Arbutus unedo Leaf Extracts as Potential Preservatives for Quark Cheese

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Abstract

The plant kingdom is an endless source of molecules that can be applied in almost all realms of society. The food industry has profited from the use of plants and their derived materials for many decades. Recently, the food industry has been looking into plants to find different ways of either preserving, coloring or sweetening foods. These natural food additives have become a recent trend, and consumers are preferring them over synthetic and artificial food additives due to harmful effects related to their consumption. Thus, in this work, leaf extracts of *Arbutus unedo* L. obtained by dynamic maceration and ultrasound assisted extraction with prior optimization through response-surface methodology, were optimized to obtain the highest yields and incorporated in quark cheese, then analysed over 8 days. Both extracts showed antioxidant and antimicrobial activity with no toxicity towards cell lines at the maximum tested concentration, and while the maceration extract showed lower antioxidant activity, when incorporated in the cheeses it better inhibited the proliferation of bacteria. As expected from a food preservative, the nutritional, chemical and physical parameters were not drastically changed from the addition of the extracts although the ultrasound extract preserved cheese did preserve soluble sugars when compared to the other cheeses. Both extracts are excellent candidates to be added to quark cheese allowing its consumption for more than eight days after opening. The two natural extracts showed better results when compared to the widely used preservative, potassium sorbate, and thus, could be used as a cheese additive and its use is encouraged by the food industry.

Keywords: *Arbutus unedo*, antimicrobials, preservatives, antioxidants, quark cheese

Resumo

O reino das plantas é uma fonte inesgotável de moléculas que podem ser aplicadas em quase todas as vertentes da sociedade. A indústria alimentar tem lucrado bastante com a utilização de plantas e seus derivados nos diferentes setores, desde há várias décadas. Recentemente, esta indústria tem utilizado plantas como aditivos alimentares, nomeadamente como conservantes, corantes e adoçantes de alimentos. Estes compostos têm ganho bastante notoriedade e neste momento, a maioria dos consumidores prefere estes aditivos naturais face às alternativas sintéticas e artificiais, dado a crescente informação relativa aos efeitos secundários destes aditivos artificiais. Assim, neste trabalho, extratos de folhas *Arbutus unedo* L. obtidos por maceração dinâmica e extração assistida por ultrassons foram analisados e desenhado um uma experiência de otimização de extração por metodologias de superfície resposta para potenciar a extração de compostos. Depois, ambos os extratos foram incorporados em queijos quark e analisados durante oitos dias. Ambos os extratos mostraram uma forte atividade antioxidante e antimicrobiana além de não mostrarem toxicidade em linhas celulares normais à máxima concentração testada. Enquanto o extrato obtido por maceração mostrou uma atividade antioxidante mais baixa quando comparado com o extrato obtido por ultrassons, quando incorporados nos queijos, o lote incorporado com o extrato obtido por maceração mostrou uma maior capacidade de inibição de crescimento microbiano. Tal como esperado para um conservante alimentar, os perfis nutricional, químico e físico dos queijos incorporados com os extratos não sofreram alterações drásticas, ainda que o extrato obtido por ultrassons tenha ajudado a inibir o consumo de açúcares solúveis por bactérias lácticas. Ambos os extratos são fortes candidatos a serem aplicados como conservantes naturais em queijo quark durante mais de oito dias após abertura da embalagem. De realçar que os dois extratos de *Arbutus unedo* mostraram uma capacidade conservante melhor quando comparados com um dos conservantes sintéticos mais usados em queijo, sorbato de potássio, e assim deviam ser recomendados como conservantes naturais em queijo quark e explorados pela indústria alimentar.

Palavras-chave: *Arbutus unedo*, conservantes, antimicrobianos, antioxidantes, queijo quark

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List of Abbreviations

AAPH	2,2′-azobis(2-methylpropionamidine) dihydrochloride
AMM	Aerobic mesophilic microorganisms
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
APC	Aerobic Plate Count
A_w	Water Activity
BAC's	Bioactive compounds
BBD	Box-Behnken design
C	catechin
CAA	Cellular antioxidant assay
CACO2	Human Colorectal Adenocarcinoma Cell Line
CCD	Central composite design
CFU	Colony Forming Unit
CIE	International Commission's Illumination
DCFH-DA	2′,7′-Dichlorofluorescin Diacetate
DM	Dynamic maceration
DMAPP	Dimethylallyl diphosphate
DMEM	Dulbecco's Modified Eagle Medium
DRBC	Dicloran Rosa Bengala Cloranfenicol Base agar
EC_{50}	Concentration of extracts responsible for 50% of lipid Peroxidation inhibition
ECAAC	European Collection of Authenticated Cell Cultures
EDTA	Ethylenediaminetetraacetic

EFSA	European Food Safety Authority
EMM	Estimated marginal means
EU	European Union
FAME	Fatty acid methyl esters
FBS	Fetal Bovine Serum
FPP	Farnesyl diphosphate
Fw	Fresh weight
GI_{50}	Concentration that inhibits 50% of cell growth
GPP	Geranyl diphosphate
HBA's	Hydroxybenzoic acids
HBSS	Hanks' Balanced Salt Solution
HCA's	Hydroxycinnamic acids
HPLC-DAD-MS/ESI	High performance liquid chromatography with a diode array Detector coupled to mass spectrometer with electro-spray ionization
HPLC-RI	High performance liquid chromatography coupled to a refraction index detector
IOD	Isorhamnetin-*O*-deoxyhexoside
IPP	Isopentenyl diphosphate
LOD	Luteolin-*O*-deoxyhexoside
LOQ	Limit of quantification
MBC	Minimum bactericidal concentrations
MDA	Malondialdehyde
MEP	Methylerythritol phosphate
MHB	Mueller-Hinton Broth

MIC	Minimum inhibitory concentration
MRS	Man, Rogosa and Sharpe agar
MRSA	Methicillin-resistant *Staphylococcus aureus*
MUFA	Monounsaturated fatty acids
PCA	Plate agar
PDA	Photodiode array diode detector
PLAB	Psychrotrophic lactic acid bacteria
PT	Preservative type
PW	Peptone water
QOD	Quercetin-*O*-deoxyhexoside
ROS	Reactive oxygen species
Rpm	Number of revolutions per minute
Rt	Retention time
SD	Standard deviation
SFA	Saturated fatty acids
SRB	Sulforhodamine B
ST	Storage time
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive species assay
TPA	Texture profile analysis
TPC	Total phenolic compounds
TSB	Tryptic Soy Broth
UAE	Ultrasound-assisted extraction
UFC	Colony-forming units

UFLC-DAD	Ultra-fast liquid chromatography coupled to a diode array detector
UV-VIS	Ultraviolet–visible
VRBG	Purple red bile lactose agar
Y	Yeasts

1. Introduction

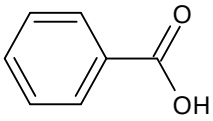
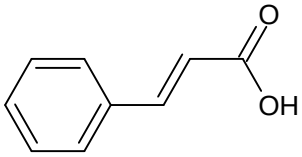
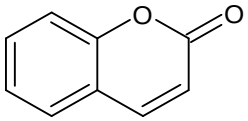
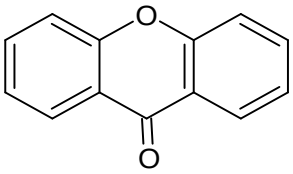
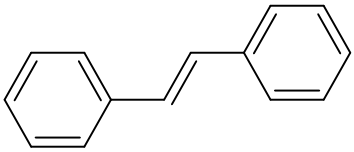
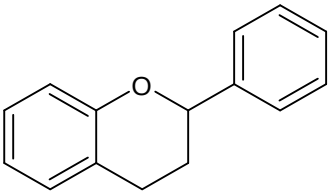
1.1. Bioactive Compounds of Plant Origin

Bioactive compounds (BAC's) in plants can be defined as secondary metabolites or chemical compounds produced by them, and are not involved directly in their normal growth, or reproduction but have many protective physiological functions, namely protection from herbivores, microbial infections, attraction of pollinators and biochemical signaling **(Zhao et al, 2015; Bernhoft et al, 2010).** They are present in small quantities in fruits and vegetables and offer many health benefits to humans (**Santos et al, 2019).** These bioactive substances can help prevent and reduce numerous chronic diseases and have many health protective effects namely an anti-inflammatory, antitumor, cytoprotective, antioxidant and antimicrobial **(Kovačević et al, 2020).**

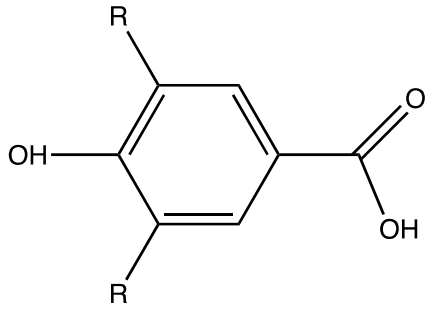
1.1.1. Phenolic compounds

Phenolic compounds are one of the most widely distributed secondary metabolites throughout the plant kingdom, depending on the plant species. They derive from the pentose, shikimate, and phenylpropanoid pathways **(Harborne, 1980; Di Ferdinando et al, 2014).** These molecules work as natural protectors from herbivores, various microbial infections (parasites and predators) and the harmful effects of ultraviolet radiation **(Yalcin & Çapar, 2017)**. Phenolic compounds (or polyphenols) are common constituents of plant foods (fruits, vegetables, cereals, among others) and beverages (tea, coffee, beer, among others) being partially responsible for the global organoleptic properties of plant foods **(Dai & Mumper, 2010)**. Polyphenols also present a strong antioxidant activity, which acts in collaboration with vitamins and enzymes as a defense against oxidative stress caused by excess of reactive oxygen species (ROS), thus preventing biomolecules (proteins, nucleic acids, lipids, and sugars) from suffering oxidative damage **(Tsao, 2010; Heleno et al, 2015)**. Their structure consists of an aromatic ring containing one or more hydroxyl substituents and they can range from simple phenolic molecules such as phenolic acids to highly polymerized compounds as tannins, with mono- and polysaccharides associated with one or more phenolic groups, as well as links to esters and methyl esters. Currently, more than 8000 structures of phenolic compounds are known and can be categorized into several classes **(Del Rio et al, 2013)**. **Carocho & Ferreira, 2013,** classified them according to their basic carbon skeleton (**Table 1**).

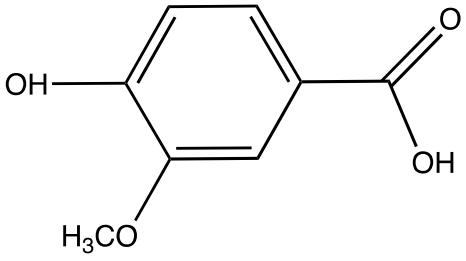
Table 1: Classification by number of carbons and basic structure within the family of phenolic compounds **(Carocho & Ferreira, 2013)**

Number of carbons	Classification	Example	Basic structure
7	Hydroxybenzoic acids	Gallic acid	
9	Hydroxycinnamic acids	*p*-coumaric acid	
9	Coumarins	Esculetin	
13	Xanthones	Mangiferin	
14	Stilbenes	Resveratrol	
15	Flavonoids	Naringenin	

The phenolic acids presented in **Figure 1**, synthesized through the shikimate pathway, have at least one aromatic ring with at least one hydrogen substituted by an hydroxyl group consisting of two groups: a) **hydroxybenzoic acids** (HBA's) C6–C1; including salicylic acid, 4-hydroxybenzoic acid, protocatechic acid, gentisic acid, vanillic acid, syringic acid, gallic acid, ellagic acid, and hexahydroxydiphenic acid; b) **hydroxycinnamic acids** (HCA's) C6–C3 where caffeic acid and *p*-coumaric acids represent between 75% and 100% of the total of HCA in fruits, respectively, while sinapic acid is abundant in cereals **(Heleno et al, 2015; Williamson, 2017; Vuolo et al, 2019)**.

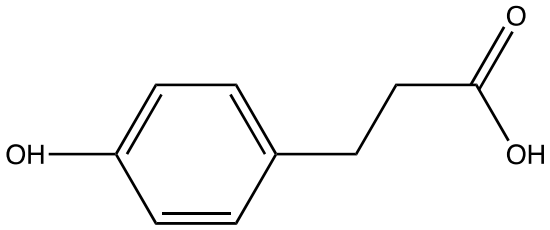


Gallic acid

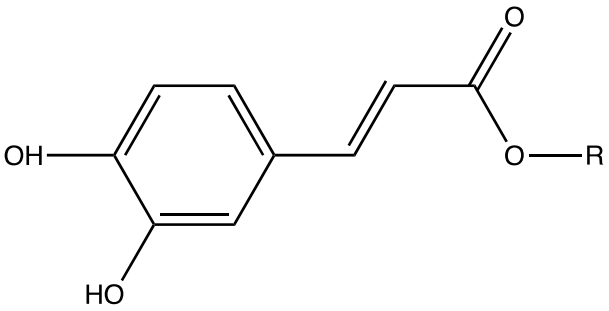


Vanillic acid

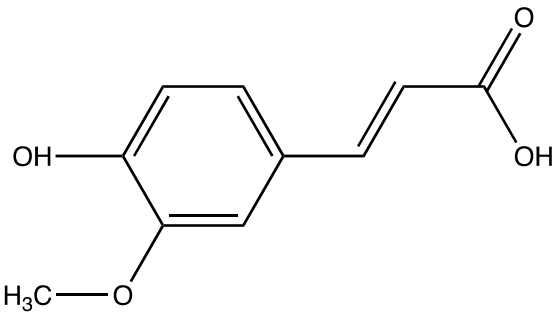
Hydroxybenzoic acids



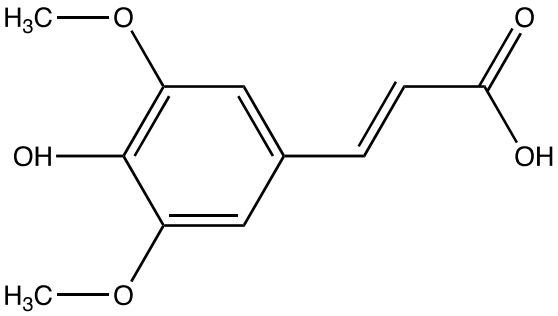
p-coumaric acid



Caffeic acid



Ferulic acid



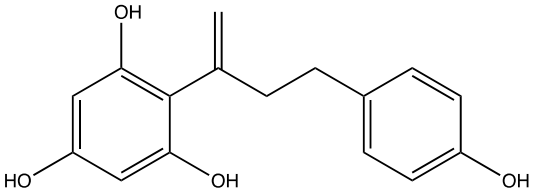
Sinapic acid

Hydroxycinnamic acids

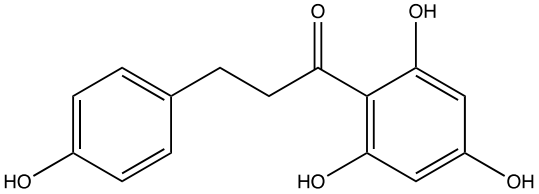
Figure 1: Structure of phenolic acids **(Tsao, 2010)**

Flavonoids constitute the largest group of phenolic compounds found in fruits, vegetables, nuts and seed, legumes and cereal grains. They are characterized by a low molecular weight and a 15-carbon skeleton, being synthesized through the phenylpropanoid pathway and showing a structure that includes two aromatic rings, A and B, joined by a three-carbon bridge, frequently in the form of a heterocyclic C ring. These C rings are mainly responsible for the varieties of flavonoid classes: **flavonols** (such as kaempferol, quercetin, isorhamnetin, and myricetin), **flavones** (apigenin, luteolin, wogonin, and baicalein), **flavanones** (naringenin and hesperetin), **flavanols or flavan-3-ols** (catechins), **isoflavones** (as phytoestrogens), and

anthocyanidins (pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin) **(Figure 2)**. The majority of flavonoids naturally occur as glycosides rather than as aglycones **(Del Rio et al, 2013; Vuolo et al, 2019)**.

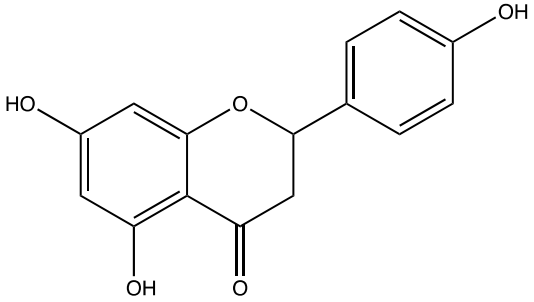


Chalconaringenin

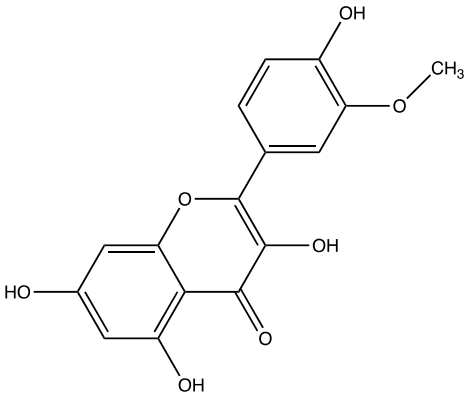


Phloretin

Chalcones

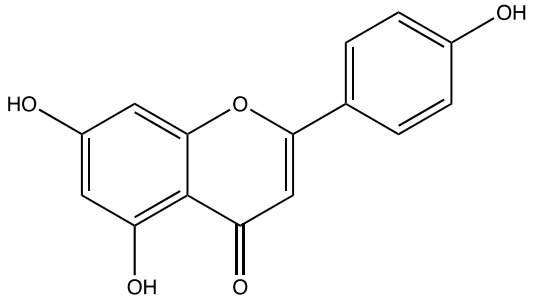


Naringenin

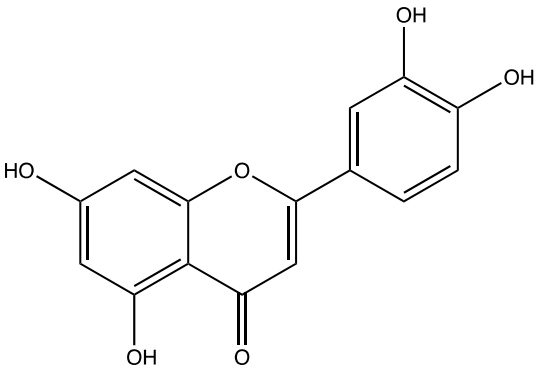


Hesperetin

Flavanones

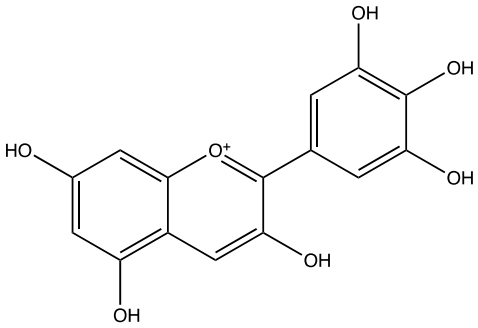


Apigenin

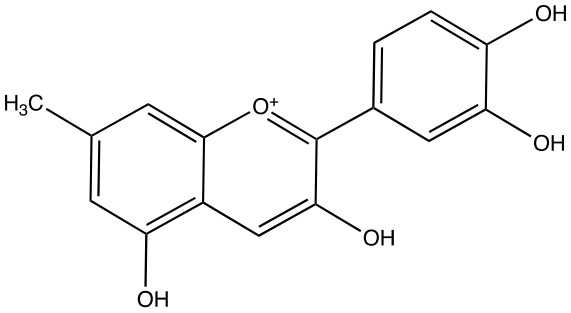


Luteolin

Flavones

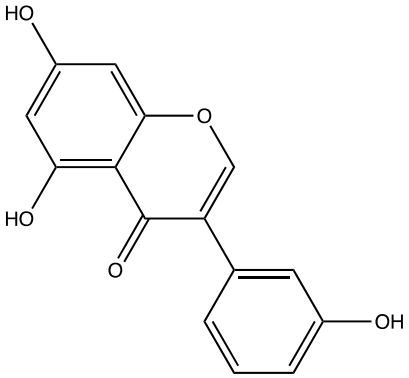


Delphinidin

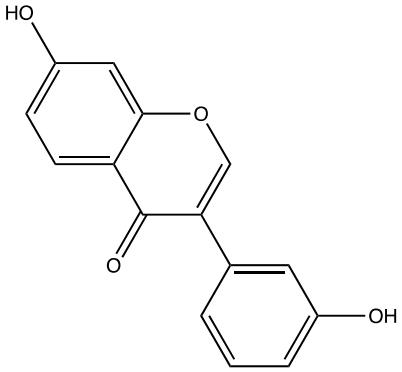


Cyanidin

Anthocyanidins

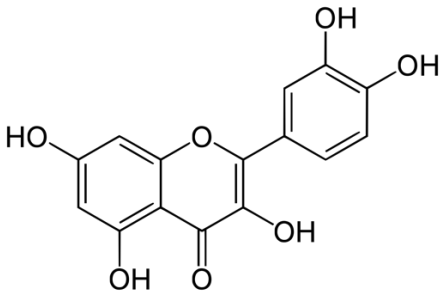


Genistein

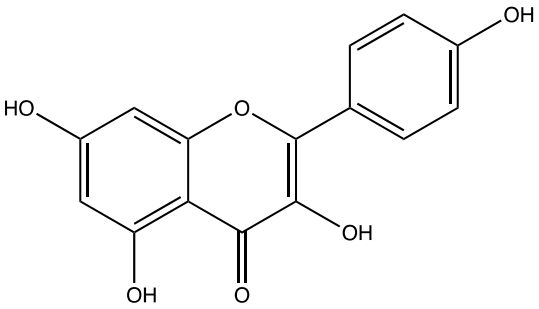


Daidzein

Isoflavones



Quercetin



Kaempherol

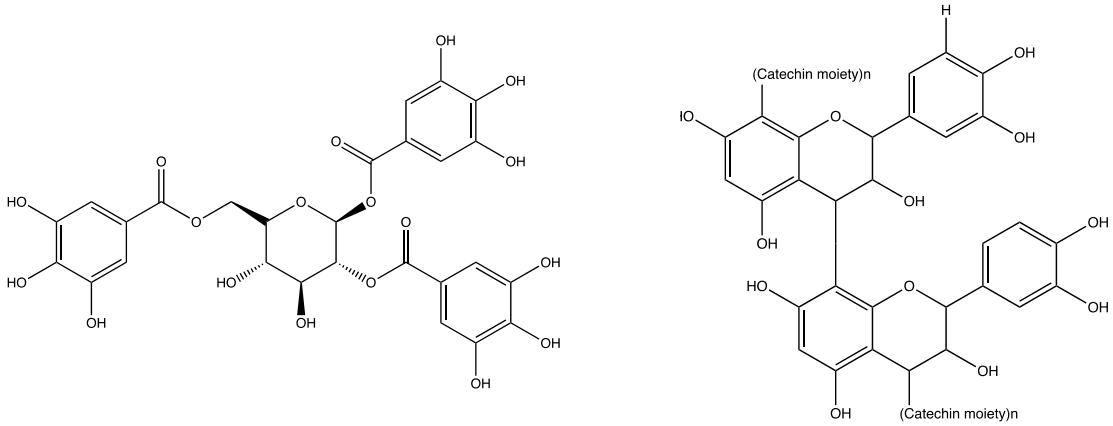
Flavonols

Figure 2: Structure of the flavonoid skeleton **(Del Rio et al, 2013)**

Due to the presence of conjugated chromophores, a large part of flavonoids has a yellow to red color, which are responsible for the color of many flowers, seeds, and fruits **(Erlund, 2004)**.

Tannins (**Figure 3)** also act against herbivores and have a role as antimicrobial agents **(Vuolo et al, 2019).** Their molecular weight varies from 500 to 3000Da and contain many hydroxyl or other functional groups in the form of esters **(Ferrer et al, 2008)**. Chemically, they can be classified into two groups: hydrolysable tannins which are polymers composed of a

monosaccharide core as gallic acid esters (gallotannins), glycosylated ellagic acid (ellagitannins), and condensed tannins which are structurally more complex and uniform than hydrolysable tannins. These molecules are usually oligomers and polymers of flavan-3-ol flavan and/or flavan-3,4-diols **(Vuolo et al, 2019)**.

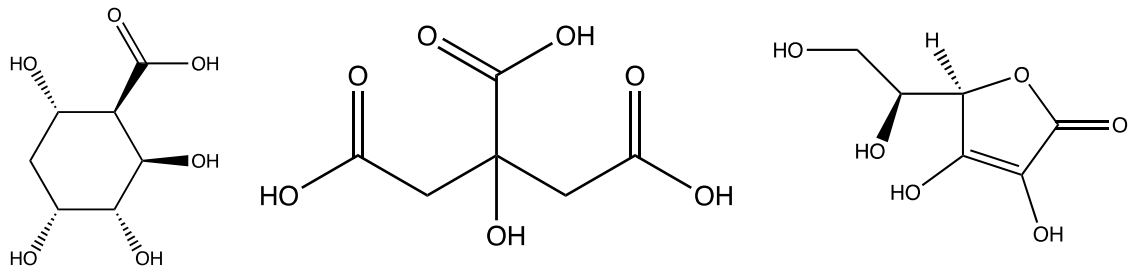


Hydrolysable tannin Condensed tannin

Figure 3: Classification of tannins **(Mingshu et al, 2006)**

1.1.2. Organic acids

Organic acids are bioactive compounds with a low molecular weight that contain carboxyl groups with a general R-COOH structure. Organic acids are known as intermediate products in the metabolism of plants: shikimic acid participates in the shikimate pathway and produces essential amino acids like L-phenylalanine and L-tryptophane; ascorbic acid is known as a strong antioxidant which is very efficient against hypertension and scurvy; quinic acid is also an antioxidant. Other organic acids such as malic, citric, succinic and fumaric acids have a key role in the Krebs cycle, being important for human metabolism. Malic acids is has a bactericidal impact; citric acid is used in bones as a crystal thickener; succinic acid is reported to have anti-diabetic effect and fumaric acid is known to help in inflammation and psoriasis **(Carocho et al, 2013; Adamczak et al, 2020; Li et al, 2020)**.



Quinic acid Citric acid Ascorbic acid

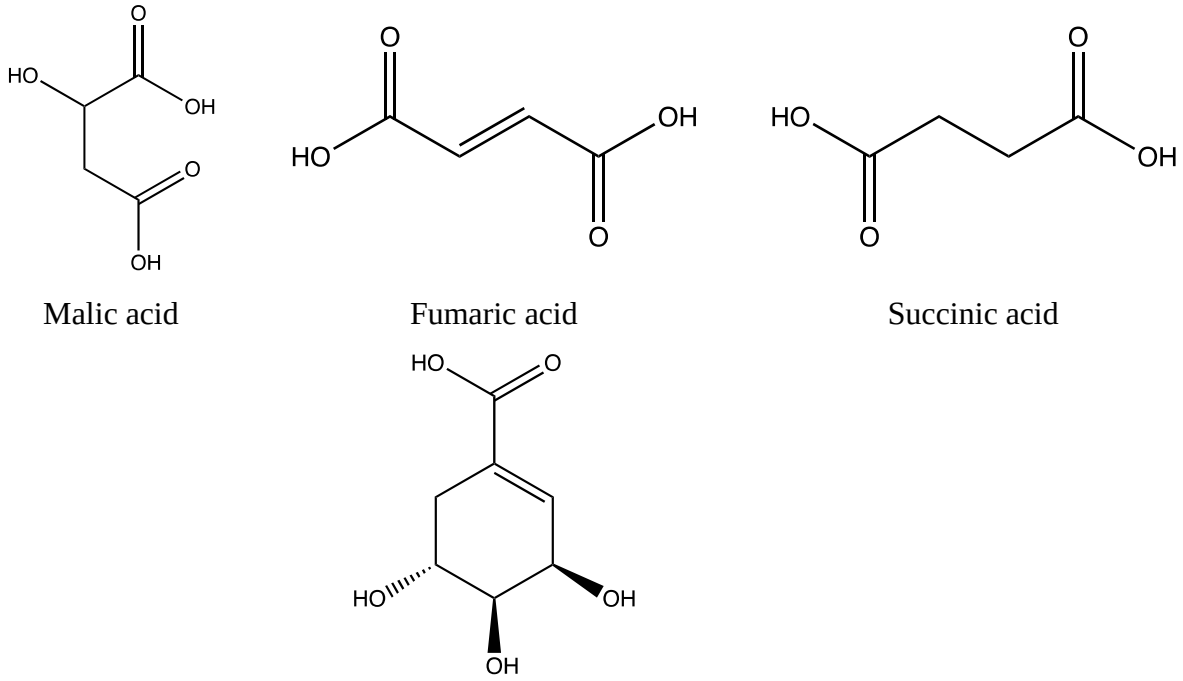
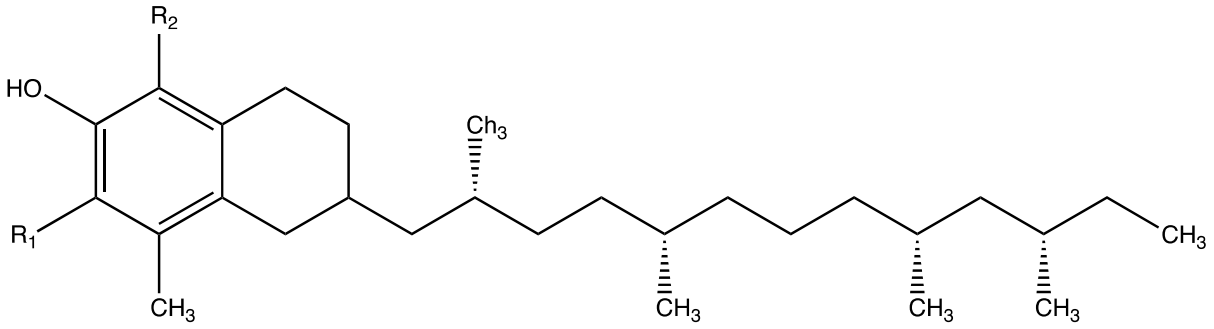


Figure 4: Chemical structures of organic acids **(Adamczak et al, 2020)**

In the food industry, organic acids, especially quinic, ascorbic and citric acid are used as acidic additives which act as buffers to regulate acidity and maintain pH at a desired level. They also act like antioxidants which are capable of delaying or preventing oxidative deterioration of lipids **(Quitmann & Czermak, 2013)**.

1.1.3. Tocopherols

Tocopherols (vitamin E) are methyl-substituted hydroxy chromans with a polar chromanol ring and an apolar phytyl side chain. There are four tocopherol vitamers **(Figure 5)** (α-, β-, γ-, and δ-tocopherol) and four tocotrienols with the same nomenclature, which differ in the number and position of the methyl groups of their chromanol ring **(Barros et al, 2008).**



$R^1=R^2=CH_3$ α-tocopherol $R^1=H, R^2=CH_3$ γ-tocopherol

$R^1=R^2=H$ δ-tocopherol $R^1= CH_3, R^2=H$ β-tocopherol

Figure 5: Chemical structures of the four tocopherol isomers

.

The main biological activity of vitamin E is its antioxidant competences which are quite effective in breaking free radical chain reactions and offers some nature oxidative protection, especially in lipidic mediums **(Barros et al, 2008).** It is also used in the inhibition of radical formation in biological systems like plasma, membranes, tissues, and in the free radical chain reactions interruption **(Sanagi et al, 2005).** As food additives, the use of tocopherols (E306 – E309) in done mostly in high fat foods, allowing the extension of food shelf-lives **(Tomassi & Silano, 1986).**

1.1.4. Terpenes and other volatiles

There are 1700 volatile compounds have been identified in over 90 plant families. Typically, they comprise a lipophilic liquid with high vapor pressures that can traverse membranes and be distributed into the atmosphere or soil in the absence of a diffusion barrier. They defend plants against pathogens, signal pathways and attract pollinators, constituting about 1% of plant secondary metabolites, being represented by three major groups: terpenoids, phenylpropanoids/benzenoids, and fatty acid derivatives. Phenylpropanoids and benzenoids are obtained from L-phenylalanine, which are converted to trans-cinnamic acid by the L-phenylalanine ammonia-lyase enzyme **(Dudareva et al, 2006)**.

Terpenoids compose the largest class of volatile compounds, being synthesized through the mevalonate pathway in the cytoplasm (sesquiterpenes) and methylerythritol phosphate (MEP) or mevalonate-independent pathways in chloroplasts (mono- and diterpenes), where the condensation of isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate

(DMAPP) precursors lead to the creation of geranyl diphosphate (GPP), prenyl diphosphates, and farnesyl diphosphate (FPP), that are responsible of the formation of all terpenes. They can be defined as hydrocarbons with a $(C_5H_8)_n$ formula, allowing hydrogenated, dehydrogenated and oxygenated derivatives. Depending on the five-carbon building blocks, there are many varieties: hemiterpenes (5 C), monoterpenes (10 C)(more than 25,000 compounds are known), sesquiterpenes (15 C), diterpenes (20 C), sesterterpenes (25 C), triterpenes (30 C)(steroids), tetraterpenes (40 C) (Limonoids and Quassinoids, Glycosides, Saponins, carotenoids, resins) and polyterpenes (n C), that are contained from a larger number of isoprene units >45 **(Carocho & Ferreira, 2013; Takshak, 2018; Petrovic et al, 2019)**. All plants produce a range of terpenoids, and while some are common to all, several are synthesized in specific taxa with adaptations to ecological conditions **(Zhou & Pichersky, 2020)**. They have a distinct odor, which can help defend injured tissues from insects, parasites and herbivores or attracting pollinators, which gives terpenes an important role in the perfume and food industry. Monoterpenes present the major constituent of terpenic oils or essential oils of the edible and aromatic species in nature. For example, orange trees include high quantities of sabinene and limonene in their foliage **(Rubulotta & Quadrelli, 2019).** Terpenes also show many bioactivities, namely antimicrobial activity, antiviral, cytotoxic, anticancer, anti-inflammatory, antineoplastic and other pharmacological properties **(Petrovic et al, 2019)**.

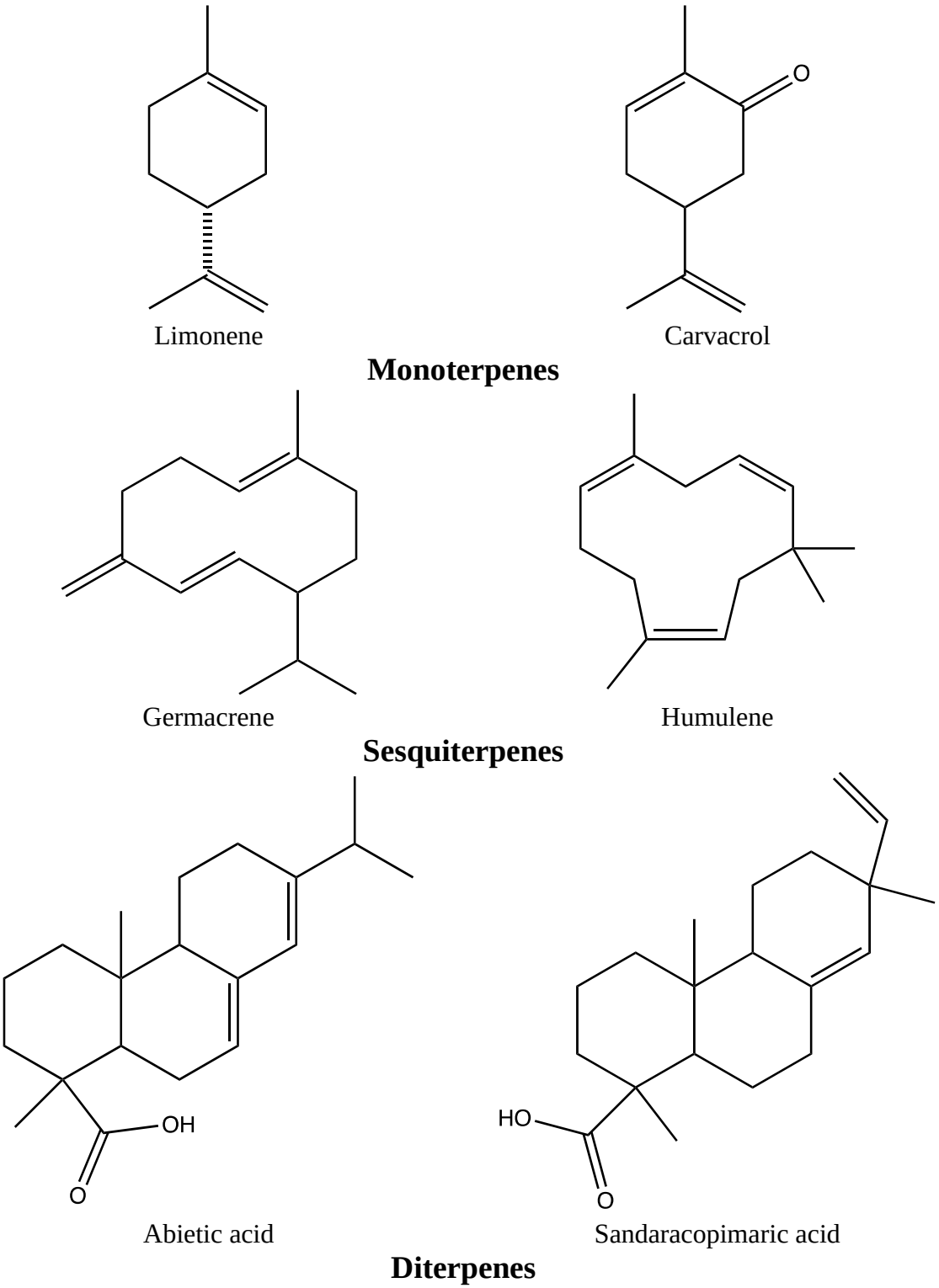


Figure 6: Chemical structures of terpenes

1.2. Strawberry-tree

Arbutus unedo L. (known as strawberry tree) **(Figure 7)** belongs to the Ericaceae family and Vaccionioideae subfamily, is a small Mediterranean plant, being found mostly in southern

Europe, north-eastern Africa, Ireland, Palestine, and the Canary Islands **(Malheiroa et al, 2012).** The *A. unedo* tree is between 1.5 m to 3 m tall, with dark green leaves, being simple and alternate **(Miguel et al, 2014).**

Figure 7: Strawberry tree

(https://br.pinterest.com/pin/515451119827070436/)

Different parts of *A. unedo* tree (roots, bark, fruits, flowers and leaves) have been studied for their chemical composition. Several secondary metabolites have been identified in the aqueous extract of the roots, namely anthraquinones, flavonoids, quinones, terpenoids, polyphenols, tannins and anthocyanins, which have strong antibacterial and antioxidant activity and hypoglycemic effect **(Miguel et al, 2014)**. Traditionally, the *A. unedo* roots have been used as anti-inflammatory and diuretic agents, in the treatment of several dermatologic, urological, gastrointestinal, dermatologic, and cardiovascular diseases **(Mrabti et al, 2018)**. Stems and flowers have been reported as anti-inflammatory agents **(Tenuta et al, 2020)**. **Isbilir et al, 2012,** proved the antioxidant activity of flower extracts rich of phenolic compounds. As many studies showed, fruits and leaves of *A. unedo* L. have been widely studied for their diverse chemical composition namely phenolic acids, flavonoids, tannins, and anthocyanins, which have been applied in traditional medicine from many years ago (**Miguel et al, 2014)**. In addition to their richness of bioactive compounds with essential antioxidant activity, they are known by their extreme contents of vitamins, particularly vitamin c, minerals, dietary fibers, and easily

digestible sugars **(Šic Žlabur et al, 2020)**. The fruit is particularly rich in calcium, high contents of malic, lactic, fumaric, suberic, protocatechuic, *p*-hydroxibenzoic, gentisic, m-anisic, vanillic, gallic and ellagic acids. The fruit also contain flavonoids namely, flavonols quercetin, anthocyanin delphinidin-galactoside and the kaempferol, which cyanidin and anthocyanins, known to be responsible for the red color **(Pallauf et al, 2008)**.

A study of *A. unedo* from the Natural Park of Montesinho showed that the leaf extracts contain flavonols (kampferol, glucosides of myricetin, quercetin), flavanols (procyanidin dimers, respective gallate esters, and catechins), several gallotannins and ellagitannins derivatives which the amount of those compounds was higher compared to the ones of the fruit extracts **(Miguel et al, 2014)**.

As stated above, the strawberry tree is mostly used in traditional medicine in several countries. In Portuguese regions the root and bark decoctions are applied as cardiotonic, anti-laxative, odontalgic, carminative, digestive, and anti-inflammatory agents. The strawberry fruit might be considered as unconventional source of catechin and flavan-3-ols compounds, which are globally applied for supplementation of cosmetic and pharmaceutical products in the nutraceutical sector, as well in functional foods. **(Orak et al, 2011, Albuquerque et al, 2017)**. **Takwa et al, (2017)**, reported that the strawberry fruit extracts have a higher antioxidant activity than some synthetic additives, which the extracts could be applied as natural preservative in new breads. Also, its extracts have been employed for the oxidation prevention of proteins and lipids and the quality preservation in the meet industries **(Miguel et al, 2014)**. **Oliveira et al, 2009** showed that strawberry tree leaves may be valuable in pharmaceutical industry and food application. Several studies on the leaf extracts are proven the strong vasorelaxant activity given by catechin gallate and oligomeric condensed tannins components, the leaves have effect on inflammatory and cardiovascular diseases, and in the treatment of diabetes, but also used for their purgative and astringent potentials **(Bento & Pereira, 2011; Malheiroa et al, 2012)**.

1.3. Quark Cheese

Quark (as known in Germany) or Tvorog (in Eastern European countries) cheese **(Figure 8)**, is classified under acid-rennet coagulated fresh cheeses. It is basically a milk protein paste and a soft fermented cheese, with white to yellowish colour, slightly acidic flavor and middle sour taste, it is slightly supple and flexible, and also quite smooth. It is produced from pasteurized skimmed or full fat milk at pH 4.6 - 4.8, with a little rennet supplement and culture

in the beginning. Its shelf-life is defined between 2 - 4 weeks at <8°C because of the elevated moisture content (82%) **(Schulz-Collins et al, 2004; Ozturkoglu-Budak et al, 2020)**. This shelf-life is reduced if no preservatives are used. The heating treatment at 90 ºC for 10 min or at 95 ºC for 15 min improves Quark moisture, yield and firmness **(Lepesioti et al, 2021).**

Figure 8: Quark cheese

(https://fr.openfoodfacts.org/produit/20001940/fresh-cheese-milbona)

Due to the advancement of food preservation technologies, fresh cheese production has been growing around the world, being this type of cheese consumed almost by all cultures and countries, making it will be one of the most attractive products in the dairy industry **(Miloradovic et al, 2018)**. According to the European Food Safety Authority (EFSA), dairy products and milk present one the most consumed foods by adults in Europe, averaging 34.2 g/day **(Ritota & Manzi, 2020)**.

1.4. Food Additives

Food additives are molecules or mixtures which are used in foods to carry out a specific task, either to sweeten, preserve, colour, or change a specific trait in food. Thus, most of these substances are of synthetic origin and an overexposure to them may constitute harm to human health, especially in a society that extensively consumes processed foods. The EFSA is the organism responsible for the certification of additives, limits of daily consumption, and monitoring their safety when used in foods within the European Union (EU) **(Carocho et al, 2015)**. Some synthetic food additives known as colorants, when used in food must have a

mention stating they can be harmful to children. Other examples of hazardous additives are Acesulfame K, which is known to cause allergic reactions, while parabens are known for their toxicity and nitrites are known carcinogens **(Carocho et al, 2013; Pellegrini et al, 2018; Bhavaniramya et al, 2019; Myszka et al, 2019)**. In the recent years, the increasing knowledge and awareness of consumers towards their diet and its relation to many diseases has led consumers to reduce consumption of foods with high amounts of additives and prefer natural alternatives. Examples of natural food additives, or natural-based additives are rosemary extract (E 392), ascorbic acid (E 300), tocopherols (E 306-E 309)), anthocyanins (E 163), chlorophylls (E 140), curcumin (E 100), steviol glycosides (E 960), glycyrrhizin (E 958), erythritol (E 968), among others, all approved by EFSA for use in the EU **(Carocho et al, 2015; Vella et al, 2018; Myszka et al, 2019)**.

Due to the propensity of cheese to spoilage and pathogenic microorganism growth, which reduce its shelf life, some synthetic preservatives have been used in Quark cheese production, namely cheese ripening salts, calcium carbonate or sodium hydrogen carbonate, sorbic acid and sorbates (E 200–203). Although these additives are considered safe by EFSA, their excessive consumption could cause several health problems, especially in diets with high amounts of processed foods, and the more prevalent use of food additives to allow longer shelf-lives **(Robinson & Wilbey, 1998)**. Consequently, scientists are focused on finding natural alternatives to these synthetic additives, mostly from plants. Thus, several plant extracts with high concentrations of phenolic compounds, as well as sesquiterpenes, terpenoids, have been reported for their antioxidant and antimicrobial activities against the common pathogenic microorganisms in cheeses, such as *Staphylococcus aureus, Listeria monocytogenes, Salmonella spp. and Escherichia coli*. Aqueous extracts of pine needles and Horseradish tree extracts, as well as oregano, rosemary, garlic, cinnamon and watercress ethanolic extracts have been applied in the cheese manufacturing process for preventing the growth of pathogens **(Ritota & Manzi, 2020)**.

This work aims to study the extracts of *A. unedo* in terms of their antioxidant and antimicrobial activity to then use them as alternative preservatives to potassium sorbate in quark cheese preservation.

2. Objectives

In the present work, the leaf extracts from strawberry tree (*Arbutus unedo* L.) will be studied regarding their profile in bioactive compounds (phenolic compounds, organic acids, soluble sugars). Furthermore, the obtained leaf extracts will be tested for their antioxidant, antimicrobial and cytotoxic activity to then be used as an antimicrobial additive in quark cheese, serving as an alternative to the widely used synthetic additive, potassium sorbate.

Thus, the specific objectives of this work consist of:

- ✓ Obtention of the leaf extracts from the *A. unedo* by dynamic maceration and ultrasound assisted extraction.
- ✓ Optimisation of extraction procedures through Response Surface Methodology by analysis of the major phenolic compounds (HPLC-DAD-ESI/MS)
- ✓ Determination of the antioxidant activity through the TBARS assay and cellular antioxidant assay
- ✓ Determination of the antimicrobial activity through the microdilution method
- ✓ Incorporation of extracts in quark cheese and analysis over 8 days
- ✓ Analysis of the nutritional profile:
    - o Moisture
    - o Proteins
    - o Crude fat
    - o Carbohydrates
    - o Ash
    - o Energy
- ✓ Individual fatty acids (GC-FID)
- ✓ Analysis of organic acids (UFLC-DAD)
- ✓ Analysis of soluble sugars (HPLC-RI)
- ✓ Analysis of the microbial load over a week
- ✓ Texture (Texturometer)
- ✓ External color (Portable colorimeter)
- ✓ pH (pH reader)
- ✓ aW (Dew point hygrometer)

3. Material and Methods

3.1. Plant Material

The leaves of *Arbutus unedo* L. (strawberry tree) used in the present work were purchased from "Cantinho das Aromáticas", founded in Vila Nova de Gaia, Portugal, retailers of aromatic, medicinal and seasoning plants. The leaves were acquired dry and upon acquisition were ground to a size of 20 mesh (~0.8mm) in a shredder (Moulinex A320, Mayenne, France) **(Figure 9).**

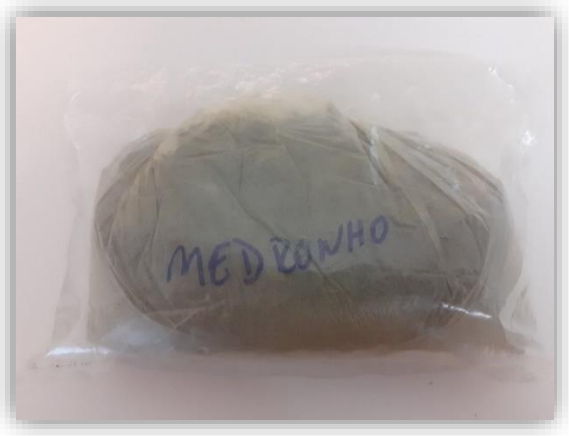


Figure 9: Leaves of Arbutus unedo L. after grounding

3.2. Optimization Conditions for *Arbutus unedo* L.

For each of the two extraction techniques ultrasound-assisted extraction (UAE) and dynamic maceration (DM), an optimization extraction was implemented, for DM, the Box-Behnken design (BBD) was used, and for the UAE the central composite design (CCD) was chosen. Three independent variables were varied [X_1 (t, min), X_2 (T, ºC or P, W) and X_3 (S, %)], where "t" represents time, "T" represents temperature, "P" represents power, measured in "W" watts and "S" represents solvent percentage, being ethanol the solvent. The BBD included 17 runs (independent combinations), with 5 repetitions of the center point to evaluate precision of the method. The CCD included 20 independent combinations and 4 repetitions of the center point. The upper and lower limits of each individual variable was selected based on reports from literature. The analyzed responses for each extraction technique were the solid residue after extractions (R1) expressed in mg, and the most abundant phenolic compounds, expressed in mg/g of extract, namely catechin (R2), isorhametin (R3), quercetin (R4), luteolin (R5) and the total amount of phenolic compounds (R6) also expressed in mg/g of extract.

3.2.1. Ultrasound assisted extraction

The extraction of bioactive compounds assisted by UAE of *Arbutus unedo* was performed using an ultrasonic device (QSonica sonicators, model CL-334, Newtown, CT, USA) **(Figure. 10)** with a titanium probe.

Figure 10: Ultrasonic probe and control device

The solid-liquid ratio (1.5 g – 50 mL) was kept constant at each repetition to maintain the same volume in the different conditions. After each extraction, the samples were centrifuged (5000 rpm; for 20 min at 10 ° C), filtered through paper filters (Whatman No. 4). Then, 1.5 mL of each extracts were filtered through a 0.2 µm nylon filter (Whatman) and collected in vials for chromatographic analysis. 5 mL of each extract were placed in weighted crucibles, left to dry in an oven at 100° C for 4 days. After reaching a constant weight the dry residue calculated and used as a response (R1) in the optimization protocol.

Figure 11: Powdered extract from UAE

3.2.2. Dynamic maceration extraction

The maceration extraction was performed using a thermostated water bath **(Figure 12)** under continuous electro-magnetic stirring for the different extraction times and solvents percentages as defined by the experimental design, in amber airtight containers at the specific temperatures.

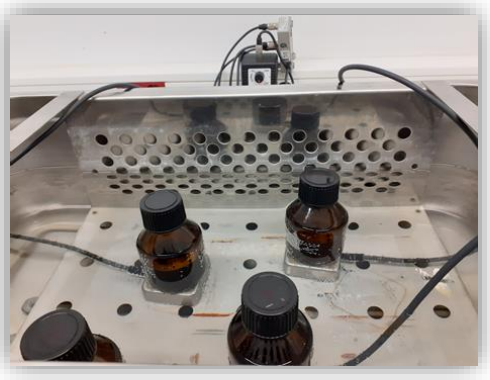


Figure 12: Thermostated water bath under continuous electro-magnetic stirring

The obtained extracts were filtered through a Whatman paper filter nº4. Then 1.5 ml of each extract was filtered through a 0.2 µm nylon filter (Whatman) and collected in vials for chromatographic analysis. 5 mL of each extract was placed in weighted crucibles and left to dry in the oven at 100 °C for 4 days, after the crucibles were weighted again, and the amount of dry residue was used as a response (R1) for the optimization protocol **(Figure 13)**.

Figure 13: A) Maceration preparation; **B)** Pipetting to crucibles; **C)** Weighing the crucibles

After analysing all the optimization runs, an ideal point (where the highest residue was determined) was sought for both extractive technologies. Those conditions were then used to

extract a high quantity of molecules for subsequent use in further steps. The obtained extracts were lyophilized (-47 ºC, 0.100 bar; FreeZone 4.5, Labconco, Kansas City, MO, USA) and kept away from light at cool temperatures for subsequent use **(Figure 14)**.

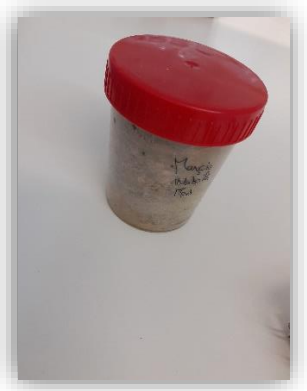


Figure 14: Extract obtained at the optimal point.

3.2.3. Identification and quantification of phenolic compounds

Chromatographic analysis was carried out in a Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA) system **(Figure 15)** equipped with an auto-sampler (kept at 5 ºC), a degasser, an automated thermostat column compartment, and a quaternary pump. The high-performance liquid chromatography (HPLC) was coupled to a diode array detector, and an electrospray ionization mass detector (LC-DAD-ESI/MSn) allowing the detection of compounds at wavelengths of 280 nm, 330 nm and 370 nm. 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 were (A) 0.1% formic acid in water and (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). MS detection was performed in negative mode, using a Linear Ion Trap LTQ XL mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an electrospray ionization source (ESI). Nitrogen served as the sheath gas (50 psi); the system operated with a spray voltage of 5 kV, a capillary voltage of -20 V, and a source temperature of 325 °C. The tube lens offset was kept at a voltage of -66 V. The collision energy used was 35 (arbitrary units). The full scan covered the mass range from m/z 100 to 1500. Data acquisition was carried out with Xcalibur® data system (ThermoFinnigan, San Jose, CA, USA). The identification of compounds was performed through the comparison of their UV-VIS, mass spectra and retention times to

available standard compounds or using literature data. For quantitative analysis, a calibration curve of standard phenolic compounds was plotted based on the UV signal. The results were expressed in mg/g of extract.

Figure 15: High performance liquid chromatography coupled to diode array detector and to an electrospray ionization mass detector (LC-DAD-ESI/MSn).

3.3. Evaluation of Antioxidant and Antimicrobial Activities of Extracts

3.3.1. Antioxidant activity

3.3.1.1. Thiobarbituric acid reactive substances (TBARS) assay

TBARS is a colorimetric assay that measures the malondialdehyde (MDA) production via lipid peroxidation, in which the thiobarbituric acid (TBA) reacts with MDA to produce oxidative MDA-TBA complex, responsible to the formation of a pink pigment which can be measured spectrophotometrically at 532 nm **(Kaur & Geetha, 2006).**

For the assay, porcine brains were obtained from a local slaughterhouse, weighted **(Figure 16)** and dissolved in Tris-HCl buffer (20 mM, pH = 7.4). The solution was then centrifuged at 3500 g for 10 minutes. Each dilution of the sample solutions (200 µL) was pipetted into Eppendorf tubes, adding to them 100 µL of FeSO4 (10 mmol/L), 100 µL of ascorbic acid (0.1 mmol/L), and 100 µL of the supernatant of brain tissue homogenate. Two blanks were prepared, one with tris-HCl buffer and the other with deionized water. The Eppendorfs were incubated at 37.5 °C for 1h. After the incubation, trichloroacetic acid (500 µL, 28% w/v) was added to stop the reaction, and at the same time 380 µL of thiobarbituric acid (TBA, 2% w/v) was added, followed by the subsequent incubation of tubes at 80 °C for 20 min. Then, to eliminate the precipitated protein, the tubes were centrifuged at 3000 rpm for 5 min, and the supernatant samples were measured at 532 nm

(Figure 16). The equation, inhibition ratio (%) = [(A−B)/A] × 100; (A- absorbance of the control and B- absorbance of the sample solution) was used to calculate the percentage of inhibition, then transformed to EC_{50} values (concentration of extracts responsible for 50% of lipid peroxidation inhibition) in mg/mL of extract solution.

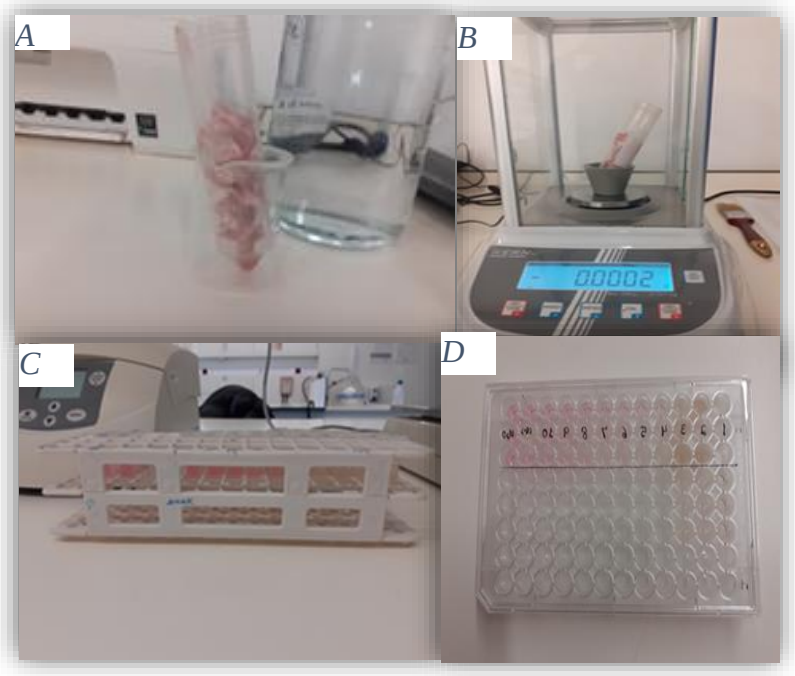


Figure 16: TBARS assay – A) Porcine brain; B) weighting the brain; C) preparation of mixtures in Eppendorf tubes; D) Spectrophotometrical reading

3.3.1.2. Cellular antioxidant assay

The cellular antioxidant assay was also used to analyze the extracts due to using live cells. For this assay, a cell culture (RAW264.7) was obtained from the ECAAC - European Collection of Authenticated Cell Cultures and grown in DMEM medium containing 10% heat inactivated FBS, glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37 ° C in a humidified air incubator containing 5% CO_2. For determination of intracellular ROS, the cells were incubated with antioxidant compounds and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), a compound that causes oxidative stress in order to promote the formation of free radicals. Thus, 2′,7′-Dichlorofluorescin Diacetate (DCFH-DA) was used as a fluorescent marker **(Wolfe et al., 2008)**, DCF-DA is a compound which, once in the cell medium is easily oxidized by peroxide radicals, to a fluorescent compound, resulting in DCFH-DA. The DCF-DA enters the cell and is targeted by esterase enzymes, the diacetate present in the molecule being cleaved. The molecule, by the action of ROS is oxidized, thus forming

DCFH-DA which has fluorescence. Oxidation can be inhibited by the action of antioxidant compounds that will reduce the antioxidant potential of ROS. The decrease in fluorescence emission compared to control cells indicates the antioxidant activity of the compounds tested **(Wolfe and Liu, 2007; Kellett et al., 2017)**. Cellular antioxidant measurements were done following the method of **Wolfe and Liu (2007)** with modifications. After RAW cells reached confluence in the culture flasks, they were washed twice with sterile Hanks' Balanced Salt Solution (HBSS) (pH 7.4) and then separated from the surface using 0.05% trypsin-EDTA. Cells were plated (3.0 x 104) in 100 µl of cell / well culture medium in 96-well black background culture plates and incubated until confluency (24-48 h). The perimeter wells were left empty to reduce any variation due to the location of the plate. The growth medium was removed after confluence and the cells were then washed with HBSS. Then, 200 µL of different extracts concentrations was added to each well in triplicate with 50 µM DCFH-DA prepared in ethanol and diluted in HBSS. As a negative control 200 µL of DCFH-DA applied in triplicate was added. Cells were incubated for 1 h at 37 ºC. Quercetin was used as a positive control. The cells were washed 3x with HBSS to ensure that any antioxidant effects observed later in the assay were due exclusively to the compounds incorporated by the cells. Then, 100 µL of AAPH was added. Cells were immediately placed on a microplate reader (FLX800 Biotek), where real-time fluorescence was read initially and every 5 min for 40 min. Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Regarding the quantification of the cellular antioxidant assay (CAA), the efficacy of the antioxidant treatments for both cell lines was quantified by examining the percentage reduction in fluorescence. Briefly, curves were generated by the 14 fluorescence response readings over a 40 min assay. The area under each curve was calculated, using Trolox as a control. The percentage reduction (or the CAA unit) was calculated from the following formula:

$$CAA = \%reduction = 1 - \frac{Antiox.sample}{Antiox.control} \times 100$$

3.3.2. Antimicrobial activity

For the antimicrobial activity, samples (100 mg) were prepared and analysed using the microplate microdilution method to obtain the minimum inhibitory concentration (MIC) which is known as the lowest concentration that inhibits visible bacterial growth, and minimum bactericidal concentrations (MBC) that present the lowest concentration that effectively kill bacteria. The bacterial strains were isolated from patients hospitalized at the Hospital of Trás-os-Montes and Alto Douro (Vila Real, Portugal) and in various departments of the North eastern

local health unit (Bragança, Portugal). Five Gram-negative bacteria were clinical isolated, *Escherichia coli* (isolated from urine), *Klebsiella pneumoniae* (isolated from urine), *Proteus mirabilis* (isolated from wound exudate), *Morganella morganii* (isolated from urine) and *Pseudomonas aeruginosa* (isolated from expectoration) and three Gram-positive *Listeria Monocytogenes* (isolated from cerebrospinal fluid), *Enterococcus faecalis* (isolated from urine), and methicillin-resistant *Staphylococcus aureus* (MRSA) (isolated from expectoration) were tested. To obtain the exponential growth phase, all these microorganisms were incubated in appropriate fresh medium at 37 ºC for 24 h before analysis.

Determination of MIC and MBC

The preparation of the stock solution with 20 mg/mL as the final concentration consisted in dissolving of the extracts initially in 5% (v/v) dimethyl sulfoxide and 95% of Mueller-Hinton Broth/Tryptic Soy Broth (MHB/TSB). Then, in duplicate, 190 μL of this solution was added to a well in a 96-well microplate, with subsequent addition of 90 μL of medium MHB or TSB were placed in the remaining wells. The concentration scale of samples (20 at 0.15 mg/mL) were obtained through a serial dilution. The final step was the addition of 10 μL of inoculum (standardized at 1.5×10^8 Colony Forming Unit (CFU) /ml) in all wells. Ampicillin and vancomycin were used as positive controls for *Enterococcus faecalis* and MRSA. Ampicillin and Imipenem were selected for *Listeria monocytogenes* and the five Gram- negative bacteria. The microplates were incubated at 37°C for 24 h. The detection of MIC of samples was obtained after adding 40 μL of 0.2 mg/ml p-iodonitrotetrazolium chloride and subsequent incubation at 37 °C for 30 minutes following the MIC detection. The wells that did not acquire color were used to obtain the MBC, which 10 μL of them was plated on solid medium, blood agar (7% sheep blood) and incubated at 37°C for 24 h.

3.3.3. Cytotoxicity activity

The cytotoxicity assay was performed using the sulforhodamine B (SRB) assay for the evaluation of the effects of the extracts in cell lines to precise a subtoxic concentration. Human adenocarcinoma cell line (CaCo2) obtained from European Collection of Cell Cultures (ECACC) CaCo2 cells was used, as well as a porcine liver non-tumor cell line. In brief, the two cell lines were maintained as adherent cell cultures in RPMI-1640 containing heat inactivated FBS (10%), penicillin (100U/mL), glutamine (2mM), and streptomycin (100μg/mL), incubated at 37 °C with 5% with CO_2 and humidified air. The cell lines were placed in 96-well microplates to which varying concentration (dilutions) of the extracts were added. Then, the plate was

incubated at 37 °C with 5% CO_2 for 48h, and directly after the incubation, an addition of 10% of earlier refrigerated trichloroacetic acid (100µl) was added to allow the fixation of the adherent cells, followed by another incubation at 4°C for 60 minutes. The last step consisted of the solubilization of the adhered cell lines with 10mM Tris (200µl), and measurement at 540 nm using the microplate reader (ELX800). The results were expressed in GI_{50} values (sample concentration inhibiting cell growth by 50%).

3.4. Quark Cheese Preservation

3.4.1. Incorporation of Arbutus unedo extracts (DM and UAE) as natural preservatives in Quark cheese.

After analysing the bioactive properties of *A. unedo* extracts obtained through DM and UAE, these extracts were incorporated in quark cheese to understand their preserving effect on this dairy product and compare their effects against a synthetic dairy food preservative (potassium sorbate) (**Figure 17**). All three preservatives (*A. unedo* from UAE, *A. unedo* from DM, and potassium sorbate) were each added to a lot of cheese at 0.1 g/100 g of cheese. A fourth lot of cheese was prepared, and no additive was added to it, serving as the negative control. The four lots were further divided in three, to have cheeses to analyse immediately after preparation (T0), after three days (T3) and after 8 days (T8). The samples were kept in air-tight containers until analysis and kept at a constant temperature of 4 ºC.

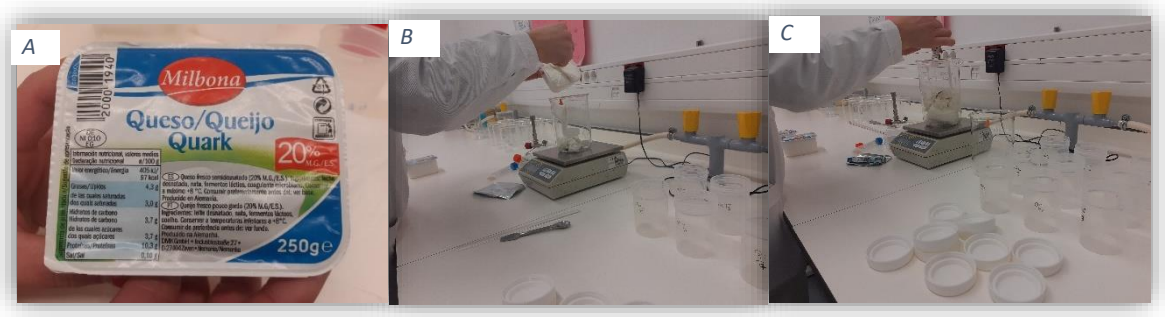


Figure 17: Incorporation process – **A)** Quark cheese; **B)** Weighing the cheese; **C)** Incorporation of the *A. unedo* extract.

3.4.2. Chemical analysis

3.4.2.1. Nutritional profile

Following the standard AOAC procedures (AOAC, 2016), the nutritional profile (moisture, proteins, fat, and ash) was determined. The nutritional profile was expressed in g/100g of fresh weight.

Moisture analysis

A sample of each cheese (2 g) was placed on the metal plate of a moisture analyser Adam Equipment company (model PBM 163, Oxford, USA). The equipment was heated, allowing the moisture in the cheese to evaporate. When a constant weight was reached, the moisture content was calculated using the following equation: % Moisture= (mi-mf)/ mi × 100, in which mi is the initial value, and the mf is the final value **(Figure 18).**

Figure 18: Moisture analysis – A) Weighing 2g of cheese on the balance; B) Evaporation of moisture; C) Final weight

Ash content

Ash content was calculated according to the AOAC 923.03 method, in which 250 mg of lyophilized cheese was weighed in porcelain crucibles and transferred to the muffle furnace **(Figure 19)** (Optic Ivymen System, N-8L, Barcelona, Spain) for incineration at 550º C for 5 h, which involves the burning of organic matter. After incineration, the percentage of total ash was expressed according to the equation: Ash= (mf/mi) ×100, which mf present the final mass and the initial mass represented by mi.

Figure 19: Muffle for sample incineration

Crude protein content

The AOAC 920.87 method was used for determining protein content **(Figure 20)**, in which the quantification of proteins is based on the amount of nitrogen (N) in the sample, with prior destruction of organic matter by means of a strong acid. The conversion factor 6.38 (N x 6.25) was used in the Macro-Kjedahl method for protein analysis. The cheese lyophilized samples (200 mg) were weighed and placed into digestion tubes, with addition of 15ml of sulfuric acid and two selenium discs (K_2SO_4 / $CuSO_4$) to catalyze the digestion. Then, the tubes were inserted in the digester which boiled the tubes at 400 ºC for 70 min. After cooling, the tubes were introduced in the Kjeldahl apparatus (Pro-Nitro-A model, JP Selecta, Barcelona), where an alkaline distillation and a titration were completed. Finally, the protein amount was determined by multiplying the conversion factor by the nitrogen amount read by the equipment.

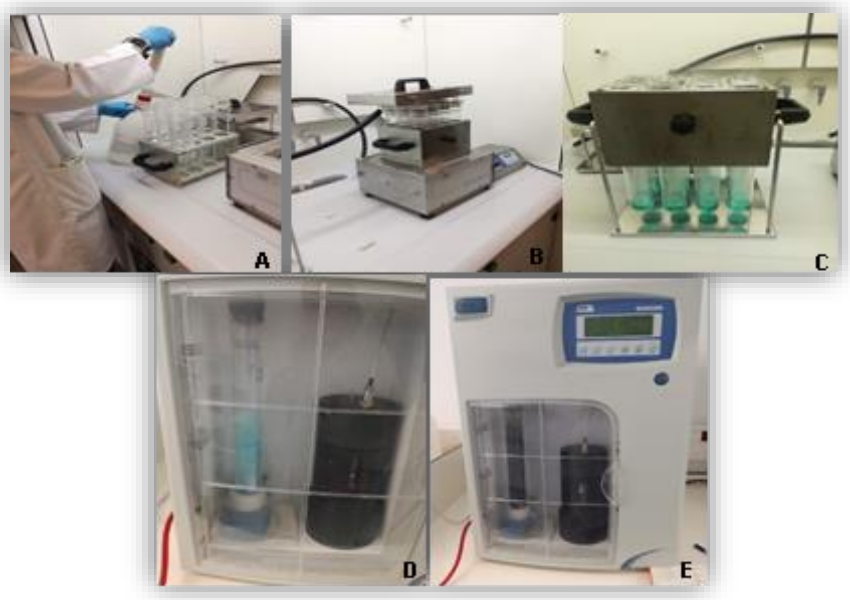


Figure 20: Crude protein analysis – A) Addition of sulfuric acid; B) Digestion; C) Sample cooling; D) Distillation; E) Titration

Crude fat content

The AOAC 920.85 method was used to determine the crude fat content, based on the extraction of fat **(Figure 21)** by petroleum ether in a Soxhlet apparatus. 2 g of lyophilised cheese samples were weighed and introduced in cartridge made of filter paper, covered with cotton. The cartridges were then placed in the equipment and extracted with petroleum ether for 6 hours at approximately 80 ºC. The obtained fat solution was placed in previous weighed tubes, which were left to evaporate and then re-weighed to allow the crude fat to be calculated by difference.

Figure 21: Fat extraction

Energy

Energy was calculated based on the following formula, approved by the European parliament and Council in 2011:

$$Energy\ (\text{kcal}/100\text{g}) = 4\ x\ [protein\ (g) + \text{carbohydrates}\ (g)] + 9\ x\ \text{fat}\ (g)$$

3.4.2.2. Organic acids

Lyophilized cheese sample were weighed (1 g) and placed in beakers covered with aluminium foil with 25 mL of metaphosphoric acid (4.5%). Then, the beakers were placed under magnetic stirring for 20 min **(Figure 22)**.

Figure 22: Extraction under magnetic stirring

The solutions were filtered through filter paper into tubes, transferred into 1.5 mL dark vials and filtered again through a HPLC nylon filter prior to injection in an ultra-fast liquid chromatograph (UFLC - Shimadzu 20A series, Shimadzu Corporation, Kyoto, Japan) coupled to a photodiode array diode detector (PDA) **(Figure 23)**. The elution solvent employed was sulfuric acid (3.6 mM) using a flow rate of 0.8 mL/min. The separation of the compounds relied on a SphereClone (Phenomenex, Torrance, CA, USA) reverse phase C18 column (250 mm x 4.6 mm, 5 μm, Phenomenex) thermostated at 35 ºC, in which the detection was carried out at 215 and 245 nm wavelengths. The identification as well as the quantification of organic acids was based on the commercial standards, retention times, and their respective standard calibration curves. The results were expressed in g/100 g of fresh weight (fw).

Figure 23: UFLC-DAD

3.4.2.3. Soluble sugars

To determine the sugar content in the cheese samples, 1g of the sample was mixed with 40 mL of 80% aqueous ethanol and 1 mL of melezitose (internal standard, IS, 25 mg/mL), then the mixture was placed in a thermostated water bath at 80 °C for 1 h 30 min with shaking every 15 min. After, the suspension was centrifuged (Centurion K24OR-2003 refrigerated centrifuge) at 15.000 g for 10 min and placed into a 250 mL round glass flask. Next, the ethanol was removed by means of a rotary evaporator (Buchi R-210 rotary evaporator, Flawil, Switzerland), the suspension was washed 3 times with 5 mL of ethyl ether to remove fat excess. Later, the aqueous phase was transferred into a tube and left in the oven overnight to remove any remaining ether **(Figure 24).**

Figure 24: Extraction of soluble sugars – **A)** Filtration; **B)** Ethanol evaporation; **C)** Washing

The samples were then diluted in distilled water in 5 mL volumetric flask and 1.5 mL was transferred to a vial for HPLC-RI analysis. The chromatographic separation was done with a Eurospher 100-5 NH_2column (4.6 × 250 mm, 5 µm, Knauer) operating at 30 °C (7971 R Graceoven). The mobile phase was acetonitrile/deionized water (70:30, v/v) at a flow rate of 1 mL/min (oven 7971 R Grace) **(Figure 25)**. The identification and quantification were performed through the comparison of the retention times corresponding to sample peaks with standard curve with the aid of Clarity 2.4 Software (DataApex, Prague, Czech Republic). The results obtained were expressed in g/100 g of fw.

Figure 25: HPLC-RI

3.4.2.4. Fatty acids

After the soxhlet extraction step that allowed to calculate the crude fat, the lipid fraction was used to determine the individual fatty acid profile. For this, the determination of fatty acids consisted of a transesterification process, performed with 5 mL of methanol: sulfuric acid: toluene 2: 1: 1 (v: v: v:) reagent, added to the fat for 12h at 50°C and 60 rpm. Following the esterification step, two different phases were obtained by adding 3 mL of distilled water. The fatty acid methyl esters (FAME) were recovered by adding 3 mL of diethyl ether, vortexed and extracted with a glass Pasteur pipette. The samples were filtered through a 0.2 µm nylon filter (Whatman), transferred to 1.5 mL vials for analysis using gas chromatography (DANI 1000, Contone, Switzerland) coupled with a flame ionization detection (GC-FID)/capillary column (**Figure 26**). Analysis was achieved with FID at 260 °C, a split splitless injector and a Zebron-Kame column (30m x 0.25mm id x 0.20 µm film thickness, Phenomenex, Torrance, CA, USA). The carrier gas flow rate (hydrogen) was maintained at 1.1 mL/min at 100 ºC. The fractional injection (1:50) was performed at 250 ºC. The identification and quantification of fatty acids was obtaining through the comparison of the relative retention times of fatty acid methyl ester peaks with the commercial standards. The results were treated and presented as a relative percentage for each fatty acid by the aid of CSW 1.7 software (DataApex 1.7, Prague, Republic Czech).

Figure 26: GC-FID

3.4.3. Physical parameters

3.4.4.1. External colour

The external color was analyzed for the cheese samples in distinct points with three repetitions, using a portable CR400 colorimeter from Konica Minolta (Chiyoda, Tokyo, Japan) **(Figure 26)**. The international Commission's Illumination (CIE), D_{65} illuminant with 8 mm aperture and 10º degrees of observation were used, which characterize the midday light in Europe. According to the colour space of the CIE L* a* b* of 1976, L* represented lightness (L = 0 black, L = 100 white), a* represented greenness-redness (-a = 0 green, + a = red), and b* represented blueness-yellowness (-b = blue; + b = yellow).

Figure 27: Portable CR400 colorimeter reading a sample

3.4.4.2. Texture

To perform the texture profile analysis, a Stable Micro Systems (Vienna Court, Godalming 191 UK) TA. XT Plus Texture Analyzer with a 30 Kg load cell was used, along with the P/45 45 mm aluminium cylinder probe, in order to comprehend the extract effects on the texture profile of the cheese. A standard texture profile analysis (TPA) was carried out, which intends to imitate human chewing by compressing the sample twice, managing to extract enough parameters for analysis, namely firmness, consistency, cohesiveness and work of cohesion **(Figure 27)**. The TPA was performed using a pre-test speed of 10 mm/s, a test speed of 5 mm/s and a post-test speed of 5 mm/s. The mode was set to strain 5% of the samples that were compressed twice for 5 seconds with additional 5 seconds timeout, and a trigger force of 3g.

Figure 28: Texture analysis

3.4.4.3. Water activity

The water activity "aw" determination was based on the dew point methodology with an absolute error of 0.003, at 20 °C for 5 min on the surface of each sample, using the activity measuring instrument AQUALAB 4TE (MeterGroup, München, Germany) **(Figure 27)**.

Figure 29: Water activity measurement

3.4.4.4. pH

The pH of the cheese samples was measured in three different points with a portable food pH meter 99161 (Hanna Instruments, Woonsocket, Rhode Island, USA) **(Figure 28)**.

Figure 30: pH measurement

3.4.4. Microbial load

3.4.4.1. Sample preparation

As described by **Carocho et al., 2019,** the preparation of cheese samples **(Figure 29**) was performed according to the procedures of the International Organization for Standardization (ISO) 6887-1:2003, in which 10 g of cottage cheese was added to 90 mL of peptone water (PW) in stomacher bags, and then homogenized in a stomach equipment (ECN 710-0873, Italy) for 1 min at 300 units. From the obtained suspension, the 10^{-1} to 10^{-12} dilutions were obtained in peptone water and analyzed in duplicate.

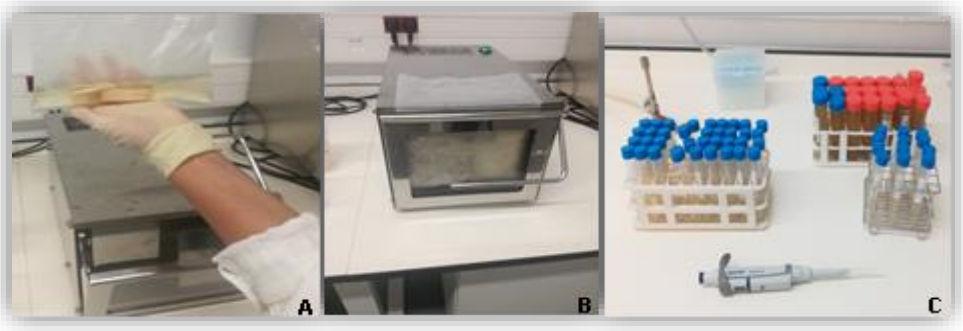


Figure 31: Sample preparation – **A)** Addition of peptone water; **B)** Homogenization; **C)** Serial dilutions

3.4.4.2. Microorganism analysis

Based on data regarding the most relevant microorganisms present in cheese, several ones were analyzed, namely enterobacteria, yeasts, molds, aerobic mesophiles, *Staphylococcus aureus* and psychrotrophic bacteria with aid of selective culture medium.

Aerobic Plate Count (APC): corresponding to the pour plate method, in duplicate (LOQ = 1 log CFU / g), 1ml of each prepared suspension was combined with 20 ml of plate agar (PCA). Subsequently and according to ISO 4833-2: 2013, the plates were incubated at 30 ºC for 72 h in an inverted position and after were counted.

Enterobacteria: corresponding to the pour plate method and in duplicate (LOQ = 1 log CFU / g), 1ml of each prepared suspension was combined with 20 ml of purple red bile lactose agar (VRBG). Subsequently and according to ISO 4832: 2006, the plates were incubated at 30 ºC for 48 h in an inverted position and after counted.

Psychrotrophic bacteria: corresponding to the pour plate method and in duplicate (LOQ = 1 log CFU / g), 1ml of each prepared suspension was combined with 20 mL of Man, Rogosa and Sharpe (MRS) agar. Then further 5 ml of medium was added to create anaerobiosis, after solidification of the medium. Subsequently and according to ISO 4832: 2006 (ISO 2006). The plates were then incubated at 22 ºC for 5 days in an inverse position and counted.

Yeasts and molds: corresponding to the pour plate method and in duplicate (LOQ = 1 log CFU / g), 0.2 mL of each suspension was distributed in Petri dishes containing 20 mL of Dicloran Rosa Bengala Cloranfenicol Base (DRBC) agar. After and according to ISO 21527-2: 2008, The plates were incubated at 25 ºC for 72 h for yeasts and 120 h for molds in an upright position, and then counted.

Staphylococcus aureus: corresponding to the pour plate method and in duplicate (LOQ = 1 log CFU / g), 0.2 mL of each suspension was distributed in Petri dishes containing 20 mL of Baird Parker (BP) agar. After and according to ISO 688-2:1999, the plates were incubated at 37 ºC for 24 h in an inverse position, and then counted.

Corresponding to the subsequent formula, the colonies count was expressed in colony forming units (CFU) per gram:

$$N = \frac{\Sigma c}{(\ v\ (n1 + 0.1n2)/d\ )}$$

In which:

N: number of colonies per g or mL of sample;

Σc: sum of the colonies in the counted plates;

V: Volume of the suspension used;

n1: number of plates counted in the first countable dilution;

n2: number of plates counted in the second countable dilution;

d: first countable dilution.

3.4.4. Statistical analysis

Throughout the whole document, all data was expressed as mean ± standard deviation. The optimization protocol was performed using Design Expert 11 (Stat-Ease, Minneapolis, MN, USA), and relied on the Response Surface Methodology using Central Composite Design and Box-Behnken models for the design of experiments followed by an optimization of the solid residue response.

Furthermore, after incorporating the preservatives in the cheese, to better understand the effects of these different preservatives (PT) and their storage time (ST), a two-way ANOVA with type III sums of squares using the SPSS Software, version 25 was used for the analysis. This multivariate general linear model treats the two factors, PT and ST as independent, thus allowing the effect of each one to be analyzed independently, providing more insight on their contribution towards the changes. If a significant interaction (<0.05) was recorded among the two factors (PT×ST), these were evaluated simultaneously, and some general conclusions and tendencies were extracted from the estimated marginal means (EMM). If there was no significant interaction (>0.05), each factor was evaluated independently using a Tukey's multiple comparison test when the means were homoscedastic, and a Tamhane's T2 for non-homoscedastic samples. Homoscedasticity was evaluated using a Levene's test. All analyses were carried out using a significance level of 0.05.

4. Results and Discussion

4.1. Extraction Optimization

4.1.1. Dynamic Maceration (DM) Extraction Optimization

Analyzing of the different runs, 4 major phenolic compounds were identified by HPLC-DAD-ESI/MS through their retention time, UV-VIS spectra and atomic mass, as seen on **Table 2**. Thus, the first peak was identified as being catechin, having a λ_{max} at 279 nm and a pseudomolecular ion $[M-H]^-$ at *m/z* 289, being positively identified according to it retention time, mass and UV-vis characteristics in comparison with the commercial standards. The second compound was tentatively identified as isorhamnetin-*O*-deoxyhexoside, according to the pseudomolecular ion $[M-H]^-$ at *m/z* 461 and MS^2 fragment released at *m/z* 315 ($[M-H-146]^-$, loss of deoxyhexoside moiety). Similarly, the third ($[M-H]^-$ at *m/z* 447) and forth compounds ($[M-H]^-$ at *m/z* 431) were tentatively assigned as quercetin-*O*-deoxyhexoside and luteolin-*O*-deoxyhexoside, respectively.

Table 2: Identification of the four phenolic compounds by HPLC-DAD-ESI/MS

Peak	Rt (min)	λmax (nm)	$[M-9-H]^-$ (*m/z*)	MS2 (m/z)	Tentative identification
1	6.6	279	289	245(199),205(39),179(12)	(-)-Catequin
2	12.29	349	461	315(100)	Isorhamnetin-*O*-deoxyhexoside
3	21.793	348	447	301(100)	Quercetin-*O*-deoxyhexoside
4	24.134	343	431	282(100)	Luteolin-*O*-deoxyhexoside

The first experimental design was performed for the DM of *A. unedo* using the Box-Behnken model which only considers points within the given range of the different factors. Thus, the three varying factors were extraction time, ranging from 10 to 60 minutes (F1), extraction temperature, which varied from 30 to 80 ºC (F2), and finally percentage of ethanol, ranging from 0 (only water) to 100% ethanol. The analysed responses included the solid dry residue of the extraction solution after complete evaporation of the solvent (R1), the quantity of catechin (C) (R2), isorhamnetin-*O*-deoxyhexoside (IOD) (R3), quercetin-*O*-deoxyhexoside (QOD) (R4), luteolin-*O*-deoxyhexoside (LOD) (R5) and the total amount of phenolic compounds detected in each extraction (R6) (**Table 3**). R1 to R6 were obtained by HPLC-DAD-ESI/MS analysis. The experimental design included three levels and 17 independent combinations (runs) and 5 replicates of the central position. The runs were executed randomly to avoid errors arising from variability of the observed responses.

The main objective of the optimization protocol of DM extraction was to obtain the highest yield of R1 (solid residue), based on the principle that a higher amount of solid residue would translate into more polyphenols, and thus, a higher preserving capacity. For this, an optimization analysis was carried out by applying an inverse transformation prior to applying a quadratic model which showed a non-significant lack of fit.

Table 3: Experimental design and responses for DM of A. unedo.

Run	Time (min)	Temperature (ºC)	Solvent (%)	R1 (mg/mL)	R2 (mg/mL)	R3 (mg/mL)	R4 (mg/mL)	R5 (mg/mL)	R6 (mg/mL)
1	10	30	50	10.64	0.331	0.196	0.190	0.141	0.859
2	35	55	50	11.94	3.353	3.032	4.963	1.384	12.732
3	35	55	50	12.46	3.199	3.075	5.228	1.372	13.874
4	35	30	0	8.0	0.641	0.271	0.273	0.156	1.341
5	60	80	50	13.78	2.849	3.816	4.610	1.470	12.744
6	35	55	50	12.98	3.241	3.606	0.870	1.346	13.063
7	35	80	50	9.72	0.351	0.138	0.143	0.000	0.632
8	35	30	100	2.08	1.162	4.019	4.884	1.391	11.457
9	10	55	0	8.10	1.636	1.085	2.173	0.492	5.386
10	60	55	0	3.64	3.374	2.153	2.468	0.981	8.976
11	60	30	50	11.28	1.024	3.468	4.744	1.447	10.685
12	35	80	100	7.90	0.551	0.297	0.843	0.361	2.052
13	35	55	50	12.86	3.857	3.011	4.653	1.817	14.338
14	35	55	50	12.74	3.788	3.556	5.111	1.388	13.843
15	10	55	100	2.52	3.212	3.696	5.505	1.668	14.082
16	60	55	100	8.46	0.356	0.204	0.194	0.145	0.900
17	10	80	50	12.90	2.896	3.705	4.243	1.336	12.180

R1: solid residue; R2: catechin-O-deoxyhexoside: R3: isorhamnetin O-deoxyhexoside; R4: quercetin O-deoxyhexoside; R5: luteolin O-deoxyhexoside; R6: total phenolic compounds. Highlighted in grey are the center point replicates.

Regarding R1, which was the response used for the subsequent extractions of *A. unedo* by DM to incorporate in the cheeses, the responses varied between 2.08 to 13.78 mg/mL. The optimal point, where the highest extractability yield is obtained was calculated through the maximize function of the Design-Expert program, attributing similar importance to all three factors (time, temperature and solvent) resulting in an optimal time of 60 minutes, at a temperature of 73 ºC and a percentage of solvent around 67% (**Figure 32a**). In **figure 32b**, the optimal point that yields the highest quantity of dry residue is represented visually. While time seems to increase the extractability, the higher amounts of ethanol over 70% do not, and thus, the best percentage of ethanol is set at 66%, and the temperature was also shifter to the highest limit, being the best point around 74 ºC.

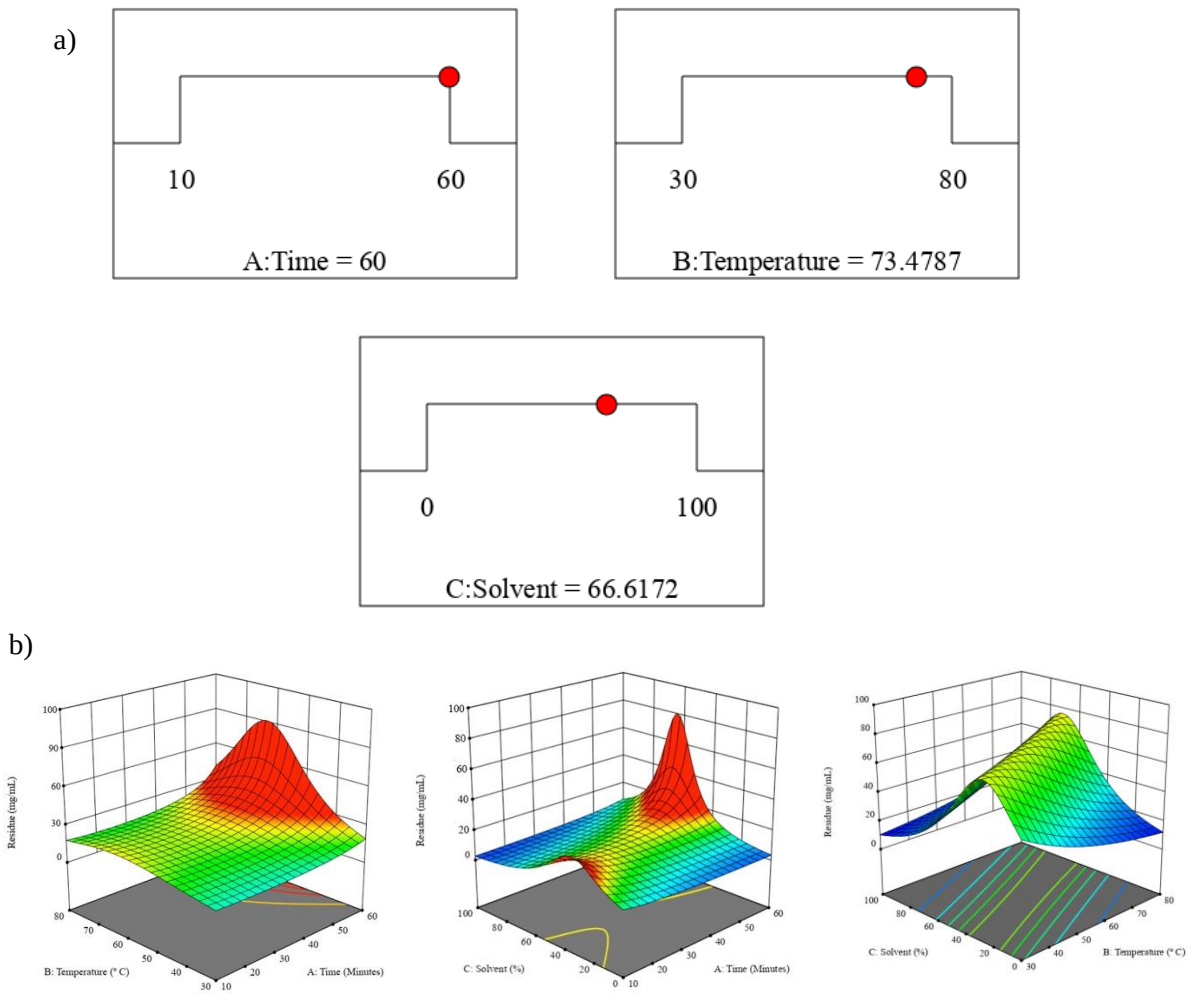
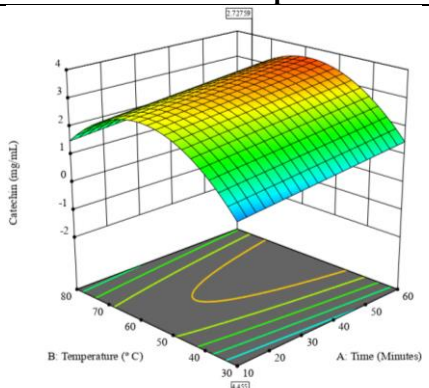
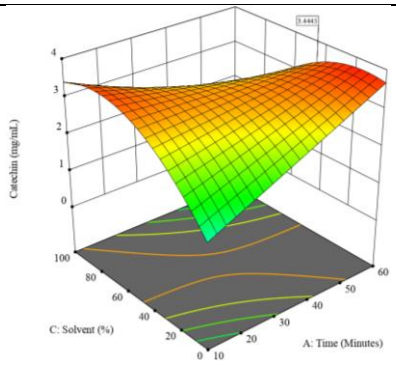
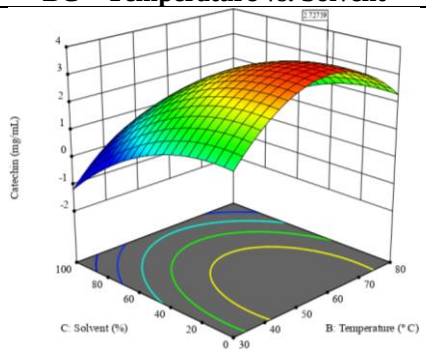
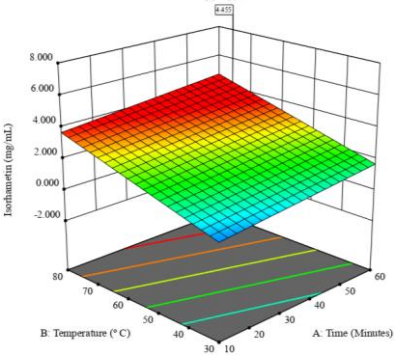
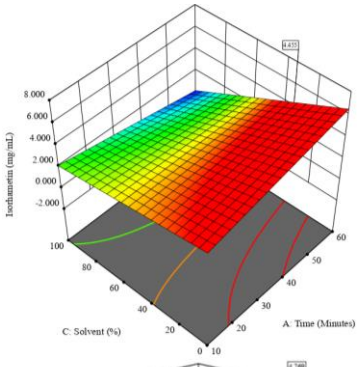
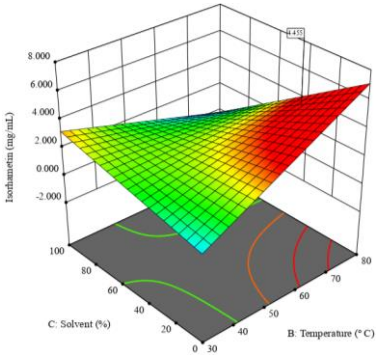
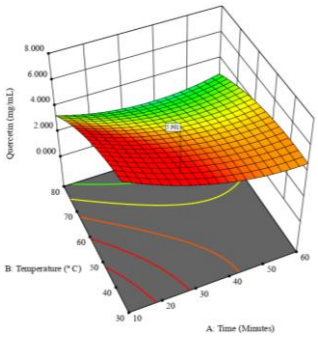
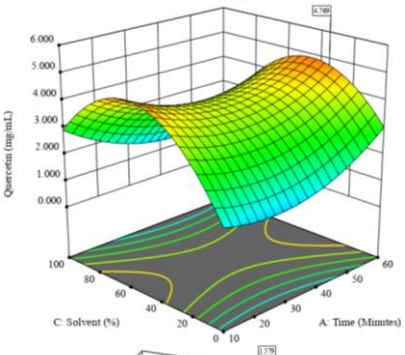
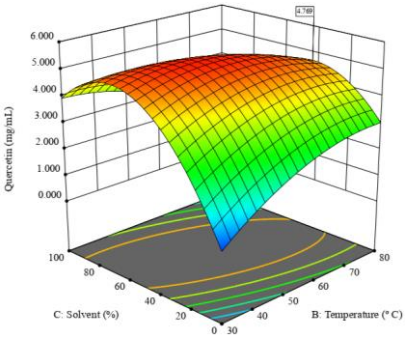
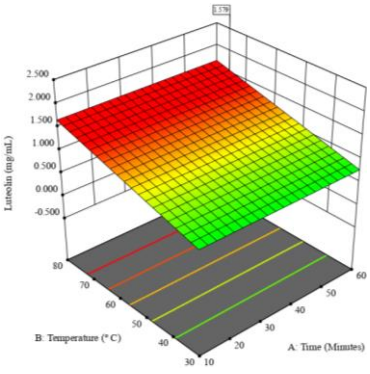
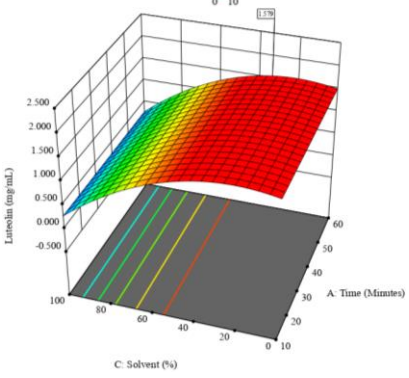
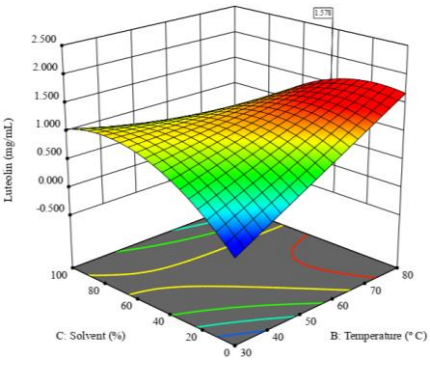


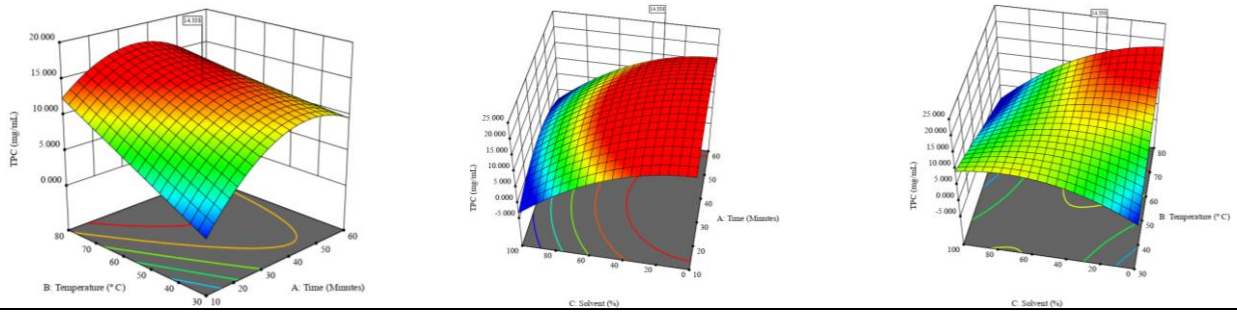
Figure 32: Graphical representation of the optimal point for the DM extraction – a) Representation of the optimal points. b) Representation of the 3D surface-response.

Table 4 shows 15 response surface 3D charts, 3 for each response. Column 1 represents time vs. temperature when the solvent is fixed at the optimal point. Thus, considering catechin, temperature near 50 to 60 ºC renders higher amounts of this phenolic compound, while the for the AC graph reveals that the extractability is promoted over time, but is not verified in graph AB. IOD extracted amount is clearly promoted by temperature while time is not a very important factor, revealing that is easily removed from the extract. Inversely, QOD is very influenced by ethanol concentration, being the best condition near 60%, while it is also promoted by lower temperature and short extraction time. LOD seems to show the most linear behavior, being promoted by higher temperature and low ethanol, while extraction time does not seem to have a high influence. Finally, for the total phenolic compounds (TPC), high temperature clearly promotes the quantity of molecules extracted, while time seems to have influence with the 30 to 40 minutes being the optimal point, although some conflicting results were sought for the ethanol percentage.

Table 4: Representation of the different response surfaces for the different responses in DM. Each 3D graph shows two of the three factors in the X axis relative to the response (Y axis) when the third response is fixed.

	AB – Time vs. Temperature	**AC – Time vs. Solvent**	**BC – Temperature vs. Solvent**
C			
IOD			
QOD			
LOD			

Total Phenolic Compounds (TPC)



4.1.2. Ultrasound Assisted Extraction (UAE) Optimization

The second experimental design was performed for UAE using the Central Composite Design, which allows for axial points to be placed outside the desired range. This model was chosen due to higher uncertainty pertaining to the extraction of *A. unedo* by ultrasound. Time and solvent were also used as varying factors, while temperature (used in DM optimization) was swapped for ultrasonic power, measured in watts, which ranged from 50 to 500 w. The analysed responses were the same as the ones used for DM (**Table 5**).

Table 5: Experimental design and responses for UAE of A. unedo

Run	Time (min)	Power (w)	Solvent (%)	R1 (mg/mL)	R2 (mg/mL)	R3 (mg/mL)	R4 (mg/mL)	R5 (mg/mL)	R6 (mg/mL)
1	5	500	50	11.22	1.867	0.490	0.675	0.351	3.384
2	11.25	50	100	0.82	1.961	1.943	2.762	1.097	7.764
3	5	500	100	2.72	0.417	0.179	0.219	0.158	0.975
4	30	387.5	100	1.94	1.434	0.508	0.630	0.242	2.816
5	23.75	162.5	100	0.6	0.433	0.245	0.195	0.143	1.017
6	17.5	275	50	12.06	2.169	1.445	2.273	0.617	6.506
7	23.75	500	25	10.38	3.303	1.978	2.733	0.784	8.799
8	23.75	500	25	9.5	2.362	1.763	2.592	0.824	7.543
9	17.5	500	75	10.62	0.343	0.175	0.207	0.164	0.890
10	17.5	275	50	10.02	0.420	0.210	0.220	0.141	0.993
11	17.5	275	50	13.06	0.352	0.139	0.000	0.000	0.491
12	30	275	0	7.58	0.705	0.607	0.746	0.423	2.482
13	30	50	75	2.64	1.4	0.7	1.000	0.99	4.000
14	17.5	50	0	2.1	2.017	1.421	2.220	0.636	6.296
15	5	387.5	0	9.8	2.910	0.472	1.238	0.464	5.085
16	5	50	0	4.38	1.235	0.309	0.705	0.206	2.456
17	5	50	50	2.7	0.426	0.148	0.150	0.000	0.725
18	17.5	275	50	11.88	1.441	0.834	1.288	0.487	4.052
19	30	50	75	2.72	0.591	0.522	0.507	0.200	1.822
20	17.5	275	0	7.74	0.286	0.138	0.143	0.000	0.567

R1: solid residue; R2: catechin: R3: isorhamnetin; R4: quercetin; R5: luteolin; R6: total phenolic compounds. Highlighted in grey are the center point replicates.

The optimal point, where the highest extractability yield is obtained was calculated through the maximize function of the Design-Expert program, attributing similar importance to all three factors (time, power and solvent) resulting in an optimal time of 12 minutes, using 402 watts of ultrasound power, at 36% ethanol (**Figure 33a**). Clearly, due to the power of the ultrasound probe, time was the least important factor.

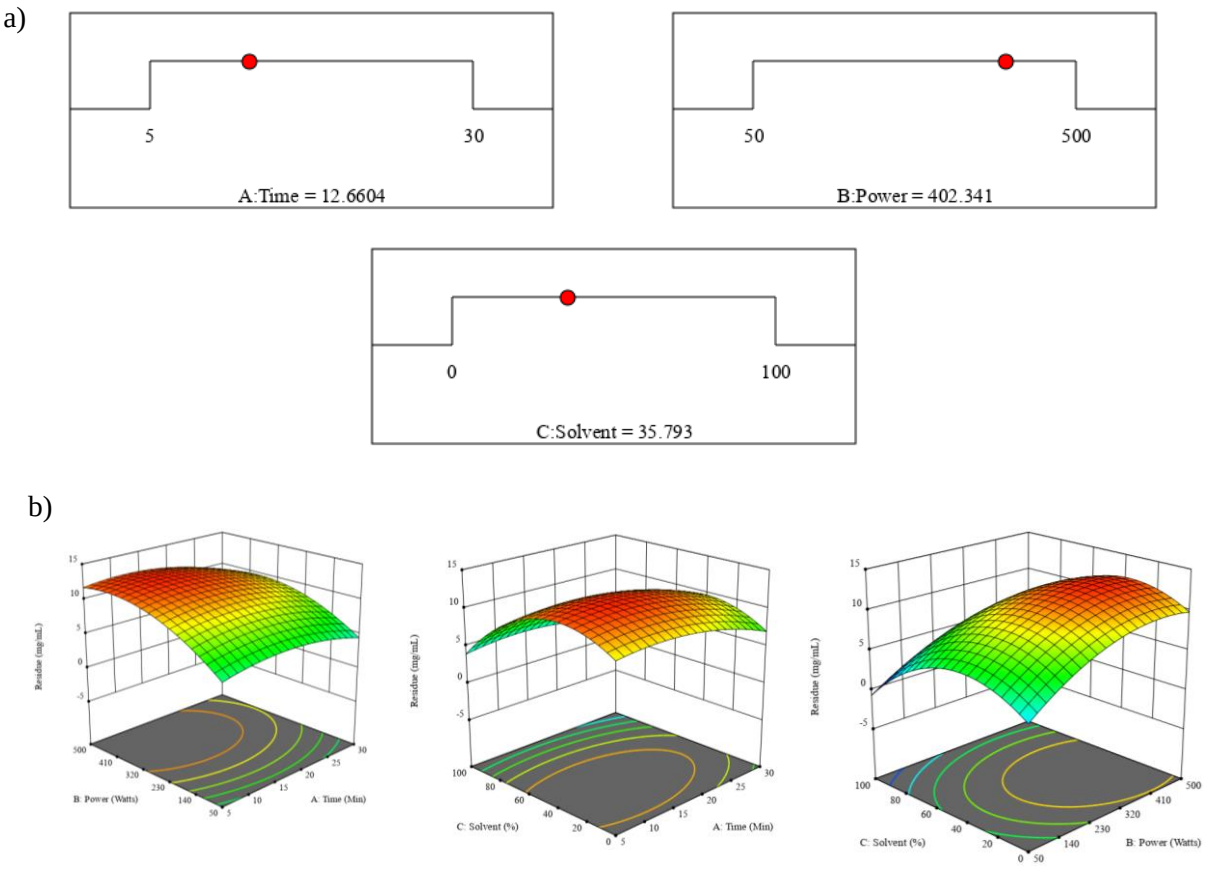


Figure 33: Graphical representation of the optimal point for the UAE – a) Representation of the optimal points. b) Representation of the 3D surface-response.

In **figure 33b**, the optimal point that yields the highest quantity of dry residue is represented visually, where it is clearly visible in the left graph the highest yield (red zone) close to the 400 watts of power, as well as the general extraction increase tendency with ethanol at a concentration of 30% in the center graph and a maximum extractability in the first 15 minutes of extraction. **Table 6** shows the 15 response surface 3D charts for the four most abundant individual phenolic compounds which had been identified by mass spectrometry in DM extraction. Overall, the UAE showed lower differences in extractability when compared to DM, visible through the lower number of red regions which represent higher extractability as

well as a higher number of planes rather than three-dimensional response surfaces. This could be due to an unbalance in the factors, where one has a clear higher influence than the others. In this case the unbalance could be due to a very high ultrasonic power. Still, considering catechin, its extractability is promoted by higher ultrasound wattage and lower amounts of ethanol while time did not seem to excerpt a significant difference. Contrarily, longer time increases the extractability of IOD along with power, while the concentration of solvent has lower impact. Considering QOD, power and solvent at maximum promoted the extractability of this polyphenol. LOD and TPC seemed to be the responses that least showed interaction among the three factors, with lower amounts of ethanol promoting luteolin's extractability as well as TPC. Previous studies have shown that for *A. unedo* fruits, dynamic maceration proved to extract more bioactive molecules than the ultrasound extraction and also higher amounts of dry residue, which are confirmed in this work for the leaves (**Albuquerque et al., 2018; López et al., 2018**).

Table 6: Representation of the different response surfaces for the different responses in UAE. Each 3D graph shows two of the three factors in the X axis relative to the response (Y axis) when the third response is fixed.

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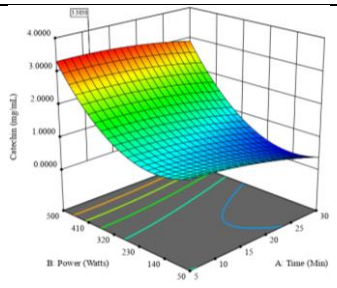
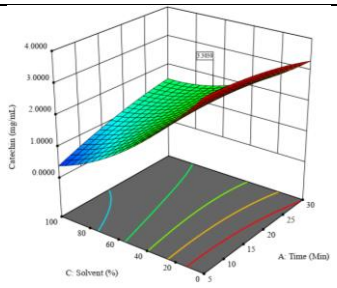
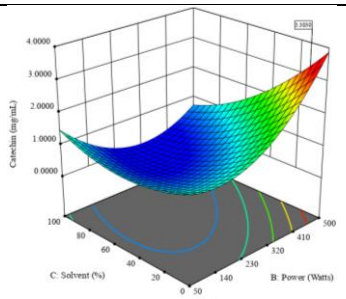
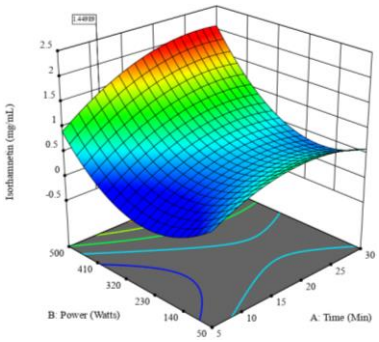
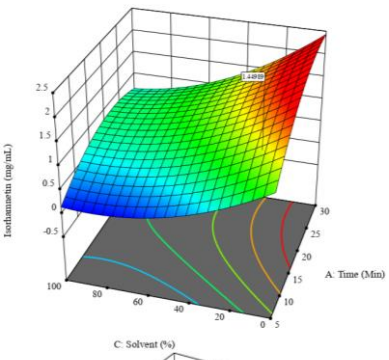
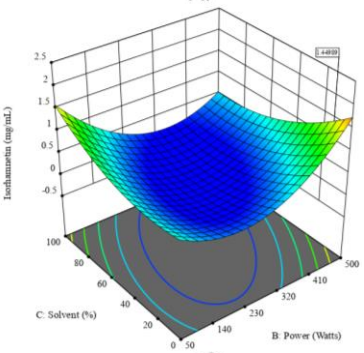
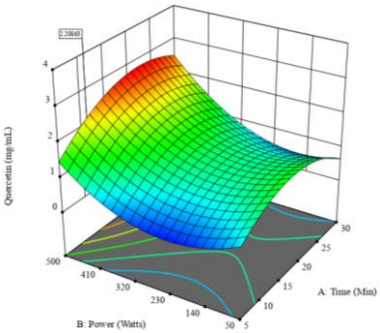
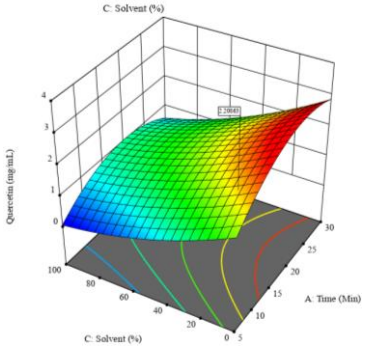
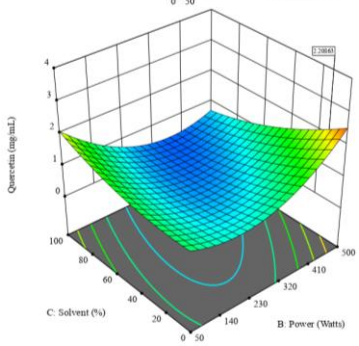
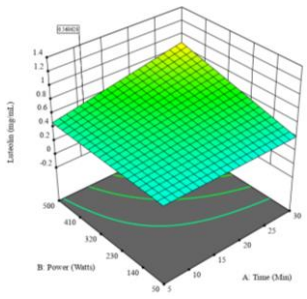
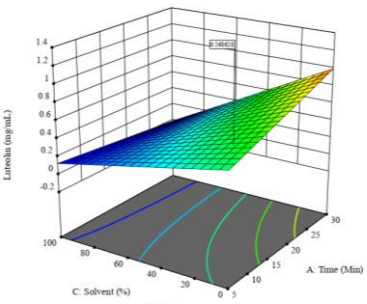
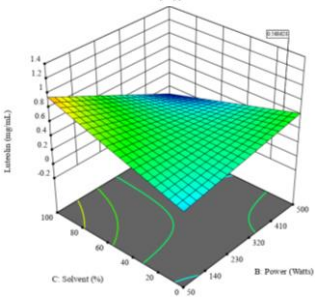
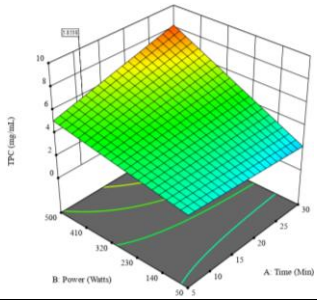
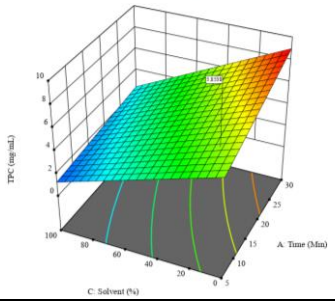
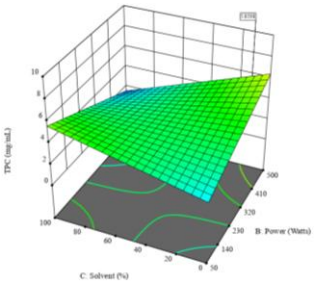
C			
IOD			
QOD			
LOD			
TPC			

4.2. Analysis of Extracts at Optimal Points

Having obtained the optimal extraction points for both the dynamic maceration and ultrasound assisted extraction, an extract was obtained at those optimal points and further characterized in terms of antioxidant and antimicrobial activities, as well as an analysis of their cytotoxicity.

4.2.1. Antioxidant Activity

4.2.1.1. TBARS assay

The antioxidant activity was analysed through the TBARS assay, and the results are displayed in **Table 7**. Considering both optimal points and the fact that the results are expressed in EC_{50} values (value that inhibits 50% of the oxidation of cells), the dynamic maceration showed a higher antioxidant activity, showing the lowest EC_{50} values, namely 140 µg. These results are in agreement with previous reports pointing out the good antioxidant capacity of leaves of *A. unedo* (**Pabuçcuoğlu et al; Mendes et al., 2011**).

Table 7: TBARS assay (EC50 values µg/mL)

	Dynamic Maceration	Ultrasound Assisted Extraction
TBARS	140±1	230±11

4.2.1.2. Cellular antioxidant activity

Regarding the cellular antioxidant assay, both samples revealed capacity to inhibit the cellular oxidation. The values of inhibition are shown in **Figure 34**, which shows the inhibition of the negative control where 100% of oxidation was achieved.

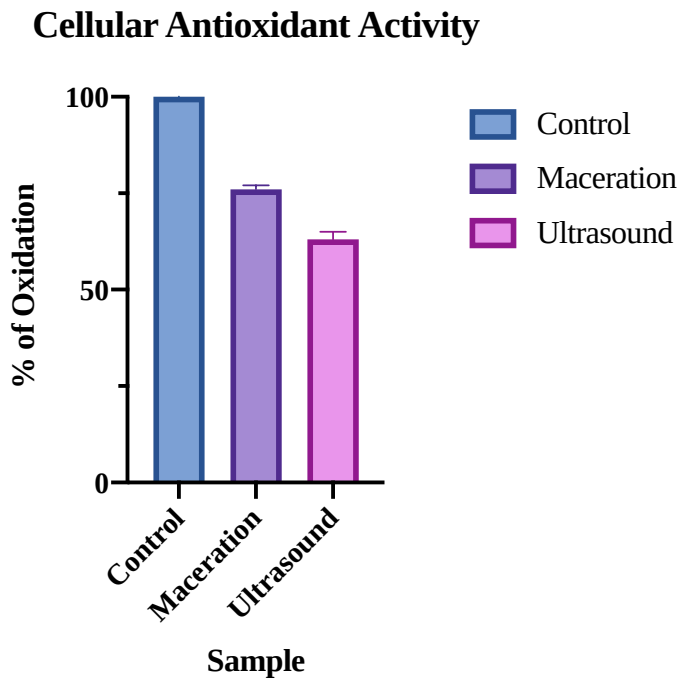


Figure 34: Comparative graph of cellular antioxidant activity of both samples

Both *A.unedo* samples revealed antioxidant capacity for the maximum tested concentration of 2000 µg/mL. The ultrasound sample revealed the highest potential by reducing the oxidation in 37±2% (63% of oxidation), while the maceration sample was able to reduce the oxidation in 24±1% (76% of oxidation). These results seem to be the inverse of the TBARS assay, where the maceration showed a better antioxidant potential. While this could seem strange, it is normal due to the different antioxidant mechanisms that each method detects. Still, both extracts showed antioxidant activity which can contribute towards the preserving capacity of the extracts when incorporated in foods.

4.2.2. Antimicrobial Activity

The antimicrobial activity was analyzed through the microdilution method, allowing for the determination of the minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC). **Table 8** shows the results of the antimicrobial activity.

Table 8: MIC and MBC values of the antimicrobial activity of the two extracts.

	DM		**UAE**		**Ampicillin (20mg/mL)**		**Imipenem (1mg/mL)**		**Vancomycin (1mg/mL)**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Gram-negative bacteria										
Escherichia coli	2.5	>20	10	>20	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Klebsiella pneumoniae	10	>20	>20	>20	10	20	<0.0078	<0.0078	n.t.	n.t.
Morganella morganii	10	>20	20	>20	20	>20	<0.0078	<0.0078	n.t.	n.t.
Proteus mirabilis	10	>20	>20	>20	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Pseudomonas aeruginosa	>20	>20	>20	>20	>20	>20	0.5	1	n.t.	n.t.
Gram-positive bacteria										
Enterococcus faecalis	2.5	>20	10	>20	<0.15	<0.15	n.t.	n.t.	<0.0078	<0.0078
Listeria monocytogenes	10	>20	10	>20	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
MRSA	2.5	>20	2.5	>20	<0.15	<0.15	n.t.	n.t.	0.25	0.5

n.t. – not tested

Considering **Table 8** DM and UAE extracts revealed activity against some of the bacterial strains. DM showed the most promising results, with inhibition capacity of all the bacteria, except *P. aeruginosa*. For this extract, the most sensitive bacteria were *E. coli*, *E. faecalis* and *MRSA* with MIC values of 2.5 mg/mL. On the other hand, the UAE extract presented MIC values of 10 mg/mL against the majority of the bacteria, except for MRSA that revealed a MIC of 2.5 mg/mL. It is important to highlight that these extracts presented interesting inhibition of MRSA, which is multi resistant bacteria. Still, this analysis was carried out to understand if *A. unedo* could have interesting application in inhibiting these pathogenic bacteria. Overall, the DM extraction showed better results when compared to UAE.

4.2.3. Cytotoxicity assay

Both samples, maceration and ultrasound extracts revealed no toxicity for the normal cell line PLP2 (Porcine Liver Primary Culture) and CaCo2 at the maximum tested concentration of 2000 µg/mL, further ruling the use of *A. unedo* leaves as safe to be used in food.

4.3. Proximate composition

After analysing both extracts and extracting them in the optimal points, they were incorporated in different lots of quark cheese, which were analysed during 8 consecutive days, being analysed after incorporation, at the third and eighth day. Then, a two-way ANOVA was used to interpret the results, resulting in the following tables. **Table 9** is divided in two sections,

the upper represents the storage time (ST) but, included on each day all different types of the preservatives, and, at the bottom, for each type of preservative extract, the three analysis times are also included. When each of the factor can be analysed independently (*p*-value ST × PT > 0.05), the classification is made using post-hoc testes (Tukey's test for homoscedastic samples and Tahmane T2 for non-homoscedastic ones); however, when *p*-value ST × PT < 0.05, then, no classification can be performed and thus, for some cases, only general trends can be obtained through the estimated marginal means plots (EMM).

The nutritional profile, along with the energy values of the quark cheeses incorporated with the different preservatives are presented in **Table 9**. After moisture, proteins are the most prevalent nutrient, followed by carbohydrates and crude fat, making these cheeses quite desirable for modern diets due high protein content and low fat and carbohydrates. Regarding the effect of each factor (time and preservative), for moisture, proteins and carbohydrates, a significative interaction was sought and thus, although no significative changes were detected over time and for all the incorporated preservatives, their influence was limited. For carbohydrates, the influence of each factor was determined, showing that over time there was a decrease in carbohydrates, with significative difference from T0 to T3, which could be explained by the consumption of lactose by lactic acid bacteria present in the cheese.

Table 9: Nutritional profile of the quark cheeses incorporated with the different preservatives, represented in g/100 g of fresh weight.

		Moisture	**Proteins**	**Crude Fat**	**Carbohydrates**	**Ash**	**Kcal**	**Kj**
Storage Time (ST)	0 Days	78.0±0.7	10.0±0.6	4.3±0.3	7.0±0.2^{b}	0.8±0.2^{a}	107±4^{b}	448±16^{b}
	3 Days	78.1±.05	11.3±0.9	3±1	6.6±0.5^{a}	0.8±0.1^{a}	100±7^{a}	420±10^{a}
	8 Days	78.4±0.5	11.1±0.2	4.2±0.2	5.7±0.6^{a}	1.0±0.2^{b}	105±3^{a}	439±5^{a}
***p*-value (n=3)**	Tukey's test	0.361	0.011	0.109	0.001	<0.001	0.027	0.027
Preservative Type (PT)	Control	78.0±0.5	9.9±0.6	4±1	7±2^{b}	0.9±0.1^{a}	104±7	437±30
	Maceration	78.4±0.8	10.2±0.7	4.0±0.8	6±1$^{a, b}$	0.89±0.07$^{a, b}$	102±5	429±22
	Ultrasound	78.2±0.6	11.1±0.2	4.1±0.2	5.5±0.3^{a}	1.0±0.3^{b}	103±3	433±13
	Potassium Sorbate	78.1±0.4	11.2±0.4	4±1	6±1$^{a, b}$	0.7±0.2^{a}	103±6	431±25
***p*-value (n=12)**	Tukey's test	0.401	<0.001	0.740	0.008	0.001	0.870	0.870
ST×PT (n=36)	*p*-value	0.895	0.007	0.357	0.135	0.076	0.470	0.470

In each row, different letters represent statistical significative differences with a significance level of 0.05. The SD were calculated from results obtained in different conditions, and thus should not be considered as precision measurements, but rather an interval of values.

Furthermore, for the ash content, a slight increase over time with significant statistical difference from T3 to T8 was sought, while also showing significant statistical difference between the preservatives, although very slight variations were sought. Finally, in terms of energy values, time showed a reduction in the energy values, with significant differences being sought from T0 to T3. Overall, the different preservatives did not influence the nutritional

quality of the cheeses, which is expected from food additives. Furthermore, longer storage periods should be further researched to understand the influence over a longer period. Still, due to the high moisture, quark cheese has a very limited storage time which could be improved by these natural preservatives.

4.4. Individual molecules

4.4.1. Fatty acids

Regarding the individual profiles of specific molecules found in the cheeses preserved with the different additives over the 8 days, the fatty acids arise as some of the most important. This relevance is due to the changes sought in unsaturated fatty acids over time that tend to lose their instaurations due to oxidation mechanisms. Thus, in **Table 10**, the profile in relative percentage of the fatty acids is shown. Only the fatty acids over 1% of relative percentage are shown in the table, where it is clear that the most abundant fatty acid is C16:0 (palmitic acid), followed by C18:1 (oleic acid). Overall, nine fatty acids showed values over 1 % with the saturated fatty acids (SFA) showing about 78 to 83% while the monounsaturated fatty acids (MUFA) representing about 16 to 21%. Regarding the effects of the preservatives and storage time, no significant changes were sought for the cheeses, except for C14:0 (myristic acid), in which the different preservatives showed a higher influence than the storage time. The control cheese and the one preserved with the ultrasound extract showed the lowest amount. Overall, no drastic changes were observed for the fatty acids profile, in accordance with what is expected for a preservative. Still, once again, eight days might not be enough time to allow the degradation of enough quantities of fatty acids to be observable.

Table 10: Fatty acid profile of the quark cheeses, presented in relative percentage.

		C4:0	C6:0	C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	SFA	MUFA
Storage Time (ST)	0 Days	8.5±0.9	4.1±0.8	2.5±0.3	6±1	4.4±0.5	11±1	33±1	10.8±0.9	15±1	82±2	17±2
	3 Days	8±2	4.0±0.4	2.4±0.4	5±1	4.5±0.3	11±1	33±1	11.2±0.4	16±2	82±3	18±2
	8 Days	8±2	4.1±0.6	2.0±0.2	5±1	4.3±0.5	11±1	33±1	11.4±0.5	15±2	81±3	18±2
***p*-value (n=3)**	Tukey's test	0.014	0.473	<0.001	<0.001	0.415	0.829	<0.001	0.001	0.136	<0.001	0.005
Preservative Type (PT)	Control	9.6±0.2	3.5±0.5	2.3±0.6	6.3±0.7	4.4±0.3	10.8±0.1^{a}	33.6±0.4	11.6±0.3	14.6±0.3	83±1	16.4±0.5
	Maceration	6±1	3.9±0.5	2.2±0.2	4.7±0.2	4.8±0.2	12.3±0.3^{b}	32±1	10.4±0.8	18.6±0.8	78±1	21.2±0.8
	Ultrasound	8.9±0.5	4.4±0.4	2.6±0.4	6.7±0.4	4.0±0.2	10.4±0.4^{a}	33.2±0.7	11.2±0.5	14.3±0.4	84.4±0.8	16.5±0.2
	Potassium Sorbate	8.1±0.7	4.6±0.1	2.1±0.2	4.1±0.4	4.4±0.6	12.4±0.2^{b}	34.5±0.4	11.4±0.2	14.8±0.1	82.7±0.6	16.8±0.2
***p*-value (n=12)**	Tukey's test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
ST×PT (n=36)	*p*-value	<0.001	0.004	<0.001	<0.001	0.140	0.446	<0.001	0.016	0.001	<0.001	<0.001

In each row, different letters represent statistical significative differences with a significance level of 0.05. The SD were calculated from results obtained in different conditions, and thus should not be considered as precision measurements, but rather an interval of values.

4.4.2. Organic acids and soluble sugars

Table 11 shows the profile in organic acids and soluble sugars, both obtained through HPLC detection. Four organic acids were detected, namely oxalic, shikimic, lactic and citric acid, with the latter being the most abundant. Overall, the profile in organic acids did not show any change over the 8 days of storage, regardless of the preservative used, showing a significant interaction among the two factors. Inversely, the two detected soluble sugars, glucose and lactose did show significant changes. In the case of these sugars, the preservatives showed a significant effect on the profiles, while storage time showed minimal effects.

Table 11: Organic acid and soluble sugar profile of the cheeses, expressed in g/100 g of dry weight.

		Oxalic acid	Shikimic acid	Lactic acid	Citric acid	Glucose	Lactose
Storage Time (ST)	0 Days	0.05±0.02	0.006±0.004	48±15	1.6±0.6	2.0±0.6	17±4
	3 Days	0.04±0.03	0.004±0.002	46±7	1.5±0.6	1.8±0.4	16±3
	8 Days	0.02±0.01	0.005±0.001	52±3	1.9±0.7	2.0±0.6	17±4
***p*-value (n=3)**	Tukey's test	<0.001	<0.001	<0.001	<0.001	0.084	0.211
Preservative Type (PT)	Control	0.05±0.02	0.0047±0.0001	56±4	2.2±0.1	1.9±0.2^{b}	17±2^{b}
	Maceration	0.05±0.02	0.009±0.003	54±4	2.08±0.09	1.7±0.1$^{a, b}$	14.8±0.9$^{a, b}$
	Ultrasound	0.02±0.02	0.004±0.001	47.8±0.8	0.8±0.2	2.7±0.5^{c}	22±3^{c}
	Potassium Sorbate	0.026±0.001	0.002±0.001	37±13	1.5±0.5	1.5±0.1^{a}	13.7±0.9^{a}
***p*-value (n=12)**	Tukey's test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
ST×PT (n=36)	*p*-value	<0.001	<0.001	<0.001	<0.001	0.208	0.181

In each row, different letters represent statistical significative differences with a significance level of 0.05. The SD were calculated from results obtained in different conditions, and thus should not be considered as precision measurements, but rather an interval of values.

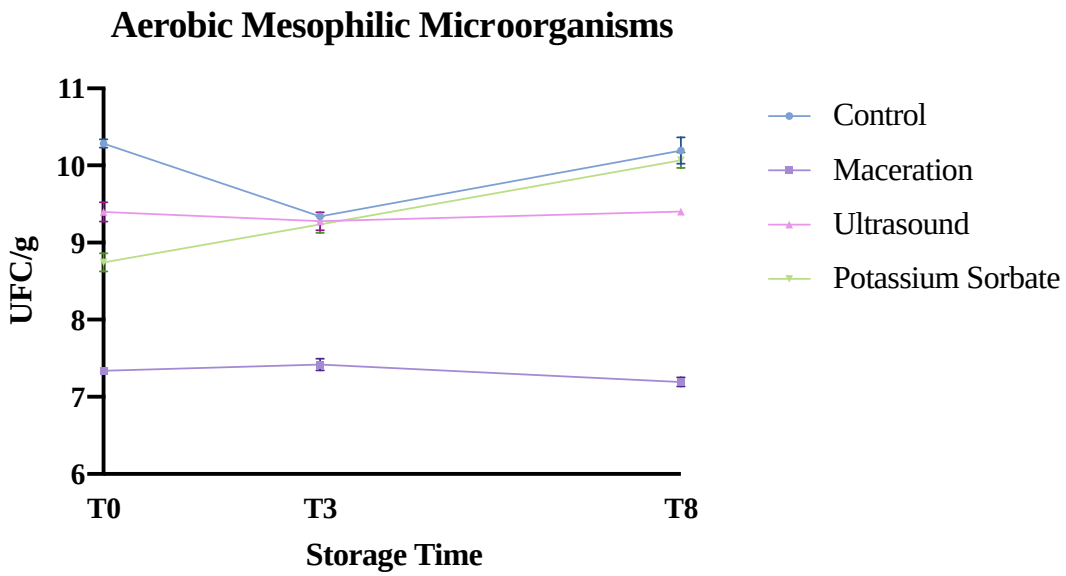
The cheeses incorporated with potassium sorbate and the maceration extracted *A. unedo* showed the lowest amount of glucose and lactose, followed by the control cheese, showing significant difference. The control sample showed and average of 17 g/100 g of lactose and 1.9 g/100 g of glucose, statistically lower than the values obtained for the ultrasound extract incorporated cheese. These high values sought for sugar may be due to higher extraction of these molecules during the ultrasound extraction, and thus contributed to a higher quantity of overall sugars in the cheese. Still, the maceration extract did not seem to alter the profile in any manner for either the organic acids or soluble sugars.

4.5. Microbial load

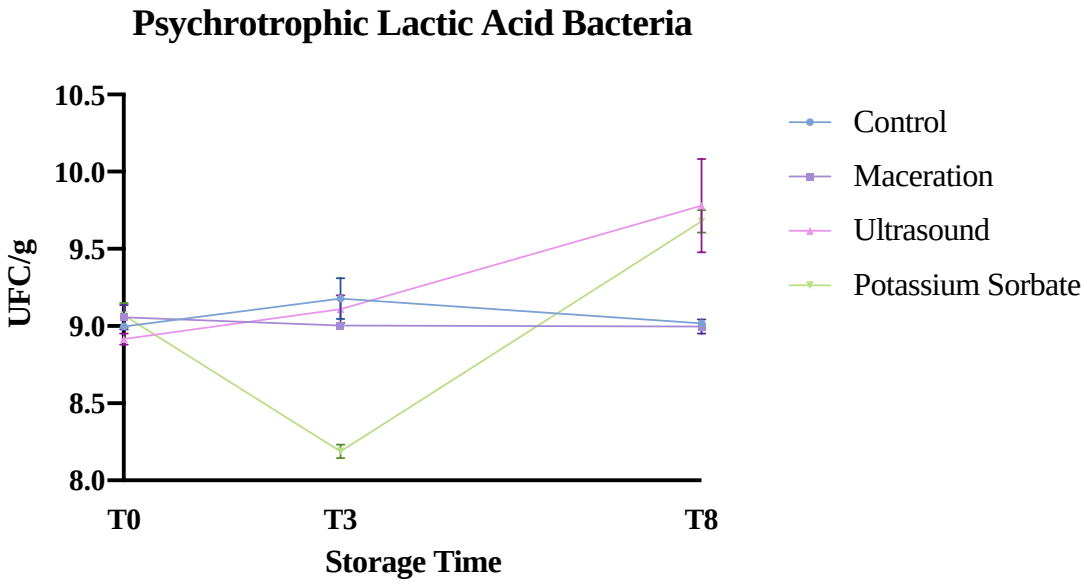
Figure 35 shows the three graphs sought for the microbial load, including the aerobic mesophilic microorganisms (AMM), the psychrotrophic lactic acid bacteria (PLAB) and the yeasts (Y), since *S. aureus* was not detected in any of the cheeses. Considering AMM (**Figure 35a**), at T0 the cheese incorporated with macerated extract showed significantly less UFC,

while the control sample showed the highest value, over 10 UFC/g. Over time, the cheese with the maceration of *A. unedo* did not show statistically significant changes, maintaining the same load of these microorganisms. Interestingly, although the cheese with ultrasound showed the same constant behaviour over time, although at a significantly higher microbial load was verified when compared to the maceration incorporated cheese. The cheeses preserved with potassium sorbate showed a statistically significant increase over time while the control cheese did significantly reduce the UFC from T0 to T3 but increased from T3 to T8. Overall, maceration, due to maintaining a constant lower value of UFC/g over the 8 days of storage was the best preserving extract, while potassium sorbate was the less efficient for these microorganisms by showing no statistical differences at T8 with the control cheese. Considering the graph of the PLAB (**Figure 35b**), no statistical differences could be sought at T0, where all cheeses showed a UFC/g of around 9. Then, from T0 to T3, the cheese preserved with potassium sorbate significantly reduced the UFC/g while the control cheese and the one incorporated with the maceration maintained the same value, with no statistically significant differences. Finally, at T8, the potassium sorbate preserved cheeses drastically increase their UFC/g and showed no statistically significant differences from the control sample, which had consistently been increasing is bacterial load over the 8 days. Inversely, the natural extracts maintained their constant values from T3 to T8, showing no statistical differences between each other. Overall, the natural preservatives which did not significantly alter over time seem to better preserve the cheese against these bacteria, which, if left to grow freely will convert all lactose to lactic acid, thus changing the sensorial qualities of the cheese. Finally, for the Y (**Figure 35c**), a very homogenous behaviour was sought for all quark cheeses except for the control sample. This latter sample significantly increased over the 8 days. Although a very slight decrease over time was sought for the incorporated cheeses, the one incorporated with the maceration of *A. unedo* has significant but slight reduction when compared to the other two from T0 to T3 and to T8 showing a shallow yet better preservation against yeasts. Considering all the microorganisms, the quark cheese incorporated with the maceration showed better preserving capacity when compared to the ultrasound incorporated cheese, and a much better effect when compared to the cheese preserved with potassium sorbate.

a)


Aerobic Mesophilic Microorganisms
11
10
9
8
7
6
UFC/g
T0
T3
T8
Storage Time
Control
Maceration
Ultrasound
Potassium Sorbate

b)


Psychrotrophic Lactic Acid Bacteria
10.5
10.0
9.5
9.0
8.5
8.0
UFC/g
T0
T3
T8
Storage Time
Control
Maceration
Ultrasound
Potassium Sorbate

c)

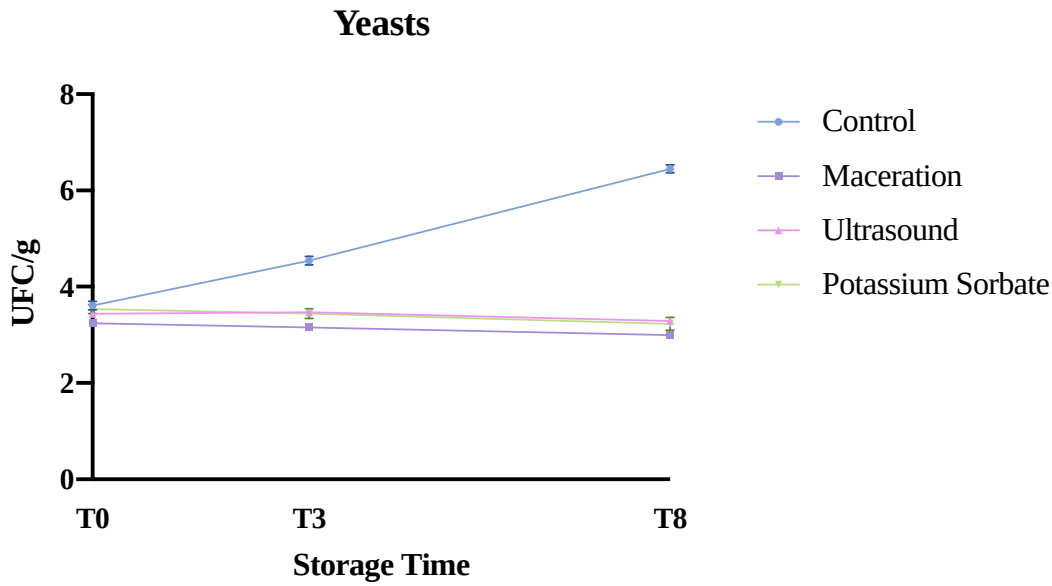

Yeasts
8
6
4
2
0
UFC/g
T0
T3
T8
Storage Time
Control
Maceration
Ultrasound
Potassium Sorbate

Figure 35: Microbial load of the cheese over the 8 days: **A)** Aerobic mesophilic microorganisms; **B)** Psychrotrophic lactic acid bacteria; **C)** Yeasts (Y)

4.6. Physical properties

The physical properties of foods are paramount to understand the effects that any preservative or ingredients has on food. Thus, the texture dimensions, external color, water activity and pH were analysed for all cheeses, show in **Table 12**.

Table 12: Texture, color profiles, water activity and pH of the cheese samples preserved with extracts over the storage time

		Firmness	Consistency	Cohesiveness	Work of Cohesion	L*	a*	b*	aW	pH
Storage Time (ST)	0 Days	365±25	458±38^{a}	-263±20	-334±23	92±4	-2.3±0.4	11.1±0.7	0.986±0.001	4.657±0.006
	3 Days	420±18	525±49$^{a, b}$	-282±20	-373±44	80±16	-2±1	13±3	0.981±0.01	4.62±0.03
	8 Days	383±30	481±63$^{a, b}$	-273±23	-339±44	92±2	-2.4±0.2	11.0±0.6	0.986±0.01	4.64±0.02
***p*-value (n=3)**	Tukey's test	<0.001	0.014	0.017	0.061	0.004	0.142	0.064	0.041	<0.001
Preservative Type (PT)	Control	370±17	467±50	-263±16	-353±47	87±12	-2.5±0.3^{a}	12±2	0.990±0.002^{b}	4.61±0.02
	Maceration	379±31	503±64	-275±26	-361±46	86±17	-2±1^{b}	12±4	0.989±0.001^{b}	4.61±0.01
	Ultrasound	389±33	484±60	-273±26	-330±36	90±3	-2.1±0.2$^{a, b}$	11.7±0.3	0.970±0.009^{a}	4.68±0.01
	Potassium Sorbate	388±33	498±57	-279±24	-350±34	88±11	-2.5±0.2^{a}	11.0±0.7	0.987±0.003^{b}	4.67±0.05
***p*-value (n=12)**	Tukey's test	0.065	0.479	0.191	0.448	0.668	0.009	0.588	<0.001	<0.001
ST×PT (n=36)	*p*-value	<0.001	0.522	<0.001	0.963	0.100	0.138	0.424	0.215	<0.001

In each row, different letters represent statistical significative differences with a significance level of 0.05. The SD were calculated from results obtained in different conditions, and thus should not be considered as precision measurements, but rather an interval of values.

Concerning the texture dimensions, firmness, consistency, cohesiveness and work of cohesion were analysed due to the cheese being a semi-solid food. Thus, the backward extrusion test showed that while no changes were sought for cohesiveness and work of cohesion, the storage times had an influence on consistency, thus showing a significant increase over time. For the color of the cheeses, an important parameter that can determine the success or failure of a food additive, lightness (L*) showed a significant interaction among the two factors, while yellowness-blueness (b*) did not show any changes. Redness-greenness (a*) did show significant changes, especially for the different preservatives, in which the control cheese and the cheese preserved with potassium sorbate revealed lower values with significant difference towards the two natural preservatives. While this is expected due to the almost transparent color of potassium sorbate and the dark color of the natural extracts, these values are only separated by 0.5 values in a total range of 200, thus being quite negligible. Water activity, an important parameter that shows the available water in the food, which could be related to an increase in the development of contaminants was also assessed. For this parameter, only the cheese preserved with the ultrasound extract showed a significant lower value, with all other cheeses showing no statistical differences. Once again, the preservatives seemed to have a higher influence than the storage time on this parameter. Finally, pH was also evaluated as a

determinant parameter due to its importance in the development of off flavours and other sensory alterations in foods. Although a significant interaction was sought for both parameters, no statistical differences were found.

A. unedo fruits had shown capacity to be used as food preservatives for loaf breads, showing potential to be used in the food industry (**Takwa et al., 2018**). Thus, the leaves could also have this potential, which was a driving force for the use of leaves in this work, using an agricultural residue as a food preservative.

A limitation this work shows is perhaps only analysing 8 days of storage time which could not have been enough to reveal the effects of the natural extracts. This limitation will be overcome by extending the analysis for a period of 15 days. While quark cheese does not have any preservative in its composition and also because the recommended shelf life of this cheese is about 3 days after opening, only seven days were considered in this study, and thus, by adding these preservatives the shelf-life could be extended probably for an extra week.

5. Conclusions

The main objective of this work was to find alternative preserving molecules for the famous quark cheese, which is known to be a very healthy cheese that offers high protein and low fat as a semi-solid. The chosen natural extracts were obtained from *A. unedo* leaves, which are known to have interesting antioxidant and antimicrobial properties. Thus, the optimization designs showed that the maceration had a higher amount of dry residue which did correlate with a higher antioxidant activity in the TBARS assay, but lower in the cellular antioxidant activity. The antibacterial assay revealed significative inhibition of some pathogenic strains, highlighting the inhibition of MRSA. Still, further analysis against food contaminants will to be carried out very soon, since these bacterial strains were clinical isolates rather than food contaminants. Regarding the incorporation of the extracts in quark cheese, overall neither of the extracts showed deep changes in the nutritional chemical and physical profiles of the cheeses. Still, the ultrasound extract did seem to preserve lactose and glucose over the 8 days, probably by inhibiting lactic bacteria. In terms of the physical analysis, the *A. unedo* extracts showed higher consistency and a slight but significative increase in redness when compared to the control and potassium sorbate preserved samples. Considering the microbial load, the extract that better kept the microbes in check was the maceration extract, showing better results when compared to the others.

Overall, while the ultrasound extract showed a higher antioxidant activity, the antimicrobial activity was highest for the maceration extract. Due to none of them having toxicity or changing the profile of the foods, both are good candidates to be used in quark cheese and perhaps other foods. Due to both extracts showing good activities that are complementary to each other, a study of a mixture of both could be carried out to potentiate their use in cheese and other foods.

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PUBLISHED WORK RESULTING FROM THIS THESIS

1. Article

Nabila Ben Derbassi1, Mariana C. Pedrosa1, Sandrina Heleno1, Marcio Carocho1, *, Lillian Barros1(2021). Plant Volatiles: Using Scented Molecules as Food Additives (submitted in Trends in Food Science & Technology journal)

2. Conference participation as Poster communication

Nabila Ben Derbassi, Mariana C. Pedrosa, Sandrina Heleno, Márcio Carocho*, Isabel C.F.R. Ferreira, Lillian Barros Centro. Februry 2021. Response surface analysis of ultrasound and dynamic maceration extractions of arbutus unedo l. at the 1st Online International “Congresson Natural products application: Health, Cosmetic and Food” Poster communication 4-5 Feb 2021 de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Portugal.