Development of a natural preservative obtained from chestnut flowers through the optimization of an ultrasonic assisted extraction technique

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ABBREVIATIONS

DAD: Diode Array Detector
DCCC: Circunscribed Central Composite Design
DMEM: Dulbecco's Modified Eagle Medium
DW: dry weight
EU: European Union
EFSA: European Food Safety Authority
ESI: Electrospray ionization
FAO: Food and Agriculture Organization
FDA: Food and Drug Administration
FAME: Fatty acids methyl ester
FBS: Fetal bovine serum
FOSHU: Foods for Specified Health Use
FT: total flavonoid
FUFOSE: Functional Food Science in Europe
HPLC: High Pressure Liquid Chromatography
HHDP: Hexahydroxydiphenic acid
GC-FID: Gas Chromatography Coupled to Flame Ionization Detector
GI_{50}: Concentration that inhibited 50% of the net cell growth
LPS: Lipopolysaccharides
LC: Liquid chromatography
LCCA : life-cycle cost analysis
Min: Minute
MS: Mass Spectrometer
NADPH: Nicotinamide adenine dinucleotide phosphate
NS: non-significant
P : ultrasound potencial
R : residue
R^2 : coefficients of determination
RI: Reflexion Index
RNS: reactive nitrogen species
ROS : reactive oxygen species
RSM: Surface Methodology of Response
S: Solvent
TT: Total tannin
T: Time
UAE: Ultrasound assisted extraction
USA: United State of America
USD: United States dollar

UV: Ultraviolet
UV-vis: Ultraviolet–visible
UPLC: Ultra Performance Liquid Chromatography
W : Watt
WHO: Word health Organization

ABSTRACT

The chestnuts are a group of nine species of deciduous trees and shrubs in the genus Castanea, in the beech family Fagaceae. Due to the nutritional properties, chestnuts have historically been used for human and animal consumption. Several by-products are produced during chestnut industrial processing, such as chestnut wood, flowers, leaves, shells, barks, and burs. Such by-products are a significant source of antioxidant compounds and can be used as food additives to be incorporated in food products to optimize nutritional and quality characteristics and to prevent oxidation processes. The aim of this research work was to optimize the conditions for the extraction of phenolic compounds from male chestnut flowers using ultrasonic-assisted extraction in order to develop an extract rich in phenolic compounds and, study it potential as a natural ingredient with potential industrial application. Time (*t*), ultrasound potencial (*P*) and solvent (*S*) were the conditions 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 being trigalloyl-HHDP-glucoside the major compound found. The conditions that maximized the total phenolic content was found at *t*= 23.47±2.90 min, *P*= 258.78±16.09 W and *S*= 50.51±7.11% ethanol producing an extract with 21.86±8.84 mg of phenolic compounds per g of extract. In this way, this study allowed to define the best conditions for the extraction of bioactive compounds and confirm the potential of the extract of male chestnut flowers as a natural ingredient for functional foods, providing health benefits.

Keywords: *Castanea sativa Mill., Phenolic Compounds, Ultrasonic-assisted extraction, Response surface methodology, Circumscribed central composite design, Preservative/Functional properties.*

RESUMO

Os castanheiros são um grupo de nove espécies de árvores caducas e arbustos do gênero Castanea, da família das faias Fagaceae. Devido às propriedades nutricionais, as castanhas têm sido historicamente utilizadas para consumo humano e animal. Vários subprodutos são produzidos durante o processamento industrial da castanha, nomeadamente a madeira de castanho, flores, folhas, cascas e, ouriços. Esses subprodutos são uma fonte significativa de compostos antioxidantes e podem ser utilizados como aditivos alimentares a serem incorporados em produtos alimentares de forma a otimizar as características nutricionais e de qualidade e prevenir processos de oxidação. O objetivo deste trabalho de pesquisa foi otimizar as condições de extração de compostos fenólicos de flores masculinas de castanheiro através de extração assistida por ultrassons visando o desenvolvimento de um extrato rico em compostos fenólicos e, estudar seu potencial como ingrediente natural com potencial aplicação industrial. Tempo (t), potência (P) e solvente (S) foram as condições otimizadas pela metodologia de superfície de resposta usando um desenho experimental de 5 níveis. As respostas utilizadas como critérios foram a quantificação dos compostos fenólicos individuais identificados por HPLC-DAD-ESI / MS e o rendimento de extração do resíduo obtido. Com base nas características cromatográficas, UV-vis e espectros de massa, foram identificados catorze compostos, dos quais sete taninos hidrolisáveis e sete flavonóides, sendo o trigaloil-HHDP-glucosídeo o principal composto encontrado. As condições que maximizaram o conteúdo fenólico total foram encontradas em t = 23,47 ± 2,90 min, P = 258,78 ± 16,09 W e S = 50,51 ± 7,11% de etanol, produzindo um extrato com 21,86 ± 8,84 mg de compostos fenólicos por g de extrato. Desta forma, este estudo permitiu definir as melhores condições para a extração de compostos bioativos e confirmar o potencial do extrato de flores masculinas de castanheiro como ingrediente natural para alimentos funcionais, proporcionando benefícios à saúde.

Palavras-chave: *Castanea sativa* Mill., *Compostos fenólicos,* Metodologia de superfície de resposta, *Desenho composto central circunscrito, Propriedades conservantes/funcionalizantes.*

I. Introduction

In the past, food was seen only as a source of nutrition and hunger satisfaction. However, there is a growing concern among consumers in choosing foods labelled as healthier and more natural. There is awareness that these choices will have a direct consequence on health and well-being (Bearth et al., 2014). Such trend and demand from consumers have been a direct influence on the food industry, as it tries to meet consumer's expectations. Food additives have emerged in the food industry due to a need to ensure food preservation over a longer shelf life in an industrialized age with a rapidly growing population. Although, this use is a common practice and recognized as extremely important, some recent scientific studies have raised some concerns regarding the daily consumption of food additives (Carocho et al., 2014a).

The use of natural additives as a substitute for food synthetic additives is seen as an excellent alternative due to the beneficial health effects associated to these ingredients, which have been linked to the prevention of various chronic diseases (Carocho and Ferreira, 2013b). Furthermore, natural ingredients are also described in literature as composed by molecules with antimicrobial activity, capable of retarding and/or inhibiting the growth of pathogenic and/or toxin-producing microorganisms in food and therefore with a great potential to be applied by the industry food (Delves-Broughton, 2012). In this sense, there are several plants that due to their interesting composition in compounds associated to their beneficial health effects, have been explored as alternative sources of new natural preservatives and functional ingredients with potential application in the food industry (Okino Delgado and Fleuri, 2016).

However, the processing of natural ingredients is a very complex and time-consuming process that requires detailed research in order to establish the correct conditions and methodologies to be used for each plant matrix and that guarantee maximum yield and purity (Jiménez et al., 2018; Lokesh et al., 2015). In this way, mathematical models and optimization methodologies have been recognized to establish the ideal conditions of extraction that contribute to the best response values. In addition, several extraction parameters, such as the solvent, time and energy, as well as the potential loss of natural compounds, must also be taken into account (Chemat et al., 2017). The selection and optimization of the correct extraction conditions are necessary to guarantee an optimal yield for the least time, solvent and energy used (Diouf et al., 2009).

1.1. Functional foods

1.1.1. Definition and health benefits

The main function of food is to provide nutrients to meet the needs of human metabolism and give consumers a feeling of satiety, satisfaction and well-being. However, it is now known that in addition to this basic function, food can have specific physiological functions in the human body (Hetherington et al., 2013). Some studies have shown that food, in addition to helping to achieve optimal health and development, can play an important role in reducing or preventing the risk of some diseases. According to the World Health Organization (WHO) and the Food and Agriculture Organization (FAO), different eating patterns, combined with habits and lifestyles, are the main risk factors in relation to the development of coronary diseases, some types of cancer, diabetes, obesity or osteoporosis (Carocho et al., 2016).

In 1981, foods with these properties were regulated for the first time in Japan in a specific category called Foods for Specified Health Use (FOSHU) (Hasler, 1998). Later, to scientifically assess the evidence that these types of specific food components act beneficially on consumer's health, the Functional Food Science in Europe (FUFOSE) project was created (Carocho et al., 2016). However, in the European Union there are still no specific categories of functional food (Gulati and Berry Ottaway, 2006). In addition to the required legislation for all foods, scientific evidence of health claims is required (Astley, 2019). In the United States, the federal agency responsible for defining product categories is the Food and Drug Administration (FDA), which depends on their characteristics and abides by various safety issues, health claims and labeling. This type of food is usually defined as "functional food", however, a universally accepted definition for this food category is not established (Bagchi, 2019).

According to literature, a food can be defined as "functional" when, in addition to having adequate nutritional effects, demonstrating beneficial functions for consumer health, namely in reducing the risk of disease. However, these beneficial effects should be demonstrated when ingested in amounts normally consumed in the usual diet (Konar et al., 2018). It is important to mention that a functional food can be functional for all members of a population or for a particular population group. Although there is no universal definition or classification and there are no universal regulatory members for this type of food, some functional food classes can be defined as: (a) unchanged products: foods which naturally

have a higher content of nutrients and/or health promoting compounds; (b) fortified products: foods in which the content of certain components, normally existing, has been added; (c) enriched products: foods to which a normally nonexistent component is added to provide benefits; (d) altered products: foods in which a component is removed or replaced by an alternative component with favorable properties; or (e) improved products: the composition of the food is altered, one of the components is improved through special growing conditions or biotechnological means (Bagchi, 2019).

Thus, the incorporation of natural ingredients in food comes from a clear perspective of health promotion and there are already several sources pointed and explored, such as certain bioactive compounds (**Table 1**). The area of functional foods is evolving and recognizing functional effects. And the food industry has been investing in technology to be more widely exploited. However, further exploration in genetics is considered necessary in order to understand how genetic factors may influence the relationship between diet and diseases and the ways in which different protective and risk factors may act (Forbes-Hernández et al., 2014).

Table 1. Sources of bioactive compounds and potential benefits (Fernandes et al., 2018).

COMPOUNDS	SOURCE	POTENTIAL BENEFITS
Carotenoids		
α-Carotene/β-carotene	Carrots, fruits, vegetables	Neutralize free radicals, which may cause damage to cells
Lutein	Green vegetables	Reduce the risk of muscular degeneration
Lycopene	Tomato products (ketchup, sauces)	Reduce the risk of prostate cancer
Dietary fiber		
Insoluble fiber	Wheat bran	Reduce risk of breast or colon cancer
β-Glucan	Oats, barley	Reduce risk of cardiovascular disease; protect against heart disease and some cancers; lower LDL and total cholesterol
Soluble fiber	Psyllium	Reduce risk of cardiovascular disease; protect against heart disease and some cancers; lower LDL and total cholesterol
Fatty acids		
Long chain omega-3 fatty acids-DHA/EPA	Salmon and other fish oils	Reduce risk of cardiovascular disease; Improve mental and visual functions
Conjugated linoleic acid	Cheese, meat products	Improve body composition; decrease risk of certain cancers
Phenolic compounds		
Anthocyanidins	Fruits	Neutralize free radicals; reduce risk of cancer
Catechins	Tea	Neutralize free radicals; reduce risk of cancer
Flavonones	Citrus	Neutralize free radicals; reduce risk of cancer
Flavones	Fruits/vegetables	Neutralize free radicals; reduce risk of cancer
Lignans	Flax, rye, vegetables	Prevention of cancer; renal failure

Tannins (proanthocyanidins)	Cranberries, cranberry products, cocoa, chocolate	Improve urinary tract health; reduce risk of cardiovascular disease
Soy phytoestrogens		
Isoflavones: daidzein and Genistein	Soybeans and soy-based foods	Menopause symptoms, such as hot flashes; protect against heart disease and some cancers; lower LDL and total cholesterol
Prebiotics/probiotics		
Fructo-oligosaccharides	Jerusalem artichokes, shallots, onion powder	Improve quality of intestinal microflora and gastrointestinal health
Lactobacillus	Yogurt, other dairy products	Improve quality of intestinal microflora and gastrointestinal health
Plant sterols	Corn, soy, wheat, wood oils	Lower blood cholesterol levels by inhibiting cholesterol absorption

1.1.2. Trends and development of functional foods

The exploration for new functional ingredients has forced the food industry to invest in new studies and development of new methodologies and technologies which consequently requires a search for approvals and certifications in regulatory bodies. There are several natural matrices that have been pointed as excellent sources of bioactive compounds. There are currently some bioactive compounds and functional ingredients that are already routinely added to food products namely probiotics, prebiotics, dietary fibers, phytosterols, carotenoids, phenolic compounds and fatty acids (Salgado, 2017). In this sense, different plant matrices characterized by demonstrating excellent bioactive properties have been studied as potential bioactive ingredients in order to be applied in the food industry (Olszewska et al., 2020).

The high number of food-borne outbreaks caused by pathogens associated with high rates of resistance to antibiotics has been increasingly worrying the population (Alejo-Armijo et al., 2018). Consequently, interest has aroused in the use of biviative compounds obtained from plant extracts that produce less antimicrobial resistance (Gupta and Birdi, 2017). The bioactive effects described by a large number of plant extracts, with direct consequences on consumer health, have been widely studied in order to explore their potential for application in the industry (Ouedrhiri et al., 2017).

Consumers are increasingly aware of the composition of foods and favor products labeled as healthier and more natural where the number of artificial additives is minimal or even zero. Thus, the functional food market has been growing exponentially by launching new functionalized products designated as beneficial to health (Bigliardi and Galati, 2013). All commercially available functional foods which are guaranteed to improve consumer

health must be strictly controlled, particularly in what concerns the information on the label. The use of claims in any commercial food communication (labeling, presentation or advertising) shall be allowed under strict conditions: (1) claims shall apply to ready-to-eat food, (2) The nutrient or substance that has previously been shown to be missing or present in the product concerned (including its reduced content) has a beneficial nutritional or physiological effect and must be assimilable by the organism and, most significantly, (3) the reported beneficial effects must be easily understood by the average consumer in order to (a) protect consumers and prevent misunderstandings about the health and/or nutritional adequacy of certain foods, and (b) promote creativity and equal competition in the food industry (Cámara et al., 2020).

The functional food market is growing worldwide (Mohamad et al., 2019). Around the world sales of naturally healthy foods come to $253 billion in 2017; functional/fortified nourishments totaled $247 billion. Organic foods and beverages led global retail esteem growth with a compound annual development rate of 7% from 2012–2017, followed by free-form products at just over 6%; healthy, 3%; and fortified/functional, 2%. In developing countries, natural foods and drink sales developed 9%, and free-form product sales were up 5% (Euromonitor, 2018). The main market for functional foods is Japan, followed by the United States of America (USA) and finally the EU. Japan was the first country to introduce the word “functional food”, it’s classification and regulation. It was also the origin of the functional food industry. As for functional food products, the US and Europe are both considered important global forces with considerably different approaches to food and claims regulation (de Boer and Bast, 2015). The evolution of this market is directly related to consumer awareness and acceptability of new products. For example, American and Japanese consumers accept the concept of functional food products with health claims more easily than Europeans and, can integrate such products into their daily diets (Domínguez Díaz et al., 2020). Next to Japan, the US has a significant share of the functional food market of around 2-3% and is constantly growing (Euromonitor, 2018). In Europe, however, the population still shows some skepticism about functional foods, because of their suspected safety, but also because of the ingrained food and cultural traditions that influence demand for functional food classes. Finland, Sweden, Netherlands, Poland and Cyprus are considered the European countries more open-minded are more interested in buying these products (Küster-Boluda and Vidal-Capilla, 2017). Dairy products, pastry, bakery and baby food lead the functional food market. However, there are several sectors that have shown high

investments in order to launch on the market products labeled healthier and more natural, which meet the expectations of consumers (Birch and Bonwick, 2019). Nestlé®, Danone® Group, Kraft Foods®, Unilever®, PepsiCo®, Coca-Cola® and Heinz® are examples of great food companies recognized worldwide as betting much of their investment on products referred to as "healthier".

1.1.3. The use of plants as natural ingredients

The use of plants in the preparation of infusions or in the preparation of food products is a traditional practice that comes from ancient times in several cultures that combines their consumption with benefits in the treatment of some symptoms (Wyk and Wink, 2017). Thus, the functionalization of foods using plants seems to be the best form for the food industry to meet the current demands of consumers. Currently, there are still a limited number of plant foods that have exceeded the stringent standard required by the FDA for authorization in health claiming products (Carocho et al., 2014a). However, there is a growing clinical research that supports the potential health benefits of various plant foods (including wild plants), which currently have no approved health claims and is therefore described as having "moderately strong evidence" (Carocho et al., 2015b).

Edible wild plants are important sources of physiologically active ingredients that are associated with different beneficial health effects. Several berries, such as elderberry, blueberry, blackberry, raspberry and wild strawberry stand out as a source of anthocyanins, proanthocyanidins, flavonols, phenolic acids and vitamins, among other bioactive compounds. These molecules, alone or in combined extracts, have antioxidant, anti-inflammatory, anticarcinogenic, cardioprotective and antibacterial properties (Pinela et al., 2016a). Thus, it is possible to find in the market various functional herbal foods, with functional properties. Scientific research in recent years proves its effectiveness as healthy foods, and the food and pharmaceutical industries are increasingly interested in developing new products based on these plants. In the future, the combination of popular knowledge, research and technological development is expected to lead to the development of functional foods and new preventive and therapeutic approaches (Carocho et al., 2015b). **Table 2** presents a list of some plants that were studied due to the claimed functional properties that they had. Different parts of plants can have functional properties with potential for exploitation by different areas, namely food, pharmaceutical and cosmetic industry.

Table 2. Edible plants with described functional properties (Pinela et al., 2016a).

Plant specie	Common name and used part	Evaluated extract	Functional compounds	Potential health benefits
Allium ampeloprasum L.	Wild leek (bulb)	Aqueous and ethanolic extracts.	Fibre, zinc, polyunsaturated fatty acids (mainly palmitic acid), polysaccharides (glucofructan) and steroidal saponins.	Antioxidant, anti-inflammatory, antiulcerogenic and gastroprotective.
Beta spp.	Beet (root)	Aqueous, hydro-ethanolic, methanolic and betalain-rich extracts and juice.	Phenolic acids (ferulic, vanillic, *p*-hydroxybenzoic, caffeic and protocatechuic acids), flavonoids (catechin, epicatechin, rutin and vitexin), betalains (betanin, isobetanin and vulgaxanthin I), minerals (potassium, magnesium, iron, zinc, calcium, sodium), folic acid, biotin and soluble fibre.	Antioxidant, hepatoprotective, anticancer, antiproliferative activity in MRC5 and MCF-7 cell lines, antihypertensive and hypoglycemic.
Capparis decidua (Forssk.) Edgew.	Caper or kair (fruit)	Aqueous, methanolic, acidified methanolic, hydroalcoholic and ethanolic extracts.	N-pentacosane, β-sitosterol, β-carotene, alkaloids, phenolic compounds including flavonoids and minerals (manganese, copper and iron).	Antioxidant, antidiabetic, diuretic, hypercholesterolemic, anti-hypertensive, anti-atherosclerotic, hypolipidaemic, antimicrobial and anthelmintic.
Dimocarpus longan Lour.	Longan (fruit)	Aqueous, hydromethanolic (80%), acetone:ethanol (1:1, v/v), hydroacetone (70%) and polyphenol-rich extracts.	Phenolic compounds (corilagin, gallic and ellagic acids, flavone glycosides, glycosides of quercetin and kaempferol and epicatechin), vitamin C, fibre and minerals.	Antioxidant, anti-inflammatory, anti-tyrosinase, anti-glycated, anticancer and memoryenhancing effects.
Eugenia uniflora L.	Pitanga or brazilian cherry (fruit)	Ethyl acetate and ethanolic extracts.	Anthocyanins, carotenoids and flavonols.	Anti-diarrheic, diuretic, anti-rheumatic, anti-febrile, antidiabetic, antimicrobial and anti-trypanosoma.
Euterpe oleracea Mart.	Acai, assai or açaí (fruit)	Ethyl acetate, *n*-buthanolic, hydromethanolic (50%) and hydroacetonic (70%) extracts.	Anthocyanins, flavonoids, phenolic acids, procyanidin, lignans and stilbenes.	Antioxidant, anti-allergic, anticancer, anti-inflammatory, atheroprotective, improves the endothelial function and platelet aggregation, vasodilator and prevents cardiovascular disease.
Ficus carica L.	Fig (fruit)	Hexane, methanolic and hydromethanolic extracts.	Phenolic acids (chlorogenic acid), anthocyanins, flavonols, flavones (luteolin), minerals (iron, potassium, sodium and calcium), fibre, sugars and vitamin A.	Antioxidant, anti-cholinesterase, anticarcinogenic, antiproliferative activity in several cancer cell lines, digestive, antifungal and anti-helminthic.
Fragaria vesca L.	Wild strawberry or European strawberry (fruit)	Aqueous extracts and combined extract of *n*-buthanolic and to HCl (1 mol/dm^3).	Flavonoids, phenolic acids, anthocyanins and salicylic acid.	Antioxidant.
Garcinia mangostana L.	Mangosteen or purple mangosteen (fruit)	Aqueous, methanolic, ethanolic, hydro-ethanolic (40 and 50%) and juice extracts.	Xanthones (α-, β-, and γ-mangostins, garcinone E, 8-deoxygartanin and gartanin).	Antioxidant, antitumor, anti-proliferative, pro-apoptotic, anti-inflammatory, anti-allergic, antibacterial, antifungal, antiviral, antimalarial, antidiabetic, antihiperlipidemic and anti-atherogenic, cardioprotective, hepatoprotective, immunomodulator and antiulcer.
Gardenia jasminoides J. Ellis	Gardenia (fruit)	Hydromethanolic (80%), hydro-ethanolic (60%) and	Caffeoylquinic acid derivatives (chlorogenic acid, dicaffeoylquinic acid and other caffeoyl-conjugate	Antioxidant and anti-inflammatory.

		cetone:ethanol (1:1, v/v) extracts.	quinic acid derivatives), flavonoids (rutin), iridoids (geniposide) and carotenoids (crocin).	
Litchi chinensis Sonn.	Litchi or lychee (fruit)	Methanolic (70 and 80%), acetone:ethanol (1:1) and juice extracts.	Phenolic compounds (cinnamic acid and procyanidins), carotenoids and vitamin C.	Antioxidant, anti-apoptotic and hepatoprotective.
Lycium barbarum L.	Goji berry (fruit)	Aqueous, methanolic and crude and purified polysaccharide extracts.	Polysaccharides, carotenoids (zeaxanthin), betaine, cerebroside, beta-sitosterol, *p*-coumaric acid and vitamin C.	Antioxidant, anti-aging, anti-inflammatory, anticancer, cytoprotective, neuroprotective, metabolism stimulator, glucose regulator in diabetics, glaucoma (eye health benefits), immunomodulatory, antibacterial and cardioprotective.
Malpighia emarginata DC.	Acerola or wild crepe myrtle (fruit)	Methanolic, hydromethanolic (50%), hydroacetonic (70%) and aqueous extracts and juice.	Vitamin C, carotenoids (β-carotene), riboflavin, thiamine, fibre, minerals (phosphor, calcium and iron) and flavonoids (anthocyanins (cyanidin-3-rhamnoside and pelargonidin-3-rhamnoside) and flavonols (quercetrin)).	Antioxidant, anti-aging, anti-inflammatory and prevents weight gain and dyslipidemia.
Myrciaria cauliflora (Mart.) O. Berg.	Jaboticaba or guapuru (fruit)	Methanol:formic acid (9:1, v/v), methanol:water:acetic acid (85:15:0.5, v/v/v/), methanolic, hydromethanolic (50%), ethanolic, acetonic and hydroacetonic (70%) extracts.	Anthocyanins, ellagic and gallic acid, carotenoids, depsides, tannins, rutin and vitamin C.	Antioxidant, anti-inflammatory, inhibits the IL-8 production, antiproliferative effects against tumour cells, protective effect in cardio vascular disease and type 2 diabetes mellitus.
Myrciaria dubia (Kunth) McVaugh	Camu-camu, cacari or camocamo (fruit)	Hydromethanolic (50%) and hydroacetonic (70%) extracts.	Anthocyanins, myricetin and conjugates, ellagic acid and conjugates, ellagitannins, flavanols, proanthocyanidins and vitamin C.	Antioxidant, anti-inflammatory and inhibits the LPS-induced NO release in RAW 264.7 cells.
Nasturtium officinale W.T. Aiton	Watercress (aerial parts)	Methanolic and hydromethanolic (70%) extracts.	Phenolic compounds and minerals (phosphorous, potassium, calcium and manganese).	Antioxidant, anticarcinogenic and chemopreventive.
Physalis spp.	Physalis or golden berry (fruit)	Hydro-ethanolic (70%) extracts.	Physalins, withanolides, sterols, polysaccharides and flavones.	Anti-inflammatory, antioxidant, antitumor, hypoglycemic and analgesic.
Prosopis cineraria (L.) Druce	Ghaf, khejri, sami or golden tree of Indian deserts (pod)	Aqueous and methanolic extracts.	Triterpenoids (3-benzyl-2-hydroxy-urs-12-en-28-oic acid and maslinic acid-3 glucoside), fatty acid (linoleic acid), piperidine alkaloid (prosophylline) and polyphenols (5,5'-oxybis-1,3-benzenediol, 3,4,5-trihydroxycinnamic acid 2-hydroxyethyl ester and 5,3',4'-trihydroxyflavanone 7-glycoside).	Antioxidant and anti-inflammatory.
Psidium cattleianum Sabine	Strawberry guava (fruit)	Hexane, ethyl acetate, acetonic, aqueous, ethanolic and methanolic extracts.	Phenolic compounds (ellagic acid, ellagic acid deoxyhexoside and epicatechin gallate), carotenoids, vitamin C and fibre.	Antioxidant, anti-inflammatory and antimicrobial.
Psidium guajava L.	Guava (fruit)	Methanolic, hydromethanolic (80%), acetone:ethanol (1:1, v/v), hexane, ethyl acetate and	Phenolic acids (chlorogenic acid), flavonoids (catechin), anthocyanins (delphinidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside).	Antioxidant, anti-inflammatory and antimicrobial.

		ethanol/water/formic acid (70:25:5, v/v/v) extracts.		
Punica granatum L.	Pomegranate (fruit)	Aqueous, ethyl acetate, acetonic and methanolic extracts.	Anthocyanins, gallotannins, ellagitannins (ellagic acid, gallic acid and punicalagin), gallagyl esters, hydroxybenzoic and hydroxycinnamic acids and dihydroflavonol.	Antioxidant, anti-inflammatory, anti-allergic, chemopreventive, anticancer, cardioprotective, gastroprotective, antimicrobial and antihelminthic.
Rhodomyrtus tomentosa (Aiton) Hassk.	Rose myrtle (fruit)	Hexane, methanolic, hydromethanolic (80%), acetone:ethanol (1:1, v/v), acetone:water:acetic acid (50:49:1, v/v/v) and flavonoids-rich extracts.	Flavonoids (gallocatechin gallate, dihydromyricetin, quercetin, kaempferol, anthocyanins and vitexin), organic acids, polysaccharides, fibre, vitamin E (α-tocopherol), minerals (manganese and copper) and essential fatty acids (mainly linoleic acid).	Antioxidant.
Rubus spp.	Blackberry and raspberry (fruits)	Hexane, ethyl acetate and methanolic extracts.	Anthocyanins, flavonols, phenolic acids (ellagic acid), vitamins C and E, folic acid and β-sitosterol.	Antioxidant, anti-inflammatory and chemopreventative.
Sambucus nigra L.	Elderberry (fruit)	Methanolic, acidified methanolic, ethanolic and hydro-ethanolic (80%) extracts.	Polyphenols (anthocyanins, flavonols, phenolic acids and proanthocyanidins), terpenes, lectins, unsaturated fatty acids, fibre, vitamins A, B, C and E and minerals.	Antioxidant, cardiovascular protection, antidiabetic and anti-obesity, reinforces the immune system, antiviral, antibacterial and UV radiation protector.
Syzygium cumini (L.) Skeels.	Jambul or jambolan (fruit)	Methanolic, hydromethanolic (50%), hydroacetonic (70%) and hexane extracts.	Anthocyanins, ellagic acid, quercetin, rutin, carotenoids, vitamin C and manganese.	Antioxidant, antiscorbutic, diuretic and antidiabetic.
Theobroma cacao L.	Cacao tree or cocoa tree (seed)	-	Polyphenolic compounds (catechins and anthocyanins).	Antioxidant, anti-inflammatory, chemopreventive and anticancer, increases blood flow to cutaneous and subcutaneous tissues and contributes to skin appearance and texture, increases cerebral blood flow, stimulates the nervous system, facilitates digestion and improves kidney and bowel function.
Vaccinium myrtillus L.	Bilberry (fruit)	Acidified methanolic, ethyl acetate, hexane, anthocyanins and proanthocyanidins-rich extracts.	Flavonoids (proanthocyanidins and anthocyanins), carotenoids (lutein and zeaxanthin) and sterols.	Antioxidant, antibacterial (inhibition of urinary tract infections), anticarcinogenic and antiproliferative activity in two human breast cancer cell lines MCF-7 and BT-20.
Vaccinium spp.	Cranberry (fruit)	Hydroacetonic (80%), ethyl acetate and phenolic extracts.	Phenolic acids and flavonoids (anthocyanins, proanthocyanidins and flavonols).	Antioxidant, anti-inflammatory and cardiovascular and urinary tract protection.
Zingiber officinale Rosoe	Ginger (rhizome)	Aqueous and methanolic extracts.	Gingerols (6-gingerol), shogaols (6-shogaol), fibre and flavonoids.	Antioxidant, anti-inflammatory, antithrombotic and cholesterol-lowering, analgesic, antipyretic and hypotensive.
Ziziphus jujuba Mill.	Jujube or red date (fruit)	Aqueous, hexane, methanolic and hydromethanolic extracts.	Saponins, tannins, terpenoids, flavonoids and iron.	Antioxidant, anti-inflammatory and gastrointestinal protector.

The use of plant extracts rich in bioactive compounds as alternatives to synthetic additives is a promising trend in the food industry, due to their high antimicrobial properties, especially because the market for clean label ingredients is projected to be value 47.50 billion USD in 2023, largely due to increased customer demand for all-natural products (Newswire, 2018).

Thus, it is possible to find several functional plant-based foods on the market. Scientific research carried out in recent years proves its effectiveness as healthy foods, and the food and pharmaceutical industries are increasingly interested in developing new products based on these plants. Therefore, medicinal and aromatic plants play an important role in the development of new or improved functional foods. It is hoped that in the future, the combination of popular knowledge, research and technological development will lead to the development of functional foods and new preventive and therapeutic approaches.

1.2. Phenolic compounds as bioactive ingredients

1.2.1. Classification of phenolic compounds

Around 200,000 chemicals are isolated and associated with different systems and classes from higher plants around the world. There are two major classes of these chemicals: primary and secondary metabolites. The primary metabolites, such as fatty acids, proteins, carbohydrates, and nucleic acids are essential for cell maintenance. The secondary metabolites are no less important, although they are considered to be necessary for plant survival despite not participating directly in photosynthetic or respiratory metabolism (Chikezie, 2015). Compared to primary metabolites, their structures and chemicals are diverse, and are responsible for plant defense. Secondary metabolites also serve as signal compounds for seed dispersion attracting pollinators or animals, as well as protecting the plant from oxidants and ultraviolet radiation (Jurić et al., 2020).

Phenolic compounds are one of the more widely distributed secondary metabolites in plants (Lin et al., 2016). The main dietary sources of this type of compounds are fruits and plant-derived beverages, such as fruit juices, teas, coffee and red wine, thus also vegetables, cereals, chocolate and pulses also contribute to the total intake of phenolic compounds. Normally phenolic compounds in nature are conjugated with sugars and organic acids (Yamagata et al., 2015). Phenolic acids, flavonoids, and tannins are the most important types

of phenolic compounds present in human diet. Chemically, phenolic acids have at least one aromatic ring, where a hydroxyl group substitutes at least one hydrogen (Taofiq et al., 2015).

These compounds can be classified according to two main types, flavonoids and non-flavonoids and into different classes, which depend on the number of phenolic rings and the structural elements connecting these rings, as can be seen from **Figure 1**.

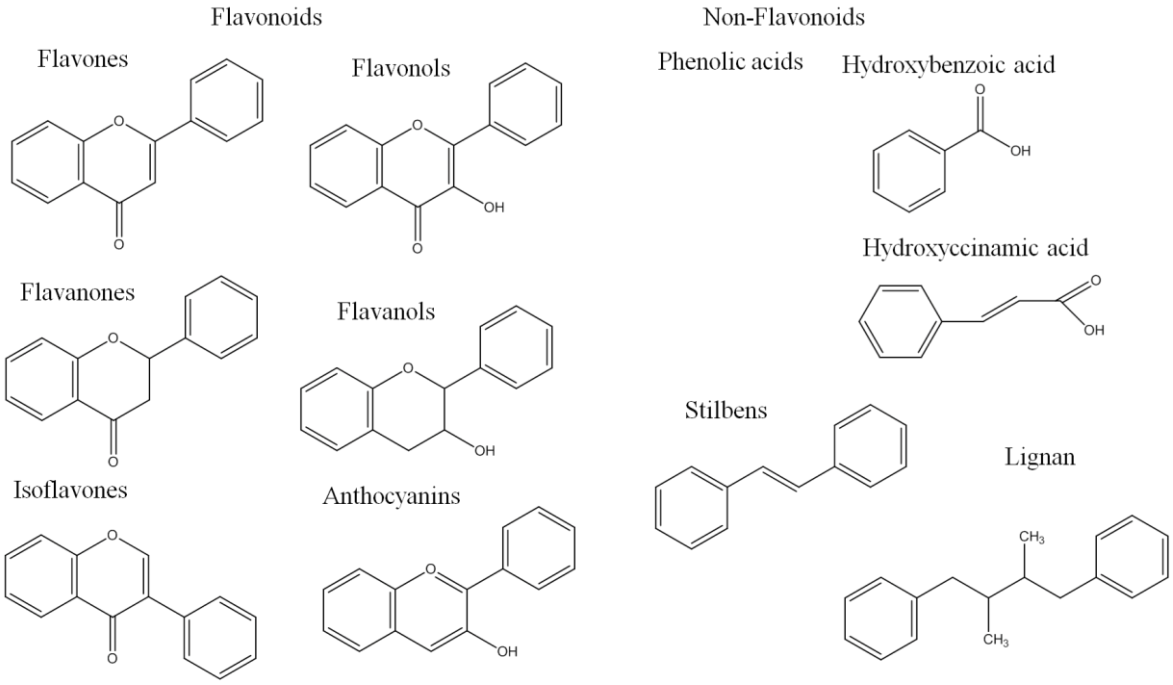


Figure 1. Classification of phenolic compounds (Caleja et al., 2017).

Some authors have described different bioactivities associated with this type of compounds namely antioxidant, antimicrobial, anti-inflammatory, antitumor and hepatoprotective effects, among others. These compounds are described in literature as being active in neutralizing reactive species and preventing oxidative stress, thus acting in various diseases. Some of these compounds exhibit high antioxidant activity as individual compounds, while others rely on synergisms effects to have bioactive effects (Giuberti et al., 2020).

Several scientific studies have described anti-inflammatory properties of phenolic compounds (Mizgier et al., 2016). During the inflammation process, the formation of several reactive oxygen species (ROS) and reactive nitrogen species (RNS) result in increased pro-inflammatory agent activity. Phenolic compounds have the ability to reduce or prevent these

harmful effects in the human body by reducing these pro-inflammatory enzymes (Bowen-Forbes et al., 2010). Phenolic compounds have been studied extensively for their capacity to counteract oxidative stress, once persistent oxidative stress is linked with various metabolism disorders and a number of pathologies, such as obesity, diabetes and cardiovascular diseases (Vuolo et al., 2018). Also, the antifungal activity of phenolic compounds has been described in literature, expressed through an interaction with the lipid bilayer of the fungal cell membrane. Phenolic acids, flavonoids and stilbenes are the most studied phenolic compounds in terms of antifungal properties (Martins et al., 2015). Several studies have also reported antimicrobial properties of some plants against different strains, this property being normally associated with the presence of phenolic compounds (Pereira et al., 2020; Qin et al., 2019).

So, some authors ensure that these phenolic compounds may be used as potential natural additives. It contributes to increasing the shelf-life of food products as naturally produced antimicrobials and antioxidants (Lopes et al., 2018).

1.2.2. Factors affecting stability and contributing to its degradation

The loss of bioactivity of phenolic compounds may occur due to a set of adverse conditions regarding processing steps, endogenous enzyme action, water activity, oxygen pressure as well as thermal/mechanical action. Additionally, the chemical structure of phenolic compounds and their interaction with other molecules present in the food product and the organism may influence the stability and, consequently, the bioavailability of these compounds (Dias et al., 2015).

There are several external factors that lead to the degradation of phenolic compounds, in which some authors have shown that different pH and high temperatures can cause changes in the chemical structure of these compounds resulting in a decreased of their stability. Also, the light radiation is pointed in some studies as responsible for accelerating the degradation of these compounds (Minatel et al., 2017). Due to the influence established by these different factors, optimization of phenolic extraction techniques and conditions is a point that has to be routinely applied in order to guarantee maximum yield with maximum purity, while ensuring the industry with minimum expenses.

When studying the incorporation of phenolic compounds in food products it is important to consider the interactions that may occur between these compounds and other

molecules in food products, which may lead to their degradation, since some biochemical and chemical processes are involved in these steps. The quantity of the several phenolic compounds is positively related to preserving the quality of food products during shelf life from oxidation (Trapani et al., 2017). Throughout the extraction process, biochemical, chemical and physical processes affecting the phenolic profile of products including enzymatic oxidative reactions occur (Cory et al., 2018).

Thus, the bioavailability and stability of phenolic compounds after consumption, has become one of the topics most addressed by the scientific community, in order to better understand the mechanisms of action of these compounds in the body (Bilal Hussain et al., 2019). The applicability of phenolic compounds as a functional ingredient in the food industry depends on research that proves its bioactive behavior in the body when consumed, as well as its stability, functionality and, consequently, bioavailability.

1.2.3. Extraction techniques

About extraction methods, these can be varied depending on the natural matrix and the final objective of the work. In addition, at industrial level this choice has a direct economic and environmental impact and therefore less costly extraction methods and extraction conditions are required using environmentally friendly solvents, while ensuring a higher yield extraction level and purity (Płotka-Wasylka et al., 2017). The techniques can be classified as conventional or non-conventional. Conventional techniques include the use, temperature, and agitation of organic solvents. New or non-conventional techniques are green or clean techniques due to lower energy usage and the use of organic solvents that are beneficial to the environment (Rodríguez-Pérez et al., 2015).

Maceration stands out as one of the most widely used techniques, as it is one of the simplest and most economical techniques. Additionally, this technique effectively promotes the extraction of active compounds using organic solvents or water/alcohols mixtures and can be performed with or without temperature and agitation (Albuquerque et al., 2017).

Currently, new methodologies have emerged to ensure shorter extraction times with lower solvent and potency to increase the extraction yield of compounds of interest. Supercritical Fluid, Microwave assisted extraction (MAE), Ultrasound assisted extraction (UAE), Enzyme Assisted Fluid, and others are some examples of these technologies (Ongkowijoyo et al., 2018). Regardless of the extraction procedure chosen, its optimization

is fundamental, since normally the optimization parameters focus on the solvent and the extraction time/temperature relationship. Aiming at a possible application at food level, it is essential to choose the solvent that should be applied in the extraction system and in these cases water, ethanol (considered non-toxic in the food industry and in clinical practice) or mixtures of both are the most appropriate solvents (Martín et al., 2017).

Among the advanced extraction techniques UAE and MAE are the most common. Compared to conventional extraction, UAE has a multitude of advantages such as high reproducibility in short time intervals, easy handling and reduced solvent and energy consumption. Regarding the mechanism of action, UAE is based on the acoustic cavitation process that causes swelling of cells and/or breakage of cell walls, thus facilitating solvent diffusion and consequently increasing extraction efficiency (Chemat et al., 2017). In turn, MAE is an effective extraction method that offers high extraction yields in a shorter time while requiring simpler manipulation, lower solvent consumption and lower energy input (Li et al., 2010). This technique acts through the use of microwave energy, causing molecular motion by ion conduction and dipole rotation which consequently induces the increase of temperature and pressure, affecting the cellular structure and stimulating the solvent penetration in the sample matrix (Angiolillo et al., 2014).

The technique and conditions of extraction depend on the type of matrix and the compounds to be extracted. In this way, optimization processes that allow the determination of optimal conditions is essential for obtaining convenient extraction rates (Ferarsa et al., 2018).

1.2.4. Application of phenolic compounds as natural preservatives and functional ingredients

Bioactive ingredients obtained from natural sources have been used all over the world since ancient times, due to the belief that they have beneficial properties for consumer's heath, being able to act in the prevention and improvement of certain diseases symptoms (Nedović et al., 2016). This has been arousing growing interest from the scientific community, but also from the food industry, which is looking for alternatives to the use of artificial additives and for new functional ingredients to meet consumer’s expectations. For this type of enrichment different natural sources of plant, animal or mycological origin can be used. Some examples of bioactive ingredients are vitamins, amino acids, fiber, fatty acids and phenolic compounds (Vieira da Silva et al., 2016).

Currently, natural compounds represent an important fraction in the composition of new drugs developed by the pharmaceutical industry. Some studies have found that the anticancer effects of bioactive plant ingredients on cell lines are even more efficient than some synthetic compounds. Additionally, the antitumor capacity of phenolic compounds has also been proven with both tumor cell lines, *in vivo* models and human tests (Carocho and Ferreira, 2013a). However, consumer's acceptance of a specific functional ingredient depends on the knowledge described about its beneficial effects. Thus, popularly known functional ingredients, such as minerals, vitamins, fiber and fatty acids, reach considerably higher levels of acceptance than the latest ones, such as phenolic compounds (Kanekanian, 2014). However, the disclosure of the beneficial effects that these compounds have on the prevention of various diseases may justify the increase in the consumption of foods rich in these compounds (Alirezalu et al., 2020).

The biological activities of phenolic compounds in the human body are mainly related to their ability to exert antioxidant actions. The main antioxidant activity of the polyphenols is to enhance the cellular detoxification systems, such as superoxide dismutase systems, catalase or glutathione peroxidase, and inhibiting the generating enzymes ROS, for example xanthine oxidase and NADPH oxidase (nicotinamide adenine dinucleotide phosphate oxidase) (Martins et al., 2016). However, recent studies suggest that the mechanisms by which phenolic compounds exert their protective action against cardiovascular disease and cancer are not simply due to their redox properties, but due to their ability to bind directly to proteins (Lutz et al., 2019). This may justify the growing demand for such compounds not only from the food industry but also from the pharmaceutical and cosmetic industries. The scientific community has been able to prove the beneficial health effects of these molecules, which the food and pharmaceutical industries have been disputing, making possible the production of some new products. Medicinal and aromatic plants play an important role in this area due to the presence of high amounts of these compounds. Currently, some functional foods that capture consumer interest can be found commercially (Caleja et al., 2017). In addition, there are several examples of studies that have revealed the potential of incorporating phenolic extracts into food matrices and the following mentions same examples. Pomegranate phenolic extracts were incorporated by (Robert et al., 2010) and (Pillai et al., 2012) in yogurt and pasta, respectively, to improve antioxidant activity. (Ezhilarasi et al., 2013) and (Pasrija et al., 2015) incorporated phenolic extracts of garcinia and green tea, respectively, into bread, resulting in a new product with higher antioxidant

activity. (Chouchouli et al., 2013) and (Martins et al., 2014) used grape seeds and mulberry flowers, respectively, to add value and antioxidant activity to functional yogurts. More recently, fennel and chamomile extracts were also incorporated into yogurts and showed superior results in comparison to yoghurt without additives and yoghurt with an artificial additive (potassium sorbate - E202) (Caleja et al., 2016).

At the industrial level it is also possible to find some products successfully launched in the market, namely Compal (Sumol + Compal, SA) has developed products defined as functional and marketed as nectar, such as Compal Vital AntiOx®, ensuring antioxidant properties due to the presence of a selected range of red fruits (Sumol + Compal, 2020).

Although the development of functional and nutraceutical foods is considered a rapid process, these products often require solid scientific evidence to be introduced in the market. Clinical studies are often very expensive and therefore not accessible to most companies dealing with the manufacture of this type of food product, which cannot afford to perform all tests (Wang and Li, 2014). Phenolic compounds obtained from natural sources are known to exhibit various bioactivities and thus their introduction into food matrices will provide greater added value to the product (Caleja et al., 2017). However, these compounds are associated with various reactions/instabilities when introduced into food as a result of processing (e.g. manufacturing temperatures), long storage periods in contact with the food matrix, among other factors (Dias et al., 2015). Thus, advances in technology have to be introduced in the food industry, enabling innovation in the development of new food products (Paini et al., 2015).

1.3. Chestnut flower as a source of bioactive compounds

Chestnuts are large trees that grown in different regions of the world. Its fruits are consumed and appreciated worldwide, its leaves are used in the preparation of infusions and its wood used in the production of furniture (**Figure 2**). China is the main producer, accounting for 83% of fruit production (FAOSTAT statistical database, 2019). In turn, Portugal is the third largest producer in Europe, accounting for an average production of 30,000 tons per year (INE, 2018). The production in Portugal is mainly in the Trás-os-Montes region, where the fruits are almost all exported representing a strong economic impact and hence called the "gold" of the region. The fruits of the chestnut tree have been eaten since ancient times, however, in ancient times they were only seasonal fruits. These fruits can now be found in the supermarket all year round and are found in frozen, paste and

flour form. In gourmet cuisine these fruits are used in the ornamentation of exquisite dishes. Some industries, notably bakery and pastry, have been exploiting these fruits as an alternative source of starch in the development of new products suitable for the celiac population (Corregidor et al., 2020). These fruits are recognized for being sources of carbohydrates, fiber, fatty acids, proteins, vitamins and minerals, among other compounds (de Vasconcelos et al., 2010).

Figure 2. Image of *Castanea sativa* Mill.

(Available from: https://www.researchgate.net/figure/Figura-6-Castanea-sativa-Mill-Koehler-1887_fig5_27727799, accessed 10 May, 2020)

Despite the great economic importance of the fruits, there are several elements that result from trees such as wood, flowers and leaves, but also other byproducts resulting from the industrial processing of nuts such as films and bark. During the processing of chestnut fruits, between 8.9 and 13.5% of outer shells and 6.3-10.1% of inner shells are generated (de Vasconcelos et al., 2010).

Table 3. Main components present in each of the elements of *Castanea sativa* Mill.

Main components	**Elements of *Castanea sativa* Mill**
Carbohydrates Proteins Fatty acids Polyphenols Minerals	**Fruits** 

Organic acids	
Carbohydrates Lignins Polyphenols	**Shell** 
Polyphenols Carbohydrates	**Peel** 
Polyphenols Carbohydrates	**Leafs** 
Sugars Polyphenols Tannins Tocopherols Fatty acids	**Flowers** 
Glucans Lignins Xylans Arbinians Proteins Polyphenols	**Hedgehog** 

Based on (Echegaray et al., 2018).

Currently, large quantities of this material are wasted and have potential for use in various industries such as food, pharmaceutical and cosmetic. They can be valued in the food industry by extracting high added value compounds as natural antioxidant compounds, considering their chemical composition, availability, renewable character and low cost. **Table 3** presents the main components present in the composition of different products resulting from chestnut. Recent research has shown that male chestnut flowers possess a high abundance of phenolic compounds that can be used in the preservation of foods due to their antioxidant and antimicrobial activity (Caleja et al., 2019; Carocho et al., 2014b) and therefore can be used as a natural ingredient as well as for enhancing the health of consumers – functional capacity (Carocho et al., 2015b). These properties as well as the medicinal effects referred to above have been related to their composition in phenolic compounds (Carocho et al., 2014b).

In order to explore the potential of chestnut flowers as a natural ingredient, there are several studies that have been carried out in which chestnut flowers have been incorporated

into different Portuguese products, namely the “Serra da Estrela” cheese, dry cakes “Económicos” and “Pastel de nata” (Caleja et al., 2020; Carocho et al., 2016, 2015a). The results confirm the potential of this natural matrix as a preservative and functionalizing ingredient in the development of new food products that meet new consumer expectations.

II. Objectives

The aim of this work was to optimize the process of extracting phenolic compounds from male chestnut flowers for futher application as a natural ingredient in the food industry as illustrated visually in **Figure 3**.

The specific objectives achieved of this work were:

- Identification and quantification of phenolic compounds present in male chestnut flowers;
- Optimization of ultrasound assisted extraction (UAE) leading to higher yield of a natural preservative rich in phenolic compounds.

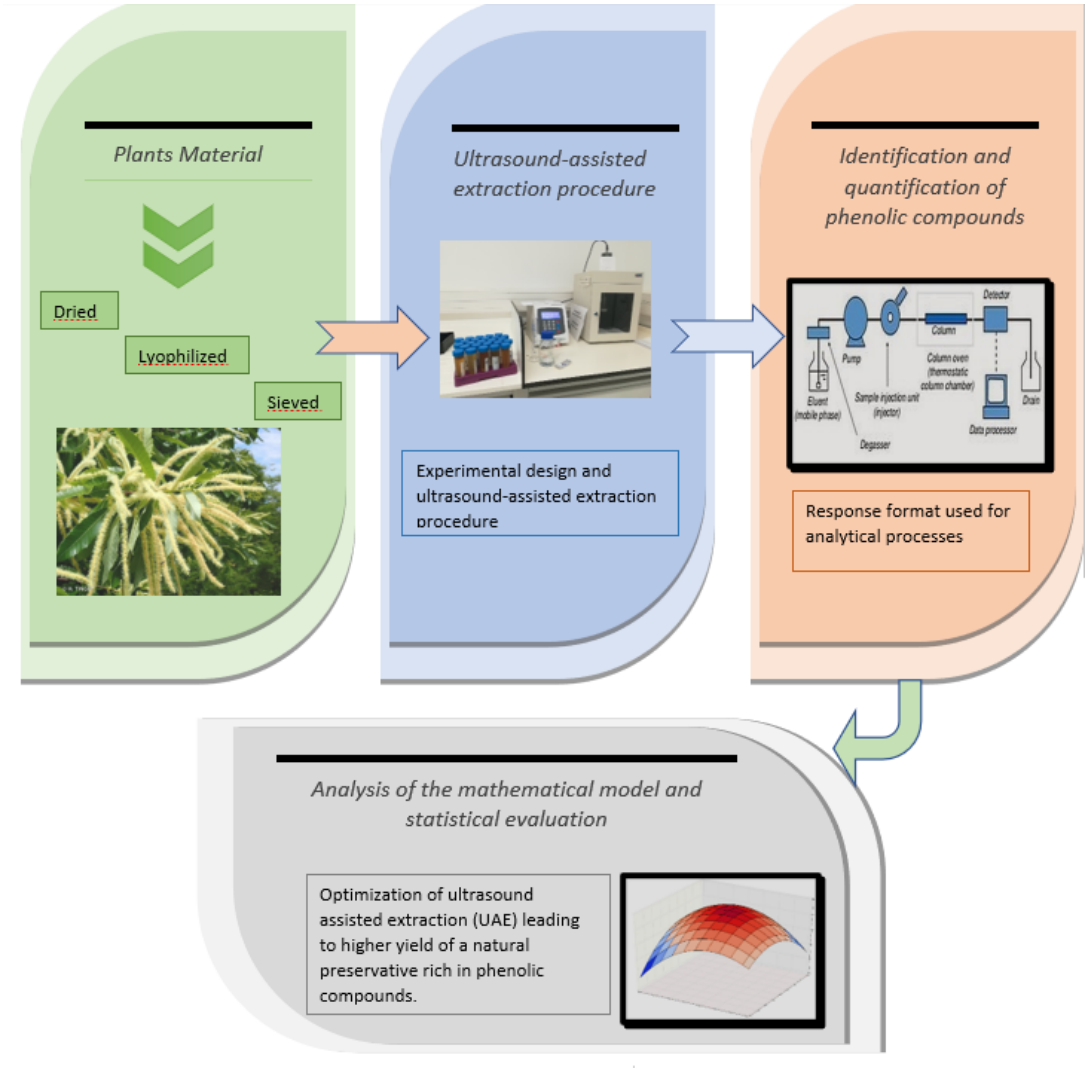


Figure 3. Explanatory scheme of the work.

III. Materials and Methods

3.1. Standards and reagents

HPLC-grade acetonitrile was obtained from Merck (Darmstadt, Germany). Phenolic standards were bought from Extrasynthèse (Genay, France). All other reagents were purchased from specialized retailers. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

3.2. Plants material

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 flowers were lyophilized, crushed and reduced to a fine, homogeneous powder

3.3. Optimization of the extraction process to obtain an extract rich in phenolic compounds, from male flowers of *Castanea sativa* Mill.

In order to optimize the extraction of phenolic compounds from male chestnut flowers, ultrasound-assisted extraction was used and an experimental design called the circumscribed central composite design (*CCCD*) was developed, applying the surface methodology of response (RSM), in order to simplify and reduce the operational costs of the process, decrease the extraction process times and, reduce the energy spent as well as the solvent consumption. Using the RSM methodology it is possible to optimize several factors (time, percentage of solvent and applied power) simultaneously, obtaining graphic models and polynomial equations that allow to describe the ideal conditions that maximize the response criteria (Roriz et al., 2017b).

Tests were performed based on individual analysis of the variables and those that caused significant effects were selected along with the relevant intervals. The effects of the three defined variables were studied using a *CCCD*, associated with five levels (Heleno et al., 2016), which generated 28 combinations of responses, performed in order to obtain a greater predictive capacity of the model (**Table 4**).

Table 4. Experimental domain and codification of independent variables in the *CCCD* factorial design with 5 range levels.

CODED VALUES	NATURAL VALUES		
	t (min)	*P (W)*	*S (%)*
-1.68	1.0	50.0	0.0
-1	8.7	141.2	20.3
0	20.0	275.0	50.0
+1	31.3	408.8	79.7
+1.68	39.9	500.0	100.0

3.4. Ultrasound-assisted extraction procedure

The extraction procedure was performed as previously described by (Jiménez et al., 2018), using ultrasound equipment (QSonica sonicators, model CL-334, Newtown, Connecticut, EE.UU). 1.5 g of samples were extracted with 50 ml of solvent. In this case, the variables and their intervals were: time (*t*, 1 to 39 min), UAE power (*P*, 50 to 500 W) and ethanol/water extraction solvent (*S*, 0 to 100%), while the temperature was controlled by the equipment to remain below 30ºC.

3.5. Preparation of extracts obtained by ultrasound-assisted extraction

After the procedure of each extraction mentioned above, the samples were centrifuged (5000 rpm; for 20 min at 10°C) and, to remove suspended solids, they were filtered through filter paper (Whatman nº 4). The supernatant was collected and divided into two fractions: one for HPLC-DAD analysis and the second for determining the extraction yield. The fraction separated for HPLC analysis (2 mL) was filtered through an LC syringe filter (0.22 µm) and then injected; the second fraction, used to determine the extraction yield (5 mL) was subjected to drying at a temperature of 105ºC for 48 hours, for subsequent weighing of the solid extract.

3.6. Identification and quantification of phenolic compounds by HPLC-DAD

The extracts obtained with were dissolved in 20% aqueous ethanol at 5 mg/mL and filtered through a 0.22-µm disposable LC filter disk. Chromatographic analysis were performed in a Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA) system equipped with a diode array detector coupled to a electrospray ionization mass

detector (LC-DAD-ESI/MS), a quaternary pump, an auto-sampler (kept at 5ºC), a degasser and an automated thermostat column compartment. Chromatographic separation was achieved with a Waters Spherisorb S3 ODS-2 C18 (3 μm, 4.6 mm × 150 mm, Waters, Milford, MA, USA) column working 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. 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 (Thermo Finnigan, 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 (Thermo Finnigan, San Jose, CA, USA).

The relevant compounds identified in the flowers of *C. sativa* Mill. by HPLC-DAD-ESI/MS were the hydrolysable tannins of pentagalloyl-glucoside (T1) and trigalloyl-HHDP-glucoside (T2), and flavonoids like Myricetin-3-*O*-glucoside (F1), Quercetin-3-*O*-glucuronide (F2), Quercetin-3-*O*-glucuronide (F3), Quercetin-3-*O*-glucoside (F4) and Kaempferol-3-*O*-rutinoside (F5) (Caleja et al., 2020). Considering that important biological activities such as antioxidant and antimicrobial are derived from those compounds, the extracts rich on then could be of interest to the industry sector.

Phenolic compounds were identified by comparing their retention times, UV-vis and mass spectra with those obtained from standard compounds. For quantitative analysis, a calibration curve was constructed based on the UV signal. The results were expressed as mg of compound detected per g of extracted residue (mg/g R).

3.7. Analysis of the mathematical model and statistical evaluation

3.7.1. Mathematical model

The RSM data were fitted by means of least-squares calculation using the following third-order polynomial equation with complex interactive terms of Eq. (1):

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{iijj} X_i^2 X_j^2 \sum_{i=1}^{n} b_{iii} X_i^3 \quad (1)$$

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 that describes linear individual effect of each variable, b_{ij} is the coefficient responsible for describing the linear interactive mechanisms between two variables, b_{ii} the coefficient responsible of quadratic effect of each variable, b_{iijj} is the coefficient responsible for describing the quadratic interactive mechanisms between two variables, b_{iii} the coefficient responsible of cubic effect of each variable, and *n* is the number of variables.

The response (*Y*) results used to optimize phenolic composition of the extracts were the residue (*Yield*, g R/g DW), flavonoids content (individual analysis of F1-5 and total of TF, mg/g R) and hydrolysable tannins content (individual analysis of T1-5 and total of TT, mg/g R) according to the *CCCD* shown in **Table 4**.

3.7.2. Procedure for optimization of variables

In order to optimize the forecasting model and, consequently, maximize the extraction yield, a *simplex* method was used, which allows solving non-linear problems (Vieira et al., 2017). To avoid variables with unnatural and unrealistic physical conditions, some limitations were imposed on the coded variables (namely $t \geq 0$; $0 \leq S \leq 100$).

3.7.3. Numerical methods, statistical analysis and graphic illustrations

The adjustment procedures, coefficient estimates and statistical calculations of the experimental results were performed according to a procedure previously described by (Prieto et al., 2014). 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) was used to assess 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 (Murado and Prieto, 2013); and iii) the R^2 was interpreted as the proportion of variability of the dependent variable explained by the model.

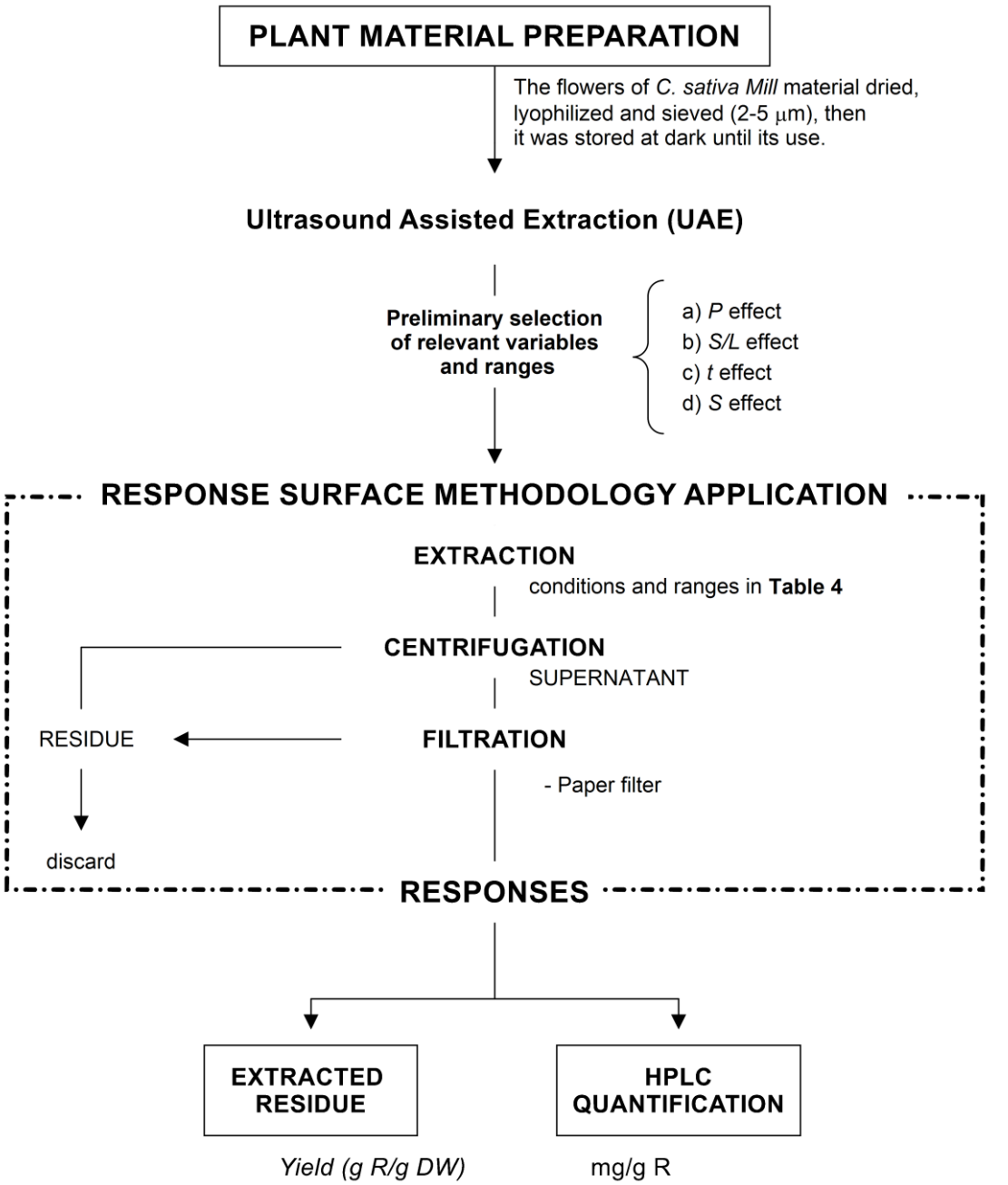
IV. Results and Discussion

4.1. Theoretical response surface models of the used response criteria for the RSM analysis and statistical verification

Although some previous studies on the extraction of phenolic compounds from the flowers of *C. sativa* Mill. can be found in literature, no reports detailing the optimal conditions maximizing their extraction are presently available. In addition, the compositional diversity of phenolic compounds in natural sources (e.g., fruits, flowers, leaves, stems and roots) does not allow to directly extrapolate the extraction conditions of these compounds from previously studied sources. Therefore, it is important to conduct independent studies to maximize the extraction of phenolic compounds from *C. sativa* Mill., by selecting the relevant variables for each selected extraction method.

The description of the compounds presents in flowers of *C. sativa* Mill. and other major sources, as well as the conditions used for their extraction have already been described (Pinela et al., 2016a; Caleja et al., 2020). Although important conclusions can be derived from this summary, the results may be highly dependent on dissimilarities not foreseen in these studies, where certain variables remaining constant, together with raw-material's variability, can definitely influence the extraction process. Therefore, the first approach to optimize the efficiency of the UAE process to recover phenolic compounds the flowers of *C. sativa* Mill. consisted of the application of RSM coupled to a CCCD design with five levels of variation for the three independent variables of *t* (1-39 min), *P* (50-500 W) and *S* (0-100 %). A detailed description of the coded and natural values of the selected variables for each extraction method in the CCCD design is presented in **Table 4**.

Response surface methodology (RSM) is a useful tool to evaluate the effects of multiple variables and their interactions on one or more responses such as the extraction of phenolic compounds. The *CCCD* is a popular form of RSM and has been applied by a number of researchers in the optimization of various food processing methods (Bezerra et al., 2008). **Figure 4** shows a comprehensive summary of the different steps carried out in the optimization recovery of phenolic compounds from the flowers of *C. sativa* Mill.



Hydrolysable tannin	Pentagalloyl-glucoside	T1
	Trigalloyl-HHDP-glucoside	T2
Flavonoid	Myricetin-3-*O*-glucoside	F1
	Quercetin-3-*O*-glucuronide	F2
	Quercetin-3-*O*-glucuronide	F3
	Quercetin-3-*O*-glucoside	F4
	Kaempferol-3-*O*-rutinoside	F5

Figure 4. Diagram of the different steps carried out for optimizing the conditions that maximize the extraction responses.

In a previous study, relevant compounds were identified in the flowers of *C. sativa* Mill. by HPLC-DAD-ESI/MS (Caleja et al., 2020; Dias et al., 2015; Echegaray et al., 2018; Pinela et al., 2016a; Yamagata et al., 2015). In this regard, can be noted hydrolysable tannins such as pentagalloyl-glucoside (T1) and trigalloyl-HHDP-glucoside (T2), and flavonoids like Myricetin-3-*O*-glucoside (F1), Quercetin-3-*O*-glucuronide (F2), Quercetin-3-*O*-glucuronide (F3), Quercetin-3-*O*-glucoside (F4) and Kaempferol-3-*O*-rutinoside (F5). Considering that important biological activities such as antioxidant and antimicrobial are derived from those compounds, the extracts rich on then could be of interest to the industry sector.

The response results used to optimize phenolic composition of the extracts were the residue (*Yield*, g R/g DW), flavonoids (individual analysis of F1-5 and total of TF, mg/g R) and hydrolysable tannins content (individual analysis of T1-5 and total of TT, mg/g R) according to the *CCCD* shown in **Table 4**.

The results of the experimental design for the extraction of phenolic compounds from chestnut flowers as a function of the three main variables involved in the UAE are displayed in **Table 5**. The experimental values obtained under the 28 runs of the five-level CCCD design applied used in the recovery of phenolic compounds from the flowers of *C. sativa* Mill. are of interest for industrial sectors dealing with the recovering of high added-value compounds from plant materials to be used as natural antioxidants, or other bio-based ingredients, providing information concerning the amount of plant material needed to obtain a certain quantity of the target compounds, and the concentration of these compounds in the produced extracts.

Once responses are produced, the next step is to fit the response values (**Table 5**) to the third-order polynomial model of Eq. (1) using a nonlinear algorithm. By performing these analytical solutions, researchers can translate the response patterns by mathematical models, simplifying complexity of the possible scenarios.

Table 5. Experimental design for the extraction of phenolic compounds from chestnut flowers. Experimental RSM results of the *CCCD* for the optimization of the three main variables involved (X_1, X_2, and X_3) in the UAE for the residue (*Yield*, g R/g DW), flavonoid (individual analysis of F1-5 and total of TF, mg/g R) and tannin content (individual analysis of T1-5 and total of TT, mg/g R).

	EXPERIMENTAL DESIGN						RESIDUE	FLAVONOID CONTENT						TANNIN CONTENT		
	CODED VALUES			NATURAL VALUES				INDIVIDUAL					TOTAL	INDIVIDUAL		TOTAL
	X_1	X_2	X_3	X_1: *t* min	X_2: *P* W	X_3: *S* %	*Yield* g R/g DW	*F1* mg/g R	*F2* mg/g R	*F3* mg/g R	*F4* mg/g R	*F5* mg/g R	*Ft* mg/g R	*T1* mg/g R	*T2* mg/g R	*Tt* mg/g R
1	-1	-1	-1	8.7	141.2	20.3	0.10	0.15	0.06	0.14	0.13	0.21	0.69	3.67	9.67	13.34
2	-1	-1	1	31.3	141.2	20.3	0.10	0.09	0.12	0.14	0.12	0.22	0.68	3.10	3.73	6.83
3	-1	1	-1	8.7	408.8	20.3	0.12	0.11	0.06	0.17	0.15	0.28	0.78	5.82	9.00	14.82
4	-1	1	1	31.3	408.8	20.3	0.12	0.13	0.14	0.16	0.15	0.23	0.82	3.00	4.41	7.41
5	1	-1	-1	8.7	141.2	79.7	0.11	0.11	0.06	0.16	0.11	0.18	0.63	4.49	11.75	16.24
6	1	-1	1	31.3	141.2	79.7	0.10	0.10	0.15	0.19	0.13	0.22	0.79	4.00	10.06	14.06
7	1	1	-1	8.7	408.8	79.7	0.17	0.13	0.06	0.12	0.11	0.16	0.59	4.00	13.57	17.57
8	1	1	1	31.3	408.8	79.7	0.16	0.11	0.13	0.16	0.15	0.19	0.74	3.75	11.57	15.32
9	-1.68	0	0	20	275	100	0.11	0.12	0.07	0.14	0.12	0.17	0.63	4.24	9.43	13.67
10	1.68	0	0	20	275	0	0.10	0.07	0.07	0.07	0.10	0.10	0.42	1.70	4.74	6.44
11	0	-1.68	0	20	50	50	0.11	0.18	0.09	0.23	0.16	0.21	0.88	4.94	11.82	16.76
12	0	1.68	0	20	500	50	0.16	0.25	0.12	0.24	0.22	0.26	1.08	5.42	18.73	24.15
13	0	0	-1.68	1	275	50	0.10	0.13	0.04	0.22	0.15	0.24	0.79	5.07	10.85	15.92
14	0	0	1.68	39	275	50	0.15	0.20	0.10	0.22	0.20	0.36	1.08	5.64	16.13	21.77
15	-1.68	-1.68	-1.68	1	50	0	0.04	0.03	0.03	0.04	0.04	0.02	0.16	0.95	2.46	3.41
16	-1.68	-1.68	1.68	39	50	0	0.06	0.05	0.04	0.05	0.06	0.06	0.26	1.14	3.09	4.23
17	-1.68	1.68	-1.68	1	500	0	0.04	0.04	0.03	0.04	0.04	0.05	0.21	1.00	2.53	3.53
18	-1.68	1.68	1.68	39	500	0	0.13	0.11	0.09	0.11	0.12	0.14	0.57	1.14	4.74	5.88
19	1.68	-1.68	-1.68	1	50	100	0.08	0.12	0.06	0.14	0.11	0.17	0.60	5.22	9.65	14.87
20	1.68	-1.68	1.68	39	50	100	0.11	0.15	0.09	0.19	0.15	0.24	0.82	5.58	13.42	19.00
21	1.68	1.68	-1.68	1	500	100	0.08	0.12	0.04	0.12	0.10	0.15	0.52	4.26	10.50	14.76
22	1.68	1.68	1.68	39	500	100	0.13	0.07	0.04	0.08	0.07	0.01	0.27	0.97	7.05	8.02
23	0	0	0	20	275	50	0.14	0.20	0.08	0.22	0.24	0.35	1.09	5.46	15.56	21.02
24	0	0	0	20	275	50	0.15	0.20	0.10	0.23	0.22	0.36	1.10	4.25	16.80	21.05
25	0	0	0	20	275	50	0.12	0.25	0.10	0.25	0.22	0.35	1.16	5.08	16.35	21.43
26	0	0	0	20	275	50	0.14	0.21	0.10	0.25	0.23	0.30	1.11	4.34	15.46	19.81
27	0	0	0	20	275	50	0.14	0.25	0.09	0.23	0.22	0.25	1.03	4.68	16.10	20.78
28	0	0	0	20	275	50	0.14	0.26	0.14	0.23	0.24	0.28	1.15	4.96	15.47	20.43

The parametric values of the second-order polynomial model of Eq. (1) obtained after fitting the extraction response format values and the corresponding statistical information (α = 0.05) are presented in **Table 6**. The fitting procedure of Eq. (1) applied to the experimental responses was performed using nonlinear least-squares estimations and those that were non-significant (*ns*) values were excluded. These values translate the response patterns and are useful for developing mathematical models, which indicate the complexity of the possible scenarios. The statistic lack-of-fit, used to test the adequacy of the obtained models, revealed that no considerable improvement was achieved by the inclusion of the statistically *ns* parametric values. The agreement between the experimental and predicted values provided an acceptable explanation of the obtained results (**Table 5**). Additionally, residues were randomly scattered around zero and no grouped data or autocorrelations were observed. The obtained coefficients of determination (R^2) were higher than 0.81 in all cases (**Table 6**), which indicates that the variability of each response can be explained by the independent variables involved in the process. Therefore, the models proved to be applicable and were used in the later prediction and optimization steps. Although the obtained model coefficients are empirical and cannot be associated with physical or chemical significance, they are useful to predict the outcome of untested experimental conditions (Ranic et al., 2014). Moreover, the sign of the parametric values determines part of the response; for positive effects, the response is higher at the high level, and when a factor has a negative effect, the response is lower at the high level. The higher of the parametric value, the more significant the weight of the governing variable is.

Certain features regarding the overall effects of the independent variables were inferred from the complexity of the parametric values, *i.e.* the variables were ordered in a decreasing form as a function of its significance in the extraction processes as $S>P>t$. It was also possible to observe that all the evaluated responses were significantly affected by linear and quadratic effects, whose values were particularly higher for the variable *S*. The parametric values also revealed that strong interactions occurred in the UAE process, mainly between the variables $t\times P$. In turn, the interactions $P\times S$ were of minor relevance. These results justify the use of RSM as an optimization tool, since one-variable-at-a-time approaches do not allow to assess the existence of interactive effects, which makes it difficult to determine optimum values. To make all these combined effects more explicit and to visually describe the extraction trends, the results were presented in the response surface graphs discussed below.

Table 6. Parametric results of the third-order polynomial equation of Eq. (1) in terms of the extraction behaviour for the residue (*Yield*, g R/g DW), flavonoid (individual analysis of F1-5 and total of TF, mg/g R) and tannin content (individual analysis of T1-5 and total of TT, mg/g R), according to the CCCD with 5 range levels (**Table 4**). The parametric subscript 1, 2, and 3 stands for the variables involved *t* (X_1), *P* (X_2), and *S* (X_3), respectively. Analysis of significance of the parameters ($\alpha = 0.05$) are presented in coded values. Additionally, the statistical information of the fitting procedure to the model is presented.

PARAMETERS		RESIDUE	FLAVONOID CONTENT						TANNIN CONTENT		
			INDIVIDUAL					TOTAL	INDIVIDUAL		TOTAL
		Yield	*F1*	*F2*	*F3*	*F4*	*F5*	*TF*	*T1*	*T2*	*TT*
Intercept	b_0	0.140±0.004	0.224±0.011	0.100±0.006	0.233±0.010	0.227±0.012	0.289±0.016	1.106±0.036	4.923±0.273	15.786±1.306	20.312±0.861
Linear Effect	b_1	0.010±0.002	-0.017±0.017	*ns*	*ns*	-0.016±0.016	-0.045±0.032	-0.083±0.049	*ns*	*2*.061±0.531	2.744±0.452
	b_2	0.026±0.007	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
	b_3	-0.011±0.007	-0.018±0.017	0.037±0.012	0.006±0.004	*ns*	*0*.009±0.009	0.043±0.015	-0.207±0.164	*ns*	-3.754±1.515
Quadratic Effect	b_{11}	-0.014±0.003	-0.045±0.009	-0.006±0.006	-0.051±0.007	-0.046±0.008	-0.064±0.009	-0.206±0.026	-0.827±0.154	-3.077±0.864	-3.628±0.745
	b_{22}	*ns*	*ns*	*ns*	-0.005±0.007	-0.018±0.008	*ns*	-0.045±0.026	*ns*	*ns*	*ns*
	b_{33}	-0.005±0.003	-0.020±0.009	*ns*	-0.011±0.007	-0.024±0.008	*ns*	-0.061±0.026	*ns*	-0.812±0.864	*ns*
Cubic Effect	b_{111}	*ns*	0.012±0.007	*ns*	*0*.008±0.002	0.010±0.006	0.023±0.013	0.055±0.019	0.294±0.064	*ns*	*ns*
	b_{222}	-0.006±0.003	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
	b_{333}	0.009±0.003	0.009±0.007	-0.009±0.005	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	1.462±0.596
Interactive Linear Effect	b_{12}	*ns*	-0.005±0.004	-0.005±0.003	-0.010±0.003	-0.007±0.003	-0.016±0.007	-0.043±0.010	-0.260±0.116	*ns*	-0.498±0.321
	b_{13}	*ns*	-0.004±0.004	*ns*	*ns*	-0.003±0.003	*ns*	-0.015±0.010	*ns*	*ns*	*ns*
	b_{23}	0.003±0.002	*ns*	*ns*	*ns*	*ns*	-0.007±0.001	*ns*	-0.173±0.116	*ns*	-0.380±0.321
	b_{123}	-0.002±0.001	-0.004±0.002	-0.002±0.002	-0.004±0.002	-0.003±0.002	-0.006±0.004	-0.018±0.006	-0.077±0.072	*ns*	-0.307±0.198
Interactive Quadratic Effect	b_{1122}	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	-4.273±0.864	-5.336±0.952
	b_{1133}	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	-0.135±0.071	*ns*	*ns*	*ns*
	b_{2233}	*ns*	-0.069±0.023	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
	b_{112233}	*ns*	0.026±0.008	-0.001±0.001	0.002±0.001	0.005±0.002	*ns*	0.057±0.022	*ns*	1.594±0.176	1.849±0.657
Statistics (R^2)		0.9331	0.9066	0.8160	0.9408	0.9036	0.8473	0.9641	0.8305	0.8426	0.9206

4.2. Effect of the extraction variables on the target responses

The extraction results as function of the combination of the three main variables involved (X_{1-3}: *t*, *P*, and *S*) can be observed in **Figure 5** and **Figure 6**. In a more detailed form, **Figure 5** shows the 3D surface plots of the extraction of the flavonoid individual analysis content of F1-5 (mg/g R) and tannin individual analysis content of T1-5 (mg/g R).. Meanwhile, **Figure 6** shows the optimized the 3D surface plots of the extraction yield (g R/g DW), total flavonoid content of FT (mg/g R) and total tannin content of TT(mg/g R). The net surfaces were predicted with the third-order polynomial model of Eq. (1), whose model equations are presented in **Table 6**.

The binary actions between the variables are displayed when the excluded variable is positioned at the center of the experimental domain (**Table 4**). The individual phenolic content in **Figure 5** and the global analysis in **Figure 6** are helpful to visualize the tendencies of each response and guide the selection of the most favourable conditions, considering simultaneously all responses.

Additionally, in each single response of **Figure 5** and **Figure 6**, the subsection **part B** illustrates the capability to predict the obtained results and the residual distribution as a function of each of the considered variables. In fact, the goodness of fit of the model is illustrated by the ability to simulate response changes between the observed and predicted data, and the residual distribution as a function of each variable.

Regarding statistical terms, the distribution of residues (**Figure 5** and **Figure 6**) presents, for the majority, more than 90% of reliability, showing good agreement between experimental and predictive values. This is also verified by the high values of R^2 (**Table 6**), indicating the percentage of variability explained by the model.

It is possible to perceive small differences in the behaviour of the extraction variables both when comparing the flavonoid *vs* the tannin content extraction (as individual or global values). However, in both extraction responses the *S* variable was the most significant variable in the levels of extracted compounds. The negative effect of quadratic terms of *S* can be observed in all plots. The ethanol concentration indicates a saddle point and the highest yield is observed at approximately 50% ethanol concentration. The negative impact of quadratic term of ethanol concentration can be explained by the fact that the addition of water to ethanol improves the extraction rate.

The negative effect of the interaction between *P* and *S* may suggest the further use of lower energy, in combination with higher *S*, which will prevent the degradation of the phenolic compounds. Inclined surfaces to the side of the higher investigated *P* are in accordance with the statement, that higher system energies could increase the solubility of target compounds, and consequently improve their liberation from the sample matrix by destroying the integrity of connective and structural tissues, as already described by other authors (Oludemi et al., 2018; Pinela et al., 2016b; Roriz et al., 2017a).

As discussed, the extraction of phenolic compounds from the flowers of *C. sativa* Mill. is differently affected by the extraction technique employed. Generally, higher yields are obtained using the non-conventional UAE method, which promotes the rupture of the plant tissue through cavitation forces and enhances the solvent entrance into the cells with consequent release of the intracellular compounds into the solvent, thus intensifying mass transfer phenomena (Antonio et al., 2016; Misra et al., 2017).

In order to determine the most cost-effective option, it would be interesting to perform a life-cycle cost analysis (LCCA), not only of the sole extraction process as an individual unit operation, but considering the entire supply chain, including the production and harvesting of the plant material, equipment investment, natural resources and energy consumption, and environment hazardous emissions (Marco et al., 2012).

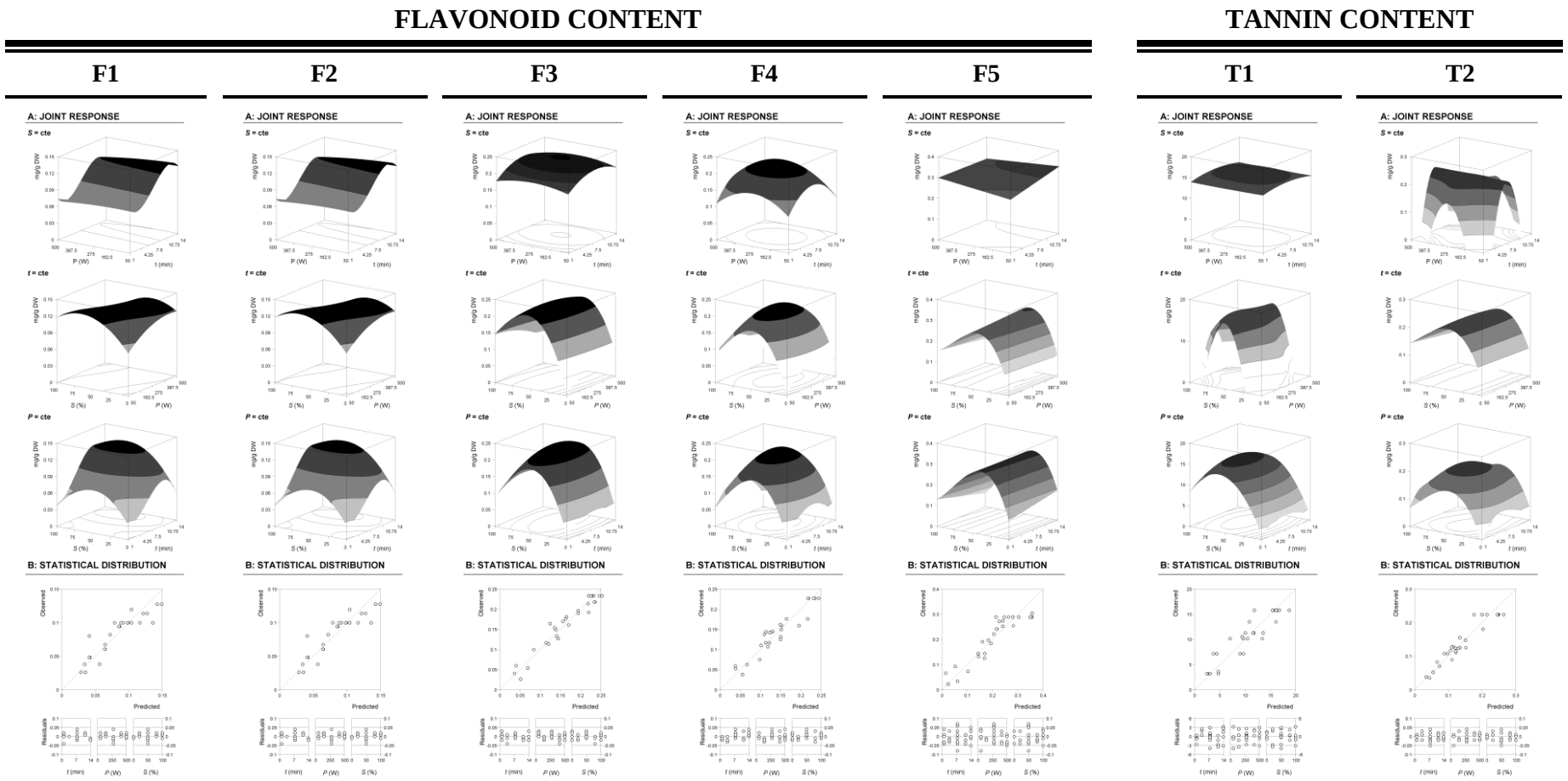


Figure 5. Shows the graphical results of phenolic compounds from chestnut flowers as a function of the three main variables involved (X_1, X_2, and X_3) for the flavonoid individual analysis content of F1-5 (mg/g R) and tannin individual analysis content of T1-5 (mg/g R). Each figure is divided in two parts. *Part A*: Shows the graphical analysis by net surfaces that represents the 3D 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 (**Table 7**). The statistical design and results are described in **Table 4**. *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.

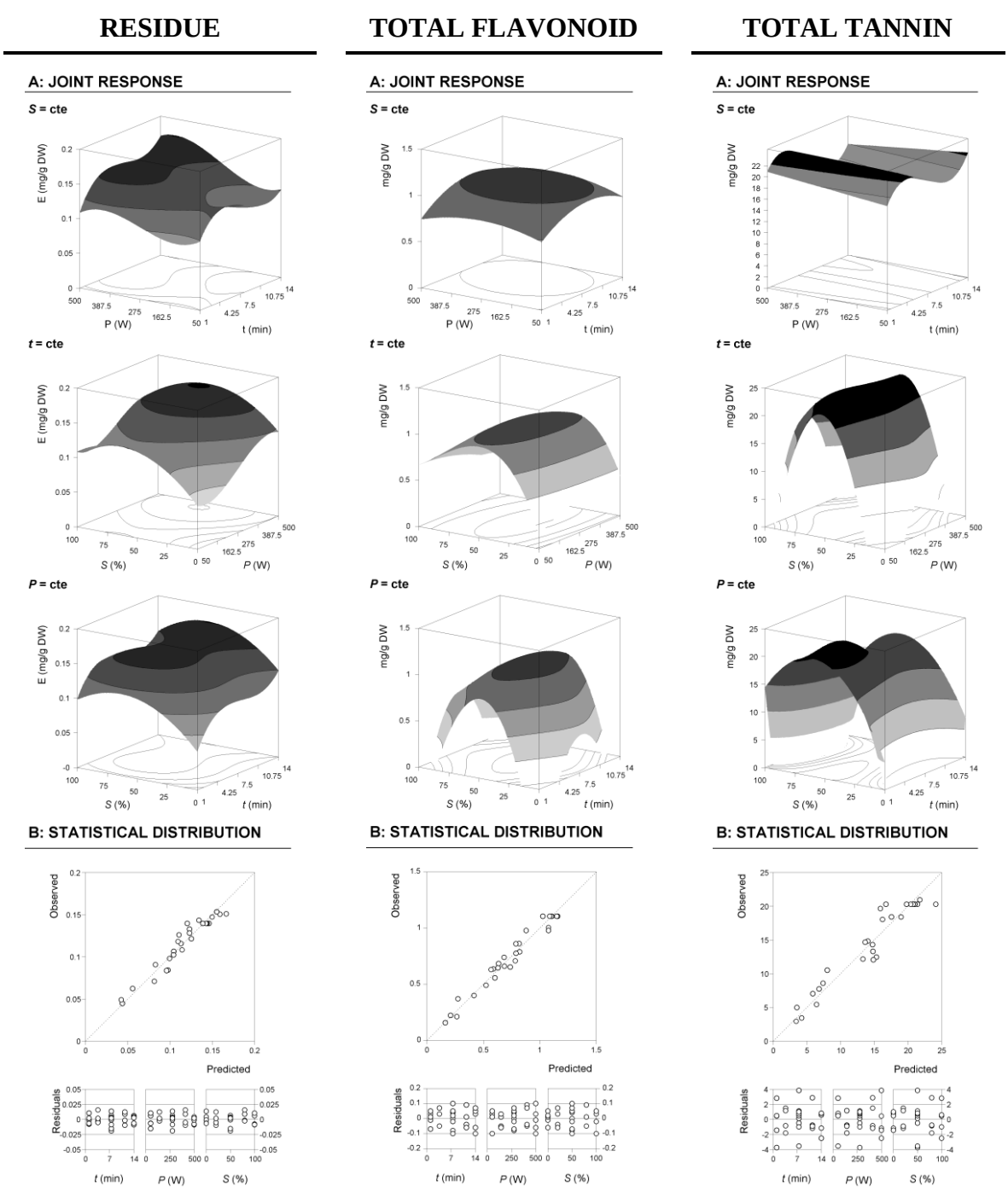


Figure 6. Shows the graphical results of phenolic compounds from chestnut flowers as a function of the three main variables involved (X_1, X_2, and X_3) for the yield (g R/g DW), total flavonoid content of FT (mg/g R) and total tannin content of TT(mg/g R). Each figure is divided in two parts. Each figure is divided in two parts. *Part A*: Shows the graphical analysis by net surfaces that represents the 3D 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 (**Table 7**). The statistical design and results are described in **Table 4**. *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.

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

The aim of this study was to maximize the extraction yield of total flavonoids and tannins from the male flowers of *C. sativa* by the application of UAE technique, within extraction parameters range. Based on the experimental results and statistical analysis, numerical optimizations have been conducted to establish the optimum level of the independent variables with desirable response levels. To verify the predictive mathematical model of the investigated process, the experimental confirmation was performed on the estimated optimal conditions. The predicted results matched well with the experimental results obtained at optimal extraction conditions, which were validated by the RSM model with good correlation.

Once the models are validated by statistical analysis (**Table 6**), it is possible to determine the absolute/relative optimal values of the variable conditions to maximize the responses individually and globally in order to obtain the most efficient extraction. **Table 7** shows the individual and global optimal values for each of the response values assessed the residue (*Yield*, g R/g DW), flavonoid (individual analysis of F1-5 and total of TF, mg/g R) and tannin content (individual analysis of T1-5 and total of TT, mg/g R).This table shows the individual and global optimal variable conditions for each group of compounds extraction and the respective amounts of the extracted compounds.

- For the individual response, optimal variable conditions of 39.00±3.74 min, 446.34±21.13 W and 58.08±7.62 % of ethanol were found to produce a maximum yield of the extracted residue of 0.18±0.04 g R/g DW.
- Regarding flavonoids, individual variable conditions were found for F1 at 17.64±2.59 min, 291.45±17.07 W and 43.28±6.58 % of ethanol, producing maximum response values of 0.23±0.13 mg F1/g R. For F2, variable conditions at 33.06±3.46 min, 50.00±7.07 W and 65.80±8.11 % of ethanol were found to produce a maximum response values of 0.13±0.04 mg F2/g R. In the case of F3, variable conditions were found at 23.37±2.94 min, 275.00±16.58 W and 50.00±7.07 % of ethanol, producing a maximum response values of 0.23±0.05 mg F3/g R. Variable conditions at 20.11±2.75 min, 279.01±16.70 W and 45.20±6.72 % of ethanol were found to produce a maximum response values of 0.23±0.05 mg F4/g R in the case of F4. Variable conditions were found for F5 at 9.31±1.96 min, 500.00±22.36 W and 46.12±6.79 % of ethanol for F5,

producing maximum response values of 0.30±0.05 mg F5/g R. At last, for total flavonoids, individual variable conditions at 23.92±2.97 min, 289.36±17.01 W and 44.18±6.65 % of ethanol were found to produce maximum response values of 1.12±0.08 mg FT/g R.

- In respect of tannins, individual variable conditions were found at 1.00±0.24 min. 500.00±22.36 W and 46.31±6.81 % of ethanol, producing maximum response values of 5.78±0.39 mg T1/g R for T1 whereas individual variable conditions at 20.00±2.74 min, 275.00±16.58 W and 59.96±7.74 % of ethanol were found to produce maximum response values of 16.13±0.90 mg in the case of T2/g R. Finally, for total tannins content, 8.52±1.89 min, 500.00±22.36 W and 52.71±7.26 % of ethanol were the individual variable conditions found to be able to produce a maximum response values of 23.35±0.47 mg TT/g R.
- Regarding the relative optimal response values for flavonoid content, the global optimal variable conditions were found at 26.32±3.07 min, 285.57±16.90 W and 44.80±6.69 % of ethanol, achieving maximum response values of 0.21±0.06 mg F1/g E, 0.12±0.04 mg F2/g R, 0.23±0.08 mg F3/g R, 0.22±0.07 mg F4/g R, 0.30±0.05 mg F5/g R, respectively from F1 to F5 compounds. In the case of tannins under optimal response values for flavonoid content, their maximum response values were 4.79±0.19 mg T1 /g R and 15.12±1.89 mg T2/g R, respectively for each tannin. Focusing on the yield of the extracted residue, it was lower than in the case of optimizing individual variables, but still reaches 0.13±0.07 g R/g DW.
- In respect of the relative optimal response values for tannin content, global optimal variable conditions at 11.44±2.03 min, 500.00±22.39 W and 51.22±7.16 % of ethanol were found to produce maximum response levels of 5.35±0.31 mg T1/g R and 15.23±0.90 mg T2/g R, respectively. In the case of flavonoids, responses values ranged from 0.07±0.01 mg F1/g R, 0.07±0.02 mg F2/g R, 0.20±0.05 mg F3/g R, 0.16±0.03 mg F4/g R and 0.29±0.04 mg F5/g R. Furthermore, the maximum yield obtained at these fixed conditions was 0.15 g R/g DW.
- At the end, the global optimal variable conditions for the optimization of both, flavonoids and tannins content and also yield, were found at 23.47±2.90 min, 258.78±16.09 W and 50.51±7.11 % of ethanol, thus producing the following maximum response values: 0.13±0.07 g R/ g DW, 0.22±0.07 mg F1/g R, 0.11±0.03

mg F2/g R, 0.23±0.08 mg F3/g R, 0.23±0.07 mg F4/g R, 0.29±0.04 mg F5/g R, 4.88±0.21 mg T1/g R and 15.77±0.97 mg T2/g R.

Table 7. Variable conditions in natural values that lead to optimal response values for RSM using a CCCD for each of the response values assessed the residue (*Yield*, g R/g DW), flavonoid (individual analysis of F1-5 and total of TF, mg/g R) and tannin content (individual analysis of T1-5 and total of TT, mg/g R).

		OPTIMAL VARIABLE CONDITIONS			OPTIMUM RESPONSE	
		X_1: *t* (min)	X_2: *P* (W)	X_3: *S* (%)		
A) INDIVIDUAL OPTIMAL RESPONSE VALUES						
Yield		39.00±3.74	446.34±21.13	58.08±7.62	0.18±0.04	g R/g DW
FLAVONOID CONTENT	F1	17.64±2.59	291.45±17.07	43.28±6.58	0.23±0.13	mg F1/g R
	F2	33.06±3.46	50.00±7.07	65.80±8.11	0.13±0.04	mg F2/g R
	F3	23.37±2.94	275.00±16.58	50.00±7.07	0.23±0.05	mg F3/g R
	F4	20.11±2.75	279.01±16.70	45.20±6.72	0.23±0.05	mg F4/g R
	F5	9.31±1.96	500.00±22.36	46.12±6.79	0.30±0.05	mg F5/g R
	FT	23.92±2.97	289.36±17.01	44.18±6.65	1.12±0.08	mg TF/g R
TANNIN CONTENT	T1	1.00±0.24	500.00±22.36	46.31±6.81	5.78±0.39	mg T1/g R
	T2	20.00±2.74	275.00±16.58	59.96±7.74	16.13±0.90	mg T2/g R
	TT	8.52±1.89	500.00±22.36	52.71±7.26	23.35±0.47	mg TT/g R
B) RELATIVE OPTIMAL RESPONSE VALUES FOR FLAVONOID CONTENT						
Yield		26.32±3.07	285.57±16.90	44.80±6.69	0.13±0.07	g R/g DW
FLAVONOID CONTENT	F1				0.21±0.06	mg F1/g R
	F2				0.12±0.04	mg F2/g R
	F3				0.23±0.08	mg F3/g R
	F4				0.22±0.07	mg F4/g R
	F5				0.30±0.05	mg F5/g R
TANNIN CONTENT	T1				4.79±0.19	mg T1/g R
	T2				15.12±1.89	mg T2/g R
C) RELATIVE OPTIMAL RESPONSE VALUES FOR TANNIN CONTENT						
Yield		11.44±2.03	500.00±22.39	51.22±7.16	0.15±0.09	g R/g DW
FLAVONOID CONTENT	F1				0.07±0.01	mg F1/g R
	F2				0.07±0.02	mg F2/g R
	F3				0.20±0.05	mg F3/g R
	F4				0.16±0.03	mg F4/g R
	F5				0.29±0.04	mg F5/g R
TANNIN CONTENT	T1				5.35±0.31	mg T1/g R
	T2				15.23±0.90	mg T2/g R
D) GLOBAL OPTIMAL RESPONSE VALUES						
Yield		23.47±2.90	258.78±16.09	50.51±7.11	0.13±0.07	g R/g DW
FLAVONOID CONTENT	F1				0.22±0.07	mg F1/g R
	F2				0.11±0.03	mg F2/g R
	F3				0.23±0.08	mg F3/g R
	F4				0.23±0.07	mg F4/g R
	F5				0.29±0.04	mg F5/g R
TANNIN CONTENT	T1				4.88±0.21	mg T1/g R
	T2				15.77±0.97	mg T2/g R

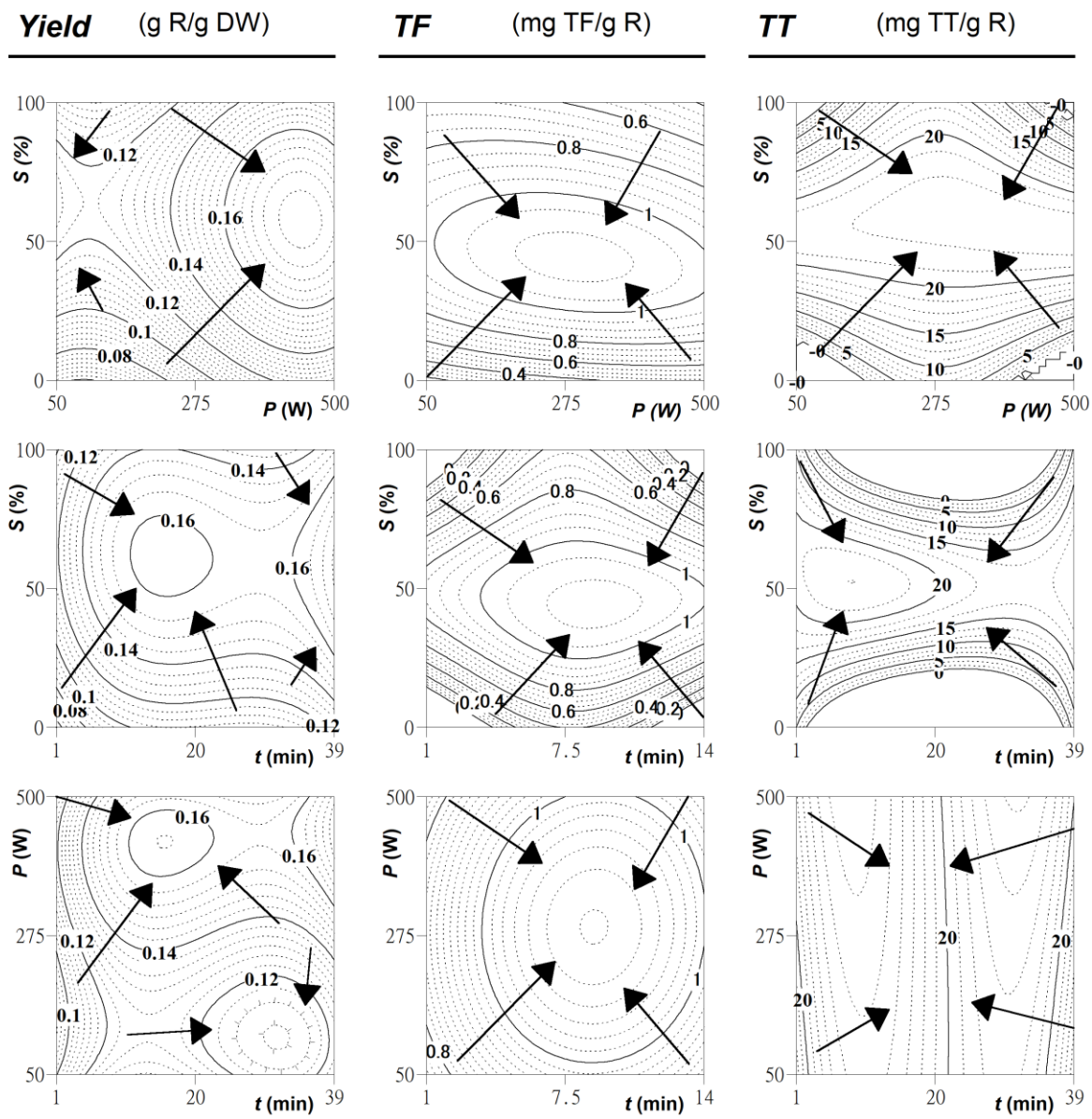


Figure 7. Shows the optimized isolines projections of the yield of extraction (g R/g DW) and the phenolic compounds of total flavonoid content of FT (mg/g R) and total tannin content of TT(mg/g R) from chestnut flowers as a function of the three main variables involved (X_1, X_2, and X_3). The figures describe visually the tendencies of each response and guide the selection of the most favourable conditions, considering 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 (**Table 7**). The statistical design and experimental results are described in **Table 5**. Estimated parametric values used are shown in **Table 6**.

Although the parametric values show the responses and can be used to understand the patterns of the responses, the best way to express the effects of any independent variable on the extraction of any type of response, is to generate 3D surface and/or contour plots, varying two variables in the experimental range under investigation and holding the other two variables at their fixed level. In this regard, **Figure 7** show the 3D surface and contour plots, respectively, representing the influence of the investigated effects of UAE parameters on the extraction behaviour of the yield of extraction (g R/g DW) and the phenolic compounds of total flavonoid content of FT (mg/g R) and total tannin content of TT(mg/g R) from chestnut flowers as a function of the three main DW variables involved (X_1, X_2, and X_3). The plots enable to visualize the influence and interaction between the variables. Visual analysis of 3D surface and contour plots are in accordance with parametric values derived from the multiple regression analysis described in **Table 6**.

Regarding individual conditions, the ideal solvent concentration ranged between 40-65% in all cases, energy applied varied in almost all scenarios from 250 to 500 W whereas time reached up to a maximum of 39 min. Considering both the individual and the global values for each group of compounds (flavonoids and tannins) and yield, the largest amounts of extracted total phenolic compounds were obtained with the global optimal conditions of 23.47±2.90 min, 258.78±16.09 W and 50.51±7.11 % of ethanol by the application of UAE technique. The central values of time, energy and solvent proposed by the experimental design were like the optimal obtained conditions values. The results here obtained are in accordance with similar conclusions previously found (Tomšik et al., 2016), in which UAE proved to consume less energy, time and provide higher extraction values while increasing the purity and, additionally, aiding to meet the requirements of a green extraction concept (Chemat et al., 2017; Montesano et al., 2008; Zhu et al., 2016).

UAE is a modern green alternative for plant-based chemistry applications that has shown to be an economically viable alternative to conventional techniques. The main benefits of applying this technique are the reduction of time, energy and solvents used, and consequently, industrial emissions (Chemat et al., 2017), which is the principal objective of sustainable ‘‘green” chemistry. The extraction process is completed in minutes with high reproducibility, simplifying manipulation and work, giving higher purity of the final product, and eliminating further treatment, contrarily to what occurs in conventional extraction methods such as Soxhlet or maceration extractions (Chemat et al., 2017).

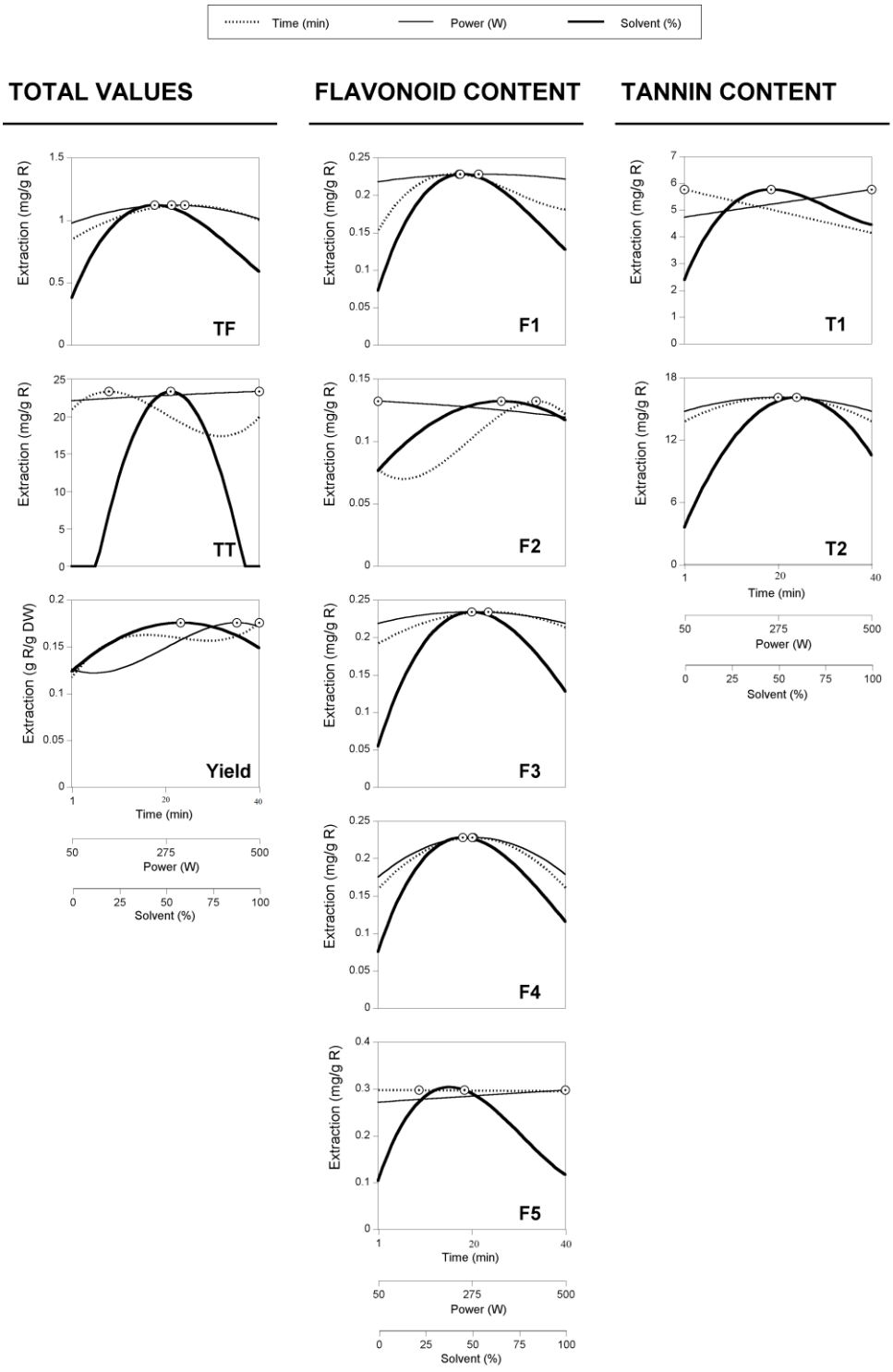


Figure 8. Final summary of the effects of all variables assessed. *Part A*: Shows the individual 2D responses of all studied responses as a function of all the variables assessed. The variables in each of the 2D graphs were positioned at the individual optimal values of the others (**Table 7**). The dots (⊙) presented alongside each line highlight the location of the optimum value. Lines and dots are generated by the theoretical second order polynomial (**Table 6**).

V. Concluding Remarks

The simplicity of using conventional methods (HAE or maceration) *versus* the advantages of new non-conventional technologies (microwave, ultrasound, cold pressing, squeezing, etc.) to recover compounds from plant materials and by-products, from an environmental and economical point of view, is in the topic-agenda of many industries (*e.g.*, in food, pharmaceutical, cosmeceutical, and nutraceutical areas). Nowadays, it may be difficult to find a production line that do not apply technological advances to increase profitability by decreasing energy costs, while reducing greenhouse gas emissions to meet legal requirements. Additionally, non-conventional technologies favour the safety of the processes and quality of the products, as well as functionality and product standardization. In this field, recent scientific findings provide opportunities for effective reuse of food outputs leading to the development of innovative products of high economic importance. The bottleneck that should be overcome is the technology transfer gap from researchers to stakeholders due to scale-up risks and long time required for commercialization of each new ingredient, due to safety precautions and market rules.

In addition, the scientific literature shows clear evidences that extraction procedures of target compounds from plant-based matrices must be assessed individually and avoid using conditions from similar scenarios. Therefore, a laborious and continues work needs to be performed, since almost all the agricultural and food industries are looking for products from pulp or flesh of fruits and vegetables to produce value-added products. Extracts from plant-based matrices are sources of primary and secondary metabolites as antioxidants, essential oils, proteins, fats, dietary fibres, colours, or even polysaccharides. The current food processing industry is aware of these extracts miscellaneous properties, such as their texturing, preservative, or colouring features, and their wide application as active compounds in cosmetics, food supplements, or pharmaceuticals. However, to take full advantage of the technological advances, the extraction conditions need to be optimized accurately, because otherwise, the efficiency and consequent profitability of the process may not be achieved. The use of mathematical models, such as RSM tools, can be an option to replace conventional extraction methods, providing optimal condition values to minimize or maximize the target responses.

Antioxidants such as the phenolic compounds within its massive diversity are one of the most important additives in terms of marketing since their presence is considered to

influence customer's perceptions, choices and preferences. Male flowers of *C. sativa* Mill. have been little explored and, to the best of the authors knowledge, the potential industrial use of their phenolic compounds has not been deeply investigated. In this paper, a new rapid method to extract phenolic compounds from flowers of *C. sativa* Mill. is proposed. RSM and other mathematical strategies were successfully employed to optimize the extraction conditions that maximize the phenolic compounds recovery to produce a rich extract with potential industrial application as natural antioxidant additive.

The efficiency of the UAE was higher than that obtained with HAE. The main phenolic compounds identified and quantified were Pentagalloyl-glucoside and Trigalloyl-HHDP-glucoside. Through the optimization of the extraction process, it was possible to reach total phenolic compounds at the global optimal conditions of 23.47±2.90 min, 258.78±16.09 W and 50.51±7.11 % of ethanol producing 0.13±0.07 g R/g DW, 0.22±0.07 mg F1/g R, 0.11±0.03 mg F2/g R, 0.23±0.08 mg F3/g R, 0.23±0.07 mg F4/g R, 0.29±0.04 mg F5/g R, 4.88±0.21 mg T1/g R and 15.77±0.97 mg T2/g R. In all cases, statistical validation was made by the high values of the adjusted coefficient of determination.

In conclusion, the present study contributed to the valorisation of the extracts produced from flowers of *C. sativa* Mill. as a source of valuable phenolic compounds with potential application as natural preservative. For that purpose, an optimized extraction method was designed using advanced and efficient extraction systems.

The present work presented a complete study on the optimal conditions that maximize the extraction of phenolic compounds from male flowers of *C. sativa* Mill. using a UAE technique considered green and of high interest for industrial application. It is considered that an evaluation of the bioactivities of the optimized extract would be relevant in a future perspective of the continuity of this study. The study of the antioxidant, antimicrobial, antitumor and toxicity potential would be of high interest for a possible food, cosmetic or pharmaceutical application. In addition, the evaluation of the potential of this optimized extract as a natural ingredient for the food industry could be tested on a food and the effect of this incorporation was evaluated over the shelf life.

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