **Figure 29** (part A) illustrates the 2D graphical response of the effects of the independent variables for all the response values assessed of yield, Hta, Fla, Phe and major (P5, P7, P8, P9, P11, P13 and P14) and minor (P1, P2, P3, P4, P6, P10 and P12) compounds. The lines in all graphs of **Figure 29** (part A) are generated using the theoretical values (**Table 35** part A) predicted with Eq. (1). By applying a simplex method to solve nonlinear problems, the optimum individual condition maximizing the recovery of PC were determined and presented in part B of **Table 35**. The dots (□) in **Figure 29** (part A) represent the optimal values for an easier interpretation of the effects of the independent variables on the extraction process. In conclusion, it can be summarized the optimal conditions that lead to maximum responses are as follow:

- For yield, the optimal conditions were: t= 120.0±12.4 min, T= 85.0±6.7 ºC and S= 44.5 %, producing 48.87±2.99 % of R.
- For Hta, the optimal conditions were: t= 20.0±3.3 min, T= 25.0±3.7 ºC and S= 44.5±9.7 % of ethanol (v/v), producing 41.14±0.96 mg/g R.
- For Fla, the optimal conditions were: t= 20.0±1.7 min, T= 85.0±14.7 ºC and S= 100.0±17.7 % of ethanol (v/v), producing 14.38±0.33 mg/g R.
- For Phe, the optimal conditions were: t= 20.0±3.7 min, T= 25.0±5.7 ºC and S= 0.0±8.7 % of ethanol (v/v), producing 55.37±2.20 mg/g R.

Similarly, the extraction of the major (P5, P7, P8, P9, P11, P13 and P14) and minor (P1, P2, P3, P4, P6, P10 and P12) compounds were affected in a different way by the variables tested, with the majority being favoured by lower times as follows:

- For P5, P14 and sum of the grouped minor compounds (P1, P2, P3, P4, P6, P10 and P12) the optimal conditions were: t= 20.0 min, T= 85.0 ºC and S= 100.0 %, originating 6.00±0.85, 1.31±0.57, and 18.53±1.33 mg/g R, respectively. Compound P7 showed similar optimum values, but S= 0.0 % producing 33.14±0.10 mg/g R.
- For P9 and P11 the optimal conditions were: t= 20.0 min, T= 25.0 ºC and S= ~0.0%, originating 4.47±0.73 and 2.21±0.56, respectively. Compound P8 showed similar optimum values, but t= 120.0 min producing 6.65±0.83 mg/g R. Compound P13 showed similar results, but S= 100.0 ±4.7% min producing 1.55±0.60 mg/g of R.

The simplicity and efficiency of using optimized extraction systems to recover bioactive compounds from natural matrices, from an environmental and economical point of view, has received special attention in the last decades [20,32]. The optimized results herein obtained are relevant for an eco-friendly alternative to industries. The main benefits are the time and energy reduction, and type of applied solvents, consequently minimizing industrial emissions [33], which is an objective of the sustainable ‘‘green” chemistry. The extraction processes are completed in minutes with high reproducibility, simplifying manipulation and work conditions,

giving products with higher purity and eliminating further treatment [33]. The results showed that the optimum conditions to maximize PC lied in the selected experimental domains and were t= 20.0±3.7 min, T= 25.0±5.7 ºC and S= 0.0±8.7 % of ethanol (v/v) producing 55.37±2.20 mg/g R. Therefore, these conditions are used for the optimization of the S/L effect by dose-response and described below.

Dose-response analysis of the solid-to-liquid effect at the optimum conditions

As mentioned in the bibliography [34,35], the idyllic S/L should be the one that permits the solvent to appropriately enter into the structure of the plant-based material but dissolving the major target compounds possible. Consequently, a study aiming to assess the S/L pattern was directed at the conditions predicted by the polynomial models described above for the maximization of the PC content. Crucial tests were achieved to discover the limit value of S/L at lab-scale conditions. The results exhibited that over 120 g/L the process could not be normalized accurately, thus the S/L dose-response process was planned from 5 to 120 g/L.

The dose-response results to S/L effects of all the response values assessed was performed by fitting the Eq. (2) (increasing or decreasing form) to the experimental responses. All fitting responses showed statically consistent parametric coefficients and robust mathematical models. The obtained parametric values are presented in **Table 37**. The effects of all the response values assessed caused by the S/L are explicitly shown in **Figure 29** part B, in which the experimental data are the dots (□) and predictions made by the mathematical model of Eq. (2) are the lines. Overall, a non-linear effect is detected for all responses as the S/L dose-response increases, causing a saturation-increasing (↑) and decreasing effects (↓). For the Hta, Phe, minor compounds and major compounds of P5 and P7 a saturation-increasing (↑) effect was found, while a saturation-decreasing effect (↓) was identified for the extraction yield, Fla and major compounds of P8, P9, P11, P13 and P14. The analysis of the results can be interpreted by means of the two main parameters K and mn (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 mn 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. A summary of the solutions found can be seem bellow:

- For the dose-responses that caused saturation-increasing (↑) effects (Phe, Hta, minor compounds and major compounds of P5 and P7), which means that initially increases as the S/L increases, but when a certain S/L level is reached (parametric value m99% from Eq. (2)), the response remains constant (parametric value K from Eq. (2)). Under this pattern, it was

possible to find a maximum of Phe of 205.4 mg/g of R (value K) at 187.8 g/L (value m99%). The Hta presented a maximum value of 85.7 mg/g of R (165.7 g/L), the compounds P5 and P7 a maximum value of 8.6 mg/g of R (112.8 g/L) and 47.6 mg/g of R (124.3 g/L). Meanwhile, the sum of all minor compounds (P8, P9, P11, P13 and P14) showed a maximum of 116.6 mg/g of R at 182.2 g/L.

- For the dose-responses that caused saturation-decreasing (↓) effects (extraction yield, Fla and major compounds P8, P9, P11, P13 and P14), which means that the response initially decreases to zero as S/L increased. The maximum extraction level is obtained at relatively low S/L (parametric value K from Eq. (2), as described in Table A3), which may probably reflect the total available response content in the CF. In this scenery, the response of extraction yield showed a maximum value of 54.96 %, the content in Fla showed values of 35.35 mg/g of R and the major compounds P8 (4.31 mg/g of R), P9 (4.31 mg/g of R), P11 (3.12 mg/g of R), P13 (3.58 mg/g of R) and P14 (2.58 mg/g of R).

In consequence, by applying a routine to solve all equations, the solution that globalize all responses and maximize the S/L dose-response will be 82.8 g/L producing a total PC of 86.5 mg/g of R.

Table 37. Parametric results of the dose-response model of Eq. (2) for of all the response values assessed in terms of the variation of the *S/L* ratio: *Extraction yield* (%), T. hydrolysable tannins (*Hta*), T. Flavonoids (*Fla*), T. Phenolics (*Phe*) and major (*P5*, *P7*, *P8*, *P9*, *P11*, *P13* and *P14*) and minor (*P1*, *P2*, *P3*, *P4*, *P6*, *P10* and *P12*) compounds. The underlined values were obtained with an increasing dose-response model of Eq. (2) using the $m_{99\%}$ as response criteria.

	K	$m_{50\%}$ or $m_{99\%}$	a	R^2
Extraction yield	54.96±3.85	158.40±25.24	0.22±0.02	0.9241
Hydrolysable tannins (Hta)	85.73±5.14	165.69±7.12	0.86±0.01	0.9836
Flavonoids (Fla)	35.35±4.45	31.01±3.89	0.44±0.03	0.9436
Total Phenolics (Phe)	205.40±18.49	187.80±11.56	0.33±0.01	0.9482
Compound P5	8.65±0.78	112.81±3.39	0.84±0.08	0.9479
Compound P7	47.62±2.38	124.35±7.39	0.79±0.08	0.9696
Compound P8	17.92±1.43	10.28±0.72	0.43±0.01	0.9189
Compound P9	4.31±0.30	159.82±25.19	1.00±0.05	0.9457
Compound P11	3.12±0.09	143.13±24.25	1.02±0.09	0.9249
Compound P13	3.58±0.29	17.62±0.18	0.44±0.01	0.9563
Compound P14	2.58±0.13	28.93±0.29	0.94±0.09	0.8648
Compound P1,2,3,4,6,10,12	116.59±8.16	182.16±8.89	0.79±0.04	0.9676

Comparison with other studies involving the extraction of phenolic compounds in C. sativa

Studies have indicated that the bioactive properties (mainly antioxidant and antimicrobial activity) presented in extracts obtained from plant-based material are related to the major PC composition and exacerbated by potential synergistic interactions between them and other relevant compounds [36]. In recent years the demand for natural additives from plant-based materials has increased exponentially, and PC, specifically Fla have been given a great interest, probably due their ability to inhibit the growth of relevant microbial strains [37,38]. Different chestnut products such as leaves, wood, fruits and bark have already been studied and characterized, presenting a great potential as source of bioactive PC, specifically hydrolysable and condensed tannins [39].

The use of CF as infusions and decoctions for medicinal purposes has been recognized since ancient times for the treatment of diverse symptomatology, namely, in the treatment of colds, coughs and diarrhoea [4,40]. The characterization of the phenolic compounds, especially in relation to their bioactivity potential, is indispensable to draw conclusions regarding the possibility of applying them at a food study level as natural ingredients [41]. Previous studies have analysed the nutritional and bioactive properties of CF [2,3,29], and some authors report that the most bioactive molecules are normally found in flowers rather than in the fruits [2,29,42].

The results found in this work are in line with the findings in other studies, in which Hta were found the predominated compounds over the Fla [29], and trigalloyl-HHDP-glucoside is the major compound [8]. Thus Barros et al. (2013), showed the phenolic characterization of a methanolic extract of male flowers of *C. sativa* at soft extracting conditions, finding a total amount of 18.97±0.04 mg PC/g of fresh weight material, in which the PC were composed of Hta, Fla, and phenolic acids. The compounds detected by Barros et al. (2013) were different from those found in the present study, but the major compounds present were Hta and the trigalloyl-HHDP-glucoside was also the predominate compound, which is in agreement with those presented in **Table 34**. Another study of CF revealed the profile of twenty-seven PC that despite being a much higher number than the one presented in the present study, the trigalloyl-HHDP-glucoside compound was found to be the main molecule [8]. The differences found between the results of the same plant-based material are likely to be related with climatic conditions. As it has been proved, when comparing plant-based material from two different ecosystems [43], climatic conditions appear to be a determining factor in the production of PC and consequently their bioactive properties.

Conclusion

Currently, there is an intense demand from the food industry for natural additives to add to processed foods in order to meet the new demands of consumers. Chestnut flower has been exploited and revealed high antioxidant power and natural high abundance of hydrolysable tannins, which could be used as a natural ingredient to preserve food and inhibit lipid deterioration and microorganism development.

The analysis presented provides important data that allows the comparison between different extraction conditions, 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.

Given the widespread interest for natural additives, there has been an effort to modernize the extraction protocols, reducing the amount of organic solvents (ecological point of view) and improving the extraction (economic point of view). The lack of optimization approaches, specifically in what concerns PC extraction contributed to detract the use of these natural solutions in food industry. The study concludes that several conditions of extraction, reduce both economic and ecological impacts of the process, in the extraction of PC from CF in an industrial level. In conclusion, the present study contributes in the valorisation of CF by the obtainment of rich extracts in PC that potentially can be applied as natural ingredients in different industrial fields.

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4.2.2. Extrato de flores masculinas de *Castanea sativa* como conservante alternativo de um pastel típico da pastelaria portuguesa "pastel de nata"

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Castanea sativa male flower extracts as an alternative preserving agent in the Portuguese pastry delicacy "pastel de nata"

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Abstract

The substitution of artificial additives by natural compounds is a current trend in food industry. In addition to their preserving effect, the naturally obtained ingredients often present important levels of bioactivity. Generally, plant species represent better sources of natural ingredients, since their compounds are less prone to convey unpleasant taste or odour. The chestnut male flower (CMF) was previously reported as having high antioxidant and antimicrobial activity. Hence, it was tested as a potential alternative to potassium sorbate in the recipe of the most treasured Portuguese delicacy: "pastel de nata". Different nutritional, chemical, physical and bioactive parameters were compared in two different periods: baking day and two days after. Samples added with CMF maintain the nutritional and chemical profiles of the original recipe but expressed a potentially more attractive appearance and, especially, an increased antioxidant activity. Accordingly, the newly obtained products might be considered as an interesting alternative with expectably better effects over consumers' health, besides rendering a novel (economically profitable) application to CMF.

Keywords: Chestnut male flowers; natural preservatives; "pastel de nata"; antioxidant activity; linear discriminant analysis.

Introduction

The main concerns about pastry products are currently focused in the improvement of nutritional composition, namely by reducing fat and sugar contents (Biguzzi, Lange, & Schlich, 2015; Biguzzi, Schlich, & Lange, 2014). Likewise, there is an increasing number of studies reporting a direct relationship between the excessive consumption of certain synthetic additives and their adverse effects on consumers' health, who are increasingly preferring food products as natural as possible, *i.e.*, with "cleaner" labels (Carocho & Ferreira, 2013). Under this new scenario, the extracts and bioactive compounds obtained from plants have been increasingly used as natural ingredients with the potential of replacing synthetic additives, mainly in result of their antioxidant and antimicrobial properties, which may render benefits for consumers' health by preventing certain diseases (*e.g.*, cancer or cardiovascular diseases) or inhibiting the growth of pathogenic food microorganisms (Carocho & Ferreira, 2013; Frank, 2014).

These natural ingredients, simultaneously acting as preserving and functionalizing agents, are particularly valued when obtained from plant species, especially due to the lower probability of affecting the organoleptic properties of the final product, or, in case they do, the potential changes tend to please the consumers (Krystyjan, Gumul, Ziobro, & Korus, 2015; Pasqualone et al., 2014).

Chestnut male flowers (*Castanea sativa* Mill.) are an important by-product resulting from the most important crop in the northeast of Portugal (representing also a relevant economic source nationwide) (Neves, Matos, Moutinho, Queiroz, & Gomes, 2009). In addition to their natural high availability, several authors describe the excellent antioxidant, antimicrobial and antitumor capacity revealed by infusions and decoctions of these flowers, which, among other functions, represent a potential preserving action (Carocho et al., 2014a; Carocho et al., 2014b).

From food application point of view, our group already incorporated aqueous extracts of chestnut male flowers in dairy and pastry products, verifying that this natural-based ingredient provided important benefits to the final products (Carocho et al., 2016a; Carocho et al., 2016b; Carocho et al., 2015a; Carocho et al., 2015b).

In the current study, we have considered the hypothesis of using the aqueous extract of chestnut male flowers as a preserving agent to be incorporated in the most acknowledged Portuguese pastry delicacy, globally known as "pastel de nata". This custard tart was created

in the beginning of XVIII century and has been recently recognized as the 15th tastiest delicacy in the world (Fox, 2009). Owing to its phenolic richness, it is expected that the incorporation of these natural extracts may also provide antioxidant and antimicrobial activities to "pastel de nata". All the analyzed chemical and nutritional parameters were measured in the baking day (after 6 months frosting) and after 2 days, the maximum period during which "pastel de nata" maintains its organoleptic features acceptable.

Material and methods

Standards and reagents

Fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA), as also sugar standards and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Acetonitrile (HPLC grade) was purchased from Fisher Scientific (Lisbon, Portugal). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, Massachusetts, USA). All other solvents and reagents were obtained from common sources and water was treated in Milli-Q water purification system (Millipore Corporation, Billerica, Massachusetts, USA).

Characterization of chestnut male flower extracts

Preparation

The male flowers of *Castanea sativa* Mill. (cv. Judia) were collected in the Northeast of Portugal (Oleiros, Bragança, Trás-os-Montes) from the orchard's ground (the natural flower drop occurs around July-August). The flower extracts rich in phenolic compounds (CFE) were obtained by heat-assisted extraction (Caleja et al., sub. Ind crop prod). In brief, the dried powdered flowers samples (600 mg) were extracted with 20 mL of a mixture of water:ethanol (33:67), for 36 minutes at 69 ºC. After evaporating the ethanol, samples were lyophilized and stored in a desiccator at room temperature (average 25 ºC), protected from light, until further analysis.

Evaluation of antioxidant properties

To determine the corresponding EC_{50} values (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay), the lyophilized samples were re-dissolved in ethanol (5 mg/mL) and successively diluted.

The antioxidant activity was determined by four different assays (Barros et al., 2013a): DPPH radical-scavenging activity and reducing power were respectively evaluated at 515 and 690 nm, using ELX800 microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA); β-

carotene bleaching inhibition and lipid peroxidation inhibition, were evaluated by measuring the colour decay of a β-carotene solution as induced by a linoleate solution, at 470 nm and by using the thiobarbituric acid reactive substances (TBARS) assay, at 532 nm, respectively. Trolox was used as positive control in all assays.

Evaluation of toxicity

The hepatotoxicity was evaluated using a cell line obtained from porcine liver acquired from certified abattoirs (Guimarães et al., 2013). To monitor the growth of the cell cultures, which were sub-cultured and plated in 96 well plates (density of 1.0×10^4 cells/well), a phase-contrast microscope was used. Dulbecco's modified Eagle's medium (DMEM) was used, supplemented with FBS (10%), penicillin (100 U/mL) and streptomycin (100 µg/mL). Ellipticine was used as a positive control. The results were expressed as GI_{50} values in µg/mL (sample concentration that inhibited 50% of the net cell growth).

Evaluation of antimicrobial properties

Antibacterial activity was evaluated following a previously described methodology (Soković, Glamočlija, Marin, Brkić, & van Griensven, 2010). Four Gram-negative bacteria: *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), *Enterobacter cloacae* (ATCC 35030), and four Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240), and *Listeria monocytogenes* (NCTC 7973) were used. The minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations were determined and streptomycin and ampicillin were used as positive controls.

In turn, the antifungal activity was evaluated in eight reference species (Soković & Van Griensven, 2006): *Aspergillus fumigatus* (ATCC 1022), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus versicolor* (ATCC 11730), *Aspergillus niger* (ATCC 6275), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112), *Trichoderma viride* (IAM 5061) and *Penicillium verrucosum* var. cyclopium (food isolate). The MIC and minimum fungicidal concentration (MFC) were determined and bifonazole and ketoconazole were used as positive controls.

Identification of phenolic compounds by HPLC-DAD-ESI/MS

The extract 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 (Bessada, Barreira, Barros, Ferreira & Oliveira,2016). Detection was carried out by DAD, using 280, 330, and 370 nm as

the preferred wavelength. Data acquisition was carried out with Xcalibur® data system (Thermo Finnigan, San Jose, CA, USA). The phenolic compounds were characterized according to their UV, retention times, and comparison with authentic standards when available. For the quantitative analysis, a baseline to valley integration with baseline projection mode was used to calculate peak areas. For quantification, calibration curves were generated by injection of known concentration (2.5–100 μg/mL) of standard compounds: ellagic acid (y = 26719x - 317255; R^2 = 0.999), gallic acid (y = 131538x + 292163; R^2 = 0.997), and quercetin 3-*O*-glucoside (y = 34843x – 160173; R^2 = 0.999).

Characterization of "pastéis de nata"

Preparation

To prepare the "pastel de nata", the traditional base recipe was followed: 2 dl of custard were mixed with 0.5 dl of milk. Then, 6 egg yolks, 100 g of sugar and 1 tablespoon of wheat flour were added and mixed with a hand mixer at 450W (MFQ 3540, Bosch, Munich, Germany). This recipe corresponded to the control "pastéis de nata" (CPN), while other batches were prepared by adding 0.54 g of CFE (PNCL) or 0.54 g of potassium sorbate (PNPS) to sugar before mixing it with the cream. Some flavouring ingredients are commonly used, including lemon zest or cinnamon sticks; however, we opted to strictly follow the traditional recipe, avoiding any kind of interference in the evaluation of bioactivity. The mixture was slowly heated, under continuous stirring, until it started boiling. After cooling down the mixture, it was poured in individual forms previously filled with puff pastry (purchased from a local supermarket) and frozen during 6 moths. Afterwards, samples were defrosted and baked at 250 ºC (oven HE-635, Teka, Haiger, Germany) for 15 minutes. Three different lots (CPN, PNCL and PNPS) were prepared: 10 "pastéis de nata" per lot, further divided in 2 groups of 5 "pastéis de nata" for each assayed time (t0 and t2). After baking, all samples were lyophilized, milled and analysed (in triplicate), immediately after preparation and after two days of storage (samples were packed in a sealed polyethylene bag and stored in the refrigerator).

Evaluation of nutritional properties

The contents of protein, fat and ash, was determined following the AOAC methods (AOAC, 2016). Total energy was calculated using the following equation: Energy (kcal) = 4 × (g proteins + g carbohydrates) + 9 × (g lipids).

Free sugars

Free sugars were determined in hydro-ethanolic extracts obtained from defatted samples using an HPLC coupled to a refraction index (RI) detector (Barros et al., 2013b). Sugars were identified by comparison with standards and further quantified (g/100 g of "pastel de nata") based on the internal standard (melezitose) method.

Fatty acids profile

Fatty acids were determined by gas-liquid chromatography with flame ionization detection (GC-FID), after the extraction and derivatization procedures previously described by Heleno, Barros, Sousa, Martins, & Ferreira, (2009). The analysis was carried out with a DANI model GC 1000 instrument equipped with a split/splitless injector, a FID at 260 ºC and a Zebron-Kame column (30 m × 0.25 mm ID × 0.20 µm df, Phenomenex, Lisbon, Portugal). The oven temperature program was as follows: the initial temperature of the column was 100 ºC, held for 2 min, then a 10 ºC/min ramp to 140 ºC, 3 ºC/min ramp to 190 ºC, 30 ºC/min ramp to 260 ºC and held for 2 min. The carrier gas (hydrogen) flow-rate was 1.1 mL/min, measured at 100 ºC. Split injection (1:50) was carried out at 250 ºC. 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 Clarity DataApex 4.0 Software and expressed in relative percentage of each fatty acid.

Evaluation of the colour parameters and pH

The colour of custard cream was measured before and after baking. Colour parameters were measured in triplicate in each "pastel de nata" using a colorimeter (model CR-400, Konica Minolta Sensing Inc., Tokyo, Japan). The illuminate C was used, with a diaphragm aperture of 8 mm, after being calibrated against a standard white tile. The CIE *L** (lightness), *a** (greenness/redness) and *b** (blueness/yellowness) colour space values were registered using "Spectra Magic Nx" (version CM-S100W 2.03.0006) (Fernandes et al., 2012).

Likewise, pH measurements were done before and after baking. Values were obtained in triplicate directly in the samples (HI 99161, pH-meter, Hanna Instruments, Woonsocket, Rhode Island, USA).

Antioxidant activity

Using a previously described procedure (Caleja et al., 2016), 3 g of each sample were extracted with a mixture of water:ethanol (33:67) during 1 hour. After removing the solvent, the extracts were dissolved to a final concentration of 200 mg/mL. The antioxidant activity evaluation was performed using two in vitro assays: 2,2-diphenyl-

1-picrylhydrazyl (DPPH) radical-scavenging activity and reducing power (RP) in the same way as described in section *Evaluation of toxicity*.

Toxicity assay

For the evaluation of toxicity, the samples were submitted to sulforhodamine B assay, as described in section *Evaluation of toxicity*.

Phenolic compounds quantification in "pastéis de nata"

The major phenolic compound was monitored along storage in order to determine if any degradation was observed. Trigalloyl-HHDP-glucoside (main ellagitannin) and quercetin-3-*O*-glucuronide (main flavonoid) were detected and quantified through an HPLC system as described in section *Identification of phenolic compounds by HPLC-DAD-ESI/MS*.

Statistical analysis

The statistical tests were done with IBM SPSS Statistics for Windows, version 22.0. (IBM Corp., Armonk, NY, USA), considering a 5% significance level. Except for antimicrobial assays results, data were expressed as mean±standard deviation; differences in the number of decimal places are justified by the magnitude of standard deviations.

An analysis of variance (ANOVA) with type III sums of squares was performed using the general linear model (GLM) procedure. The dependent variables were analysed using 2-way ANOVA considering the factors "pastel de nata" formulation (PN) and storage time (ST). In all parameters where a statistically significant interaction among factors was found, the observed differences were evaluated in the estimated marginal means plots for all levels of each factor. In cases with no statistical significant interaction, means were compared using Tukey's multiple comparison test, after checking the equality of variances through a Levene's test.

Furthermore, a linear discriminant analysis (LDA) was used to compare the effects of PN and ST over the evaluated parameters. 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. It was intended to determine which independent variables contributed more to the differences in the average score profiles of PN conjugated with ST. To verify the significance of the canonical discriminating functions, Wilk's λ test was used. A leaving-one-out cross validation procedure was carried out to assess the model performance.

Results and discussion

The recent trend of replacing artificial by natural additives is justified not only by the higher acceptability of natural ingredients, but also by the bioactivity that is typically reported in extracts or compounds isolated from natural sources. Considering the specific case of preserving agents, the dualistic effect of natural alternatives (preserving and functionalizing) has been validated in different products (Caleja et al., 2015a; Caleja et al., 2015b, Caleja et al., 2016; Caleja, Barros, António, Oliveira, & Ferreira, 2017; Carocho, Barreira, Bento, Morales, & Ferreira, 2014c; Carocho et al., 2015b; Carocho et al., 2016a; Carocho et al., 2016b; Sciarini, Ribotta, León, & Pérez, 2012; Singh, Jha, Chaudhary, & Upadhyay, 2012), among others.

Herein, we have studied the effects of using the extracts of chestnut male flower (CMF) as a natural substitute of potassium sorbate (E202) in "pastel de nata". The male flower of chestnut tree was previously characterized as containing important quantities of phenolic compounds, particularly gallic acid derivatives, but also some flavonoids (myricetin, quercetin, kaempferol and isorhamnetin) derivatives (Barros et al., 2013c). Nevertheless, it had never been used, as far as we know, as a natural preserving agent in the Portuguese delicacy "pastel de nata".

Characterization of chestnut male flower extract

The water:ethanol (33:67) extracts of CMF were initially assayed for their antioxidant activity (**Table 38**), showing particularly good results in the cases of β-carotene bleaching inhibition (EC_{50} = 32 μg/mL extract) and TBARS formation inhibition (EC_{50} = 13 μg/mL extract), which might indicate a higher suitability of CMF extracts towards lipophilic environments. In general, the obtained EC_{50} values are lower (higher antioxidant activity) than those obtained previously in aqueous extracts of CMF (Barreira, Ferreira, Oliveira, & Pereira, 2008; Carocho et al., 2014a).

The antibacterial activity (**Table 38**) was significant, since CMF gave similar (or even lower) MIC and MBC than ampicillin for all bacterial species, except *Staphylococcus aureus* and *Micrococcus flavus.* Among the tested bacteria, CMF was particularly effective against *Bacillus cereus*, as indicated by the lowest MIC and MBC values obtained in the assays conducted with these species. The MIC and MBC values are in the same range as those obtained with infusions and decoctions of CMF (Carocho et al., 2014b).

Table 38. Antioxidant activity (values presented as mean±standard deviation) and antimicrobial activity of chestnut male flower (CMF) extract

	Antioxidant activity (EC_{50} values, µg/mL extract)			
	DPPH scavenging activity	Reducing power	β-carotene bleaching inhibition	TBARS formation inhibition
CMF extract	105±2	190±3	32±1	13±1

	Antibacterial activity (MIC and MBC values, mg/mL extract)							
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
	Gram positive bacteria							
	Escherichia coli		*Pseudomonas aeruginosa*		*Salmonella typhimurium*		*Enterobacter cloacae*	
CMF extract	0.15	0.30	0.45	0.60	0.45	0.60	0.20	0.60
Streptomycin	0.20	0.30	0.20	0.30	0.25	0.50	0.20	0.30
Ampicillin	0.40	0.50	0.75	1.20	0.40	0.75	0.25	0.50
	Gram negative bacteria							
	Staphylococcus aureus		*Bacillus cereus*		*Micrococcus flavus*		*Listeria monocytogenes*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
CMF extract	0.45	0.90	0.075	0.15	0.45	0.90	0.45	0.60
Streptomycin	0.04	0.10	0.10	0.20	0.20	0.30	0.20	0.30
Ampicillin	0.25	0.45	0.25	0.40	0.25	0.40	0.40	0.50

	Antifungal activity (MIC and MFC values, mg/mL extract)															
	Aspergillus fumigatus		*Aspergillus ochraceus*		*Aspergillus versicolor*		*Aspergillus niger*		*Penicillium funiculosum*		*Penicillium ochrochloron*		*Trichoderma viride*		*Penicillium verrucosum*	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
CMF extract	0.10	0.15	0.075	0.15	0.30	0.60	0.075	0.15	0.04	0.90	0.30	0.60	0.45	0.60	0.30	0.60
Ketoconazole	0.25	0.50	1.50	2.00	0.20	0.50	0.20	0.50	0.20	0.50	2.50	3.50	1.00	1.00	0.20	0.30
Bifonazole	0.15	0.20	0.15	0.20	0.10	0.20	0.15	0.20	0.20	0.25	0.20	0.25	0.15	0.20	0.10	0.20

A similar level of activity was found among fungal species. In fact, the antifungal activity of CMF was higher than that exhibited by ketoconazole, particularly against *Aspergillus ochraceus* and *Penicillium ochrochloron*, as evidenced by the lower MIC (twenty times lower in the case of *A. ochraceus*) obtained with CMF. In general, the water:ethanol extracts studied herein showed higher activity than the corresponding decoctions and infusions (Carocho et al., 2014b).

Owing to the measured bioactivity, CMF was hypothesized as a novel preservative with potential food application. Accordingly, it was incorporated in the Portuguese delicacy "pastel de nata" as a possible alternative to potassium sorbate. Both prepared formulations (added with CMF or potassium sorbate) were also compared with the traditional recipe (no additives included). After a 6 months storage period, samples were evaluating at the baking (after defrosting) day and after 2 days.

Characterization of different "pastel de nata" formulations

In addition to the potential health-related benefits provided by the substitution of potassium sorbate by CMF, the new "pastel de nata" formulation was assayed to fulfil the contemporary consumers' preference towards food products free of synthetic compounds.
The prepared samples were compared considering two distinct sources of variability (factors), specifically "pastel de nata" formulation (PN) and storage time (ST). In addition to evaluate the individual effect of each factor, their interaction (PN×ST) was also verified. For all parameters showing a significant (p<0.050) interaction, the statistical classification was not possible, and the results were discussed based on the estimated marginal means (EMM) plots (data might be provided upon request).

Nutritional parameters and free sugars profile

The proximate analysis results (**Table 39**) showed significant (p < 0.050) differences mainly among PN. On the other hand, ST was only significant in the case of sucrose, which tended to present higher contents in samples analysed in the baking day (36 g/100 g). However, the detected difference is more likely to be explained by a less effective sugar extraction in samples stored during 2 days, than by some potential sucrose degradation, as no increase in glucose and fructose was observed along time (glucose and sucrose were also detected, but in contents lower than 0.1 g/ 100 g).

As it might be concluded from **Table 39**, the interaction among factors was significant in all cases except protein and ash content. Therefore, these were the single parameters where the statistical classification was possible. In both cases, PN added with PS showed higher

contents. In what concerns the remaining parameters, PN with CMF tended to present a higher moisture value (28 g/100 g), but lower contents in fat (24 g/100 g), carbohydrates (38 g/100 g), sucrose (34 g/100 g) and, consequently, lower energy value (406 kcal/100 g).

The higher effect of PN is probably related with the fact that samples were stored for a short period (2 days). Nevertheless, and despite the identified differences among PN, their nutritional profile was generally similar, indicating (as it could be easily anticipated) that the incorporation of different additives did not cause any major difference in none of the assayed parameters.

Table 39. Nutritional composition (g/100 g fw) and energy (kcal/100 g fw) values for different "pastel de nata" formulations (PN) and storage times (ST). Results are presented as mean±standard deviation.1

		Moisture	Fat	Protein	Ash	Carbohydrates	Sucrose	Trehalose	Energy
PN	Control	27±1	25±1	7.9±0.3 c	0.36±0.02 b	39±1	35±1	3.0±0.2	413±3
	Chestnut male flower (CMF) extract	28±1	24±1	8.4±0.3 b	0.36±0.02 b	38±1	34±1	2.6±0.1	406±3
	Potassium sorbate (E202)	27±1	25±1	8.8±0.3 a	0.40±0.03 a	39±1	36±1	2.5±0.1	414±3
	ANOVA *p*-value (n = 18)[2]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
ST	0 days	27±1	25±1	8.4±0.4	0.37±0.04	39±1	36±1	2.8±0.2	410±5
	2 days	27±1	25±1	8.4±0.5	0.38±0.03	39±1	35±1	2.7±0.3	411±4
	t-Student *p*-value (n = 27)[3]	0.452	0.997	0.794	0.297	0.395	0.001	0.256	0.561
PN×ST	*p*-value (n = 54)[4]	<0.001	0.005	0.248	0.533	<0.001	0.009	0.030	<0.001

[1]The results presented for each PN include values measured in both ST; likewise, results for each ST were obtained considering the values measured in all PN. [2]If $p<0.050$, the corresponding parameter presented a significantly different value for at least one PN. [3]If $p<0.050$, the corresponding parameter presented a significantly different value for each ST. [4]$p<0.050$ indicates a significant interaction among factors, thereby precluding performing multiple comparison tests.

Fatty acids profile

The fatty acids profiles were also characterized in order to determine any possible alterations (**Table 40**). In line with the results discussed in the former section, a significant interaction among ST and PN was also verified (in all fatty acids except C14:1 and C16:0), indicating that the putative effects of storage vary within each prepared formulation. Regarding the individual effect of each factor, most fatty acids showed significant differences among PN, exempting the cases of C14:0 (p-value = 0.168), C14:1 (p-value = 0.604) and C16:1 (p-value = 0.097). In contrast, ST had significant effect only over C16:0, C18:1n9 and C18:2n6 (besides SFA, MUFA and PUFA).

For the previous reason, the statistical classification is only presented in the case of C16:0, which was lower in control samples and in the baking day.

Despite the impossibility of presenting the statistical classification, some overall trends were found for specific fatty acids: butyric acid (C4:0), stearic acid (C18:0), oleic acid (C18:1n9), saturated (SFA) and monounsaturated fatty acids (MUFA) presented higher percentages in "pastel de nata" added with PS, which, in turn, presented the lowest values in linoleic acid (C18:2n6) and polyunsaturated fatty acids (PUFA); control samples, on the other hand, had the lowest percentages in caproic acid (C6:0), palmitic acid (C16:0) and linolenic acid (C18:3n3) and the highest in lauric acid (C12:0); the most noticeable change in "pastel de nata" added with CMF was the higher percentage in arachidonic acid (C20:4n6).

In what concerns the differences resulting from ST, stored samples were mostly characterized by higher contents in C16:0, C18:1n9, SFA and MUFA, and lower percentages of C18:2n6 and PUFA.

Table 40. Major (detected above 1%) fatty acids (relative percentage) in "pastel de nata" formulations (PN) and storage times (ST). Results are presented as mean±standard deviation.[1]

		C4:0	C6:0	C10:0	C12:0	C14:0	C14:1	C16:0	C16:1	C18:0	C18:1n9	C18:2n6	C18:3n3	C20:4n6	SFA	MUFA	PUFA
PN	Control	3.1±0.1	1.7±0.1	2.3±0.1	2.9±0.2	8.2±0.4	1.1±0.1	30±1 b	1.8±0.1	9.9±0.2	13.5±0.4	17±1	3.3±0.2	1.3±0.1	62±1	16±1	22±1
	CMF extract	3.1±0.2	1.8±0.1	2.1±0.1	2.7±0.2	8.4±0.3	1.1±0.1	31±1 a	1.8±0.1	9.6±0.5	13.5±0.2	16±1	3.8±0.2	1.5±0.1	62±1	16±1	22±1
	Potassium sorbate (E202)	3.7±0.1	1.9±0.1	2.2±0.1	2.7±0.2	8.3±0.3	1.1±0.1	31±1 a	1.7±0.1	10.3±0.4	14.4±0.4	14±1	3.8±0.1	1.4±0.1	63±1	17±1	20±1
	ANOVA *p*-value (n = 18)[2]	<0.001	<0.001	0.028	0.005	0.168	0.604	<0.001	0.097	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
ST	0 days	3.3±0.3	25±1	2.2±0.1	2.7±0.2	8.4±0.3	1.1±0.1	30±1	1.7±0.1	10.0±0.3	13.6±0.5	16±2	3.6±0.3	1.4±0.1	62±1	16±1	21±1
	2 days	3.3±0.4	25±1	2.2±0.2	2.8±0.2	8.2±0.4	1.1±0.1	31±1	1.8±0.1	9.9±0.5	14.0±0.5	15±1	3.7±0.2	1.4±0.1	63±1	17±1	20±1
	t-Student *p*-value (n = 27)[3]	0.427	0.948	0.492	0.140	0.065	0.106	<0.001	0.189	0.171	0.006	0.001	0.070	0.059	0.032	0.004	0.003
PN×ST	*p*-value (n = 54)[4]	0.002	0.003	0.023	0.006	0.001	0.292	0.150	0.042	<0.001	<0.001	<0.001	0.003	0.002	<0.001	<0.001	0.008

Butyric acid (C4:0); caproic acid (C6:0); capric acid (C10:0); lauric acid (C12:0); myristic acid (C14:0); myristoleic acid (C14:1); palmitic acid (C16:0); palmitoleic acid (C16:1); stearic acid (C18:0); oleic acid (C18:1n9); linoleic acid (C18:2n6); linolenic acid (C18:3n3); arachidonic acid (C20:4n6); SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. [1]The results presented for each PN include values measured in both ST; likewise, results for each ST were obtained considering the values measured in all PN. [2]If $p<0.050$, the corresponding parameter presented a significantly different value for at least one PN. [3]If $p<0.050$, the corresponding parameter presented a significantly different value for each ST. [4]$p<0.050$ indicates a significant interaction among factors, thereby precluding performing multiple comparison tests.

Colour parameters and pH value

The external appearance (**Figure 30**) on any given food product is certainly among its main acceptability criteria. Thus, the colour parameters (*L**: lightness, *a**: redness and *b**: yellowness) were evaluated in "pastel de nata" samples before and after being baked (**Table 41**).

In general, the interaction among PN and ST was significant only in baked samples, as shown by *a**, *b** and pH values. Furthermore, PN had a much more noticeable effect (significant differences in all cases) than ST, which caused significant differences only in the case of *L** and pH, both in baked samples.

In what concerns the differences among formulations, control samples showed higher *L** values (before and after baking), indicating their higher lightness when compared to samples added with either PS or CMF; *a** values, in turn, were higher in samples added with CMF, which, on the other hand, gave the lowest *b** values, before and after baking.

Despite the great similarity among pH values, it was possible to identify a certain tendency for higher values in samples added with PS, in what concerns PN effect, and those stored during 2 days, pertaining ST effect.

Table 41. Colour parameters evaluated in in "pastel de nata" formulations (PN) and storage times (ST). Results are presented as mean±standard deviation.[1]

		*L**		*a**		*b**		pH	
		Before baking	After baking	Before baking	After baking	Before baking	After baking	Before baking	After baking
PN	Control	78±1 a	80±4 a	3.7±0.2 c	4.2±0.3	34±1 b	45±3	6.3±0.1 b	6.4±0.2
	Chestnut male flower (CMF) extract	64±1 c	58±4 c	8.8±0.4 a	9.6±0.3	24±1 c	21±2	6.2±0.1 c	6.3±0.1
	Potassium sorbate (E202)	72±1 b	77±3 b	4.8±0.3 b	3.9±0.5	39±1 a	41±1	6.4±0.1 a	6.4±0.2
	ANOVA *p*-value (n = 18)[2]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.041
ST	0 days	71±6	68±10	6±2	6±3	32±6	38±11	6.3±0.1	6.2±0.1
	2 days	71±6	75±10	6±2	6±2	32±6	34±10	6.3±0.1	6.5±0.1
	t-Student *p*-value (n = 27)[3]	0.902	0.021	0.976	0.539	0.988	0.211	0.204	<0.001
PN×ST	*p*-value (n = 54)[4]	0.708	0.131	0.982	<0.001	0.979	<0.001	0.241	<0.001

[1]The results presented for each PN include values measured in both ST; likewise, results for each ST were obtained considering the values measured in all PN. [2]If $p<0.050$, the corresponding parameter presented a significantly different value for at least one PN. [3]If $p<0.050$, the corresponding parameter presented a significantly different value for each ST. [4]$p<0.050$ indicates a significant interaction among factors, thereby precluding performing multiple comparison tests.

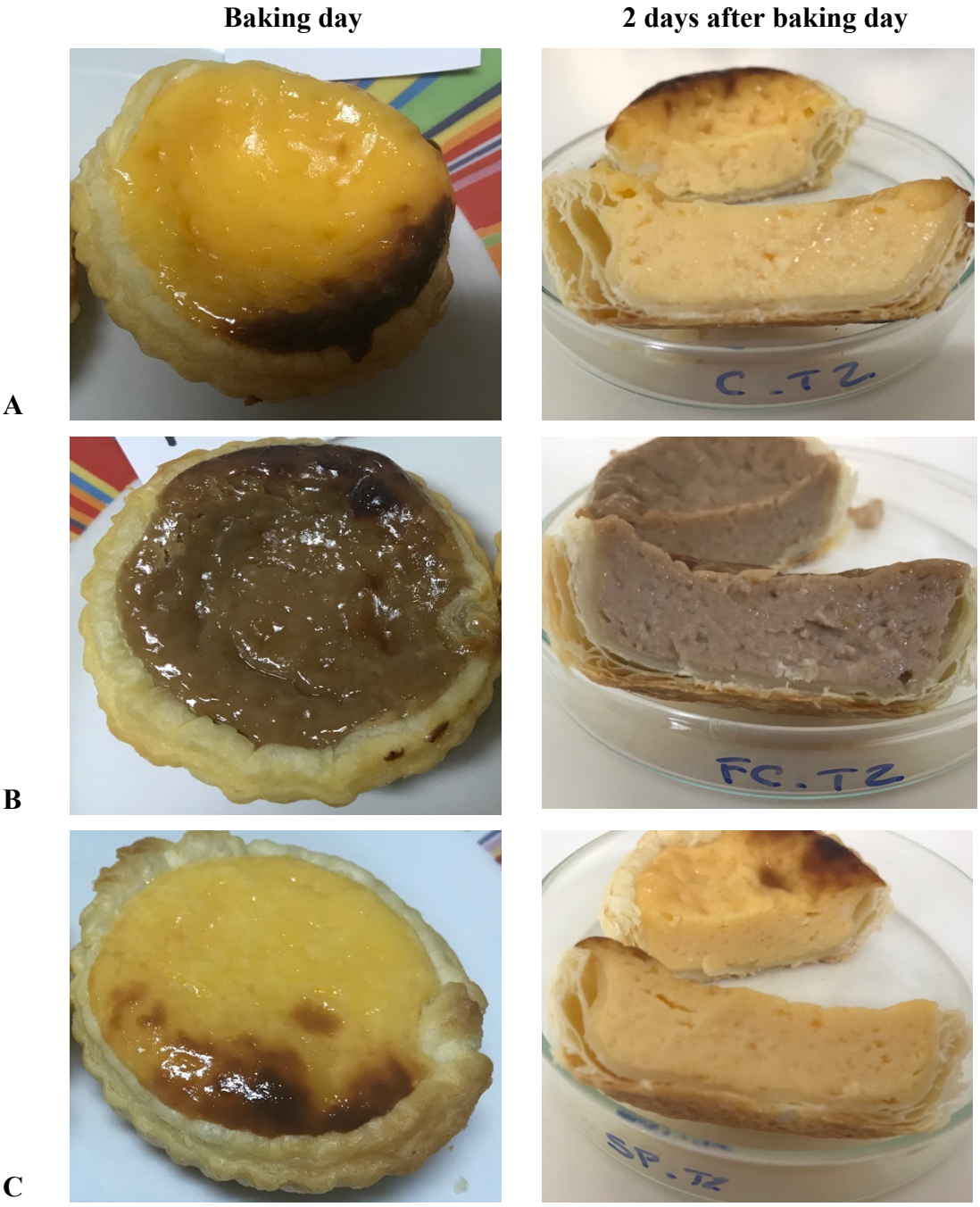

Figure 30. External appearance of different PN formulations (A) control, (B) "pastel de nata" with CMF; (C) "pastel de nata" with potassium sorbate.

Antioxidant activity

In order to verify the possible gaining of bioactivity among formulations added with CMF, the prepared "pastel de nata" formulations were submitted to an antioxidant activity screening. The average EC_{50} values for DPPH scavenging activity (considering the average values for both times) were 124±5 mg/mL of extract for "pastel de nata" with CMF and 145±16 mg/mL of extract for "pastel de nata" added with PS. The values obtained in reducing power assay were very similar: 123±8 mg/mL of extract for "pastel de nata" with CMF and 161±34 mg/mL of extract for "pastel de nata" added with PS. It should be highlighted that the EC_{50} values obtained with "pastel de nata" containing CMF were very similar to those obtained with the CMF extract itself, indicating that the activity was not lost after incorporation, frosting, baking and storage.

In what concerns the control samples, no activity was detected up to the maximum assayed concentration (200 mg/mL), thereby indicating a clear increase in the antioxidant activity of "pastel de nata" containing PS, and more noticeably in those incorporating CMF (lower EC_{50} values).

In addition, the antioxidant activity measured in the baking day was almost entirely maintained in "pastel de nata" with CMF: no loss in DPPH scavenging assay (EC_{50} = 124±5 mg/mL of extract in samples assayed in the baking day; EC_{50} = 125±5 mg/mL of extract in samples assayed 2 days after baking) and 10% less in reducing power (EC_{50} = 117±7 mg/mL of extract in samples assayed in the baking day; EC_{50} = 129±3 mg/mL of extract in samples assayed 2 days after baking), while a significant decrease was observed in those added with PS: 19% less in DPPH scavenging assay (EC_{50} = 132±7 mg/mL of extract in samples assayed in the baking day; EC_{50} = 157±13 mg/mL of extract in samples assayed 2 days after baking) and 52% less in reducing power (EC_{50} = 128±1 mg/mL of extract in samples assayed in the baking day; EC_{50} = 194±1 mg/mL of extract in samples assayed 2 days after baking).

Accordingly, CMF proved to be a better solution than PS considering the specific objective of providing antioxidant activity to the product.

Furthermore, none of the "pastéis de nata" showed cytotoxicity up to the maximal assayed concentrations (400 µg/mL) and the quantities of trigalloyl-HHDP-glucoside and quercetin-3-*O*-glucuronide ("pastel de nata" with chestnut flower extract) were also maintained along ST, which could be considered as an indicator of the maintenance of the antimicrobial activity (in line with the observed with the antioxidant activity).

Linear Discriminant Analysis

In the former sections, changes resulting from PN and ST were evaluated in each parameter individually. As explained, some statistically significant differences were found in

specific cases, but the true effects of PN conjugated with ST could be better characterized by identifying the most significant changes induced by both factors, *i.e.*, finding the variables (studied parameters) more highly correlated to the PN and ST levels.

Accordingly, a linear discriminant analysis (LDA) was performed to evaluate the association between PN and ST (categorical dependent variables) and the matrix of obtained results (quantitative independent variables). The significant independent variables were selected according to the stepwise method of LDA, considering the Wilks' λ test. Only variables with a statistically significant classification performance ($p < 0.050$) were selected in the outcome model.

The first three defined discriminant functions included 98.7% (first function: 83.9%; second function: 9.2%; third function: 5.6%) of the observed variance (**Figure 31**). From the 37 studied variables, 9 (fructose, C18:2n6, *L** before and after baking, *a** after baking, *b** before and after baking, DPPH scavenging activity and reducing power) were selected as having discriminant ability.

The first function was highly correlated with *a** after baking and DPPH scavenging activity and its main effect was the separation of markers corresponding to "pastel de nata" added with CMF (either assayed in baking day or two days after), which showed higher *a** values and the lower EC_{50} in DPPH scavenging assay. Function 2, in turn, was mostly correlated with reducing power and linoleic acid, and its most noticeable effect was the separation of markers corresponding to each assayed period in the case of control and PS-added samples, mainly due to the decrease in linoleic acid percentage and the attenuation of reducing power. Function 3 was more highly correlated with *L** before baking, which was higher in control samples, thereby contributing to separate the markers corresponding to this formulation (particularly those assayed two days after baking).

In what concerns the model performance, all samples were correctly classified, either considering original grouped or cross-validated cases.

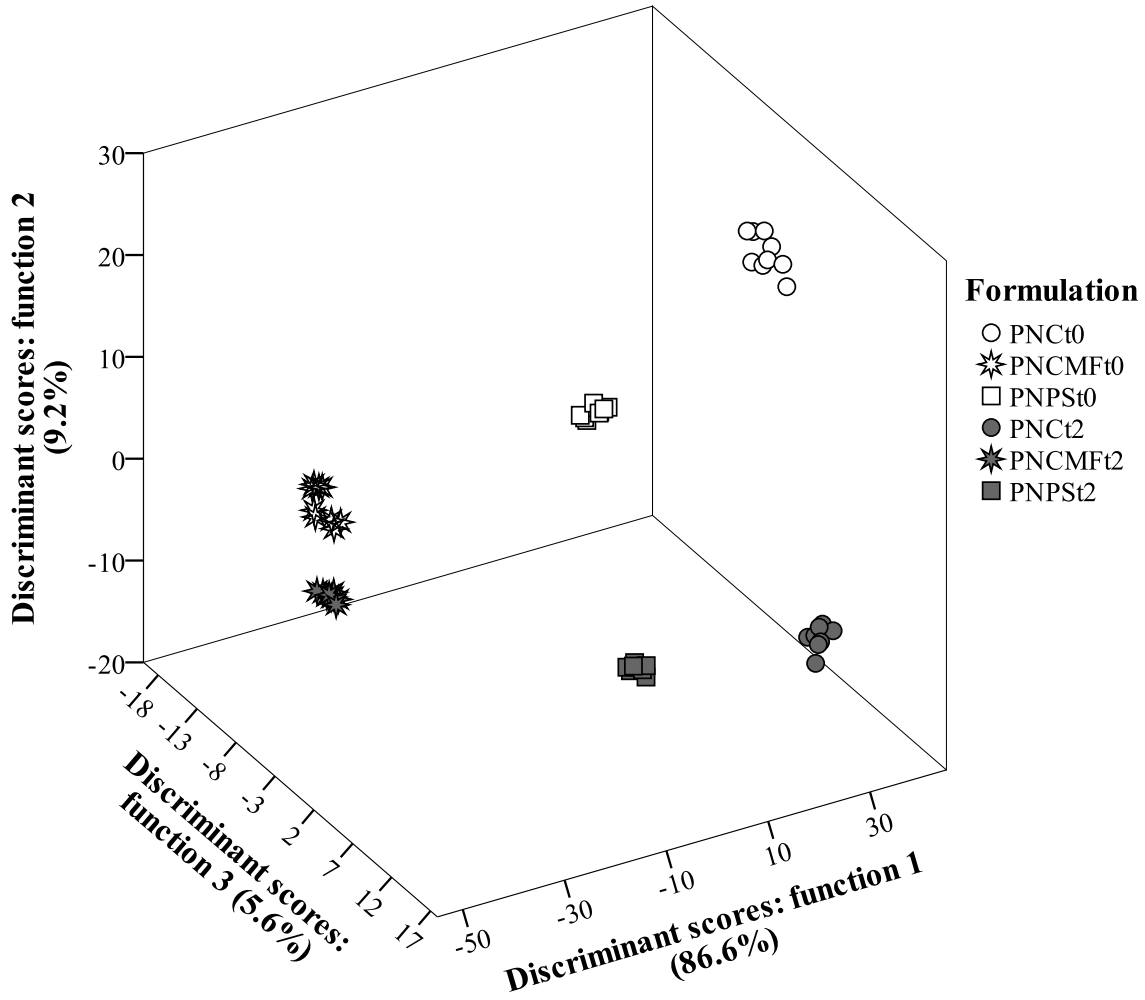

Figure 31. Canonical discriminant functions coefficients defined from the evaluated parameters considering "pastel de nata" formulation and storage time conjugated effects.

Conclusion

Overall, "pastel de nata" prepared with CMF had enough differences to be distinguished from control samples and those added with PS. Among the variables with highest changes (fructose, C18:2n6, *L** before and after baking, *a** after baking, *b** before and after baking, DPPH scavenging activity and reducing power), those presenting the most noticeable differences in CMF-added samples were *a** parameter (after baking), reducing power and DPPH scavenging activity, which validates the higher antioxidant activity presented by the newly suggested "pastel de nata" formulation. In addition, and contrarily to what was observed among control and PS-added samples, "pastel de nata" added with CMF maintain their characteristics throughout the 2-day storage period (as it has been previously pointed out for antioxidant activity), as it might be observed in the LDA output (proximity among PNCMFt0

and PNCMFt2 markers). Accordingly, CMF proved to be a suitable alternative as a natural preservative to be applied in the Portuguese delicacy "pastel de nata".

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5.

Considerações finais e perspetivas futuras

Neste capítulo final descrevem-se as conclusões obtidas em cada um dos capítulos envolvendo trabalho experimental, culminando com uma conclusão global onde se faz uma análise sobre as potencialidades dos resultados e a sua aplicação na indústria alimentar. Numa perspetiva de continuidade do trabalho, apresentam-se sugestões de trabalho futuro.

5.1. **Conclusão geral**

No ínicio desta tese apresentaram-se, como principais objetivos, estudar a utilização de diferentes plantas/extratos, muito conhecidas no nosso quotidiano e com benefícios reconhecidos ao nível de saúde dos consumidores. O funcho, camomila, cidreira e a flor de castanheiro foram as plantas selecionadas e estudadas. Após seleção e em alguns casos otimização da técnica de extração, procedeu-se à caracterização química dos extratos obtidos bem como ao estudo da sua atividade antioxidante, antimicrobiana e determinação da sua hepatotoxicidade. Todas as plantas demonstraram excelentes propriedades confirmando a sua viabilidade de utilização como ingredientes naturais em alimentos.

Os extratos de funcho e camomila obtidos e incorporados, tanto em produtos lácteos como em produtos de pastelaria, revelaram ser excelentes ingredientes naturais conferindo propriedades bioativas para além de prolongarem o tempo de prateleira dos produtos testados, revelando resultados interessantes quando comparados com aditivos tradicionalmente utilizados na indústria alimentar.

Após definição das condições ótimas para a obtenção dos extratos ricos em compostos fenólicos a partir de folhas de cidreira e flores de castanheiro, confirmaram-se as excelentes capacidades antioxidante e antimicrobiana destes, bem como a ausência de hepatotoxicidade. Apesar de a incorporação destes extratos em produtos de pastelaria modificarem o seu aspeto visual, o seu perfil nutricional não foi alterado para além de conferirem propriedades bioativas adicionais e prolongarem o tempo de prateleira. Mais uma vez, os resultados obtidos demonstram que os extratos estudados podem ser uma alternativa ao uso de aditivos artificiais correntemente utilizados pela indústria alimentar, e frequentemente associados, de acordo com vários estudos, a diversos problemas de segurança e toxicidade.

Todos os objetivos delineados para esta tese foram alcançados, evidenciando resultados promissores que poderão ser utilizados em futuros projetos, não só na área alimentar mas também na área farmacêutica e cosmética.

5.2. **Conclusões parciais**

5.2.1. **Caracterização e incorporação de *Foenicullum vulgare* Mill. e *Matricaria recutita* L. como ingredientes naturais em diferentes matrizes alimentares**

A análise química do extrato de funcho obtido por decocção, apresentou a quercetina-3-*O*-glucurónido como o composto mais abundante na amostra, sendo o ácido 5-*O*-

cafeoilquínico o ácido fenólico predominante. O mesmo extrato demonstrou efeitos bactericidas e fungicidas para os microrganismos testados sendo, *Salmonella typhimurium* e *Bacillus cereus* as bactérias mais sensíveis e, *Aspergillus niger*, *Aspergillus versicolor* e *Penicillium funiculosum* os fungos mais suscetíveis. Apesar deste extrato demonstrar uma excelente atividade antioxidante (valor EC_{50} no ensaio de DPPH, 0.75 ± 0.01 mg/ mL), são vários os estudos que apontam para uma atividade superior, no entanto estes utilizam solventes que revelam ter alguma toxicidade não sendo, portanto, muito adequados à incorporação em alimentos.

O extrato aquoso de camomila obtido por decocção apresentou um teor de ácidos fenólicos superior ao dos flavonoides, sendo os derivados do ácido cafeoil-2,7-anidro-3-desoxi-2-octulopiranosónico o composto maioritário e o luteolin-*O*-glucuronido o flavonoide mais abundante. A presença deste composto pode justificar a sua atividade antioxidante e antimicrobiana. As bactérias mais suscetíveis foram *S. aureus*, *B. cereus* e S*. typhimurium*, sendo os fungos *P. funiculosum*, *A. versicolor* e *T. viride* os mais vulneráveis ao extrato de camomila.

O requeijão é um produto alimentar com um tempo de vida útil reduzido, adquirindo uma tonalidade amarela ao longo do tempo. Os extratos incorporados parecem evitar a intensificação da cor amarelada e melhorar as propriedades antioxidantes do produto, sem alterar o seu perfil nutricional. Adicionalmente, decorrido o tempo de análise (14 dias), os únicos requeijões que apresentaram sinais de degradação foram os requeijões controlo (sem adição de extratos). Os requeijões enriquecidos conseguiram manter as propriedades antioxidantes durante o armazenamento, no entanto, e apesar de ser sempre superior aos requeijões controlo, essa atividade começou a diminuir após o sétimo dia de armazenamento. Para tentar prolongar a bioatividade, os extratos aquosos destas duas plantas foram microencapsuladas em matrizes de alginato e incorporadas também em requeijões. Os resultados mostraram, mais uma vez que, a incorporação não alterou significativamente os parâmetros nutricionais, perfil de ácidos gordos ou cor. Contudo, tanto no caso dos microencapsulados de funcho como nos de camomila, verificou-se uma maior atividade antioxidante a partir do sétimo dia. Tal parece demonstrar que os compostos microencapsulados são libertados após esse tempo de armazenamento sendo possível concluir que o encapsulamento dos bioativos auxiliou na preservação da atividade antioxidante ao longo do tempo de armazenamento.

Sendo o requeijão um produto com tempo de prateleira muito reduzido, e em que industrialmente não se aplicam aditivos artificiais, estudou-se outro produto lácteo que comercialmente contém na sua composição o sorbato de potássio (E202) como conservante. Para testar o uso de decocções de plantas para desenvolver novos iogurtes, substituindo conservantes artificiais, prepararam-se quatro grupos de amostras: iogurtes controlo (sem

qualquer aditivo), iogurtes com extrato de funcho, iogurtes com extrato de camomila e iogurtes com E202. Os resultados obtidos demonstram que a fortificação de iogurtes com os antioxidantes naturais e os artificiais não causaram alterações significativas no pH, cor e valor nutricional do iogurte, comparativamente com as amostras controlo. No entanto, todos os iogurtes fortificados apresentaram maior atividade antioxidante que o iogurte controlo, e os adicionados com os aditivos naturais (destacando-se os iogurtes com decocção de camomila) a maior capacidade.

Por último, os extratos ricos em compostos fenólicos de funcho e camomila foram incorporados num produto de pastelaria (biscoitos caseiros), produto com um tempo de prateleira muito prolongado (60 dias). Industrialmente, é adicionado o hidroxilanisole butilado (BHA) como conservante. Foram preparados quatro grupos de amostras: biscoitos controlo (sem qualquer aditivo), biscoitos com extrato de funcho e biscoitos com extrato de camomila em duas concentrações distintas e biscoitos com BHA. Os resultados demonstraram que a incorporação de aditivos, tanto naturais como artificiais, não provocou alterações de cor ou valor nutricional dos biscoitos, quando comparados com os biscoitos controlo. Apesar dos resultados demonstrarem que tanto os aditivos naturais (na sua maior concentração), como o artificial, conferem atividade antioxidante semelhante, os extratos de plantas parecem ser uma solução de maior aceitabilidade pelos consumidores que preferem alimentos rotulados de “mais saudáveis” e "livres" de aditivos artificiais, evitando desta forma os efeitos adversos descritos na literatura.

No desenvolvimento de novos produtos é importante comparar diferentes receitas para verificar quais as necessidades e gostos do consumidor deste tipo de produtos. Assim as diferentes amostras (sendo que os biscoitos com extratos naturais foram incorporados com duas concentrações distintas) foram apresentadas a 120 provadores (com idades e sexo distintos) que preencheram um questionário para avaliar vários parâmetros dos biscoitos. Apesar da similaridade dos biscoitos apresentados, os que continham a maior concentração de camomila ou funcho foram geralmente apontados como os preferidos. Estes resultados vêm reforçar a possibilidade de integrar extratos de plantas como excelentes ingredientes naturais para preparar novos produtos alimentares promotores de saúde com aceitabilidade validada.

Numa visão mais geral, os resultados atestam que os extratos de funcho e camomila são excelentes ingredientes naturais que demonstraram capacidade conservante com benefícios para a saúde do consumidor, mostrando o seu potencial de aplicação tanto na indústria de produtos lácteos como de pastelaria.

5.2.2. Extratos ricos em compostos fenólicos para aplicação como aditivos naturais na indústria de pastelaria

Para maximizar a extração de ácido rosmarínico a partir de folhas de *Melissa officinalis* L. foram testadas e comparadas três técnicas de extração distintas: assistida por calor, por microondas e por ultrassons. Para a maximização das respostas foram testadas diferentes variáveis como a temperatura ou pressão (no caso do ultrassons), tempo e % de etanol em misturas etanol/água. A quantificação do ácido rosmarínico foi determinada por HPLC-DAD permitindo determinar o rendimento de extração do resíduo obtido. A metodologia de superfície de resposta foi aplicada e a extração por ultrassons provou ser a técnica mais eficaz com capacidade de produzir 86,3 ± 4,1 mg de ácido rosmarínico/g planta (dw, massa seca). Estes resultados foram obtidos para as condições ótimas de extração (33,0 ± 3,2 min, 371,7 ± 19,3W e 39,9 ± 1,4% de etanol). Comparando as quantidades obtidas, as técnicas de extração assistida por microondas e calor surgem como menos eficazes. Os resultados obtidos apontam para a viabilidade do uso de folhas de cidreira para extração de extratos ricos em ácido rosmarínico, seguindo diferentes técnicas e numa visão de aplicação industrial.

As atividades antioxidante, antibacteriana e antifúngica do extrato rico em ácido rosmarínico obtido a partir das folhas de cidreira (ERA), bem como a ausência de hepatotoxicidade foram comprovadas. Após incorporação do extrato em *cupcakes*, os efeitos provocados na preservação, composição química, parâmetros de cor e atividade antioxidante foram avaliados e comparados com *cupcakes* controlo (sem aditivos) e com *cupcakes* com E202. Com a incorporação de ERA em *cupcakes*, os parâmetros da cor surgem bastante alterados em relação aos restantes, resultando na alteração do aspeto visual do produto original. No entanto, e tendo em conta a forte bioatividade (atividades antioxidante, antibacteriana e antifúngica) exibida pelo ERA, os resultados observados indicam que este extrato natural tem propriedades funcionais adequadas para ser utilizafo como um potencial conservante natural, com capacidade de proporcionar propriedades benéficas a novos produtos alimentares, nomeadamente produtos de pastelaria.

Similarmente, as condições para a obtenção de um extrato fenólico a partir de flores de castanheiro foram otimizadas e os extratos obtidos posteriormente avaliados quanto à atividade antioxidante, antimicrobiana, tendo-se também confirmado a ausência de hepatoxicidade. Optou-se pela sua incorporação num produto de pastelaria (pastel de nata). As condições que maximizam a extração de compostos fénolicos são *t*= 20,0±37,7 min, *T*= 25,0±5,7 ºC, conduzindo à produção de 86,5 mg/g. No extrato fenólico obtido foram identificados, quatorze compostos dos quais sete taninos hidrolisáveis e sete flavonoides, com base nas características cromatográficas, UV-vis e espectro de massa. Os resultados revelaram o trigalloyl-HHDP-glucosídeo como o composto maioritário. Os efeitos da

incorporação deste extrato natural no perfil nutricional, parâmetros físico-químicos e atividade antioxidante foram avaliados e comparados com pasteis de nata controlo (sem aditivos) e com E202. Como no caso do ERA, também este extrato alterou o aspeto característico do produto. No entanto, os efeitos funcionais que acrescentou ao produto poderão constituir um factor atrativo, tanto para a indústria alimentar como para os consumidores.

5.3. **Perspetivas futuras**

Os alimentos funcionais têm sido apontados como uma das principais tendências da indústria alimentar. A sociedade está cada vez mais preocupada com questões de bem-estar e saúde. Conhecendo os efeitos a longo prazo de uma dieta pobre, adicionado ao consumo de tabaco e álcool, vida sedentária e altos níveis de stresse, os consumidores sentem-se cada vez mais atraídos por produtos alimentares que apresentem efeitos benéficos para a saúde comprovados. São vários os compostos fenólicos que têm sido descritos como poderosos antioxidantes, entre outras propriedades bioativas, nomeadamente, antimicrobianas ou mesmo antitumorais. Existem numerosas matrizes alimentares naturalmente enriquecidas com compostos fenólicos, sendo que, actualmente, muitos outros produtos têm sido enriquecidos com extratos naturais ou com os seus compostos individuais isolados.

Apesar de todo o potencial apresentado por estes produtos na prevenção de doenças e promoção da saúde, profissionais de saúde, nutricionistas, toxicologistas e agentes reguladores devem trabalhar em conjunto para planear a regulamentação adequada para a sua utilização. Adicionalmente, a crescente procura por alimentos funcionais à base de plantas, para promover saúde, longevidade e qualidade de vida, tem destacado a importância das plantas medicinais silvestres, o que tem levado a que algumas delas estejam ameaçadas devido ao uso irresponsável, associado a interesses económicos. Portanto, o cultivo destas plantas é apontado como uma alternativa a ter em consideração. Mais, a próxima fase do crescimento do mercado depende fortemente de uma investigação orientada para a utilização de tecnologias de produtos inovadoras e mais eficazes e do uso de estratégias de *branding* associadas a produtos naturais.

Com a conclusão deste trabalho e validação do potencial dos extratos obtidos a partir de funcho, camomila, cidreira e flores de castanheiro como ingredientes naturais, em diferentes produtos alimentares, seria importante avançar com a certificação destes como conservantes (aditivos). Este tipo de extratos pode ser introduzido em alimentos na qualidade de ingrediente, no entanto, para alcançar o estatuto de conservante alimentar (aditivo) devem passar pela avaliação da EFSA.