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**Extracción de compuestos bioactivos a partir de subproductos  
alimentarios y posterior uso como ingredientes para la  
formulación de alimentos enriquecidos**

/

**Extraction of bioactive compounds from food byproducts and  
its use as ingredients for the formulation of enriched foods**

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A mis padres,  
por enseñarme

“I like the view right now”  
The view, Stray kids

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## **RESUMEN / ABSTRACT**

## **Resumen**

La presente tesis explora tecnologías emergentes para preservar y recuperar compuestos bioactivos valiosos de subproductos agroindustriales, específicamente de naranjas y aguacates, que son ricos en compuestos fenólicos conocidos por sus beneficios para la salud. Inicialmente, la tesis describe el modelado matemático de la cinética de secado de estos subproductos para prevenir la descomposición, lo cual es crucial para una gestión eficaz de los residuos. A continuación, se evalúa la extracción de estos compuestos bioactivos para su reutilización en productos de alto valor. Los métodos de extracción tradicionales, que a menudo involucran altas temperaturas y solventes, pueden degradar compuestos sensibles y son perjudiciales para el medio ambiente. Para abordar estos desafíos, la tesis se centra en tecnologías no térmicas y escalables, como la extracción asistida por ultrasonidos combinada con pretratamientos como campos eléctricos pulsados y fermentación sumergida con bacterias ácido-lácticas. La escalabilidad de la tecnología de ultrasonidos y la purificación de compuestos fenólicos mediante filtración por membrana también se evaluó a escala piloto. Finalmente, los extractos obtenidos se utilizaron para fortificar zumo de naranja. Los hallazgos de la tesis demuestran el potencial de transformar los residuos agroindustriales en productos de alto valor, apoyando así la economía circular y reduciendo el impacto ambiental.

## **Abstract**

The present thesis explores innovative methods to preserve and recover valuable bioactive compounds from agro-industrial by-products, specifically from oranges and avocados, which are rich in phenolic compounds known for their health benefits. Initially, the thesis describes the mathematical modeling of the drying kinetics of these by-products to prevent decay, which is crucial for effective waste management. The research then assesses the extraction of these bioactive compounds for reuse in high-value products. Traditional extraction methods, which often involve high temperatures and solvents, can degrade sensitive compounds and are not environmentally friendly. To address these challenges, the study focuses on non-thermal and scalable technologies such as ultrasound-assisted extraction combined with pre-treatments like pulsed electric fields and lactic acid submerged fermentation. The scalability of the ultrasound technology and purification of phenolic compounds using membrane filtration was evaluated at a pilot scale. Finally, the obtained extracts were used to fortify orange juice. The findings of the thesis demonstrate the potential of transforming agro-industrial waste into high-value products, thereby supporting the circular economy and reducing environmental impact.



# INTRODUCTION





## 1. FOOD BY-PRODUCT VALORIZATION

Fruits and vegetables are key elements in a healthy and balanced diet as they provide nutrients including vitamins and minerals, dietary fiber, and a host of beneficial non-nutrient substances. According to the World Health Organization (WHO), adequate intake of fruits and vegetables is one of the five most important factors in a healthy diet, as it is associated with significantly lower risks of noncommunicable diseases such as obesity, heart disease, stroke, diabetes, and certain types of cancer [1]. In 2022, Spain's households consumed 6,043 million kilograms of fresh and 556.3 million kilograms of processed fruits and vegetables [2]. Although consumption is below the WHO's minimum recommendation of 400 grams, the trend is slightly increasing [1]. However, there is a wide and varied supply of minimally processed fruit and vegetable products with increasing demand, which has also boosted the market for derived products, thereby promoting the generation of **by-products** from processing. Worldwide, the agri-food industry generates more than 190 million tons of by-products annually. These by-products residues can be further divided into agricultural and process residues. Agricultural residues are the remnants left in the field after crop harvesting, such as leaves stems, seedpods, and stalks. Process residues, on the other hand, are materials that remain after the crop has been processed into another product. These include molasses, bagasse, husks, seeds, leaves, straw, stems, stalks, shells, pulp, peels, and roots [3]. Agri-food by-products from agriculture and food processing, produced at different stages of operations, are rich in nutrients, functional, and bioactive elements. The Food and Agriculture Organization (FAO) 2030 Sustainable Development Goals advocates for a comprehensive and interdisciplinary strategy to mitigate food waste emphasizing the re-use of food waste as animal feed, compost, or for energy recovery, among others [4]. This goes in line with the concept of circular bioeconomy adopted for the European Union Commission (**Figure 1**). The circular economy represents a sustainable approach to production and consumption, emphasizing activities such as sharing, leasing, reusing, repairing, refurbishing, and recycling. This strategy aims to maximize the usability of materials and products for as long as feasible, thereby prolonging their lifecycle. This model minimizes waste by ensuring that, at the end of their lifespan, products are not discarded but are recycled to keep materials within the economic loop. This reuse of materials continuously generates additional value. Such a framework marks a significant shift from the conventional linear economy, which operates on a pattern of acquiring resources, manufacturing goods, using them, and then disposing of them. This traditional approach typically depends on the availability of abundant, affordable materials and energy [5].

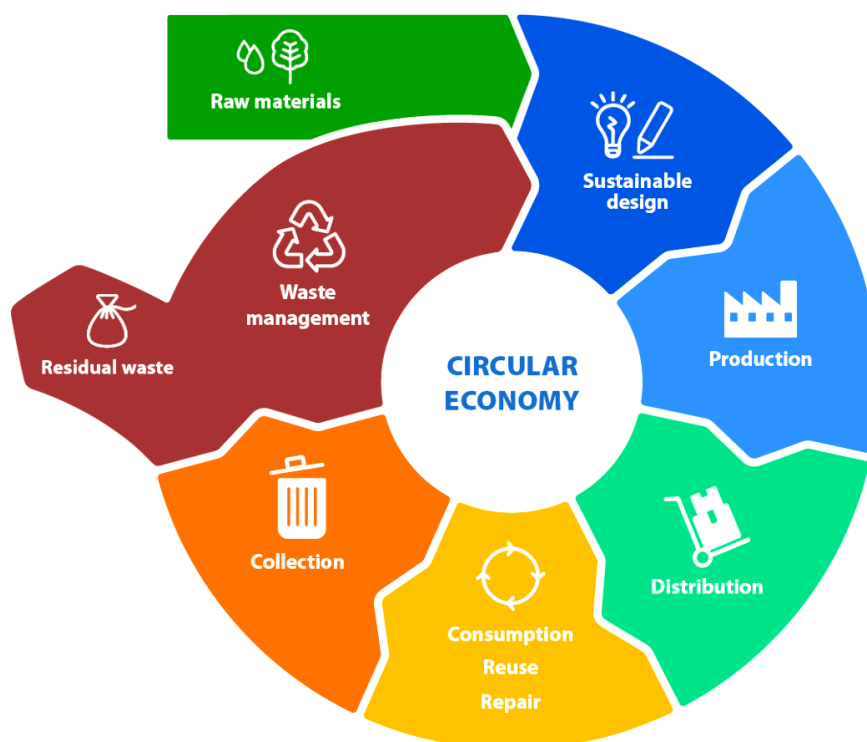


**Figure 1.** Infographic of the circular economy model (from [5]).

In this context, the valorization of agri-food waste has gained significance due to its potential to tackle several pressing challenges. Firstly, it plays a crucial role in **ensuring food security** by transforming waste that is often unsuitable for direct human consumption into valuable products. This process not only minimizes waste but also maximizes the utilization of available resources within the agri-food sector. Moreover, the valorization of agri-food waste contributes to the **sustainable management of natural resources**. By utilizing waste materials, it effectively reduces the portion of agricultural production that would otherwise go to waste, thus conserving valuable resources such as water, land, and energy. This approach aligns with the principles of sustainability by optimizing resource use and minimizing environmental impact. Additionally, the valorization of agri-food waste offers an opportunity to **reduce reliance on synthetic resources**. Agri-food waste contains a wealth of natural bioactive compounds that can be extracted and utilized for various technological and health-related purposes. By harnessing these compounds, industries can reduce their dependence on synthetic additives and ingredients, promoting a more sustainable and environmentally friendly approach to product development [6].

Thus, there has been a surge of interest in the efficient use of agri-food by-products, driven by their potential to boost sustainability, reduce waste disposal issues, and generate value-added products for the food industry. This interest has spanned researchers, industry professionals, and policymakers, catalyzing a significant uptick in scientific studies and technological innovations. This approach encourages the re-valorization or transformation of underutilized by-products.



Consequently, a variety of applications have been developed by researchers to address the issue of food and agricultural by-products. These applications include food production, nutraceuticals, cosmeceuticals, animal nutrition, plant nutrition, energy (biogas), and ethanol production. In **food applications**, the by-products of food and agriculture can be used directly or with minor modification such as drying and grinding to formulate new food products in dairy, meat, bakery and beverages industries with improved properties [3]. They can also be recycled for the extraction of oil and/or bioactive compounds [7], which can be used as food additives [8]. Besides, they can be used for the production of vinegar [9], and edible coatings [10]. Besides, another possible application of agro-industrial wastes is in animals fed for improving protein concentration, litter size, milk yield, and nest characteristics, among others. Additionally, the initial research results indicate the possibility of producing organic fertilizers from agro-industrial by-products, which can be a viable alternative to chemical fertilizers. Besides, biogas produced from agro-industrial by products such as soybean residue, papaya peel, and sugarcane bagasse had good production efficiency with methane yields [7].

## 2. BIOACTIVE COMPOUNDS

Fruit and vegetable waste products contain high concentrations of **bioactive compounds** of huge interest that can be recovered, thus reducing the volume of waste, improving the economic viability of the process (by producing high-value-added extracts), and mitigating environmental impact [3]. The term "bioactive" is derived from two components: "bio-" and "-active." From an etymological perspective, "bio-" originates from the Greek word (βίο-) "bios," which means life. The suffix "-active" comes from the Latin word "activus," signifying something dynamic, energetic, or involving activity. This term encompasses all phenomena that exhibit some form of life, function, or process. In scientific contexts, "bioactive" is often used interchangeably with "biologically active" [11]. "Bioactive compounds" refers to molecules with biological activity, which can elicit pharmacological or toxicological effects in humans and animals. These compounds, also known as phytochemicals, occur naturally in plants and food items and have the capacity to modulate metabolic processes to promote better health [12].

The terms "bioaccessibility," "bioavailability," and "bioactivity" are interconnected and crucial for understanding the impact of bioactive compounds on the living system (**Figure 2**). Before bioactive compounds can exert their beneficial effects, they must first be released from the food matrix in which they are consumed, become available within the body, and then carry out their biological functions effectively. This understanding is essential for the development of fortified and functional foods, necessitating detailed research into these three areas specific to each compound and its delivery system [13].



**Figure 2.** Representation of the interconnection of the three terms.

**Bioaccessibility** refers to the extent to which a bioactive compound is released from the food matrix during digestion within the gastrointestinal tract. The digestive process, influenced by gastric secretions, prepares the bioactive compounds for subsequent absorption into the bloodstream through the intestinal epithelium. **Bioavailability** describes the proportion of bioaccessible compounds that are absorbed by the intestinal cells and enter the circulatory system (blood or lymphatic) where they can perform their intended physiological roles. However, the actual absorption can be affected by interactions with other dietary components; for example, phytates can hinder the absorption of proteins and minerals, while vitamin D and ascorbic acid can enhance the absorption of calcium and iron, respectively. Finally, **bioactivity** refers to the actual physiological effects elicited by the bioactive compounds, such as antioxidant, anti-inflammatory, or antidiabetic actions, when they interact with specific target tissues in the body. The bioactivity level depends not only on the presence of the compound at the targeted site but also on its interactions with other biomolecules during absorption and circulation, and the influence of metabolic processes. In some cases, such as with nondigestible polysaccharides, oligosaccharides, and dietary fibers, bioactivity may be independent of the digestibility and absorption of the compounds. Understanding these three aspects allows for the effective incorporation of bioactive compounds in food products designed to deliver specific health benefits [13].

The task of systematizing and characterizing the extensive array of food bioactive compounds, which certainly numbers in the dozens, presents a significant challenge that researchers around the globe are addressing. In this regard, three primary databases compile and organize the information from various studies on the bioactive components of different foods: the USDA flavonoid database [14], Phenol-Explorer for polyphenols [15], and eBASIS (Bioactive Substances in Food Information Systems) [16]. The latter, an EU-funded multinational initiative, not only covers polyphenols but also includes information on a broader range of bioactive compounds. Based on that, bioactive compounds can be considered to encompass a diverse range of molecules, including polyphenols, phytosterols, terpenoids, polysaccharides, carotenoids and tocopherols, glucosinolates, triterpenes, alkaloids, bioactive peptides, and polyunsaturated fatty acids (Figure 3), exhibiting varied chemical structures (hydrophilic or lipophilic) and widespread distribution in nature [12]. It's important to note that food bioactive compounds are not limited to plant-based foods; they are also found in animal-derived foods like meat and dairy, as well as in fungi, particularly edible and wild mushrooms, which are emerging as valuable sources of non-plant bioactive compounds [17].

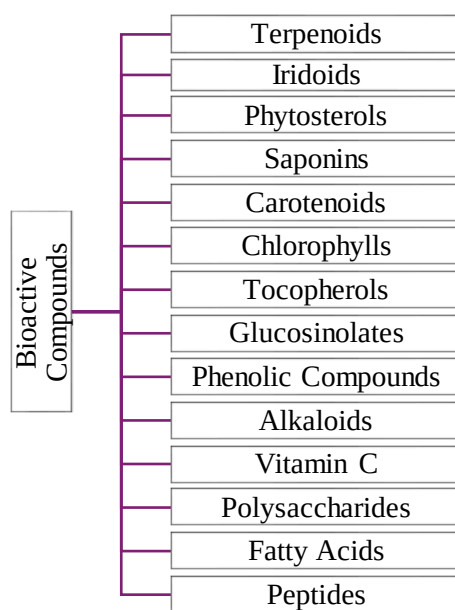


**Figure 3.** Scheme of the main classes of bioactive compounds.

Incorporating these described bioactive compounds into food products will help address and mitigate a range of current and emerging lifestyle diseases. Observing the contemporary and forthcoming lifestyle demands, numerous industries, including pharmaceuticals, food production, and chemicals, are venturing into the extraction of bioactive compounds. Beyond the benefits previously mentioned, these substances also function as natural food additives and processing aids for the food industry (**Figure 4**) [13].

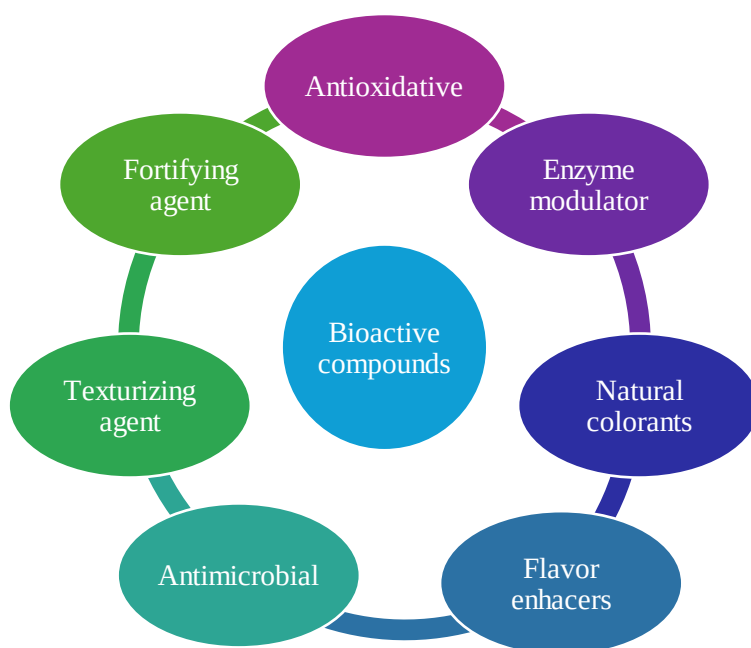


**Figure 4.** Possible functions of incorporating bioactive compounds in food.

Bioactive compounds serve as antioxidants to counteract the negative effects of free radicals and single oxygen molecules that arise from processing and prolonged storage of foods. Besides, during food processing, certain bioactive compounds can either inhibit or promote specific enzymatic reactions, thus controlling enzyme activity and influencing the outcome of food processing. Compounds like anthocyanins, carotenoids and curcumin offer natural alternatives to synthetic dyes, which, while stable, can be toxic and offer no nutritional benefits. Natural flavoring agents such as cinnamaldehyde and vanillin are commonly utilized to enhance the taste of sweet foods, chewing gums, and beverages. Phenolic compounds act as antimicrobial agents to prevent the growth of food spoilers and pathogens, thus maintaining the sensory and safety quality of food products. Various natural gums and dietary fibers, both nutritive and non-nutritive, are employed to improve the texture of food products through their significant water and oil retention capabilities, thereby enhancing solubility and viscosity. Otherwise, extracted and purified bioactive compounds are used to fortify staple foods and specialized products aimed at nutritionally vulnerable populations, enriching them with essential nutrients and functional benefits [13].

## 2.1.TERPENOIDS

Terpenoids, also referred to as isoprenoids or terpenes, the largest group of secondary metabolites, are ubiquitous across all living organisms and particularly abundant in plants and fruits [17]. Originally, the term "terpene" was used to describe the hydrocarbons in turpentine, with the suffix "ene" indicating the presence of olefinic bonds. These compounds are categorized based on the number of carbons they contain, which are assembled from isoprene units (2-methylbuta-1,3-diene). This assembly follows the linear alignment, cyclization, and rearrangement of these units, a concept known as the isoprene rule. Hemiterpenoids represent the simplest class, consisting of a single isoprene unit. According to the isoprene rule, terpenoids are primarily formed through a sequential, head-to-tail attachment of isoprene units, although other types of connections like head-to-head or head-to-middle also occur. These different connections are observed in classes such as triterpenoids and certain irregular monoterpenoids [18]. Thus, terpenoids can be classified into various categories based on the number of carbon atoms and the presence of isoprene units: (1) Monoterpenes, composed of 10 carbon atoms or two isoprene units; (2) Sesquiterpenes, containing 15 carbon atoms or three isoprene units; (3) Diterpenes, consisting of 20 carbon atoms or four isoprene units; (4) Triterpenes, composed of 30 carbon atoms or six isoprene units; and (5) Tetraterpenes, with 40 carbon atoms or eight isoprene units (**Figure 5**) [19]. Terpenoids are synthesized primarily via two biochemical pathways: the cytosolic mevalonate (MVA) pathway and the plastidial 2-C-methyl-D-erythriol 4-phosphate (MEP) pathway. While hemi-, mono-, and diterpenoids largely arise from the MEP pathway, sesqui- and triterpenoids generally come from the MVA pathway, though exceptions and interactions between these pathways occur. Terpenoids

are key components of essential oils [18]. Many terpenoids are volatile at room temperature and are responsible for the characteristic scents of plants and fruits, such as limonene in various citrus fruits [17]. Triterpenoids, encompass at least 18 subclasses, with well-known examples being squalene derivatives and saponins. Compounds such as lupane, oleanane, and ursane are being explored for their promising anti-cancer properties [17].

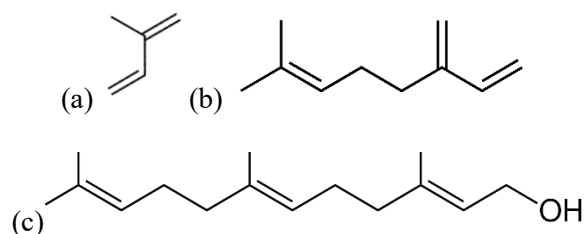


**Figure 5.** Chemical structure of isoprene unit (a), myrcene (monoterpene) (b), farnesol (sesquiterpene) (c).

## 2.2. IRIDOIDS

Iridoids, a subclass of monoterpenoids, are acetal derivatives of iridodial (**Figure 6. (a)**). These compounds are especially common in the plant kingdom, predominantly among dicotyledonous species within the *Scrophulariaceae*, *Pyrolaceae*, *Oleaceae*, *Labiatae*, *Rubiaceae*, and *Gentianaceae* families. Due to the unstable nature of their C1-OH group, iridoids frequently react with sugars to form glycosides. Based on the integrity of the cyclopentane unit, iridoids can be classified into two types: iridoid glycosides (such as geniposide, loganin, acetylbarlerin, deacetylasperulosidic acid, and brasoside) and secoiridoid glycosides (including swertiamarine, gentiopicroside, qinjiaoside A, qinjiaoside B, sweroside, and oleuropein (**Figure 6. (b)**)). The bond between the C7 and C8 atoms in the parent nucleus of secoiridoids is typically cleaved, leading to the formation of a ring. Additionally, iridoids can form dimers known as bis-iridoids (including cantleyoside, laciniatoside I-II, and sylvestroside I) and exist as unglycosylated iridoids called non-glycosidic iridoids (such as acevaltrate and valtrate). Compounds with these structures exhibit a range of physiological activities and are utilized as sedative and antipyretic agents [20].

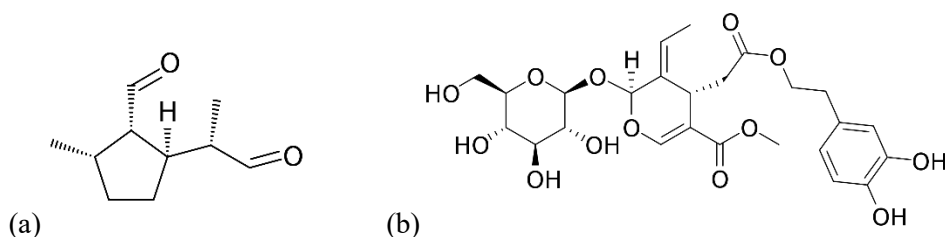


**Figure 6.** Chemical structure of iridodial (a) and oleuropein (b)

### 2.3. PHYTOSTEROLS

Derived from triterpenes, phytosterols are essential structural components in plant cells, with similar structure and function to cholesterol in animal cells. They are categorized into two groups: sterols, which possess a double bond in the sterol ring, and stanols, which do not have a double bond in the sterol ring. Humans cannot produce phytosterols, unlike cholesterol, and must obtain them through their diet. Phytosterols are present in all plant-based foods, with the highest levels typically found in unrefined plant oils, nuts, and olive oils, seeds, whole grains, and legumes [21]. Notably, dietary phytosterols like sitosterol, ergosterol, and stigmasterol (**Figure 7**) have been shown to effectively lower low-density lipoprotein (LDL) cholesterol in numerous clinical studies [17].

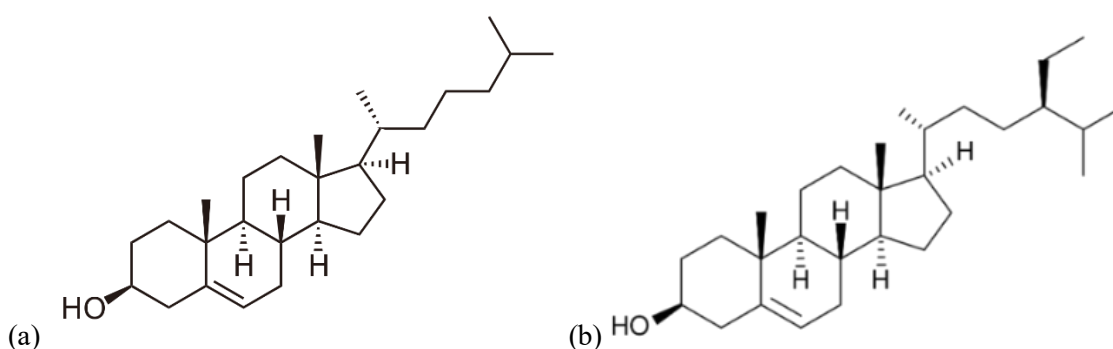


**Figure 7.** Structure of cholesterol (a) and beta-sitosterol (b).

### 2.4. SAPONINS

Saponins are a diverse group of secondary plant metabolites named for their soap-like qualities, which allow them to foam in water. These compounds are typically divided into triterpenoid and steroid saponins based on their structural backbone. Among the various plants known for their saponin content, the soybean stands out as particularly rich in these compounds (**Figure 8**). Research has demonstrated that saponins are involved in or contribute to several biological functions including hemolytic activity, anti-inflammatory properties, and antifungal, antibacterial, and antimicrobial effects. Additionally, they exhibit molluscicidal activity, cytotoxicity, and antitumor properties [21].

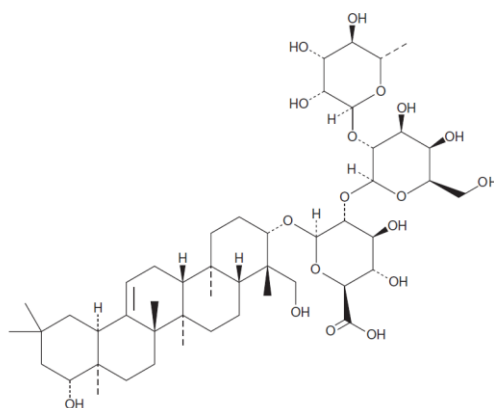


**Figure 8.** Structure soyasaponin from soybean.

## 2.5. CAROTENOIDS

Carotenoids are tetraterpenoids derived from the condensation of 5-carbon isoprene units [21] and are a group of fat-soluble natural compounds present in plants, algae, and photosynthetic bacteria. The exploration of carotenoids began in 1831 when Wackenroder isolated a pigment from carrots, which he named "carotin." In 1837, Berzelius coined the term "xanthophyll" for the yellow pigments found in autumn leaves. By 1910, the term "carotenoids" was introduced by Tswett to describe this entire group of pigments [22]. Later, in 1938, Strain differentiated carotenoids into two groups: xanthophylls, which include oxygen-containing compounds such as lutein, zeaxanthin, and cryptoxanthin, and carotenes, which are hydrocarbons like  $\alpha$ -carotene,  $\beta$ -carotene, and lycopene that lack oxygenated groups. These compounds are known for their strong antioxidant properties that can mitigate the effects of aging by preserving cellular activities and reducing oxidative stress, which may lead to conditions like osteoporosis and contribute to the aging of skin, eyes, and blood vessels [3]. Besides, carotenoids such as beta-carotene are considered precursor to vitamin A, essential for the human diet [21].

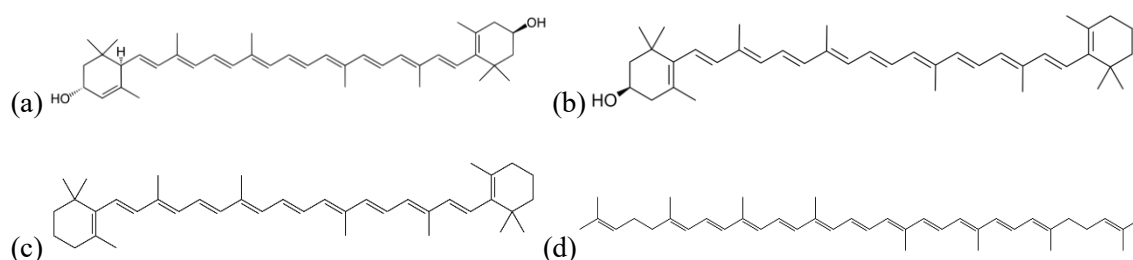


**Figure 9.** Structure of the xanthophylls lutein (a) and cryptoxanthin (b), and the carotenes beta-carotene (c) and lycopene (d).

## 2.6. CHLOROPHYLLS

Chlorophyll, a chlorin pigment prevalent in green plant parts like stems and leaves, features a magnesium ion at its core encircled by four nitrogen atoms from the chlorin ring, forming a heterocyclic compound derived from pyrrole (**Figure 10**) [23]. Chlorophylls are essential for photosynthesis and are commonly present in green fruits and vegetables, which are staples in our diets. While research on their therapeutic potential is still emerging, studies indicate that these pigments and their derivatives hold promise for various health benefits. They have been associated with antioxidant, antimutagenic, antigenotoxic, anti-cancer, and anti-obesity properties [24].

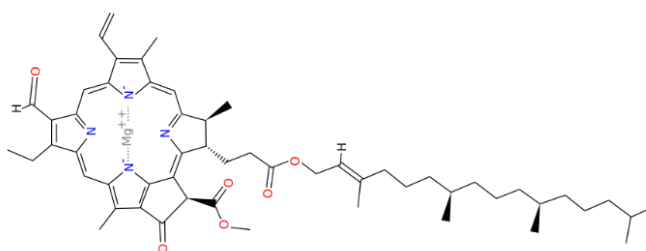


**Figure 10.** Structure of Chlorophyll a.

## 2.7. TOCOPHEROLS

Tocopherols, commonly known as vitamin E, serve as major lipid-soluble antioxidants found in cell membranes, where they act as scavengers of peroxy and alkoxyl free radicals. Naturally occurring vitamin E exists in eight different forms, comprising  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -isomers of both tocopherol and tocotrienol. Among these,  $\alpha$ -tocopherol is the most biologically active, being the only isomer maintained in human plasma and recognized as necessary to meet human nutritional needs. Dietary sources rich in  $\alpha$ -tocopherol include nuts, seeds, and vegetable oils, with smaller amounts found in green leafy vegetables and fortified cereals. Beyond its primary role as an antioxidant in the lipid portions of cell membranes,  $\alpha$ -tocopherol also influences various cellular functions such as cell growth, proliferation, apoptosis, cell adhesion, angiogenesis, and inflammation [25].

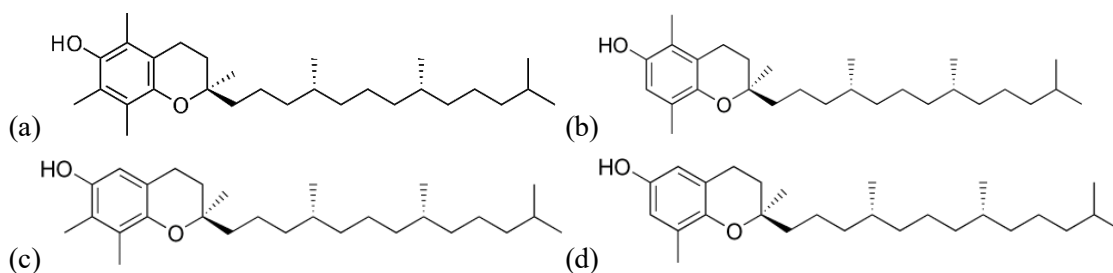


**Figure 11.** Structures of  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ - tocopherol, corresponding to (a), (b), (c) and (d).



## 2.8. GLUCOSINOLATES

Glucosinolates, with a thiohydroximate-O-sulfonate core linked to glucose (**Figure 12**), are primarily found in cruciferous vegetables like broccoli, cabbage, and cauliflower, imparting distinctive tastes and odors. Notable for their chemopreventive and anti-inflammatory properties, glucosinolates such as isothiocyanates are being studied for their anti-cancer potential [17].

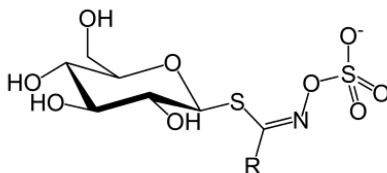


**Figure 12.** Glucosinolate skeleton.

## 2.9. PHENOLIC COMPOUNDS

Phenolic compounds, derived from botanical sources like plants, vegetables, and fruits, are essential secondary metabolites in plant biology, distinct from primary metabolites crucial for growth and development. These compounds play dual roles as attractants for pollinators and defenses against predators, shaping the physical and sensory characteristics of plants and their by-products. Moreover, phenolic compounds contribute nutraceutical and functional properties, including antioxidant effects. Synthesized through the shikimate and/or acetate pathways, phenolic compounds encompass various classes like phenolic acids, flavonoids, and others, each with unique structures and bioactivities. Among them, phenolic acids and flavonoids are widely distributed in plant tissues, exhibiting diverse bioactivities. Through their intricate functions, phenolic compounds underscore the versatility of plant-derived materials, offering potential applications in nutraceuticals, pharmaceuticals, cosmetics, and foods. They will be described extensively in the section 3.

## 2.10. ALKALOIDS

Alkaloids are characterized by their alkaline properties and a nitrogen atom, often derived from various aminoacids, within a heterocyclic ring [17]. Alkaloids are commonly classified into three groups: (1) true alkaloids, exemplified by atropine, nicotine, and morphine; (2) protoalkaloids, that are alkaloids with nitrogen in the side chain such as adrenaline and ephedrine; and (3) pseudoalkaloids, including solanine, caffeine, theobromine, and theacrine (**Figure 13**). It is estimated that approximately 14–20% of plant species contain alkaloids, with main representatives found in families such as Solanaceae, and in foods such as coffee and cocoa [19]. Alkaloids in plants are recognized as multifaceted compounds, serving as growth regulators, reserve substances, and playing crucial roles in plant physiology [21]. Additionally, alkaloids find

extensive applications in therapeutics and pharmacology, owing to their diverse biological properties such as anti-inflammatory, antidepressant, antitumor, antiviral, antihypertensive, as well as antimicrobial and antimalarial effects [19].

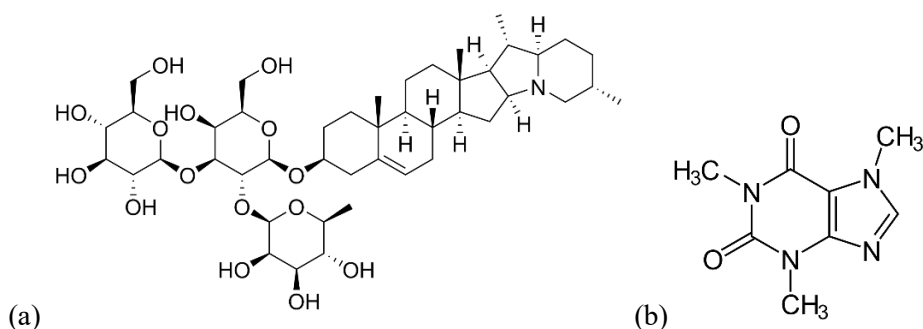


**Figure 13.** Food alkaloid's structure: Solanine (a) and caffeine (b).

## 2.11. VITAMIN C

Vitamin C, also known as ascorbic acid, is a crucial micronutrient vital for human health due to its antioxidant properties and involvement in various metabolic processes. Discovered for its efficacy against scurvy, it plays roles in collagen synthesis, neurotransmitter production, iron absorption, and immune function. While humans lack the enzyme necessary for its synthesis, adequate intake from diet or supplements is essential to prevent deficiencies. Vitamin C-rich foods like citrus fruits are recommended for maintaining optimal levels, although the vitamin's content can vary due to factors like ripening, storage, and processing. Despite its stability challenges during storage and processing, strategies like cold storage and proper handling can help preserve its potency. Studies suggest that vitamin C intake within recommended levels may offer protection against chronic diseases, and its safety for consumption, even at higher doses, has been established. It will be discussed further in the section 4.

## 2.12. BIOACTIVE POLYSACCHARIDES

Polysaccharides, alongside oligosaccharides, constitute the most prevalent group of biopolymers. They play crucial roles in numerous biological processes [26]. Chemically, they are divided into two types: homo-polysaccharides, made of a single type of monosaccharide like glucose found in cellulose and glycogen, and hetero-polysaccharides, consisting of multiple types of monosaccharides. They can also be classified based on the glycosides linked to them as proteoglycans, glycoproteins, glycolipids, and glycoconjugates. There are described bioactive polysaccharides in plants, algae, lichen, and animals [26]. Dietary fibers, defined by FAO, encompass various indigestible plant polysaccharides like cellulose, hemicelluloses, pectins, and gums. These fibers contribute to disease prevention by affecting bowel movement, cholesterol levels, and sugar absorption rates. They also play roles in preventing gastrointestinal disorders

like constipation and diverticulosis. Studies indicate that increased dietary fiber intake correlates with decreased risks of diabetes, cardiovascular diseases, obesity, and gastrointestinal cancer [26]. Some of them play significant roles in maintaining gut microbiota homeostasis and overall health, impacting factors like infant nutrition, blood sugar, and lipid metabolism [17]. Some examples of bioactive polysaccharides reported to act as prebiotics are: Fructans, such as inulin and fructo-oligosaccharides (**Figure 1**), consist of linear chains of fructose with  $\beta(2\rightarrow1)$  linkages, often terminating with glucose units similarly linked; Galacto-oligosaccharides arise from lactose extension and are divided into two subgroups: those with excess galactose at specific positions and those produced through enzymatic trans-glycosylation from lactose; and resistant starch, resistant to upper gut digestion, potentially qualifies as a prebiotic due to its ability to produce high levels of butyrate, promoting health [27]. The main food sources are grains, fruits, vegetables, nuts, legumes, rice, chicory root, wheat, onion and konjac [26].

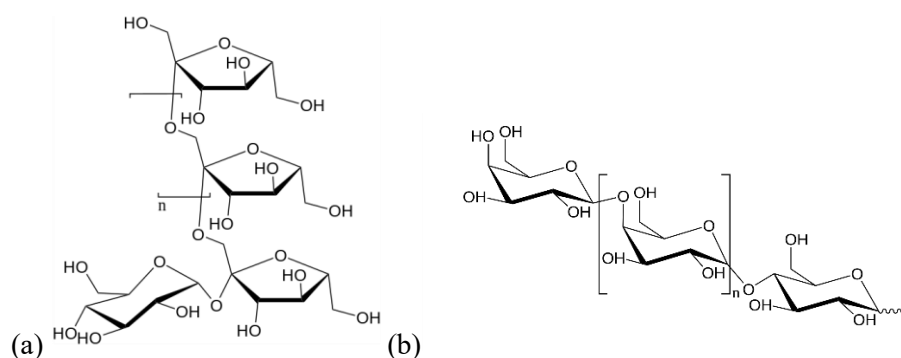


**Figure 14.** General structure of fructo-oligosaccharide (a) and galacto-oligosaccharide (b).

### 2.13. BIOACTIVE FATTY ACIDS

Polyunsaturated fatty acids (PUFAs) or omega-6/omega-3 fatty acids are characterized by containing two or more double bonds, with one located at the sixth/third carbon atom from the methyl ( $\text{CH}_3$ ) end of the carbon chain, respectively [28]. The primary types of omega-3 fatty acids found in foods include alpha-linolenic acid (ALA), Eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA), that are critical in diets lacking in omega-6 and omega-3 fatty acids, which are linked to a higher prevalence of chronic diseases [17]. They are considered as bioactive fatty acids. These fats are essential for modulating inflammatory responses at the gene expression level and within cell membrane structures. Omega-3 fatty acids offer numerous benefits in reducing the risk of heart disease and alleviating symptoms associated with chronic conditions such as hypertension, atherosclerosis, hypertriglyceridemia, and hypercholesterolemia. Additionally, these fatty acids play a crucial role in brain development, function, and mental well-being. DHA and other omega-3 fatty acids contribute to regulating neurotransmitters and maintaining the fluidity of brain membranes [28].

Sources of PUFAs include walnuts, various seeds, oils from plants like sunflower, flax, soybean, and corn, as well as fishes such as salmon and mackerel [17]. In addition, the term "conjugated linoleic acid" refers to a collection of geometric and positional isomers of linoleic acid. Meat and dairy products from ruminants are among the food groups known for being high in it. Linoleic acid conjugated forms exhibits a broad spectrum of bioactive properties, including anti-adipogenic, anti-diabetic, anti-atherosclerotic, and anticarcinogenic effects [28].

## 2.14. BIOACTIVE PEPTIDES

Bioactive peptides, which serve as both precursors and providers of milk proteins, remain inactive until they are liberated from the protein sequence through enzymatic proteolysis. These peptides are valued for their numerous health benefits, such as mitigating muscle wasting diseases, reducing metabolic syndrome risks, maintaining blood pressure, and supporting a functional gut environment. Examples include carnosine, creatine, taurine (**Figure 15**), and carnitine, identified in various meats and marine organisms [17]. Examples of bioactive peptides identified in milk include casomorphins,  $\beta$ -lactorphin,  $\alpha$ -lactorphin, lactoferroxins, casoxins, casokinin, lactokinins, immunopeptides, lactoferricin, casoplatelins, and phosphopeptides [28].

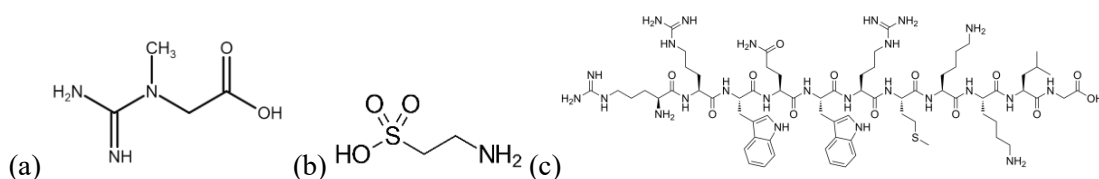


**Figure 15.** Chemical structures of creatine (a), taurine (b), and lactoferricin (c).

## 3. PHENOLIC COMPOUNDS

Phenolic compounds, derived from botanical origins such as plants, vegetables, and fruits, constitute a vital category of secondary metabolites within the realm of plant biology [29]. These compounds intricately influence the physical attributes and sensory profiles of the botanical sources and their derived by-products, thereby contributing to their distinct taste profiles and vibrant pigmentation. Moreover, phenolic compounds are recognized for their potential roles in conferring nutraceutical or functional properties to the plants or residues [29]. Several of them like flavonoids, phenolic acids etc. are found to perform different functions such as protecting a living plant against free radicals generated during photosynthesis [12]. Through their multifaceted contributions, phenolic compounds underscore the complexity and versatility of plant-derived materials, paving the way for innovative applications in nutraceutical, pharmaceutical, cosmeceutical and food scopes [29].

In plants phenolic compounds are synthesized by the shikimate and/or acetate pathways [29]. The shikimate pathway occurs through the reaction between phosphoenolpyruvate and erythrose 4-

phosphate, forming shikimic acid and leading to the formation of a variety of phenolic compounds. The acetate pathway occurs through the decarboxylation of pyruvic acid to form acetyl-CoA, which combines with malonyl-CoA units to form phenolic compounds (**Figure 16**) [30].

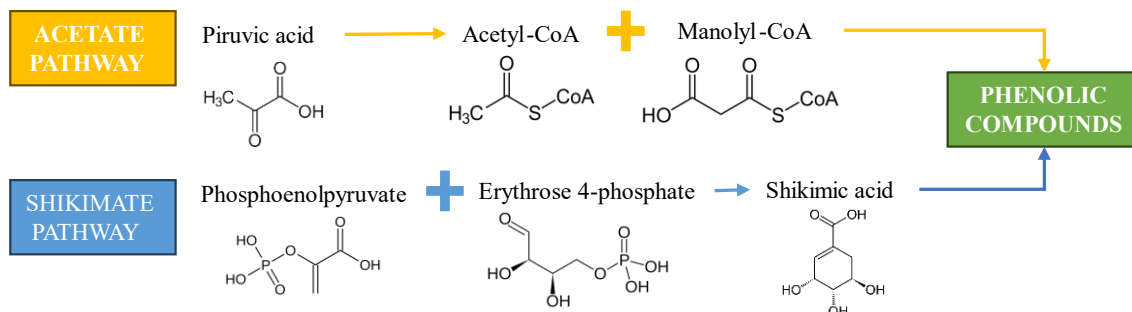


**Figure 16.** Scheme of the biosynthetic pathways of phenolic compounds.

Phenolic compounds are a class of chemical compounds consisting of one or more hydroxyl groups (OH) linked to at least one aromatic ring. They have been classified according to its chemical structure. Briefly, they can be classified in simple phenols, phenolic acids, naphthoquinones, coumarins, benzophenones and xanthenes, stilbenes, lignans, flavonoids, Alkylresorcinols, lignins and tannins (**Figure 17**) [29]. Among all the mentioned phenolic compounds phenolic acids and flavonoids are the most spread groups in plant tissues with several demonstrated bioactivities.

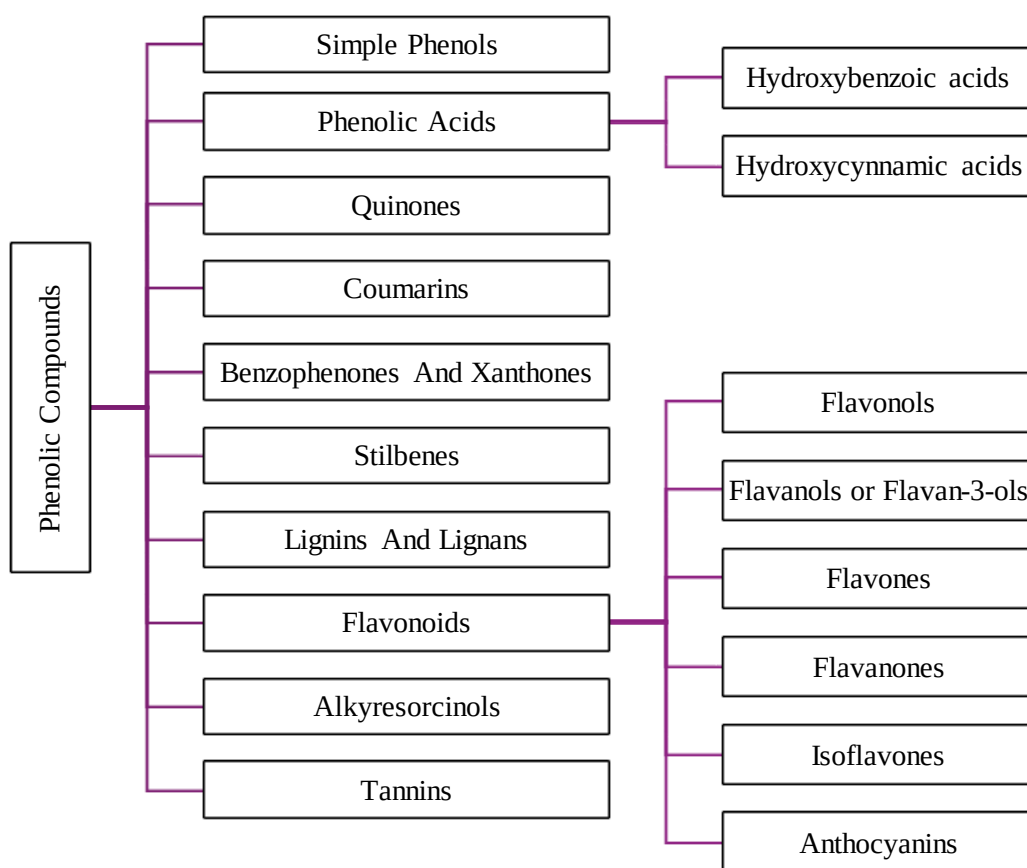


**Figure 17.** Scheme of phenolic compounds classification.

### 3.1. SIMPLE PHENOLS

Simple phenols are characterized as compounds that contain at least one hydroxyl group bonded to an aromatic ring, which serves as their fundamental structure. Notable members of this class include phenol, catechol, resorcinol, and phloroglucinol (**Figure 18**). While these phenols are not commonly found in plants on their own, phloroglucinol, resorcinol, and catechol often appear alongside cinnamic acids, playing a crucial role in the creation of various plant flavonoids such as hydroxytyrosol in olives [31].

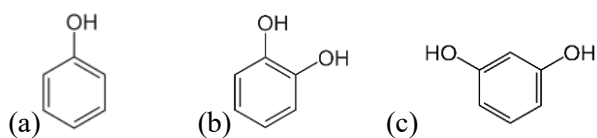


**Figure 18.** Chemical structure of the simple phenols, phenol (a), catechol (b) and resorcinol (c).

### 3.2. PHENOLIC ACIDS

Phenolic acids and their derivatives represent significant classes of phenolic compounds that are prevalent throughout the plant kingdom. These acids are characterized by a carboxyl group attached to a benzene ring, which is a common structural feature in these types of molecules [31].

Phenolic acids are widely recognized for their ability to combat oxidative damage-related diseases like coronary heart disease and cancer when consumed through fruits and vegetables. They can be classified into two groups: benzoic acid and cinnamic acid derivatives [12]. Derivatives of benzoic acid with the basic C6-C1 structure, known as hydroxybenzoic acids, are found in a variety of foods, including grapes and wines. These include protocatechuic acid, vanillic acid, gallic acid, syringic acid, salicylic acid, and gentisic acid, among others. Additionally, derivatives of cinnamic acid, or hydroxycinnamic acids (C6-C3), arise from phenylpropanoids with the most prevalent types being *p*-coumaric acid, caffeic acid, ferulic acid, and sinapic acid (**Figure 19**) [31]. Phenolic acids are typically found in various tissues in a range of conjugated forms, with free acids being rare. The most commonly reported conjugates are esters and amides, while glycosides are less common. These compounds encompass low molecular weight, water-soluble entities found in the cytosol, lipid-soluble forms associated with plant surface waxes, and bound forms esterified or etherified to cell wall polymers [32].

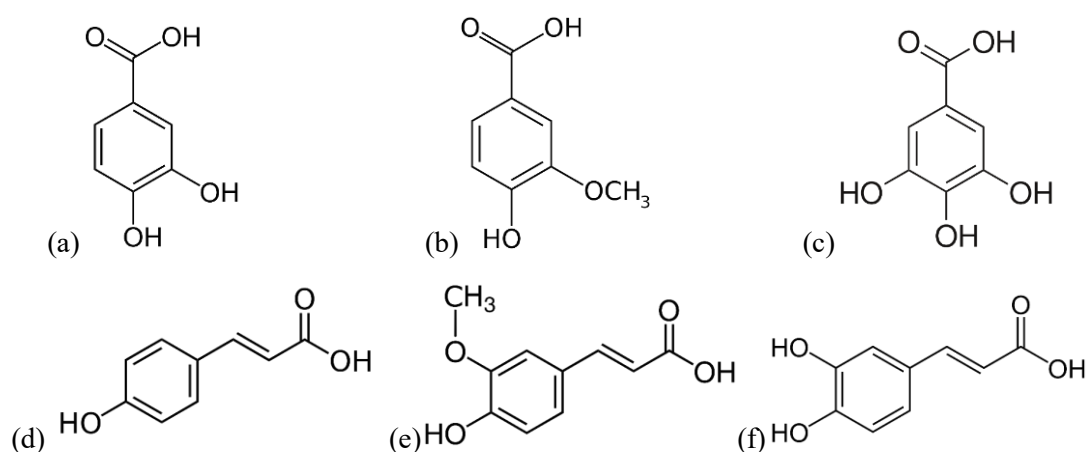


**Figure 19.** Chemical structure of hydroxybenzoic acids protocatechuic (a), vanillic (b) and gallic (c) acids, and the hydroxycinnamic acids coumaric (d), caffeic (e) and ferulic (f) acids.

### 3.3. QUINONES

Quinones are a category of quinoid compounds widely prevalent in nature. Over 1,200 quinones have been identified so far. These compounds are recognized by a characteristic structural feature: a ketone group that is either ortho or para substituted and connected to either an aromatic ring (as in benzoquinones) or a fused polycyclic aromatic structure (**Figure 20**) [33]. Benzoquinones comprises compounds featuring two carbonyl groups on a hexacyclic aromatic ring system (benzene ring), typically positioned ortho or para (monocyclic). Naphthoquinones are found to some degree in fungi and prevalent in higher plants, these compounds consist of the naphthalene nucleus bearing two carbonyl groups on a single nucleus, usually ortho or para (bicyclic). Anthraquinones, commonly occurring as fungal metabolites and in higher plants, are characterized by the anthracene nucleus with two carbonyl groups, typically located on ring B at

para positions (tricyclic). Polyquinones are dimers of various quinone types, with some being of mixed origin. Inter- or intramolecular oxidative coupling can lead to the formation of carbon-carbon or carbon-oxygen bonds [34]. In general, quinones are prevalent across numerous plant families and are also found in fungi and bacteria, and even in small quantities in animals, notably in echinoderms. However, toxic forms of quinones, such as anthraquinone and 1-hydroxyanthraquinones, may arise in the environment through the sunlight-driven photo-oxidation of polycyclic aromatic hydrocarbons (PAHs). Otherwise, vitamin K (fillo or menaquinone) is crucial for life due to its role in blood coagulation processes and its effectiveness in preventing cardiovascular diseases and treating osteoporosis. Other quinones such as thymoquinone and anthraquinone-2,6-disulfonic acid also exhibit a range of activities, including antibiotic (phaeosphenone), antimicrobial (anthraquinones), and anticancer (thymoquinone, Tanshinone IIA, emodin, doxorubicin) properties [33].

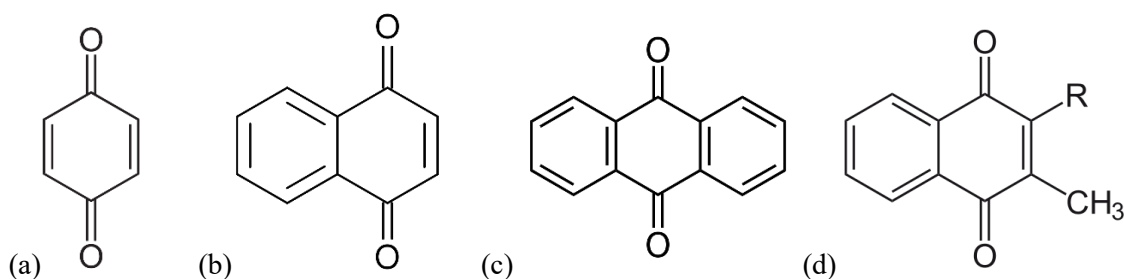


**Figure 20.** Chemical structure of the quinones: benzoquinone (a), naphthoquinone (b), anthraquinone (c), and vitamin K (d).

### 3.4. COUMARINS

Coumarin, initially isolated in 1820 from tonka beans (*Dipteryx odoranta* Wild; Fabaceae family), was also referred to as Coumarou, a term derived from French. Coumarins belong to the category of benzopyrones (1,2-benzopyrones or 2H-1-benzopyran-2-ones) and is extensively found across various natural sources [35]. They represent a group of heterocyclic, phenolic compounds characterized by the presence of both a benzene and an alpha-pyrone ring [36]. Based on chemical structure variations, coumarins can be classified into several groups including simple coumarins (**Figure 21**), furanocoumarins, pyranocoumarins, pyrone-substituted coumarins, and isocoumarins, which are isomers of coumarin with a reversed lactone ring configuration [37]. Coumarins are extracted from numerous plant species including celery, coriander, cumin, fennel, and parsley. Beyond fragrance, some of them can exhibit notable bioactivity and photosensitivity, offering a range of advantageous properties including antimicrobial, antitumor, anticoagulant, antioxidant, anti-inflammatory, anticarcinogenic, antidiabetic, analgesic, and anti-neurodegenerative effects [36].



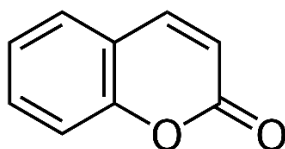


**Figure 21.** Chemical structure of coumarin.

### 3.5. BENZOPHENONES AND XANTHONES

Benzophenone (**Figure 22. (a)**), a secondary metabolite produced by type III polyketide synthases, exemplifies the diversity resulting from variations in starter substrates, condensation reactions, and ring closure mechanisms. Benzoyl-CoA, a rare starter molecule, initiates the process, with benzophenone synthase orchestrating the formation of linear tetraketide intermediates from benzoyl-CoA and three malonyl-CoA molecules. These intermediates undergo intramolecular cyclization to yield 2,4,6-trihydroxybenzophenone. Polycyclic benzophenone derivatives, prevalent in the *Clusiaceae* family, exhibit varied structures. Additionally, xanthenes, derivatives of benzophenones, demonstrate regioselective cyclization [34]. The biosynthetic pathway defines xanthenes as cyclized 2,30-dihydroxybenzophenone derivatives. Xanthenes (**Figure 22. (b)**), secondary metabolites present in select higher plants, fungi, and lichens, have garnered significant interest due to their pharmacological potential and taxonomic significance. Initially described in 1977 as a diverse group of natural compounds, xanthone glycosides have since become a subject of considerable research focus, particularly regarding their pharmacological properties. The xanthone structure, derived from the Greek word for yellow, comprises a planar, conjugated ring system connecting two aromatic rings via a carbonyl group and an oxygen atom. This class includes various subclasses such as oxygenated xanthenes, prenylated xanthenes, xanthone glycosides, and others, distinguished by their structural features and degree of oxidation. These compounds exhibit considerable structural diversity and are found abundantly in nature, with approximately 650 xanthenes identified from various sources, notably mangosteen fruit. Higher plants, including those from families such as *Gentianaceae*, *Moraceae*, *Guttiferae*, *Polygalaceae*, and *Leguminosae*, are key sources of xanthenes, with *Clusiaceae* and *Gentianaceae* exhibiting particularly high concentrations [38].

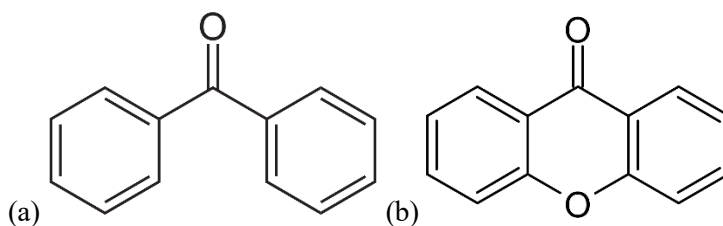


**Figure 22.** Chemical structure of benzophenone (a) and xanthone (b).

### 3.6. STILBENES

Stilbenes are characterized by having two phenyl groups linked by a two-carbon methylene bridge, with resveratrol being the most notable example of this class [39]. They are widely distributed in both dietary items such as grapes, peanuts, and other foods, as well as in medicinal plants. Natural stilbenes, which are polyhydroxyphenolic compounds, are formed through hydrogen substitution by hydroxyl groups in various positions of benzene rings, with stilbene serving as the fundamental parent nucleus. These compounds encompass stilbene monomers, polymers, and heteromers. Extensive pharmacological studies have revealed the diverse biological activities of stilbenes, including antitumor, anti-inflammatory, antioxidant, antibacterial, and antiviral properties [40].

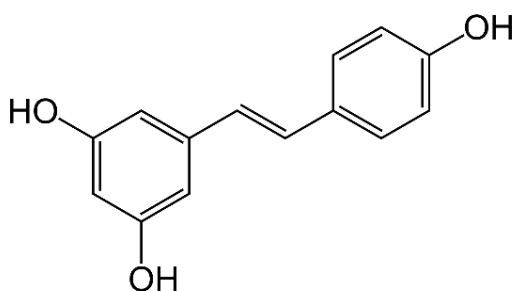


**Figure 23.** Chemical structure of resveratrol.

### 3.7. LIGNINS AND LIGNANS

Lignins, essential polymers contributing to the robustness of plant cell walls, are synthesized via the shikimate pathway. They consist of repeating units derived from coniferyl alcohol, 4-hydroxycinnamyl alcohol, and sinapyl alcohol (**Figure 24. (a)**) [41]. Otherwise, lignans, abundant in the plant world, are natural compounds distinct from lignins despite sharing similar monomers. While lignins are polymeric, lignans are dimeric, featuring a phenylpropanoid core. Lignans are identified as dimeric C<sub>6</sub>C<sub>3</sub> motifs coupled at carbons 8 and 80. Compounds with a coupling differing from C<sub>8</sub>–C<sub>80</sub> are termed neolignans. Lignans follow biosynthesis through the phenylpropanoid pathway and are categorized based on their molecular structure, including arylnaphthalene, aryltetralin, dibenzylbutane, dibenzylbutyrolactone, tetrahydrofuran, and furofuran. Biologically, lignans exhibit diverse activities such as anticancer, antioxidant, antihypertensive, antiviral, estrogenic, and insecticidal properties [41]. In the realm of dietary lignans, matairesinol and secoisolariciresinol (**Figure 24. (b)**) are converted by intestinal flora into enterolactone and enterodiols, exhibiting estrogenic activity [39].

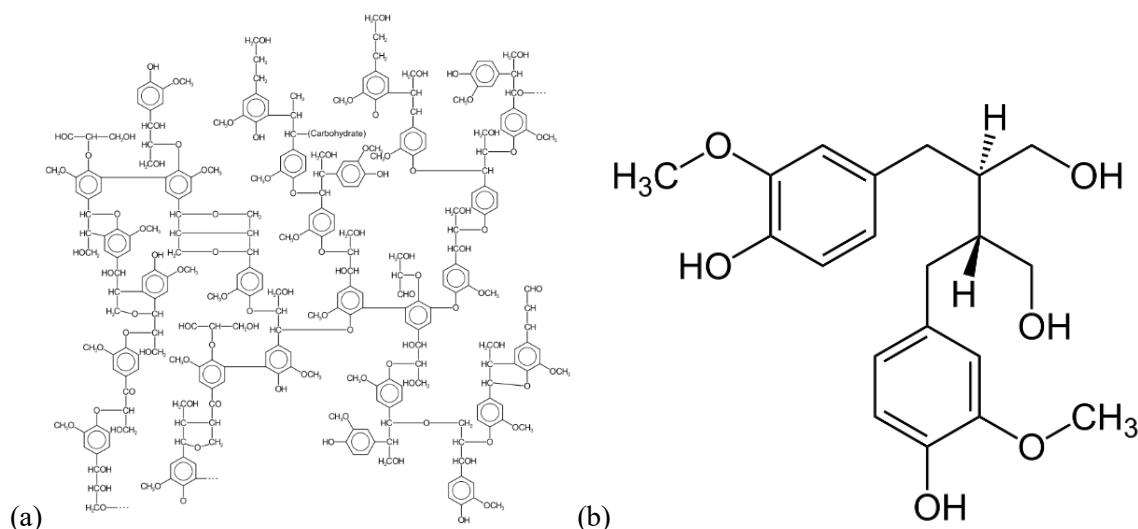


**Figure 24.** Chemical structure of lignin (a) and the lignan secoisolariciresinol (b).

### 3.8. FLAVONOIDS

Flavonoids encompass a diverse group of phytochemicals extensively distributed throughout the plant kingdom. They are notably concentrated in the epidermis of leaves and fruit skins, serving crucial roles as secondary metabolites in plants. With over 8000 distinct varieties identified, flavonoids often contribute to the vibrant hues of fruits and flowers. Their presence spans across a wide array of consumables including fruits, vegetables, tea, wine, dark chocolate, grains, roots, stems, leaves, and flowers, constituting a regular part of human dietary intake [42]. Flavonoids, which possess the diphenyl propane (C6-C3-C6) skeleton, are polyphenolic compounds categorized into various subgroups as outlined in Figure 1. Variations within each subgroup stem from differences in the number and arrangement of hydroxyl groups, as well as the nature and extent of alkylation and/or glycosylation. The degree of hydroxylation plays a crucial role in determining flavonoids' susceptibility to degradation in the colon and the resulting degradation products formed by colonic microflora. Typically, flavonoids in foods are glycosylated, with glucose or rhamnose commonly linked sugars, although galactose, arabinose, xylose, glucuronic acid, or other sugars may also be present. Glycosylation, which can occur at one, two, or three sugar positions, along with additional substitutions, significantly influences the chemical, physical, and biological properties of flavonoids. The presence of sugar molecules also appears to influence the absorption of phenolics. Phenolics that include sugar molecules are absorbed through the small intestine [32]. However, the biological properties and bioavailability of phenolic compounds depend to a great extent on the food matrix.

#### 3.8.1. Flavonols

Flavonols, a subgroup of flavonoids containing a ketone group, are essential for synthesizing proanthocyanins (flavanols) and are found abundantly in various foods such as lettuce, tomatoes,

onions, apples, grapes, berries, tea, and red wine with antioxidant properties and a reduced risk of vascular disease [32]. Prominent examples of flavonols include quercetin, kaempferol, myricetin, and isorhamnetin (**Figure 25**) [42].

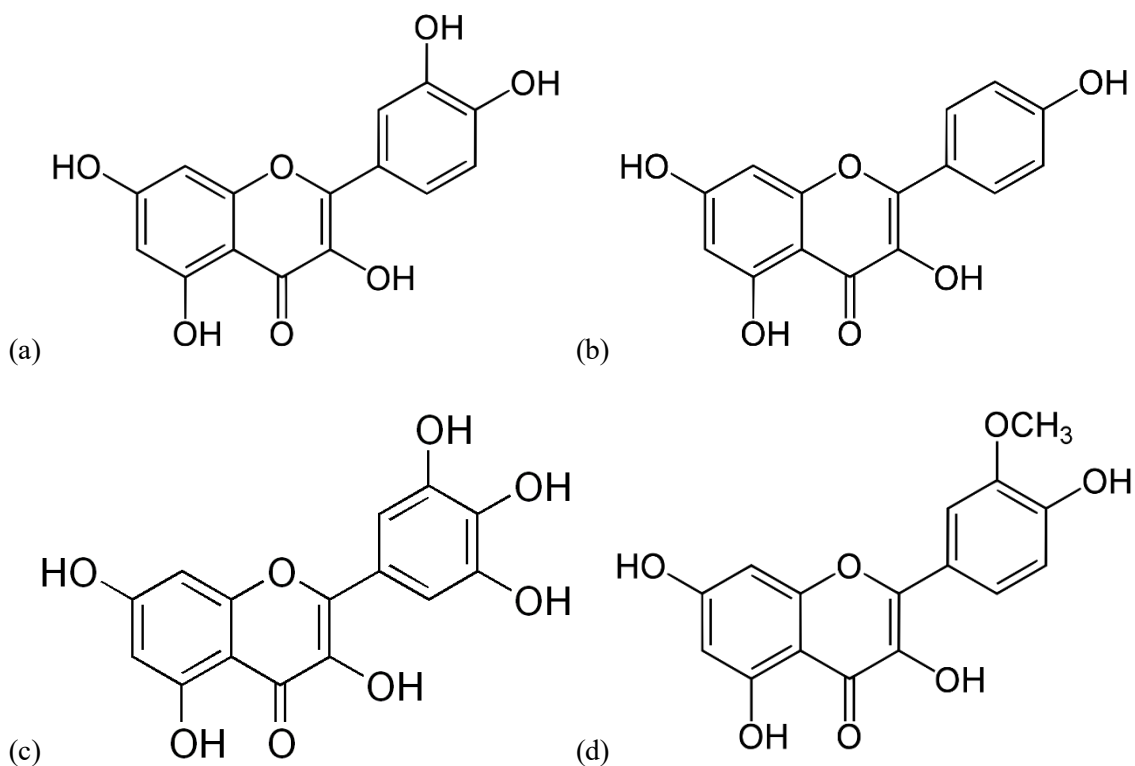


**Figure 25.** Chemical structure of the flavonols quercetin (a), kaempferol (b), myricetin (c) and isorhamnetin (d).

### 3.8.2. Flavanols

Flavan-3-ols, commonly known as flavanols, represent the most intricate subgroup within the flavonoid family due to their diverse range in size (**Figure 26**). They span from simple monomers like catechin and its isomer epicatechin to the more complex oligomeric and polymeric forms known as proanthocyanidins or condensed tannins. Proanthocyanidins can exist as polymers with up to 50 units and, when hydroxylated, can give rise to gallocatechins, or undergo esterification to produce gallic acid. While catechins are widely distributed, major dietary sources are often processed plant-based foods like grapes, avocado, tea, wine, and chocolate [42].

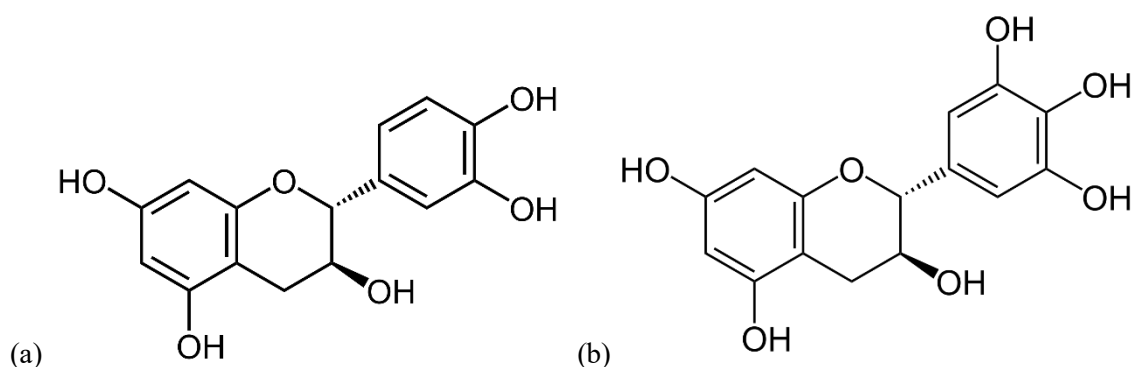


**Figure 26.** Chemical structure of the flavanols catechin (a) and gallocatechin (b).

### 3.8.3. Flavones

Flavones share a structural similarity with flavonols; however, they are less prevalent in plants. Their notable dietary sources include celery, parsley, and select herbs, primarily in the form of 7-O-glycosides such as luteolin and apigenin (**Figure 27. (a) and (b), respectively**). Polymethoxylated flavones, exemplified by compounds like nobiletin and tangeretin, have been identified in citrus fruits [42]. found in fruits and vegetables like Ginkgo biloba and citrus fruits, offer antioxidant, anti-inflammatory, and anticancer effects [32].

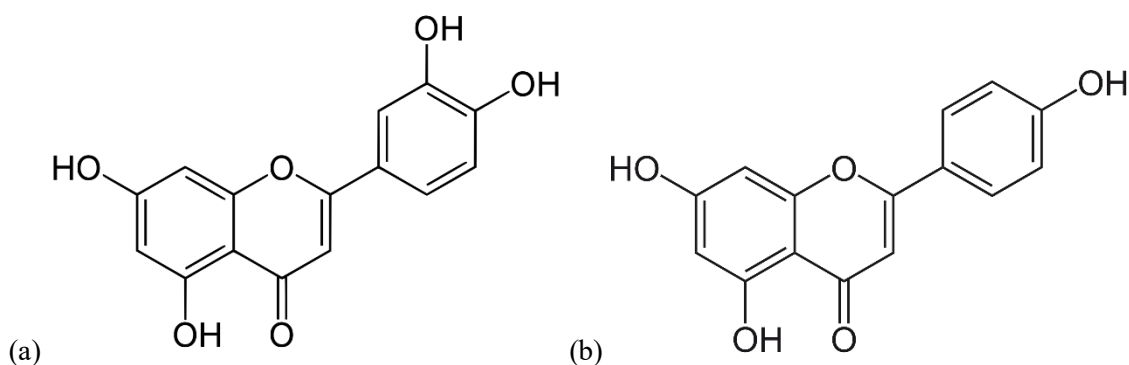


**Figure 27.** Chemical structures of the flavones luteolin (a) and apigenin (b).

### 3.8.4. Flavanones

Flavanones, specific to citrus fruits, provide antioxidant and cholesterol-lowering benefits, among others [32]. Flavanones mark the initial products of the flavonoid biosynthetic pathway. Distinguished by a chiral center at C2 and the absence of the C2–C3 bond, they possess a highly reactive structure prone to hydroxylation, glycosylation, and O-methylation reactions. Citrus fruits are notably rich in flavanones, with hesperidin (hesperetin-7-o-rutinoside) (**Figure 28. (a)**) being the predominant glycoside, particularly abundant in citrus peel. Notably, flavanone rutinosides exhibit a neutral taste, while flavanone neohesperidoside conjugates, such as neohesperidin from bitter orange and naringin (naringenin-7-o-neohesperidoside) (**Figure 28. (b)**) from grapefruit peel, impart an intensely bitter flavor [42].

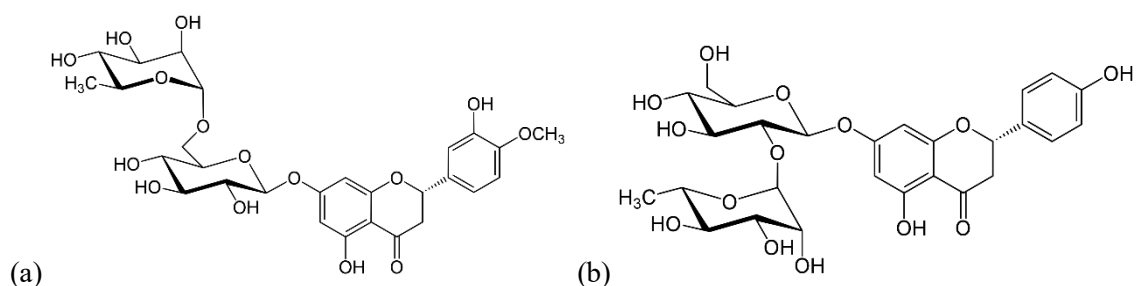


**Figure 28.** Chemical structure of the flavanones hesperidin (a) and naringin (b).

### 3.8.5. Isoflavones

Isoflavones, categorized as flavonoids, are often referred to as phytoestrogens due to their capacity to bind to estrogen receptors and exhibit mild estrogenic effects. While they share some structural resemblances with mammalian estrogens, their estrogenic potency primarily stems from their distinctive structural arrangement and the presence of hydroxyl groups on the A and B rings. Functioning as both estrogen agonists and antagonists, they compete with estrogen for receptor binding. Moreover, isoflavones have demonstrated various biological effects beyond their interaction with estrogen receptors [42]. Isoflavones such as daidzein and genistein (**Figure 29. (a) and (b), respectively**) are most abundant in soybeans [32].

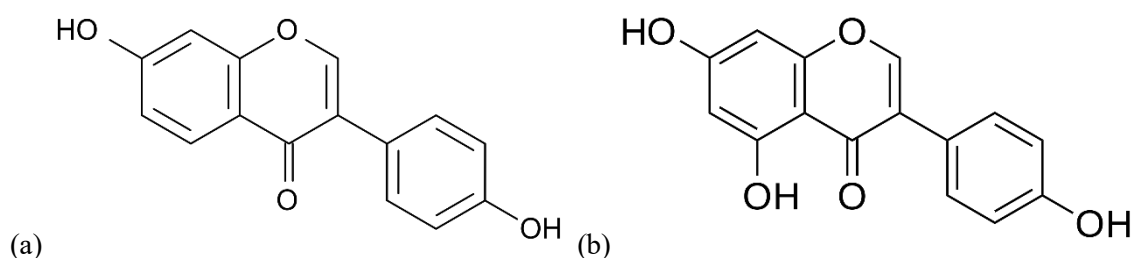


**Figure 29.** Chemical structure of the isoflavones daidzein (a) and genistein (b).

### 3.8.6. Anthocyanins

Lastly, anthocyanins, responsible for vibrant colors in plants and fruits, are abundant in berries and grapes. They are also present in stems, seeds, and root tissue [12]. In plants, their primary functions include shielding leaf mesophyll cells from excessive light and scavenging radicals. Additionally, they serve a crucial role in attracting pollinating insects. Among the most common anthocyanins are cyanidin (**Figure 30. (a)**), pelargonidin, delphinidin, peonidin, petunidin, and malvidin (**Figure 30. (b)**), typically existing in plants as sugar conjugates [42]. They have been also attributed to exhibit anti-inflammatory, antioxidant, antimicrobial, and anticarcinogenic properties, making them valuable in the food industry for various applications [12].

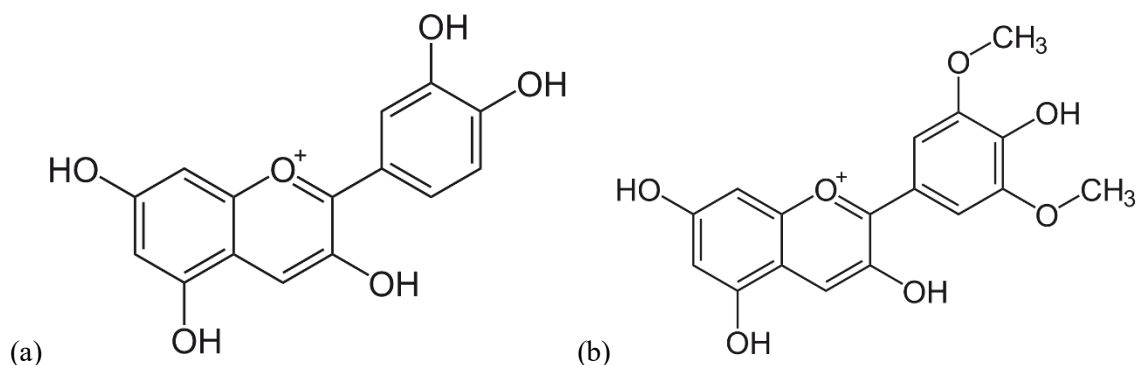


**Figure 30.** Chemical structure of the anthocyanins cyanidin (a) and malvidin (b).

### 3.9. ALKYLRESORCINOLS.

Alkylresorcinols represent amphiphilic phenolic lipids featuring a 1,3-dihydroxybenzene (resorcinol) ring and an odd-numbered alk(en)yl chain attached to C-5 of the benzene structure. The alkylresorcinol side chains may vary, being either saturated or containing one or two double bonds, resulting in approximately 30 different identified compounds. They can be found in plants such as in *Ginkgo biloba* (biobol (**Figure 31**)). Varying degrees of substitution result in a range of compounds reported to exhibit antimicrobial, antiparasitic, antitumor, and antioxidant properties [43]. In cereals, particularly rye and wheat, these side chains commonly consist of 15–25 carbon atoms, with rye containing higher total alkylresorcinol content compared to wheat. Given their presence in the outer parts of grains and stability during processing, alkylresorcinols serve as reliable biomarkers for whole-grain rye and wheat consumption. Moreover, alkylresorcinols may exhibit cancer-preventive properties and influence lipid metabolism [44].

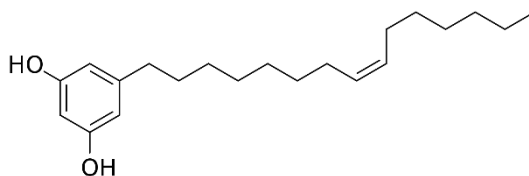


**Figure 31.** Chemical structure of the alkylresorcinol biloba.

### 3.10. TANNINS

Tannins, intricate chemical compounds often derived from phenolic acids or tannic acids, belong to the category of phenolic compounds found abundantly across various plant species worldwide, spanning diverse climates and habitats. Throughout antiquity, various cultures procured tannins from diverse sources such as willows, quebracho, maples, wattle, eucalyptus, and red mangrove [45]. Tannins are considered water-soluble phenolic compounds with molecular weights ranging between 500 and 3000 Daltons, characterized by a significant number of hydroxyl or other functional groups (approximately 1 to 2 per 100 Daltons). This structural feature enables them to

form cross-linkages with proteins and other macromolecules. Vegetable tannins possess the capability to aggregate with alkaloids, gelatin, and proteins, leading to precipitation [46]. Tannins are broadly categorized into two groups: hydrolysable and nonhydrolyzable, also known as condensed tannins. Hydrolysable tannins feature a central core comprising polyhydric alcohol like glucose, along with hydroxyl groups esterified either partially or completely by gallic acid (known as gallotannins) or hexahydroxydiphenic acid (referred to as ellagitannins). Upon hydrolysis through acids, bases, or specific enzymes, gallotannins break down into glucose and gallic acids. Condensed tannins are notably more structurally intricate compared to hydrolysable tannins, and their complete compositions remain to be fully elucidated. Primarily, they consist of polymerized products derived from flavan-3-ols and flavan-3,4-diols, or a combination of both. These polymer formations, often termed "flavanols," are commonly recognized as condensed tannins in scientific literature [46].

#### 4. VITAMIN C

Vitamin C, the L-enantiomer of ascorbic acid or antiscorbutic acid, is composed of six carbons and related to the C6 sugars. It is the aldono-1,4-lactone of a hexonic acid with an enediol group on carbons 2 and 3 [47]. Ascorbic acid, known for its remarkable effectiveness against scurvy, was discovered after centuries of search for a cure. The term itself is derived from "antiscorvy." Scottish naval surgeon James Lind in 1753 documented the potential of fresh food consumption in his "Treatise on the Scurvy," which later influenced the British navy to adopt lime juice as a standard treatment in 1795. The structure of ascorbic acid was determined by Sir Walter Norman Haworth, who was awarded the Nobel Prize in 1937 for his work [22].

It is a micronutrient that plays a crucial role in human health, most thanks to its properties as a redox system [48]. Vitamin C functions as a potent antioxidant by donating electrons. It scavenges harmful reactive oxygen and nitrogen species, reducing oxidative stress and potential damage to lipids, proteins, and DNA. It contributes to the regeneration of vitamin E ( $\alpha$ -tocopherol), enhancing antioxidant protection in lipid environments and maintaining cell integrity [48]. Ascorbic acid is essential for several hydroxylation reactions, maintaining metal ions in their reduced states, necessary for enzyme activity. It is crucial for the synthesis of collagen through its role in the activity of prolyl 4-hydroxylase, prolyl 3-hydroxylase, and lysyl hydroxylase. Vitamin C aids in the synthesis of carnitine, tyrosine, and various neurotransmitters and hormones by maintaining the required reduced state of metal ions in enzymes such as trimethyllysine dioxygenase, 4-g-butyrobetaine dioxygenase, homogentisate dioxygenase, and dopamine  $\beta$ -hydroxylase [48]. Vitamin C can degrade the histamine molecule, helping to regulate levels during inflammatory responses and potentially easing allergy symptoms and enhancing immune function [48]. It enhances iron bioavailability by maintaining non-heme iron in the ferrous state and promoting ferric reductase activity in the duodenum, thus improving dietary iron absorption.



Vitamin C also regulates ferritin mRNA translation, which increases intracellular iron storage and prevents oxidative damage [48]. In its role as a cofactor or antioxidant, ascorbic acid is oxidized to dehydroascorbic acid, which is less stable but can be rapidly recycled back to ascorbic acid by enzyme systems, such as those dependent on glutathione or NADPH [48].

However, despite being essential, humans cannot produce it internally like many animals, who synthesize it through their own biological processes. This is due to humans lacking the enzyme necessary (L-gulono-1,4 lactone oxidase) for its synthesis. Consequently, it is vital for humans to obtain adequate amounts of vitamin C from their diet or through supplements to prevent deficiencies [49]. The well-known symptom of ascorbic acid deficiency is associated with connective tissue damage, such as scurvy, which is characterized by fragile tissues and poor wound healing [47]. To avoid this, they have been established recommended daily intakes. The World Health Organization suggests 45 mg/day and the National Academy of Sciences recommending 75 mg/day in women and 90 mg/day in men. To prevent deficiency, a daily intake of 45 to 90 mg is sufficient [1]; however, higher doses (100 mg/day for tissue saturation and  $\geq 500$  mg/day for plasma saturation) may be necessary for optimal health benefits [48]. Research suggests that consuming at least 80-120 mg of vitamin C daily may reduce the risk of these chronic diseases. A daily intake of 150-200 mg of vitamin C can significantly boost its various functions preventing conditions such as coronary heart disease, cancer, and cataracts. Extensive studies confirm that these levels, and even substantially higher doses of vitamin C, are considered safe for consumption [50].

Vitamin C in foods is typically reported as the total of its biologically active forms, reduced ascorbic acid (AA) and oxidized dehydroascorbic acid (DHA), both of which have comparable biological activity [48]. The transformation of AA to DHA can occur enzymatically in the presence of oxygen or non-enzymatically during food processing [51]. Ascorbic acid is reversibly oxidized into dehydroascorbic acid (DHA) upon exposure to light, heat, transition metal ions and pH (alkaline condition) (**Figure 32**) [47]. In the human body, ascorbic acid is primarily absorbed in the ileum and jejunum via the sodium-dependent transporter. DHA is absorbed in the duodenum and jejunum through facilitated diffusion, often involving glucose transporters, though this process can be inhibited by glucose [48].

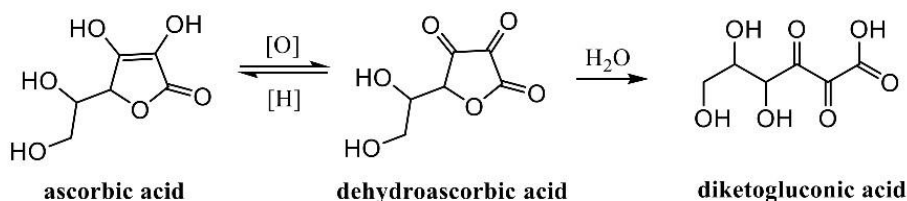


**Figure 32.** Degradation of L-ascorbic acid to dehydroascorbic acid and 2,3-diketogulonic acid (Adapted from [52])

Foods rich in vitamin C, such as pepper, oranges, lemons or tomatoes, are essential for maintaining sufficient levels of this important nutrient [49]. The vitamin C content in citrus fruits and their products varies significantly due to factors such as species variety, agricultural practices, maturity levels, climate conditions, quality of fruit, handling of fresh fruit, processing methods, packaging, and storage conditions [51]. In terms of nutritional value, citrus juices are a rich source of vitamin C. Approximately one-fourth of the vitamin C in citrus fruits is present in their juice. Overall, orange juice has been identified as the richest source of vitamin C among citrus juices, with higher levels than those found in grapefruit, lemon, and mandarin juices [51]. However, the concentration of ascorbic acid is notably higher near the peel than in the center of the fruit. Orange peel has been identified to have the highest concentration of vitamin C (flavedo 34% and albedo 19%), followed by the pulp (21%) and juice (26%) [53].

The impact of fruit ripening on vitamin C levels is debated, with some studies indicating a decrease in vitamin C content as fruits ripen, while others suggest an increase [51]. Apart from that, proper storage and handling of these foods are essential to preserve their vitamin C content [48]. As previously reported, the best strategy to keep the vitamin C content in plant and vegetable juices is to store them in glass containers at a temperature of 4 °C for the shortest time feasible [54]. Besides, the **effect of citrus processing** techniques like pasteurization, freezing, and concentration on the vitamin C content and antioxidant capacity has been studied extensively, though predominantly at experimental or pilot plant scales. Fresh orange juice, for example, has a limited shelf life that can be extended through thermal processing, which stabilizes the juice against enzyme and microbial activity. However, such processing can lead to a reduction in vitamin C and changes in the juice's flavor, aroma, and color. Pasteurization and freezing have been found to increase the levels of dehydroascorbic acid slightly but do not significantly affect the ascorbic acid content, though the vitamin C in the pulp is significantly reduced by >50% [51]. But in some cases, due to a hard treatment, DHA can further irreversibly hydrolyze to form 2,3-diketogulonic acid (**Figure 32**), an inactive form of ascorbic acid that is usually used as markers of the impact of processing and storage conditions [51].

## 5. ORANGE BY-PRODUCTS

Orange (*Citrus sinensis*) is a citrus fruit and a hybrid of pomelo (*Citrus maxima*) and mandarin orange (*Citrus reticulata*), from a tree in the *Rutaceae* family [55]. In 2022, the worldwide orange production amounted to 47.77 million metric tons. Orange is the second most produced fruit in the European Union, mainly in the countries of the Mediterranean basin where it supposes 6 million tons, almost 10% of the world production. Besides, it is the most important citrus fruit produced in Spain, accounting for 3.4 million metric tons in 2022, followed by tangerines (2.12 million metric tons), lemons (1.0 million metric tons) and grapefruits (0.08 million metric tons). In Spain, oranges are most cultivated in the province of Andalusia, reaching 1.6 million tons in

2020 [2]. The edible part of oranges is considered around 73% of the whole fruit and is composed by 88.1% water, 8.6% carbohydrates, 2% fiber, 0.8% protein, 0.2% of fat, minerals 0.2%, being the major potassium (200 mg/100g), and 0.05% of vitamins, being vitamin C the major (50-82.5 mg/100g) [56].

Fruit juice is a non-alcoholic beverage made from the extraction of the liquid contained in natural fruits such as oranges. They are often consumed for their perceived nutritional benefits. For instance, orange juice has a perceived nutritional value because it contains vitamin C, folic acid, and potassium. Although fruit juice consumption in the European Union used to be higher, the European Union has still historically consumed more fruit juice than any other region worldwide, accounting for a total of 9.1 million liters in 2018, making the region the biggest market for fruit juices and fruit nectars globally [2]. Increasingly, citrus juices have gained great popularity, representing more than 50% of the juices available in international trade. From them, orange juice is the main product processed by the beverage industry and consumed throughout the world due to its high nutritional value and desirable sensory characteristics [57]. In 2022 orange juice production reached 1.64 million metric tons. The leading producers of orange juice were Brazil, the United States, and Mexico, with the European Union occupying the fourth position. This means that around 3.8% of worldwide orange juice production originated from the European Union, amounting to 62,000 metric tons. Within this context, in 2021 Spain was the fourth producer of orange juice in the European Union, and the ninth worldwide [2]. In Spain orange juice accounts for 30% of the fruit juice and nectar sales-volume and the production was 5.1 million liters [2].

During the processing of oranges to obtain their juice, by-products are generated, mainly consisting of the peel composed by the flavedo and albedo (**Figure 33**). The peel is estimated to be 20-50% of the whole fruit and is a rich source of soluble sugars, bioactive compounds, dietary fibers (cellulose, hemicelluloses, and pectin), vitamins, and essential oils [58–65].

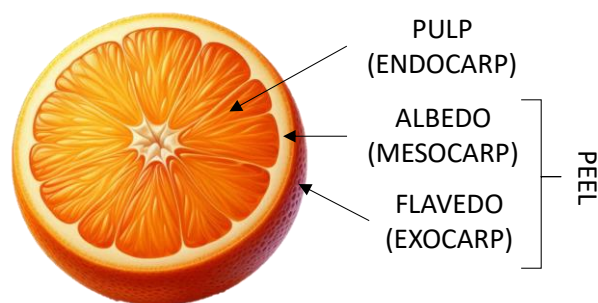


**Figure 33.** Orange parts.

Using whole orange peel without separating its components, directly or simply dried, offers a straightforward method to process this raw material. Efforts have been made to utilize them as livestock feed due to their nutritional value, although challenges such as low protein content and high sugar levels limit their effectiveness in this regard [66]. Orange peel, like many plant-based substrates, serves as a rich nutrient source for microbial communities, particularly those in the rumen. These microbes have adapted to digest plant materials, making orange peel an excellent high-energy feed for ruminants, which promotes microbial growth and lactation. Research indicates that cattle gain more weight when fed orange peel waste compared to starch-rich feeds. However, high concentrations can cause rumen parakeratosis, especially when the dietary forage is low. Additionally, careful preparation is crucial to prevent mycotoxin development due to poor preservation, which is harmful to ruminants [67]. Another viable use for waste orange peel is converting it into fertilizer through composting. The resulting compost exhibits some phytotoxicity, but within acceptable levels [67]. Besides, orange peels continue to find applications in various food products worldwide, from baked goods [68], and jams[69] to savory dishes, where they enhance flavor and aroma. Additionally, their inclusion in certain culinary traditions, such as Indian and European cuisines, highlights their versatility and culinary appeal [66].

Directly utilizing orange peel is a straightforward method requiring minimal infrastructure or investment, significantly increasing the value of this waste material. However, orange peel contains numerous high-value compounds. By adopting a biorefinery approach, we can further enhance the value of this substantial agricultural waste. Biorefinery maximizes the value of biomass feedstock by producing multiple products and minimizing waste [67].

Orange peel contains several high-value compounds that can be extracted using advanced technologies, transforming it from a problematic substrate into a valuable commodity. Among these compounds, essential oils and pectin stand out for their economic potential [67].

As it is well-known, the orange peel is a source of **essential oils**. The volatile fractions of orange peel essential oil boast a diverse array of chemical constituents, including terpenoids [70]. On average, they make up about 0.5–5% of the fresh weight of citrus peels. Additionally, most citrus peels contain monoterpene and sesquiterpene hydrocarbons, along with their oxygenated derivatives, which encompass aldehydes, ketones, acids, alcohols, and esters. Among these, limonene and  $\gamma$ -terpinene emerge as the primary constituents in the essential oils of citrus peels [66]. The monoterpene hydrocarbons like  $\beta$ -phellandrene,  $\gamma$ -terpinene, myrcene, D-limonene, p-cymene,  $\beta$ -pinene, and  $\delta$ -3-carene, oxygenated monoterpenes such as geranial, nonanal, and (Z)-neral, along with terpene alcohols like  $\alpha$ -terpineol, (E)-verbenol, linalool, (E)-carveol, and geraniol, and sesquiterpenes like (Z)- $\alpha$ -bergamotene, are the most prominent in orange peels. Aldehydes such as decanal and esters like ethyl cinnamate and ethyl *p*-methoxycinnamate contribute significantly to the chemical composition of orange peel essential oil [70]. The essential oil present in the orange peel have also a non-volatile fraction oil, comprising 1 to 15% of cold-pressed oil, consists mainly of limonoids, including limonin [70]. Limonoids, a group of natural tetracyclic triterpenoid compounds, exist in both aglycone and glucoside forms. Aglycones can be further categorized into neutral dilactones, acidic monolactones, and dicarboxylic acids, most of which are bitter and water insoluble. Conversely, limonoid glucosides, predominantly as 17- $\beta$ -D-glucopyranosides of acidic aglycones, lack bitterness and are water-soluble [66].

The global demand for **pectin**, is primarily met by citrus peels including orange peel, which yield over 124 million tons of commercial pectin per year [66]. This versatile substance serves as a crucial ingredient in the food industry, functioning as a gelling agent, emulsifier, and fat substitute [70,71].

Other high-value compounds present in the orange peels are organic acids, alkaloids, carotenoids, vitamin C and phenolic compounds. Despite its significance in food production, these bioactive compounds in orange peel also could be used for the production of nutritional dietary supplements or for giving and added value to food products [72].

**Organic acids** hold significant importance among biologically active compounds. Citrus fruits are particularly rich in these acids, some of which possess antioxidant properties. Citric and malic acids are the primary organic acids found in citrus fruits, with citric acid being the most prominent. Citric acid not only predominates but also enhances the efficacy of vitamin C. Oranges contain the citric acid mayorly in the endocarp but between 0.6 and 1.5% could remain in the by-product [72].

The primary **alkaloid** components found in citrus peels are synephrine and N-methyl tyramine. Synephrine stands out as the most prevalent alkaloid in dried citrus peels, constituting approximately 0.38% of its composition [66].

The orange color of orange fruits comes from **carotenoids** and apocarotenoids, mainly xanthophyll esters. Fully mature oranges have significantly higher carotenoid content in the peel compared to the pulp. The major carotenoids in oranges' peel are violaxanthin, along with lutein, antheraxanthin, and  $\beta$ -carotene. In orange peels, xanthophylls like  $\beta$ -citraurin are linked to fatty acids such as stearate, palmitate, caprate, oleate, palmitoleate, myristate, and laurate [70].

**Vitamin C**, or ascorbic acid, is the most plentiful vitamin in citrus fruits, making these fruits a key natural source of this nutrient. The peel of citrus fruits is especially high in vitamin C, with oranges having 34% in the flavedo, 19% in the albedo, 47% in the pulp [72].

**Phenolic compounds** of orange peel include phenolic acids, flavonoids, and anthocyanins. Regarding phenolic acids, the hydroxycinnamic acids are more common in citrus than the hydroxybenzoic acids. Caffeic, ferulic, p-coumaric and sinapic acids are the most common representatives in orange peels [73]. Among the flavonoids found in orange peels are primarily categorized into two groups: flavanone glycosides, and polymethoxyflavones. Flavanone glycosides are formed by glycoside linkage on the basic benzo- $\gamma$ -pyrone (C6-C3-C6) skeleton of flavonoids. Hesperidin and narirutin—hesperetin and naringenin metabolites, respectively—naturally present in the orange peel, exemplifies this group [74]. Polymethoxyflavones, on the other hand, are flavones with multiple methoxy groups attached to their basic benzo-c-pyrone (15-carbon, C6-C3-C6) skeleton, along with a carbonyl group at the C4 position. Their less polar and planar structure facilitates high permeability through biological membranes and efficient absorption into the bloodstream. Nobiletin is one of the most extensively studied polymethoxyflavone in orange peels [66]. Additional flavonoids, such as flavonols (quercetin, rutin, myricetin, kaempferol), and anthocyanins are also present in citrus peel, albeit in small quantities. Quercetin and kaempferol are the predominant flavonols found in orange peel, primarily in their glycosidic form. Anthocyanins, which serve as important pigments in certain flowers and fruits, are minor constituents in citrus peels and are typically only identified in blood oranges [73].

Orange peel also contains significant amounts of **cellulose, hemicellulose, and lignin**, which remain after extracting the previous mentioned high-value compounds. These components can be converted into high-value products through microbial fermentation. Ethanol production from orange peel involves enzymatic hydrolysis followed by fermentation using *Saccharomyces cerevisiae*. Methane, the main component of natural gas, can be produced anaerobically by methanogenic bacteria from various substrates, including orange peel waste. The thermophilic

anaerobic digestion of orange waste, followed by aerobic post-treatment, has been successful. This ethanol and methane processes not only enhances the economic value of orange peel waste but also promotes sustainable practices by reducing waste and producing environmentally friendly products [67].

### **5.1. Bioactivities of orange peel phenolic compounds**

It was studied a possible antiproliferative effect of the bioactive compounds of sweet orange peel against human hepatoma cells, in vitro and in vivo. It has been demonstrated that water, ethyl acetate and hexane orange peel extracts make an inhibition of HepG2 cell growth in vitro, but in 19.27, 48.68 and 62.06%, respectively [75,76]. Hesperidin exerted anti-carcinogenic activity in lung cancer demonstrated in mice with a feed supplementation of 25 mg/kg [77]. Hesperidin also was discovered to have an anticancer activity in breast cancer in vitro in a dose-dependent manner [78]. A posterior in vivo study confirmed that with a dose of 50 mg/kg daily for 8 weeks in rats of hesperidin induces the apoptosis in mammary gland tumors [79]. Hesperidin was also reported to induce apoptosis in human colon cancer cells [80]. Moreover, hesperidin was demonstrated to work reducing the cytotoxic effect and limiting the cardiac toxicity of imatinib treatment of breast cancer in mice [81]. Similarly, orange peel extract and its main compound naringin were reported to protect the side effects of doxorubicin treatment in esophageal cancer stem cells derived tumors in vivo [82]. Orange peel extracts were demonstrated to exhibit chemopreventive effects on Cyclophosphamide cancer treatment induced organ toxicity, attributed mainly to naringin, hesperidin, diosmin, rutin and neohesperidin flavonoids [83]. Thus, orange peel extracts rich in hesperidin were demonstrated to have anticancer activity against liver, lung, colon, and breast cancer, while linked to naringin, limiting the side effects of usual cancer treatments as a chemopreventive.

In vitro studies indicated that hesperidin and naringin from bitter orange peel have antiatherogenic and anti-inflammatory effects by activating peroxisome proliferator-activated receptor and up-regulating adiponectin expression in adipocytes, which plays an important role in glucose and lipid metabolism [84]. Hesperidin could reverse inflammatory conditions acting as an anti-inflammatory better than indomethacin (nonsteroidal anti-inflammatory) without tissue toxicity with a treatment of 50 mg/kg in rats with air pouch model of inflammation, acting as substitute of indomethacin and preventing ulcers [85].

Besides, hesperidin was attributed to have an antinociceptive activity and in turn the additive and supra-additive response when combined with other nonsteroidal anti-inflammatory as ketorolac, suggesting their clinical potential in pain therapy [86]. Hesperidin was reported to alleviate acetaminophen induced toxicity in rats by abrogation of oxidative stress, apoptosis and inflammation, demonstrating a hepatoprotective and nephroprotective activity [87]. Besides,

Naringin, and naringenin from orange peel hydroethanolic extracts exert their hepatopreventive effects in acetaminophen administered rats via enhancement of the antioxidant defense system and suppression of inflammation and apoptosis [88]. The renal protective effect of hesperidin was discovered too against gentamicin-induced acute nephrotoxicity in rats, thanks to its action as a potent scavenger of free radicals in the kidney preventing the toxic effects at the biochemical and histopathological levels [89]. In a recent study, it has been demonstrated through the treatment with 50 mg/kg of hesperidin during 28 days against methotrexate induced hepatotoxicity in rats, that it has a protective effect on the oxidative stress, lipid peroxidation, inflammation and apoptosis [90,91]. On the other hand, it has been studied the effect of hesperidin treatment in cigarette smoke extract induced chronic obstructive pulmonary disease in mice, with a clear alleviation of the inflammation and oxidative stress [92]. Also, hesperetin has showed a hepatic protective effect by reducing the apoptosis in mice with oxidative liver damage [79]. The treatment with orange peel water extracts in rats at 100 mg/kg for 28 days demonstrating a protective effect against CCl<sub>4</sub> induced injury in rats by attenuating oxidative stress attributed to hesperidin content of the orange peel extract [93]. More concretely, in a recent study in rats with the treatment of isolated hesperidin (100 mg/kg for 4 weeks) for the same CCl<sub>4</sub> induced injury significantly depressed CCl<sub>4</sub>-induced elevation of several biomarkers. Furthermore, the protective or therapeutic administration of hesperidin markedly restored the CCl<sub>4</sub>-induced altered hepatic architecture, depleted glycogen, and DNA contents [94]. These results confirm the role of the flavonoids hesperidin and naringin from the orange peel as a potent hepatoprotective and nephroprotective agent to combat liver disease.

One of the most prevalent complex inflammatory diseases is rheumatoid arthritis. It is expressed with high oxidative stress and inflammatory biomarkers. Its continuous administration of usual medicine is associated with deleterious side effects [95]. So, the use of natural resources in order to alleviate this issue is a new research area. One example of that is the use of orange peel compounds extracts. Hesperidin has been reported to have antirheumatic activity in collagen-induced arthritis in mice with the administration of 150 mg/kg three times a week [96] and in adjuvant arthritis in rats with the administration of 80-160 mg/kg a day [97] apart from restoring the index of immune organs. These effects are due to the inhibition of the synoviocyte activity and the modulation of the inflammatory cytokine production of synoviocytes [98]. A hesperidin derivative, 7,3'-dimethoxy hesperetin has been found to have an important antirheumatic activity in vitro inducing apoptosis in adjuvant arthritis fibroblast by modulating a group of genes than activate the caspase-3 expression at protein and transcription levels [99].

It has been demonstrated a cardioprotective effect of hesperidin in rats as an anti-aging. It is due to that the treatment of 100 mg/kg elevates the myocardial antioxidant status by increasing GSH levels, lowering the lipid peroxidation and protein oxidation, and increasing the expression of



antioxidant enzymes [100]. By this way, hesperidin seems to have a role in improving the induced hypercholesterolemia and fatty liver in rats with the diet supplementation of 20 mg/day, by inhibiting both the synthesis and absorption of cholesterol and regulating the expression of mRNA for retinol binding protein, and heart and cutaneous fatty acid-binding proteins [101]. It has been demonstrated that a daily oral consumption of 500 mg of hesperidin 3 times a week improves the endothelial function, reduces circulating biomarkers of inflammation, and favorably alters lipid profiles in subjects with the metabolic syndrome [102]. Another study in individuals with hypercholesterolemia demonstrated that, the consumption of 500 mg/day of hesperidin for 6 weeks lowers the serum triglycerides level and consequently suppress the small dense LDL formation [103]. The oral administration of 50 mg/day of naringin for 8 weeks in induced hypercholesteremic rabbits showed that naringin plays an important role in regulating antioxidative capacities by increasing the superoxide dismutase and catalase activities through regulating their gene expressions [104]. Moreover, this treatment of naringin contributes to hypocholesterolemic action via regulating the acyl-CoA cholesterol acyltransferase enzyme activity and higher excretion of fecal sterols from the diet [105].

It has been studied the possible effect of the oral administration of 50mg/kg of hesperidin or naringin against induced type II diabetic rats, and the results show that both phenols exhibit antidiabetic effects by potentiating the antioxidant defense system and suppressing proinflammatory cytokine production [106]. Orange peel hydroethanolic extract, naringin and naringenin have potent anti-diabetic effects in NA/STZ-induced type 2 diabetic rats after treatment of 100 mg/kg during 4 weeks via their insulintropic effects and insulin improving action which in turn may be mediated through enhancing insulin receptor, GLUT4 and adiponectin expression in adipose tissue [107]. Besides, naringin from sweet orange peel were reported to improve testicular function in high fat diet-induced diabetic rats [108].

These studies demonstrate that compounds from sweet orange peel, particularly hesperidin and naringin, possess a wide range of bioactivities, including anticancer, anti-inflammatory, hepatoprotective, nephroprotective, cardioprotective, and antidiabetic effects (**Figure 34**).

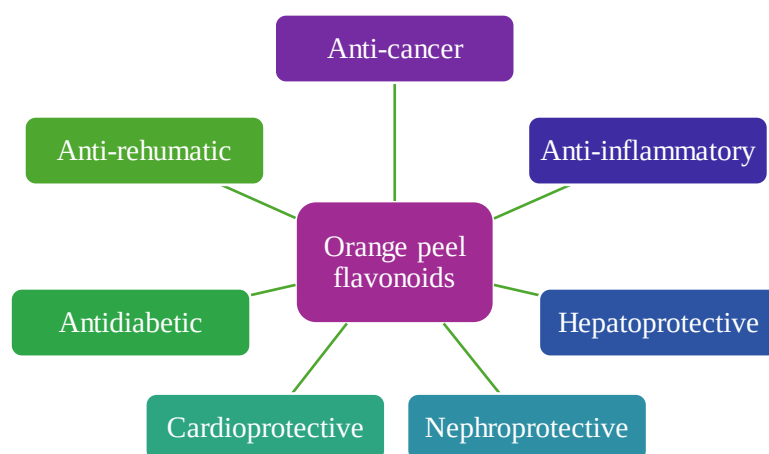


**Figure 34.** Graphical representation of orange peel flavonoids bioactivities.

## 6. AVOCADO BY-PRODUCTS

Avocado is a fruit that belongs to the *Persea* genus within the *Lauraceae* family. It is primarily cultivated in tropical and subtropical regions such as Central Mexico but has become prevalent worldwide. There are numerous varieties of avocado according to the growth climate, with Hass being the most well-known and marketed [109]. Global avocado production in 2022 reached approximately 8.98 million metric tons, marking a notable increase from the previous year and a 70% rise compared to 2000. Mexico led global production, yielding just over 2.5 million tons, while Colombia and Peru also ranked among the top producers, with volumes of about 1.1 million and 866 thousand tons, respectively. Additionally, Spain emerged as the fourth-largest exporter and importer of avocados in 2022, and it held the same rank in avocado consumption within Europe [2].

Avocado by-products are industrial waste generated during the processing of avocado fruit in the production of guacamole or essential oils [110], principally composed of peels and seeds (**Figure 35**) [111]. These wastes account for 21–39% of the whole avocado [57].

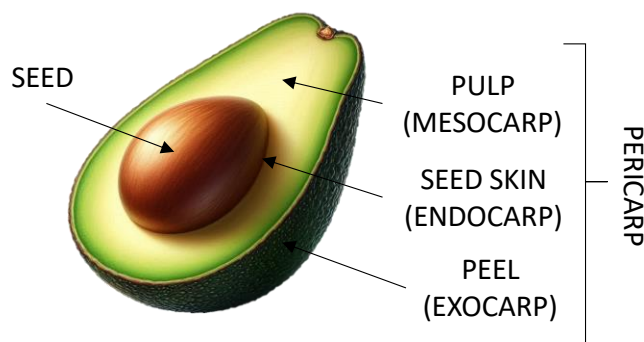


**Figure 35.** Avocado parts.

The seeds of Hass and Fuerte avocado varieties exhibit a similar proximate composition: 50-70% moisture, 1-3% ash, 1-3% protein, 13-25% starch, and 0.5-5% fat, which is significantly less than the pulp. Most of the lipids in the seeds are neutral lipids (77-80%), with glycolipids and phospholipids accounting for 7.4% and 10.9%, respectively. Additionally, the seeds have a high dietary fiber content (5-15%) [57,112]. Avocado peels, often underutilized, have a composition that includes 60-70% moisture, 0.5-5% ash, 1-3% protein, 2-5% total lipids, 10-15% carbohydrates and 20-30% fiber [57].

In this context, the valorization of those by-products is an important environmental problem that must be faced. Until now, avocado by-products have been processed using different biorefinery approaches, such as obtaining bioenergy, biogas, biofuel, biodiesel or bioethanol, as well as the

extraction of oils, fermentable sugars, starches, and colorants [57]. However, those residues are also a prospective source of bioactive compounds [109]. The primary bioactive compounds identified in avocado pulp and its by-products (peel, seed, and leaf) are phenolic compounds. Following polyphenols, the most notable compounds include carotenoids, tocopherols, and sterols [57].

The total *carotenoid* content in both avocado pulp and peel is comparable, whereas the seed contains significantly less. Most carotenoid identification studies concentrate on avocado pulp, highlighting xanthophylls (oxygen-containing carotenoids) such as lutein, zeaxanthin, and  $\beta$ -cryptoxanthin as the primary carotenoids. Regarding carotenes, also minor amounts of  $\alpha$ -carotene and  $\beta$ -carotene were found. Other carotenoids identified in avocados include neoxanthin, neochrome, lutein-5,6-epoxide, and chrysanthemaxanthin [57].

***Sterols and tocopherols*** ( $\alpha$ -,  $\gamma$ -, and  $\delta$ -) have been primarily identified in the avocado pulp, with  $\gamma$ -tocopherol being the predominant compound, followed by  $\delta$ -tocopherol and  $\alpha$ -tocopherol. Approximately 89% of the total sterol content in Hass avocado pulp is  $\beta$ -sitosterol, with smaller amounts of campesterol and stigmasterol [57].

Avocado seeds and peels exhibit significantly high total *phenolic compounds* content compared to the pulp and leaves. The main phenolic compounds identified in avocado are flavan-3-ols or procyanidins. They include types A, B, and C, with types A and B reported in avocado seed and peel, and type C in leaves. These PCs exhibit different degrees of polymerization (up to 13), and their content increases with fruit ripening due to the release of tannins bound to cell-wall structures. Other identified compounds include a variety of phenolic acids, such as hydroxybenzoic, vanillic, caffeic, ferulic, protocatechuic, gallic, sinapic, chlorogenic, resorcylic, syringic, and cinnamic acids, as well as flavonoids like luteolin, quercetin, apigenin, rutin, catechin, and epicatechin. Additionally, they have been described two iridoids, penstemide and its isomer, in the pulp, seed, and peel of avocado [57].

Thus, avocado agro-industrial wastes have huge potential use in developing high-value-added products while reducing its environmental impact. Specially, procyanidins from avocado by-products could be of huge relevance in the fields of nutraceuticals and food with several demonstrated bioactivities [57].

### **6.1. Bioactivities of avocado by-products phenolic compounds**

Agro-industrial avocado by-products have been extensively demonstrated to exhibit high radical scavenging activity against synthetic free radicals (DPPH and ABTS) and reactive oxygen species (peroxyl, superoxide, and hypochlorous acid) and high ability to reduce  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  [112–116]. The main compounds with significant contribution to the antioxidant activity were procyanidin

B2 and epicatechin in the peel and trans-5-O-caffeoyl-D-quinic acid, procyanidin B1, catechin, and epicatechin in the seed [117].

Polyphenols extracted from avocado seeds have demonstrated inhibitory effects on various human cancer cell lines, including prostate cancer (LNCaP), breast cancer (MCF7), lung cancer (H1299), and colon cancer (HT29), exhibiting inhibition rates of 19, 19.1, 67.6, and 132.2  $\mu\text{g/mL}$  respectively in a dose-dependent manner [118]. Similarly, methanolic extracts of avocado peels and seeds have exhibited analogous outcomes in breast cancer cell lines (MDA-MB-231) at a concentration of 0.1  $\text{mg/mL}$  [113]. Furthermore, ethanolic extracts of avocado peels and seeds have been found to induce apoptosis in Jurkat lymphoblastic leukemia cells [119]. Additionally, it has been observed that methanolic avocado seed extracts can stimulate proliferation of normal cells and inhibit the proliferation of immortalized HaCaT keratinocytes, potentially attributed to the presence of proanthocyanidins B1, proanthocyanidins B2, and A-type trimers [120]. Besides, ethanolic peel extracts were demonstrated to inhibit the cell metabolic activity of different cells models such as colorectal adenocarcinoma (Caco-2), lung cancer (A549,) and cervix epithelioid carcinoma (HeLa) [121]. Also in melanoma cells (B16F10), mainly attributed to its procyanidin content [122].

The inflammatory process serves as the body's defense mechanism against microbial invaders, involving various chemical mediators such as nitric oxide (NO) and pro-inflammatory cytokines like TNF- $\alpha$ . TNF- $\alpha$ , primarily released by macrophages, plays a pivotal role in inflammation development, especially in response to stimuli like bacterial lipopolysaccharides. It induces the expression of cell adhesion molecules on endothelial cells, facilitating the migration of defense cells to the site of inflammation. While inflammation is typically a defensive response, it can become excessive, leading to attacks on the host by immune cells. In such cases, drugs targeting TNF- $\alpha$  and/or NO are essential to mitigate or inhibit this exaggerated response. Into this context avocado peel and seed ethanolic extract was demonstrated to suppress the release of TNF- $\alpha$  and NO [117]. With similar mechanisms procyanidins from avocado peels with a mean degree of polymerization  $\text{DP}_m = 6.10$  have been demonstrated to have inhibitory activity against the inflammatory activity of *H. pylori* in vitro [123]. Besides, semi-synthetic adducts prepared from avocado procyanidins were reported to reduce significantly the adherence and IL-8 production in *H. pylori*-infected AGS cells [124]. Ethyl acetate avocado seeds extracts rich in procyanidins were reported to prevent and treat indomethacin-induced gastric ulcer in mice [125]. Proanthocyanidin-rich avocado peel extracts prevented the high protein diet induced alterations of barrier function in Caco-2 cells and attenuated the genotoxic effect of the CSs upon HT-29Glc-/+ , being considered as a promising product for improving colonic homeostasis [126]. Proanthocyanidin-rich polyphenol extracts from avocado peel have demonstrated to have positive effects in the microbiota composition in rats [127].

Avocado peel extracts rich in procyanidins can protect and/or prevent transgenic parkin *Drosophila melanogaster* fly against paraquat-induced oxidative stress, movement impairment and lipid peroxidation, as model of Parkinson disease [128].

Research suggests that avocado seeds may have beneficial effects in managing type 2 diabetes by interacting with the peroxisome proliferator-activated receptor-gamma, similar to certain anti-diabetic medications. Studies on spontaneously hypertensive rats fed a high-sugar diet supplemented with avocado seeds demonstrated significant reductions in blood glucose and cholesterol levels. These effects are attributed to bioactive compounds promoting glucose storage in the liver. In diabetic rats, avocado seed extract treatment led to substantial decreases in blood glucose levels and restoration of pancreatic islet cell damage. Additionally, supplementation with avocado seed extracts alongside glibenclamide showed significant blood glucose reduction, comparable to the efficacy of glibenclamide alone. Histological examination revealed preserved pancreatic islet cells in treated rats, indicating the potential anti-diabetic properties of avocado seed extracts and the need for further investigation into their therapeutic benefits [118].

These bioactivities highlight the potential health benefits of avocado by-products, particularly in antioxidant, anticancer, anti-inflammatory, gastroprotective, microbiota modulation, neuroprotective, and antidiabetic applications [129] (**Figure 36**).

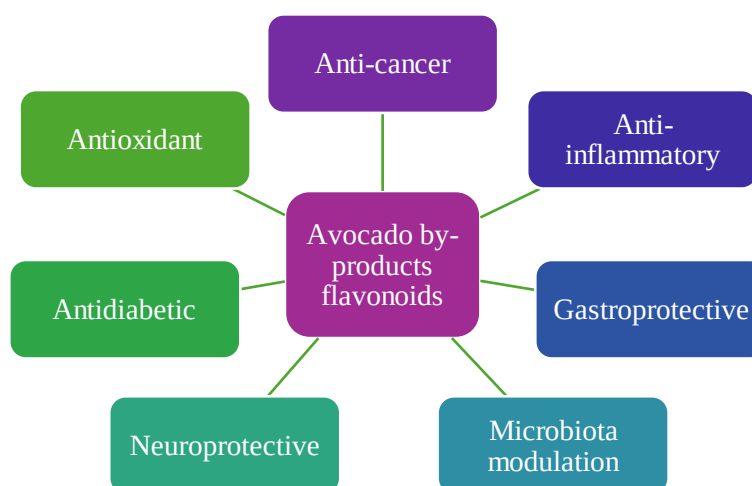


**Figure 36.** Graphical representation of avocado by-products bioactivities.

## 7. APPLIED TECHNOLOGIES

As previously described orange and avocado by-products exhibit several bioactivities, which are directly related to the phenolic compounds present in them. Thus, maximizing the recovery of them is crucial. Polyphenols are compounds highly sensitive to various environmental factors, including heat and light lacking of long-term stability [29]. Into this context, **drying** effectively preserves by-products, inhibiting spoilage by reducing moisture content, thus enhancing quality, stability, and shelf-life while minimizing storage space and shipping costs [130]. From other side, novel extraction technologies have been explored as alternatives to **traditional methods** such as solvent extraction. Various non-conventional techniques have been suggested to enhance the extraction of valuable compounds from agricultural waste and by-products. These include high voltage electrical discharges, pulsed ohmic heating, microwave-assisted extraction, subcritical fluid extraction, supercritical fluid extraction, high-pressure processing, accelerated solvent extraction, extraction assisted by hydrotropic solvents, and ultrasound-assisted extraction [131]. These extraction techniques are aligned with the concept of "green" (commonly known as "green extraction techniques" due to the use of food-grade solvents and being more environmentally friendly) and are capable of providing extracts that can be used as raw materials on an industrial scale, with optimal energy and chemical consumption [132]. Non-thermal technologies, such as ultrasound, can enable the obtaining of safe and stable plant-based extracts with minor changes and/or higher phytochemical content compared to thermal techniques. **Ultrasound-assisted extraction** stands out as a sustainable alternative that requires moderate solvent and energy investment [29]. In addition, different pre-treatments such as pulse electric fields and submerged fermentation can be applied to enhance the phenolic compounds recovery. **Pulsed electric fields** have stood out in recent years for this purpose. PEF works as a non-thermal technology producing electroporation and/or electroporabilization of the matrix, which increases mass transfer from plant tissues during the extraction of intracellular bioactive compounds. Additionally, thanks to electroporation and consequently increased extraction of compounds of interest, this technique promotes the use of higher water ratios during extraction, thus reducing the use of organic solvents [133]. **Submerged fermentation** by lactic acid bacteria is another non-thermal pretreatment that it is able to break the cell walls increasing the release of the phenolic compounds during extraction step and/or can causes bio-transformations that increase the polyphenol content and their bioavailability and bioaccessibility [134]. Besides, these approaches can be integrated with other processes to enable component separation, fractionation, and concentration. **Membrane technologies**, among others, offer an appealing avenue for addressing these processes effectively [131].

## 7.1. DRYING

By-products, due to its high moisture content, are easily spoiled. Drying is one of the most common and extended processes used to preserve quality and stability and to extend the shelf-life of fruits, vegetables, and their by-products. It inactivates the oxidant, enzymatic, and microbial degradation. Reducing the water content results in the reduction of weight and volume and it lowers the shipping cost and minimizes the space needed for storage [130]. Thus, selecting the appropriate drying process is a critical step in waste utilization. Various drying technologies utilize distinct mechanisms, leading to a range of dried food products with varying qualities. Therefore, choosing the right dryer is essential for developing dried products that meet specific objectives throughout the drying process. The objectives should be in line with economic considerations, conserving energy and reducing product loss, meanwhile maintaining the organoleptic characteristics minimizing nutritional and chemical changes [135].

When aiming to dry the by-product, firstly, temperature is an important factor, because the bioactive and antioxidant compounds are thermolabile, and high temperatures could lead to their degradation. On the other hand, from the industrial and economic point of view, the drying process should be short and low energy consuming. Moreover, drying involves the simultaneous transference of heat and mass, with convection, conduction, and diffusion processes, turning it into an easy handled but complex phenomenon (Figure 4). Therefore, the use of mathematical models to simulate its kinetics and explain the mechanism of the water transference during this operation is a useful tool to control the process [136]. Several drying techniques have been applied to the dehydration of by-products, such as sun drying, hot air drying, freezing drying, fluidized bed drying, microwave drying, infrared drying... [137]. **Fluidized bed drying**, which involves air flowing through a bed of particles, is often employed to dry particulate products. This method is particularly effective and quick due to the intense heat and mass transfer at the particle surface, facilitated by the continually renewing boundary layer. Nonetheless, its use for drying porous materials presents challenges, including low energy efficiency and extended drying times during the falling rate period. **Microwave drying**, a volumetric drying technique, can shorten drying times and enhance the quality of the final product. The rapid dissipation of energy throughout the material helps overcome the limitations of slower drying methods, enhancing the quality of the dried products, but with a high initial investment and high energy consumption. **Freeze drying** stands out among dehydration methods, especially for heat-sensitive fruits and vegetables, due to its use of low temperatures and an almost oxygen-free process. It maintains the structural integrity of the samples by keeping the substance frozen at the surface where sublimation occurs, thereby preventing structural collapse and ensuring the preservation of the porous structure and non-shrinking of the final product. However, it tends to require more time, thus increasing energy usage and capital costs. In contrast with the previous ones, **hot air drying** is the most widely



adopted because of its simplicity and cost-effectiveness. It has been the subject of numerous studies aimed at developing dehydrated food products. Among all the different techniques used in this scope, convective drying has been demonstrated to be the most scalable and cheapest at the industrial scale [138]. In convective drying hot and dry gas (usually air) is used both to supply the heat necessary for evaporation and to remove the water vapor from the surface of the product (Figure 37).

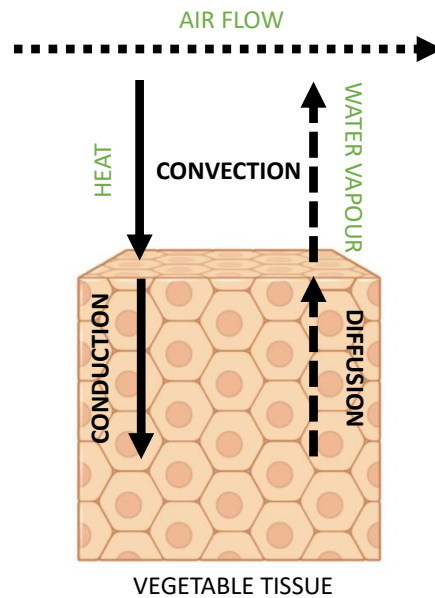


**Figure 37.** Schematic representation of convective drying process.

## 7.2. ULTRASOUND

Ultrasonication (US) holds great promise for the food processing sector owing to its ability to induce intense cavitation through high-intensity acoustic fields. This phenomenon profoundly impacts the chemical, biochemical, and mechanical properties of food matrices, particularly in liquid mediums. Consequently, since the 1990s, the food processing industry has increasingly adopted US in large-scale operations. Extensively researched, US finds application across various processes, including but not limited to mixing, freezing, thawing, homogenization, brining, filtration, enzyme and microbial inactivation, emulsification, stabilization, equipment sterilization, dispersion, hydrogenation, fermentation, particle size reduction, meat tenderization, drying, ripening, aging, oxidation, dissolution, crystallization, extraction, degassing, and food atomization [139].

Ultrasound-assisted extraction (UAE) utilizes ultrasound energy and solvents to extract target compounds from various plant matrices. These conditions expedite biochemical reactions and enhance extraction efficiency [140]. US, characterized by mechanical waves with frequencies exceeding 20 kHz, induces displacement and dislodgement of molecules through a series of compression and rarefaction cycles (**Figure 38**).

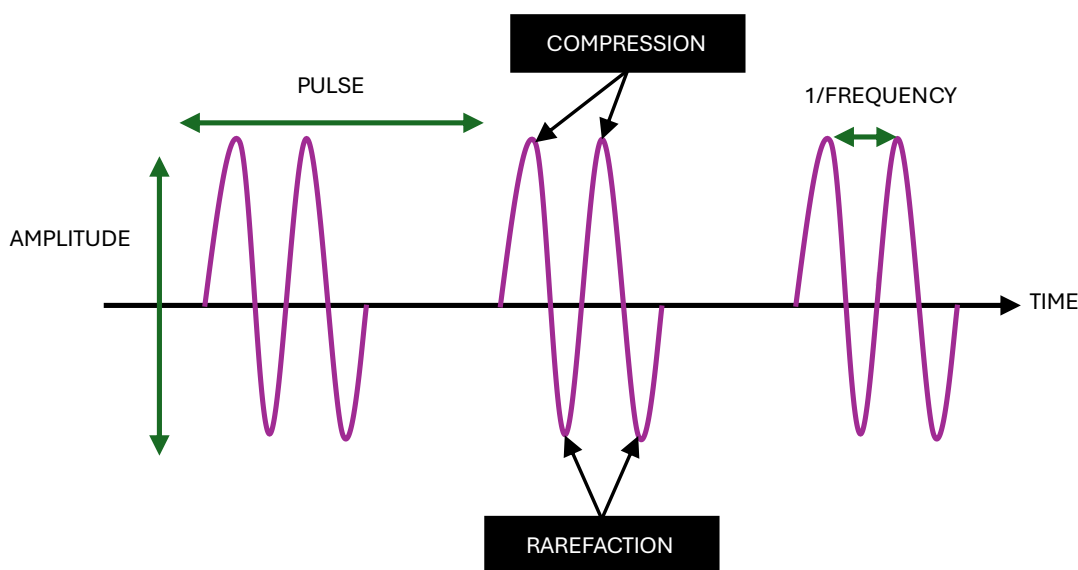


**Figure 38.** Representation of an acoustic wave with its principal parts.

These cycles cause cavitation and the formation of bubbles in liquid medium (**Figure 39**). The formation of cavitation bubbles occurs when the pressure within the liquid falls below its vapor pressure, prompting a phase transition and the formation of vapor or gas bubbles. These bubbles may contain vaporized liquid, gas, or a mixture of both. The acoustic cavitation process can be categorized as either transient or stable, contingent upon the operational factors (such as the power, amplitude, pulse, frequency and time) and the composition of the liquid medium [139].

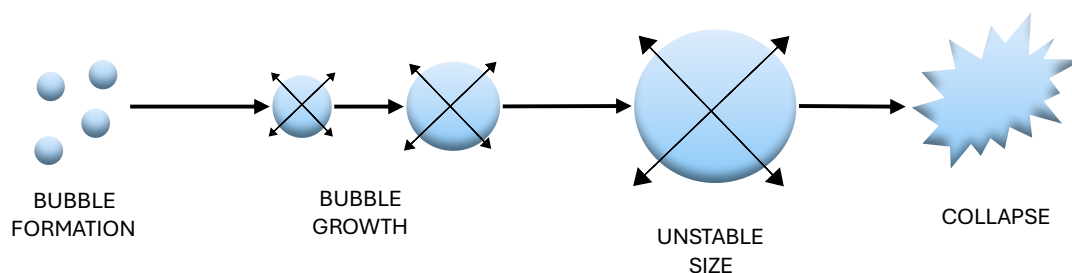


**Figure 39.** Representation of bubbles life in ultrasound treatments.

Thus, acoustic cavitation is the primary mechanism involved, inducing phenomena such as (**Figure 40**): fragmentation (1), erosion (2), pore formation (sonoporation) (3) and shear force (4) in plant cellular matrices. Fragmentation (1) due to collapsing cavitation bubbles leads to solubilization of bioactive components by decreasing particle size and increasing surface area, while erosion (2) facilitates solvent contact and extraction yield. Sonoporation (3) results in the release of bioactive compounds from cell membranes, while shear force (4) and turbulence break down cell walls, aiding in compound release. Ultrasound also enhances water absorption and swelling index of plant tissue, improving solute desorption and diffusion for increased extraction yield. The enhanced extraction yield achieved by UAE results from the combined effects of these mechanisms [140].

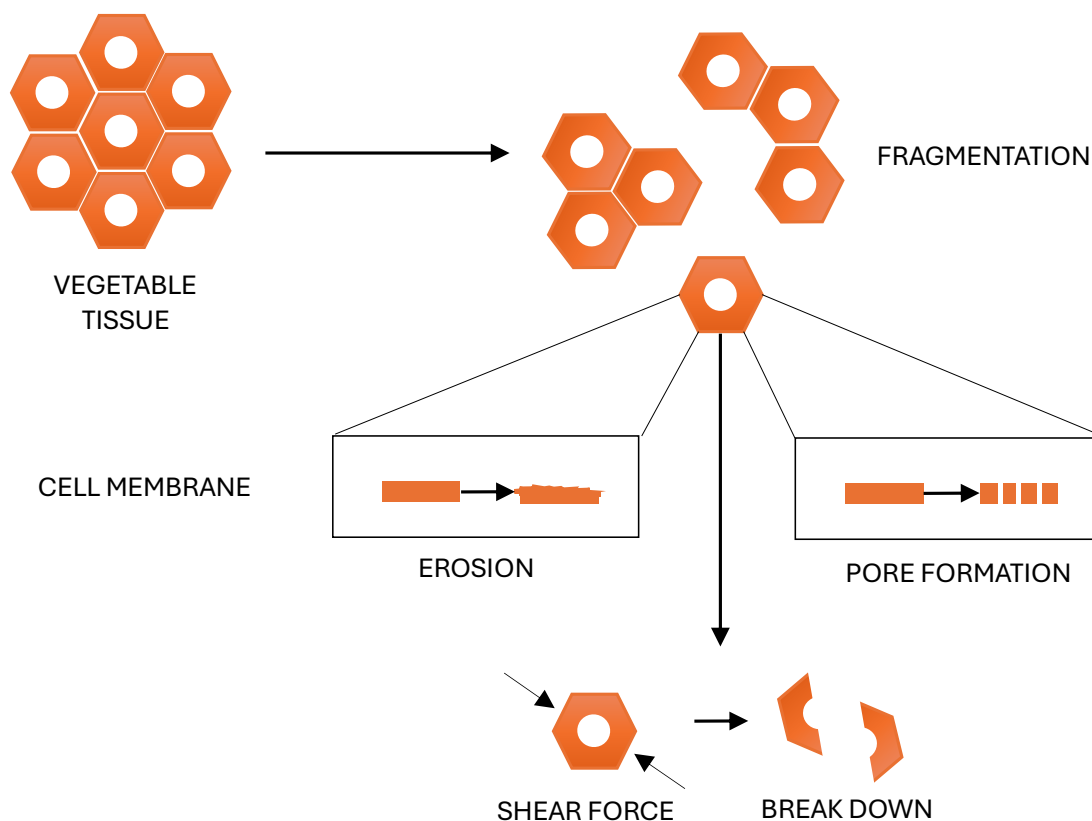


**Figure 40.** Phenomenon taking place in in vegetable matrix during ultrasound treatment.

Ultrasound-assisted extraction (UAE) is commonly performed using either **ultrasonic baths** or **ultrasonic probes (Figure 41)**, both of which rely on piezoelectric transducers as the source of ultrasound power. In ultrasonic baths, the solid matrix is dispersed in the solvent within a stainless-steel tank connected to the transducer. While ultrasonic baths are economical and easy to handle, their low reproducibility limits their use in extraction processes. Ultrasonic probes, on the other hand, consist of a probe or horn connected to a transducer, with the probe immersed in the extraction vessel to deliver ultrasound to the media with minimal energy loss. Probe-based systems are preferred over bath systems due to their higher ultrasonic intensity at the tip of the probe, making them powerful tools for extracting bioactive compounds [141]. The non-uniform energy distribution and reduction of power over time in bath systems reduce efficiency compared to probe systems, where energy delivery is concentrated in a specific sample zone, generating a more efficient cavitation effect [142]. The ultrasonic energy is radiated into the sample through the probe, with probes of smaller tip diameters generating greater cavitation effects but limited to a narrower field. Probes with larger tip diameters have lower cavitation effects but distribute energy over a larger area. Laboratory-scale probe tip diameters range from 2 mm for handling sample volumes up to 5 mL to 25 mm for samples up to 1L.

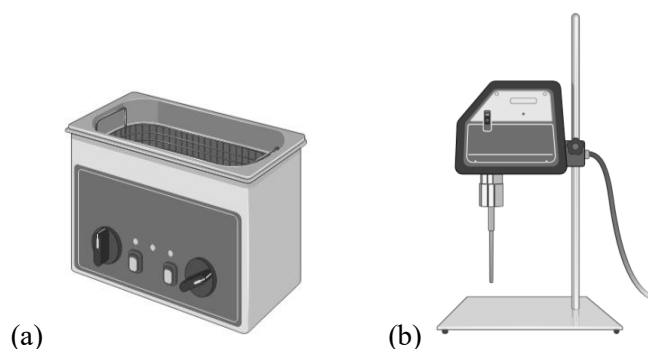


**Figure 41.** Ultrasound bath (a) and ultrasound sonotrode with probe (b) at laboratory scale.

The efficiency of ultrasonic treatments can be influenced by various factors, which can be categorized into product factors or process factors. Regarding the process factors they can be summarized as power, frequency, pulse, solvent, liquid-solid ratio, and others such as probe immersion depth or probe diameter [143].

**Power** (expressed in Watts (W)) denotes the rate at which acoustic energy is emitted per unit time. However, the actual output power fluctuates depending on factors such as sample volume and characteristics, reactor setup, and temperature changes during the process. Each ultrasonication (US) transducer is designed with a maximum input power. During operation, the power input can be adjusted by modifying the power amplitude (%) within the range determined by the manufacturer's specifications [139]. The amplitude of ultrasonication (US) is typically expressed in micrometers ( $\mu\text{m}$ ) concerning the kinematic properties of the medium, representing the

maximum height of a wave cycle or the maximum displacement of the medium's particles from their equilibrium position. US transducers commonly offer vibration displacement amplitudes ranging up to 20–25  $\mu\text{m}$  peak-to-peak (pp). In systems employing US probes, the intensity of cavitation correlates with the displacement amplitude of the incorporated US probe. The transducer's vibration amplitude is magnified by a significant reduction in the probe's cross-sectional area from input to its output tip surface. Consequently, a high-amplitude US wave is transmitted to the liquid medium. This amplification, specific to each probe type, is denoted by an amplification factor or amplitude gain. The amplitude gain of the horn is inversely proportional to the diameter of its output tip. Thus, the amplitude delivered to the solution can be computed by multiplying the transducer's amplitude by the amplitude gain of a specific probe provided by the supplier. While this amplitude gain remains constant for a given probe, the amplitude from the US generator to the transducer can be adjusted within the range of 20% to 100% in most setups [139]. The power applied for UAE of bioactive compounds from fruit and vegetables by-products depends on the compound to be extracted and the types of plant matrix selected for the extraction, varying in the range from 20 to 700 W [144,145]. The yield of the UAE extraction increases with an increase in power or power density and then decreases after reaching a peak. This can be explained by the increase in the effect of violent cavitation bubble collapse with an increase in power. The resonant bubble size is proportional to the power of the ultrasonic wave, and as the bubble size increases, their impact on implosion also intensifies [146]. However, when power is increased to a very high level, it leads to an increase in the number of bubbles formed. The cavitation effect is decreased at higher bubble volume concentration. An increased number of bubbles leads to large inter-bubble collision, deformation, and collapse in a nonspherical way, which decreases the impact of the bubble implosion [147]. In addition to that, the layer of cavitation bubbles assembled around the probe tip hinders the transmission of the energy into the extraction medium (saturation effect), reducing the yield. The high ultrasound intensity may also degrade the bioactive compound, decreasing the yield [148].

**Frequency** denotes the number of sound waves passing through a medium per second, measured in hertz (Hz). The ultrasonic generator emits a signal at a predetermined frequency, which corresponds to the operating frequency of the transducer. This frequency is typically set by the manufacturer with very limited tolerance for variations. Operating at low frequencies (20–100 kHz) results in the formation of large and transient cavitation bubbles that collapse with significant intensity. Therefore, low-frequency operation is preferable for applications requiring robust sonophysical effects, because generates strong shear and mechanical force, which is advantageous for example in the extraction process [139]. At higher frequencies, reaching up to 500 kHz, sonochemical effects become more pronounced. The increased frequency leads to a higher number of bubbles, which in turn enhances the production of free radicals through the

sonolysis of water molecules. This phenomenon boosts various applications, including chemical synthesis [139]. In UAE of bioactive compounds from fruit and vegetable by-products, the frequency typically ranges from 20 to 120 kHz. However, there are few studies investigating the effect of varying frequency on extraction yield and quality, with most studies focusing on constant frequency [140].

**Pulse or cadence.** US signal can be delivered in continuous, pulsed, or sweeping modes. In pulsed mode, acoustic energy is transmitted in short bursts or pulses, resulting in a low average intensity of output over time. During pulsed US, the output is cycled between "ON" time, representing the pulse length, and "OFF" time, which denotes the pulse interval. The cadence or pulse refers to the percentage of time during which the US signal is "ON" [140]. Pulsed mode can help to reduce bubble coalescence and enhances active cavitation zones. In contrast, continuous US (with a pulse of 100%) involves the continuous transmission of acoustic energy, maintaining a constant acoustic intensity over time and in some cases may lead to the formation of excessive bubbles, which subsequently coalesce to form clouds, aiding in degassing [139].

**Solvent.** The choice of solvent plays a significant role in the ultrasonication (US) process. For instance, in a high-viscosity liquid medium, not only does the mass transfer rate of the solvent decrease, but the cohesive forces within the medium also elevate the cavitation threshold. Moreover, high viscosity tends to diminish the active cavitation zone, resulting in greater attenuation of the acoustic wave. Conversely, low-viscosity samples may lead to heightened bubble coalescence, thereby reducing acoustic intensity. Additionally, in the nucleation phase of bubbles, liquids characterized by high surface tension offer increased resistance to mass transfer during the compression phase of the acoustic wave. However, in subsequent stages, this surface tension aids in stabilizing bubble growth [139]. Various solvents, including acidified water, ethanol, alcohols, acetone, and water, have been employed for extraction purposes during UAE. Among these, alcohols, and acetone, with varying levels of water content, are commonly utilized for extracting phenolic compounds from plant materials. Ethanol, in particular, has demonstrated a superior affinity for phenolics in numerous studied systems and is therefore often the preferred solvent for extracting phenolic compounds from fruit and vegetable waste [149,150]. Ethanol's popularity as a solvent is also attributed to its affordability, its renewable source (sugar cane), and its classification as a generally recognized as safe (GRAS) solvent [151]. The concentration of ethanol is a critical factor affecting the yield in UAE. Despite efforts to reduce or eliminate ethanol usage in extraction processes, current research has only succeeded in minimizing its volume for extraction using UAE [140].

**Liquid-solid ratio.** The concentration of solvent can be expressed either as the liquid-solid ratio (LSR), which is the ratio of the volume of solvent (mL) to the quantity of sample taken for extraction (g). The yield of UAE increases with an increase in LSR up to a certain point, after

which it decreases following a peak. At low LSR values, the solution's viscosity is high, posing greater difficulty for cavitation effects as the negative pressure during the rarefaction cycle must overcome stronger cohesive forces in the highly viscous solution. Initially, as LSR increases, the viscosity and concentration of the extraction medium decrease, leading to a greater cavitation effect. The higher concentration difference of the solute enhances diffusivity and dissolution of the solute in the solvent, thereby augmenting the extraction process. At high LSR values, the ultrasound intensity imposed on the by-product matrix increases, resulting in more fragmentation, erosion, and pore formation, thereby increasing the yield. The increase in contact area between the material and solvent may also contribute to higher yields. However, a decrease in yield at very high LSR values may be attributed to enhanced cavitation effects, leading to the degradation of the desirable solute itself [140].

**Other.** The **immersion depth of the probe**, pertinent to US probe/sonotrode systems, and the liquid height within the reactor or beaker, relevant to plate transducers in a US bath setup, have notable effects on the behavior of acoustic waves within the liquid and against the reactor wall surfaces. This parameter also influences the dynamic characteristics of liquid movement and the distribution of cavitational activity. Placing the probe in close proximity to the bottom or base of the reactor enhances the reflection of sound waves, thereby expanding the cavitation zones [139]. Moreover, the **ratio of the probe** significantly impacts the extent of cavitational activity. In larger reactor sizes, a higher ratio of the probe's immersion diameter to the reactor vessel diameter results in broader cavitational activity, leading to increased power dissipation within the medium. This effect helps overcome acoustic impedance until an optimal diameter ratio is reached, typically reported to be around 1.5 to 1.45 for notable sono-chemical efficiency. Probe diameters exceeding this optimal value may lead to a reduction in the acoustic amplitude delivered to the reactor medium [139].

Therefore, UAE can offer an advantage by enhancing extraction yield at lower temperatures, thereby reducing extraction time, and producing a higher quality product. Moreover, scaling up the UAE process is feasible [143]. The use of UAE in fruit and vegetable by-products is of huge interest in the recent years for the extraction of phenolic and antioxidant compounds. Studies have explored the extraction of these compounds from various sources, including mango peel [152], pomegranate peel [153], grape pomace [154], apple pomace [150], pomegranate seed [155], papaya seed [144], among others.

### 7.3. PULSE ELECTRIC FIELDS

Pulse electric field (PEF) is a novel non-thermal green technology that is considered promising in the food industry. Briefly, it consists of application of an external electric field to a food matrix for a short period of time (from nanoseconds to milliseconds), resulting in the formation of pores in cell membrane, a phenomenon called electroporation [156,157]. The electric field applied causes an increase of the transmembrane potential that led to the formation of hydrophilic pores that increase in size and number as the longer is the treatment or higher is the electric field applied. Into this context, PEF can cause irreversible electroporation which gives rise to the leakage of intracellular compounds, or reversible electroporation letting the recovery of the membrane integrity (**Figure 42**) [158].

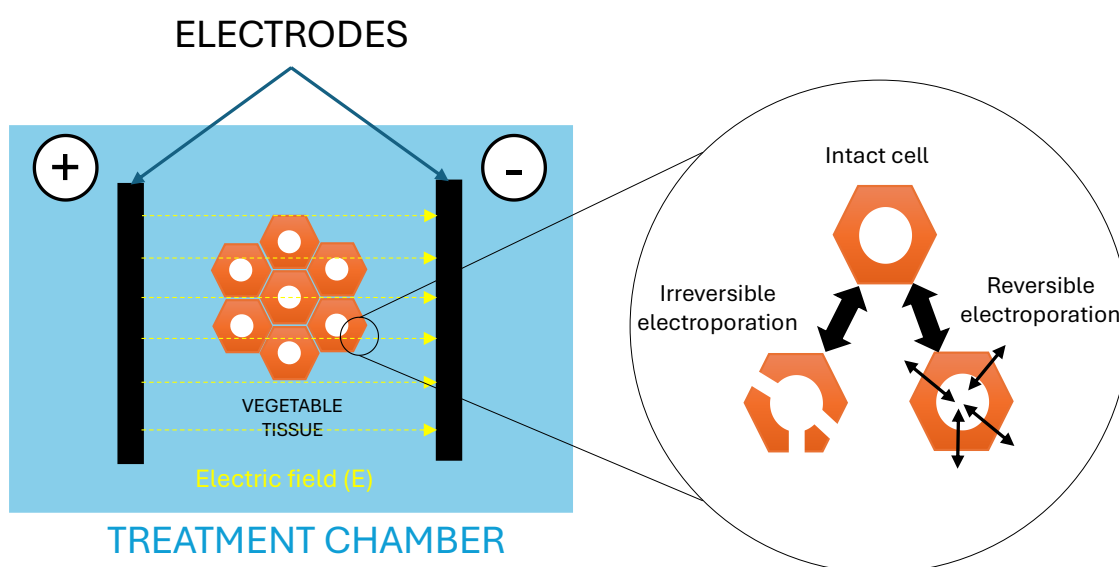


**Figure 42.** Pulse electric fields mechanism in vegetable matrix.

PEF has been widely studied as a non-thermal method for food processing, preservation, and microbial inactivation. The use of PEF in the food industry field has growth with the increased demand of consumers for safe and nutritionally high-quality food [159]. However, its application in extracting bioactive compounds from by-products is less explored but is a growing area of research. PEF technology presents significant potential for enhancing the extraction of valuable intracellular compounds [160]. It facilitates the permeabilization of cell membranes, thereby increasing the diffusivity of intracellular substances and improving extraction efficiency through enhanced mass transfer rates [143]. The efficiency of PEF depends on the process parameters, the food product characteristics, and the processing aim. Foods are complex matrices with various chemical components that have different structures and affect the conductivity and further the PEF treatment [161]. So, an adequate knowledge of these factors and their influence on PEF process is necessary to reach an optimum performance of PEF in food.



The key process parameters defining PEF technology include pulse shape, width and number, frequency, electric field, specific energy and chamber and media characteristics.

**Pulse.** Various **shapes** of electric field pulse waves influence the efficiency of PEF treatment. The most common types in PEF food processing include bipolar or monopolar, exponentially decaying, and square wave pulses (**Figure 43**).

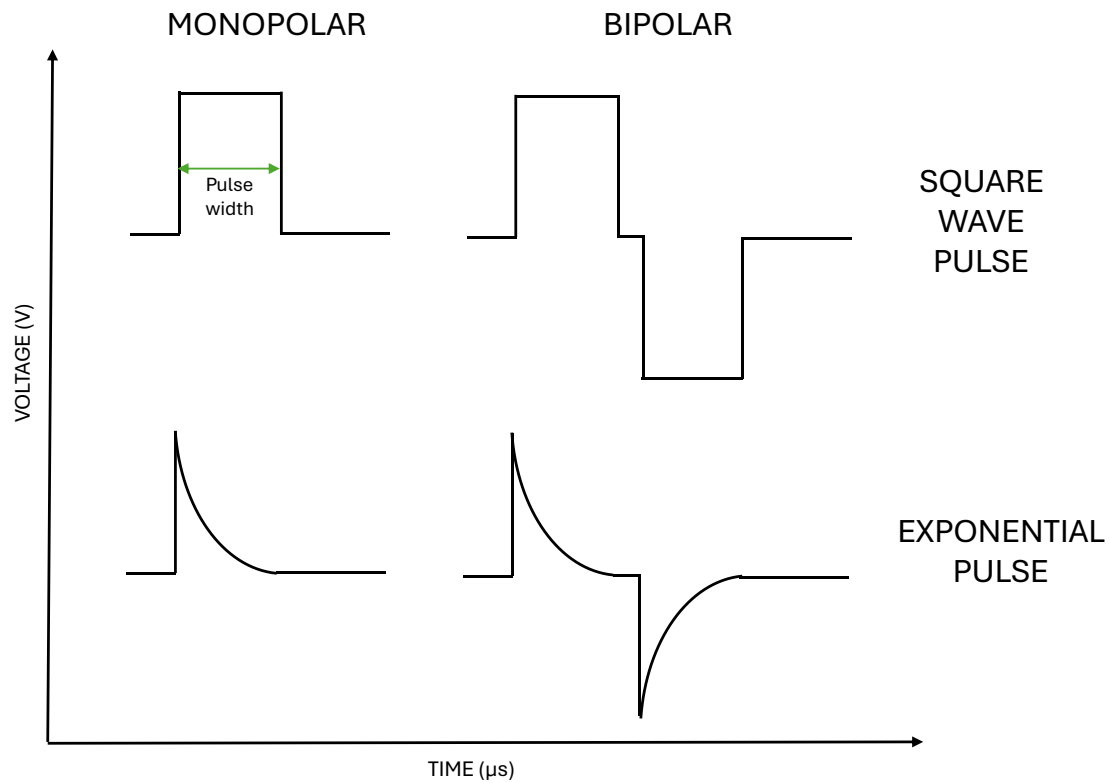


**Figure 43.** Different pulse types and shapes adapted from [162].

In exponentially decaying waves, the voltage is unidirectional, rapidly reaching its maximum value before gradually decreasing, resulting in a lingering lower field strength segment. This lower strength portion does not inactivate cells but rather generates excess heat, increasing the temperature of the food. In contrast, square wave pulses maintain peak voltage for a longer duration without a tailing of lower field strength. Thus, square wave electric field pulses are considered more effective and energy-efficient compared to exponentially decaying wave pulses. However, the production of the pulse, as well as the change in field direction, are easier in exponential decaying pulses than square wave pulses. Bipolar pulses are more effective compared to monopolar pulses of similar field strength. The reversal in the electric field direction caused by bipolar pulses results in bidirectional damage or additional stress to the cell. Additionally, the application of bipolar pulses results in a lesser deposition of solids on the electrodes. However, recent studies have found that bipolar nanosecond electric pulses are far less efficient than

monopolar pulses of similar total duration and amplitude. This inefficiency is attributed to bipolar cancellation, where the application of the second pulse of the opposite polarity cancels the effect of the first pulse [163]. The pulses in PEF system are measured as number of pulses applied and the pulse width expressed in  $\mu\text{s}$ . **Pulse width**, for square pulses denotes the duration at peak voltage. For exponential decay pulses, it signifies the time to reduce voltage to 37% of peak value. In consequence the **treatment time** signifies the cumulative effect of pulses administered multiplied by pulse width, and the **frequency** indicates pulse number per unit time, typically measured in Hz [162].

**Electric field.** The **electric field strength** pertains to the localized field strength within the treatment chamber during sample processing. It is contingent upon factors such as applied voltage between electrodes, chamber geometry, and dielectric properties distribution. The voltage (V) that can be applied is dependent of the capacitor current (I) possibilities and the electrode area ( $\text{cm}^2$ ). This electrode area is function of height (cm) and width (cm) of the treatment chamber. The electric field strength usually is expressed as  $\text{kV/cm}$  calculated with the voltage divided by the distance between electrodes (cm). **Specific energy** per pulse ( $\text{kJ/kg/pulse}$ ) denotes electrical energy received by treated product per pulse. It is influenced by product electrical properties and pulse characteristics. **Total specific energy input** ( $\text{kJ/kg}$ ) is calculated by multiplying specific energy per pulse by pulse count, offering a means to compare data across different conditions and equipment, providing insights into energy consumption [162].

**Chamber.** Characteristics of treatment **chambers**, such as electrode configurations and operational modes, play a crucial role. Typically, treatment chambers feature two electrodes secured by insulating material, forming an enclosure for the food materials undergoing treatment. Various electrode configurations exist, including parallel plates, wire-cylinder, rod-rod, rod-plate, and coaxial (concentric) cylinders. Among these, parallel plates and coaxial configurations are commonly utilized. Parallel plates offer uniform distribution of electric field strength and boast simplicity in design. The chamber size (high and width) and the distance between electrodes modifies the electric field applied to the products [164].

**Conductivity.** The **media** used, such as water and buffer systems, can affect PEF efficiency on various cell types to similar or differing extents. The electric conductivity ( $\sigma$ , Siems/m) of the medium plays a role in PEF efficiency. The ionic strength of the medium is directly correlated with its electric conductivity, meaning higher ionic strength leads to lower PEF efficiency. It has been observed that different pH levels or the presence of ions in the medium can increase or decrease the efficiency of PEF treatment. Besides, each food product or by-product to be treated by PEF has its own conductivity. When the starting sample + solvent product has a higher conductivity, it results in reduced PEF effects due to smaller peaks of the electric field within the

treatment chamber. These smaller peaks do not induce permanent electroporation effects [163]. A rise in **conductivity** has place during PEF treatment and is attributed to membrane electroporation and ion diffusion from cells into surrounding media, initially of lower conductance [162].

PEF is widely utilized for extracting bioactive compounds from various natural sources, addressing the challenge of breaking down cell walls to obtain natural extracts from target tissues or cells. This technique induces cell stress, promoting the production of polyphenols as secondary metabolites and facilitating cell membrane permeabilization. Research on PEF-assisted extraction from by-products primarily targets polyphenolic compounds, but investigations into extracting polysaccharides, proteins, isothiocyanates, phytosterols, steroidal alkaloids, seed and germ oil, among others, have also been conducted. [165–167]. Overall, studies have shown the advantages of moderate electric field pulse technology, whether as a pretreatment step or as part of a continuous extraction system, for extracting phytochemicals from by-products [143]. PEF technology has been investigated for polyphenols and antioxidant recovery from wastes and by-products such as apple peels [168], orange peel [169] mango peel [167,170], papaya peels [171], sesame seeds [172] and sesame cake [166], and grape pomace [173,174][175], stevia leaves [176], potato peels [177] and brewers' spent grain [178].

## 7.4. FERMENTATION

**Fermentation** is an ancient method employed for transforming products into value-added commodities through microorganisms [12]. Nowadays it has evolved into a novel strategy for valorizing fruit and vegetable by-products, with a focus on extracting high-value ingredients [179]. While fermentation dates back to antiquity, advancements in techniques have led to increased productivity, fostering the development of more efficient processes [134]. Currently, two predominant strategies are employed for fermentation, depending on the substrate: submerged fermentation or solid-state fermentation [134]. Both processes aim to obtain bioactive compounds of industrial significance from various substrates, including waste materials. While both processes are utilized in both research and industrial settings, certain methods yield superior results due to variations in microbial metabolism between the two approaches [12].

In **solid-state fermentation (SSF)**, a technique utilized in the absence of water, microorganisms grow on a solid substrate. This method employs microorganisms, like fungi, which thrive without requiring high water levels. SSF optimizes the use of nutrients within the solid substrate. Its benefits include increased yield, production of highly active final products, low water consumption, eco-friendliness, and enhanced resistance to contamination. Nevertheless, SSF encounters challenges such as difficulty in scaling up the process, heat buildup, and challenges in accurately controlling process parameters [134].

**Submerged fermentation (SmF)** is a fermentation method where the substrate is either liquefied or immersed in a water source [12]. It entails introducing the microbial culture into a liquid medium to produce the desired product [180]. This fermentation approach is preferred when fermenting with bacteria or yeasts, as they require high humidity. During fermentation, microorganisms secrete bioactive compounds into the liquid medium, making SmF a common choice for producing secondary metabolites in liquid form [134].

Fermentation processes are broadly categorized into **aerobic** and **anaerobic** modes. Aerobic fermentation, which involves incorporating oxygen into the liquid medium, is responsible for producing antibiotics and enzymes. Conversely, anaerobic fermentation, such as butanol production, occurs without oxygen. Some processes, like ethanol production, involve organisms like *Saccharomyces cerevisiae*, which can switch between aerobic and anaerobic pathways. Enzymes like amylases and proteases are typically produced via Submerged Fermentation (SmF), utilizing the aerobic route [180].

For **industrial purposes**, SmF involves cultivating selected microorganisms within closed vessels known as bioreactors, which contain nutrient broths. Bioreactor design facilitates the supply of oxygen necessary for aerobic microorganisms and enables the monitoring and control of various parameters such as pH, temperature, viscosity, dissolved oxygen, foam formation,

biomass formation, substrate utilization, and desired product formation. The ability to regulate parameters online has facilitated the utilization of submerged fermentation in large-scale fermenters with volumes ranging from thousands to hundred-thousand liters. Submerged fermentation may encounter challenges associated with high broth viscosity, impeding oxygen and mass transfer, and potentially affecting product output. However, these challenges can be mitigated through the implementation of various submerged fermentation processes. There are four primary types: batch culture, fed-batch culture, continuous culture, and perfusion batch culture. **Batch** cultivation, the simplest mode, involves inoculating microorganisms into a fixed volume of fermentation medium for a specified duration, after which the broth is collected for downstream processing. In **fed-batch** mode, concentrated nutrient components are gradually added to the batch culture. **Continuous** reactors continuously receive fresh medium while withdrawing broth from the bioreactor, allowing for extended fermentation periods. In **perfusion batch** cultivation, new medium is added, and cell-free broth is withdrawn in equal volumes. The selection of submerged fermentation process type depends on the end-use of production and the microorganism type. For instance, continuous reactors may offer higher productivity compared to batch reactors and can reduce viscosity for fungal cultivation; however, the prolonged fermentation time in continuous processes elevates the risk of contamination [181].

Thus the primary **advantage** of SmF processes lies in their scalability [180]. Most commercial products are manufactured using SmF processes [180]. SmF is predominantly employed in industrial processes due to its high yield, low cost, and contamination resistance. This fermentation method is also favored by researchers for its ease of process control and sterilization [12]. Another advantage is its efficient mass and heat transfer during the process which is a major drawback of SSF [134].

However, the major **drawbacks** associated with SmF processes include low productivity, high production costs, and medium complexity [180]. Additionally, SmF presents challenges such as physical space requirements and energy or water consumption [12]. Another drawback is that it is not environmentally friendly due to the high volume of waste water that is generated [134].

In a nutshell, the fundamental **difference between SSF and SmF** lies in the substrate used: solid for SSF and liquid broth for SmF [182]. Submerged fermentation is favored over solid-state fermentation in industrial settings due to its ability to control fermentation parameters using defined substrates [181]. Optimization techniques are essential for evaluating these variations and identifying more efficient approaches to reduce process redundancy [182].

The fermentation processes vary depending on starting substrate, microbial cultures, and valorization objective [179]. Thus, the selection of fermentation processes is specific to the product [12]. Extensive literature explores the fermentation of fruit and vegetable by-products

using diverse microorganisms as alternative valorization methods. Into this context, SmF processes have been found to enhance the extraction of antioxidant molecules from food matrices, including vegetable by-products. Additionally, fermentation can alter the profile and types of antioxidant bioactive compounds and may even generate new compounds with desirable biological activities. Furthermore, fermentation can impact the activity of these compounds or their bioaccessibility/bioavailability, although the bioavailability of dietary polyphenols in the human body remains a subject of ongoing research [134].

Utilizing **lactic acid bacteria (LAB)** and other microorganisms, fermentative processes have become pivotal in this endeavor [179]. LAB constitute a diverse group of microorganisms closely related phylogenetically, primarily producing lactic acid as a major or sole product during carbohydrate fermentation. These Gram-positive, nonsporulating, catalase-negative organisms are tolerant to acidity and are either non-respiring or aerotolerant [183]. They can be homo- or heterofermentative. Homofermentation primarily produces lactic acid from disaccharides, while heterofermentation yields lactic acid along with ethyl alcohol, carbon dioxide, and other compounds from lactose decomposition. Predominant LAB cultures utilized in lactic fermentation include species of *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Bifidobacterium*. These bacteria, classified as relative or obligatory anaerobes, thrive in acidic environments. With few exceptions, LAB are nonpathogenic, often possessing generally recognized as safe (GRAS) or food-grade status [151] and are extensively employed in the food industry. Moreover, they constitute a part of the natural microflora of the human intestines [184]. Agricultural processing waste contains fermentable sugars like glucose, fructose, and sucrose, which are readily accessible to LA-producing microorganisms and can be directly converted into lactic acid. The reduced need for raw material pretreatment simplifies the production process and lowers production costs [185].

LAB SmF has been previously reported to be used to enhance the polyphenol and antioxidant content of different samples such as acerola and guava fruit by-products [186], barley by-products [187], avocado leaves [188] and apple pomace [189], among others.

## 7.5. MEMBRANE FILTRATION

Nowadays, membrane processing is widely used in food processing industry over the last 40 years [190]. A membrane, in general terms, can be defined as a selective thin layer of a semipermeable material that under a potential gradient can separate fractions from a solution [191]. Membranes are selective and allow some materials to pass through (the permeate) and other materials to keep retained (the retentate) on it. Sometimes it is the retentate that is desired, in others the permeate stream, while sometimes both products are of value [190]. The potential gradient can be generated by pressure, temperature, electrical or concentration difference and the separation can be based on their sizes or affinity [191]. Membrane technology provides higher selectivity, minimum effect on temperature, it is easier to install and remove and consumes less utilities. Membranes, that have the capability of separate, concentrate, clarify, dealcoholize, pH stabilize, sugar regulate and cold sterilize beverages [192].

Briefly, **membranes can be classified attending to three main characteristics: material, configuration, and pore size.** According to its **material**, membranes can be organic or inorganic. Organic ones are the most common membranes used, mainly made of polymeric materials. Examples of that are polysulfone, polyethersulfone or polyvinylidene fluoride, usually hydrophilic materials that have two key advantages: they have higher resistance to attachment of organic material on their surface and they become wet easily, which makes them more permeable for a given pore size [193]. On the other hand, inorganic membranes can be metallic or zeolite-based, but the most used for food applications are ceramic membranes. These membranes have superior mechanical, thermal and chemical properties but are more expensive than organic membranes, so a balance between these factors must be done in order to select the more appropriate [194].

Regarding the **configuration**, there are a wide variety of geometries available in the market. According to the membrane geometrical configuration, there are principally two types of ceramic membranes available, flat, and cylindrical membranes. Flat ceramic membranes can be in a disc form with low packing density, usually used in small-scale pilot plant or laboratory applications. Also, they can be in a sheet form, the most common. They are around 6 mm of thickness and can be mono or multi-channelled. Usually, they are packed in modules which makes it easier to replace that the disc form. This shape can be used with higher turbidity feeds. Cylindrical membranes do not have to be obligatorily of circle form, other forms as hexagons are included in this type of tubular membranes. In general, they can be of three types: single-channel, multichannel or hollow fiber membranes. Single-channel membranes have mean duct diameter of 10-25 mm and are suitable for fluids with high turbidity and large amount of suspended solids. They are very easy to clean because of their diameter and robustness. Also, this type of membranes permits high crossflow velocities to control fouling. Multi-channel membranes arose as an improvement to

increase the packing density of tubular membranes. They are also called monoliths with a surface/volume ratio up to  $782 \text{ m}^2/\text{m}^3$  and have similar advantages as single-channel membranes. The exterior diameter of this type of membranes is variable and sometimes can be limited if the running configuration is inside-out. This is due to the increment of the pressure drop in the monolith because of the long and tortuous path that the liquid is forced to pass from inside to the outer surface. Some enterprises solve this problem by adding extra conducts in the tubular membrane through which the permeate can flow till the collection [195]. Finally, hollow fiber membranes are usually made of ceramic materials with 2-4 mm diameter or lower, depending on the maker. This novel system consists in more compact modules with higher effective membrane surface, avoiding the problem found with multi-channel membranes allowing the use of lower transmembrane pressures to drive permeate flow [193].

Regarding the **pore size**, we should differentiate between dense and porous membranes. For food applications the most used are porous membranes such as microfiltration ( $0.1\text{-}10 \text{ }\mu\text{m}$ ), ultrafiltration ( $0.01\text{-}0.1 \text{ }\mu\text{m}$ ), nanofiltration ( $0.001\text{-}0.01 \text{ }\mu\text{m}$ ) and membrane distillation ( $0.2\text{-}1.0 \text{ }\mu\text{m}$ ) ones, which perform separation processes according to sieving mechanisms in which molecules larger than the membrane pores are rejected while smaller ones pass through [191].

In general, **membrane performance is modulated by two parameters: flux and selectivity**. The flux is the volume of a substance that permeates through the membrane per unit of area for a period of time, meanwhile the selectivity (rejection) is the fraction that is retained on the membrane [191].

Cross-flow and dead-end filtration are the two types of filtration modes according to the direction between the feed flow and the permeate flow. They are schematically described in **Figure 44**. The main difference between them is the direction in which the feed crosses the membrane. When the direction is tangential to the membrane surface and perpendicular to the permeate it is cross-flow filtration, whereas when the direction of the flux is perpendicular to the membrane surface it is called dead-end filtration. The second one is more prone to accumulate particles on its surface and trigger fouling, meanwhile in cross-flow filtration the direction of the flux respect to the membrane helps remove deposited particles on the membrane layer [191].



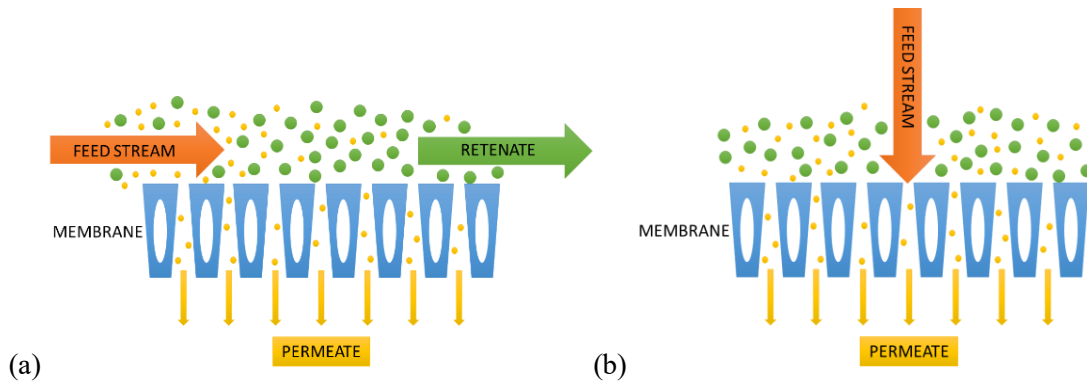


**Figure 44.** Schematic diagram of membrane filtration modes: (a) cross-flow; (b) dead-end filtration.

The principal **limiting factor** in membrane filtration is **fouling**, that includes (i) adsorption, (ii) pore blockage, sealing or constriction, (iii) precipitation and (iv) cake formation. Fouling takes place when particles are deposited on the membrane surface or in the pores of the membrane. The first and easy way to minimize fouling is implementing cleaning cycles to restore its productivity, which must be optimized to minimize costs and plant shut-downs. Reducing the permeate flux also has been reported to help to minimize the fouling [196–198]. To mitigate foulant agglomerations and to increase the effective surface, structural modifications of the membrane surface have been demonstrated to be useful by inducing secondary flow that produce eddies and in consequence reduce fouling [199]. Specifically, when using rotating disk membranes, the fouling is easy to be decreased by increasing the rotation speed and transmembrane pressure. It is due to the high centrifugal force and the radial components of drag on particles near the membrane surface [200]. Besides, sometimes particles added on membrane filtration systems or suspended in the feed flow can as well behave as fouling mitigation agents [201]. It is similar to the new concept of using a “dynamic membrane” made of particles deposited via permeation grand on the membrane surface. They act as a secondary membrane minimizing fouling enabling higher fluxes [202]. Bubbling is another technique used to enhance the filtration process by reducing fouling by injecting gas into the feed stream [203]. Novel approaches report that artificial intelligence and machine leaning are crucial techniques for better control membrane fouling in filtration processes and that reduce cleaning costs [204]. Anyway, membrane processes in the industry need to have three types of service support facilities and equipment: backwash, CIP and feed systems chemical cleaning [193].

Various membrane processes have been proposed to address several challenges encountered in the food industry, particularly in the processing of food, fruit juices, and beverages. Specifically, regarding by-products treatment, microfiltration (MF) and ultrafiltration (UF) operations have been introduced to reduce the organic load and chemical oxygen demand in wastewaters from

food processing industries. However, the adoption of these methodologies was limited until researchers recognized their advantages, such as the absence of additional phases, low recovery costs, rapid separation, and high selectivity for specific components. These membrane techniques have been employed to recover high-value compounds from aqueous extracts and by-products, offering a solution to environmental challenges posed by wastewater disposal. Currently, MF and UF are utilized for separating and recovering both macro and micro-molecules, while nanofiltration (NF) is employed for isolating and purifying recovered solutes. Due to the narrow pore size in UF membranes, they effectively serve as a border for nanofiltration, capable of separating and recovering functional solutes like sugars, phenolic compounds, pectins, and proteins. In Europe, olive mill wastewater disposal has been addressed through the recovery of polyphenols using membrane operations. As conventional membrane processes began to offer notable results beyond simple by-product treatment, they were increasingly utilized for the recovery of valuable solutes. For example, NF processes have been instrumental in separating, fractionating, and concentrating bioactive components such as phenolic compounds from by-products and natural sources [131]. Indeed, for the purpose of obtaining polyphenols, different by-products have been recently processed by integrated membrane processes, such as Olive by-products [205,206], grape by-products [207,208], and coffee by-products [209], among others.





# OBJECTIVES





## **General objective**

The aim of this thesis is to face the challenges related to the development of pretreatment, extraction, and purification technologies for obtaining bioactive compounds from orange and avocado by-products to achieve cost-effective and sustainable production of high-value functional ingredients. Therefore, this proposal aims to evaluate and develop an optimal combination of non-thermal technologies to recover antioxidant compounds from industrial fruit by-products and subsequently use them as functional ingredients to create healthy products.

## **Specific objectives**

To achieve the main objective, several specific objectives will be met:

1. Develop and optimize drying methods evaluating the impact on phenolic content for orange and avocado peels (CHAPTER 1 and CHAPTER 6, respectively).
2. Configure ultrasound-assisted extraction using a sonotrode, for extracting phenolic compounds from the agro-industrial residues of orange and avocado (CHAPTER 2 and CHAPTER 7, respectively) and to determine them using HPLC-FLD/ESI-TOF-MS.
3. Optimize pulse electric fields conditions as a pre-treatment followed by ultrasound extraction with sonotrode of phenolic compounds in the orange and avocado by-products (CHAPTER 3 and CHAPTER 8, respectively).
4. Evaluate the effect of submerged fermentation with different lactic acid bacteria as a pre-treatment followed by ultrasound extraction on the phenolic compounds of orange and avocado by-products (CHAPTER 4 and CHAPTER 9, respectively).
5. Concentrate/purify phenolic compounds from orange peel extracts using membrane filtration technology and ensure compliance with food-grade regulations (CHAPTER 5).
6. Formulate phenolic-enriched juices using purified extracts. Assess enrichment levels and stability of phenolic compounds throughout the shelf life (CHAPTER 10).





# **EXPERIMENTAL SECTIONS**



The current report encompassed all the work carried out for the PhD thesis entitled: **Extraction of bioactive compounds from food byproducts and its use as ingredients for the formulation of enriched foods**. The samples by-products under study were orange peels and avocado peels and seeds. Thus, the content has been divided into three sections for better tracking.

**Section 1** is relative to the treatments and technologies applied to the orange by-product. It is organized in five chapters:

**Chapter 1** deals with the mathematical modelling of the drying kinetics of the orange peel. Five different regression models were evaluated at three temperatures (40, 60 and 80 °C) and three different air flows (0, 0.8 and 1.6 m/s). Besides, it was studied the effect of drying in the phenolic compounds (high performance liquid chromatography (HPLC) with electrospray ionization (ESI) coupled to a time of flight (TOF) with mass detector (MS)), the vitamin C content (HPLC with ultraviolet/visible light detector (UV/VIS)) and the antioxidant activity (DPPH, ABTS and FRAP).

**Chapter 2** focuses on the optimization of phenolic compounds extraction by sonotrode in the orange peel. It was used an experimental Box-Behnken design evaluating the ratio ethanol/water, time, amplitude and pulse. The evaluated responses were the total phenolic content (Folin Ciocalteu) and the antioxidant activity (DPPH and ABTS). Besides, the optimal extract was characterized with HPLC-ESI-TOF-MS.

**Chapter 3** is based on evaluating the use of pulse electric field pre-treatment prior to ultrasound extraction in orange peel. It was performed an optimization with Box Behnken design evaluating the electric field, number of pulses and pulse width. The studied responses were the hesperidin, narirutin and total phenolic content (HPLC-ESI-TOF-MS). Besides the antioxidant activity was evaluated (DPPH and ABTS).

**Chapter 4** investigates the fermentation of orange peels by different lactic acid bacteria (LAB) strains and its impact on phenolic composition (HPLC-ESI-TOF-MS) and antioxidant activity (DPPH and ABTS). Three different LAB strains, two *Lactiplantibacillus plantarum*, and one *Levilactobacillus brevis* were utilized.

**Chapter 5** presents a pilot scale (1000 L) trial for the extraction and recovery of phenolic compounds by ultrasound assisted extraction from orange peels. Besides, it gives a first overview of different separation processes including membrane filtration (microfiltration, ultrafiltration and nanofiltration). The final product was evaluated according to European food grade regulations and characterized by HPLC-ESI-TOF-MS.

**Section 2** is relative to the treatments and technologies applied to the avocado by-products and contains four chapters:

**Chapter 6** concerns the mathematical modelling of the drying kinetics of avocado peels and its influence in the procyanidin content (HPLC with fluorometric (FLD) and diode array (DAD) detectors) and antioxidant activity (DPPH, ABTS and FRAP). They were evaluated fourteen different models at three different temperatures (40, 60 and 80 °C) and three different air-flows (0, 0.8 and 1.6 m/s).

**Chapter 7** relates the optimization of the ultrasound assisted extraction by sonotrode of procyanidins from avocado peel and seed. The conditions were established with an experimental Box-Behnken designs. The factors evaluated were ratio ethanol/water, time and amplitude and the responses evaluated were the total procyanidin content (HPLC-FLD-DAD) and antioxidant activity (DPPH, ABTS and FRAP).

**Chapter 8** focuses on evaluating the effect of pulse electric fields as a pre-treatment to ultrasound extraction of flavan-3-ols in avocado by-products. The best conditions were selected with an experimental Box- Behnken design based on the effect of frequency, electric field and number of pulses in the procyanidin content (HPLC-FLD-DAD).

**Chapter 9** investigates the fermentation of avocado seeds by different LAB strains and its impact on phenolic composition (HPLC-ESI-TOF-MS), antioxidant activity (DPPH and FRAP), and antidiabetic activity (alpha-amylase). Different LAB strains were utilized: *Leuconostoc mesenteroides*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Levilactobacillus brevis* and two *Lactiplantibacillus plantarum*.

**Section 3** contains **chapter 10** that consist of the formulation of a fortified orange juice with an extract containing the major phenolic compounds of orange peels and avocado by-products. Besides hydroxytyrosol from olive leaves was also incorporated. There were evaluated different enrichment levels and the stability of pH, total soluble solids, color, phenolic compounds, vitamin C and antioxidant activity throughout 5-days. The effect of pasteurization was also studied.

The description of the mentioned methods and other specific materials and methods used can be found inside of each chapter.





## SECTION 1. ORANGE BY-PRODUCT







# **CHAPTER 1. Mathematical modelling of convective drying of orange by-product and its influence on phenolic compounds and ascorbic acid content, and its antioxidant activity**

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Article

# Mathematical Modelling of Convective Drying of Orange By-Product and Its Influence on Phenolic Compounds and Ascorbic Acid Content, and Its Antioxidant Activity

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## Abstract

Orange peel is one of the main by-products from juice processing and is considered as a promising source of phenolic compounds with anti-carcinogenic, anti-inflammatory, anti-microbial and antioxidant properties. The drying is an essential step to ensure the storage of this by-product at an industrial level, in order to use it as a functional ingredient or as a nutraceutical. Thus, this research focuses on the evaluation of the effect of the convective air-drying process in orange by-products at three different temperatures (40, 60 and 80 °C) and air flows (0, 0.8 and 1.6 m/s) on the phenolic content (measured by HPLC-MS), the antioxidant activity (measured by DPPH, ABTS and FRAP), and the vitamin C content (measured by HPLC-UV/VIS). Moreover, the mathematical modelling of its drying kinetics was carried out to examine the orange by-product behavior. Among the tested mathematical models, the Page model reported the highest fit and the best drying conditions, which showed the lowest reductions were at 60 °C with an air flow of 1.6 m/s and taking 315 min.

**Keywords:** HPLC-MS; orange peel; vitamin C; air-drying; polyphenols; hesperidin

## 1. Introduction

The orange is the most important citrus fruit produced in Spain, accounting for 3.3 million metric tons in 2019/2022, followed by tangerines (1.81 million metric tons), lemons (0.9 million metric tons) and grapefruits (0.06 million metric tons). Moreover, the volume produced in 2020 in Spain has increased by 0.3 million metric tons since 2012. Most of them are cultivated in the province of Andalusia, reaching 1.6 million tons in 2020. Although the home consumption of oranges was decreasing from 2013 to 2019, in 2020 there was a slight increase of 6.5% with respect to the previous year. It has been estimated that the per capita consumption of oranges in Spain is around 20 kg a year [2].

Fruit juice is a non-alcoholic beverage made from the extraction of the liquid contained in natural fruits such as oranges, pineapples, strawberries, cranberries, or mangoes. They are often consumed for their perceived nutritional benefits. For instance, orange juice has a perceived nutritional value because it contains vitamin C, folic acid, and potassium. Although fruit juice consumption in the European Union used to be higher, the European Union has still historically consumed more fruit juice than any other region worldwide, accounting for a total of 9.1 million liters in 2018, making the region the biggest market for fruit juices and fruit nectars globally. Within this context, in 2021 Spain was the fourth producer of orange juice in the European Union after Germany, France and the United Kingdom, and the ninth worldwide. In fact, in Spanish orange juice accounts for 30% of the fruit juice and nectar sales-volume, followed by pineapple and peach. In 2017 the production of orange juice was 5.1 million liters [2].

However, from the process of production of orange juice there are generated residues which are often discarded as waste, which could be revalorized as a potential nutraceutical resource. The main by-product generated from the orange-juice industry is the peel, composed of the albedo, flavedo and rests of the pulp. It is rich in polyphenols, fiber and vitamins that have been attributed to antioxidant, antimicrobial, and cardioprotective activities, among others. In general, agro-industrial waste has been given special attention for its potential use in developing high value-added products as nutraceuticals and at the same time reducing its environmental impact [57].

Drying is one of the most common and extended processes used to preserve the quality and stability and to extend the shelf-life of fruits, vegetables, and their by-products. It allows a reduction in the moisture content and water activity, avoiding spoilage during management and storage [130]. Orange by-product is a matrix characterized as having a high sugar and pectin content and at the same time high moisture (>50%), making it an excellent growth substrate for microorganisms. Thus, drying is necessary to ensure stability, providing long storage-life [210]. To find the best conditions for drying the orange by-product is an important matter, and some things should be taken into account. As previously reported [59], there are some authors that have

studied the dehydration of the orange peel, comparing techniques for different purposes. Firstly, temperature is an important factor, because the bioactive and antioxidant compounds are thermolabile, and high temperatures could lead to their destruction. On the other hand, from the industrial and economic point of view, the drying process should be short and low energy-consuming. Moreover, drying involves the simultaneous transference of heat and mass, turning it into a complex phenomenon. Therefore, the use of mathematical models to simulate its kinetics and explain the mechanism of the water transference during this operation is a useful tool to control the process [136]. Although some authors have evaluated the effect of drying in some parameters of the orange by-product [211,212] they did not discuss its drying kinetics. Recently, Deng et al. [137] analyzed the orange peel drying kinetics using only the Weibull model. Moreover, Afrin et al. [213] evaluated the orange-pomace drying kinetics through other models, but not focusing on the polyphenol content or antioxidant activity. In addition, in other fruits and vegetables the mathematical modelling of the drying process has been studied for the asparagus root [214], dragon fruit [215], edamame [216], pomelo albedo [217], uvaia fruit [218] or yam [219]. There are few previous studies focusing on this aspect for orange by-products.

Considering all these facts, this research aims to evaluate the effect of the convective air-drying process in orange by-product on phenolic compounds, vitamin C and antioxidant activity (in order to establish the best drying conditions that will permit a high-quality standard for this by-product that could be used as raw material for the production of functional ingredients for foods and/or nutraceuticals. Moreover, the mathematical modelling of its drying kinetics was carried out to examine the orange by-product behavior and to find the best model. To our knowledge, it is the first time that the modelling of the drying process of the orange by-product has been carried out at this range of temperatures (40–80 °C) and at three different air-flows (0–0.8–1.6 m/s) using five different mathematical models.

## **2. Materials and Methods**

### **2.1. Chemicals and Samples**

Ascorbic acid, Trolox, DPPH, ABTS and FRAP reagents were acquired from Sigma-Aldrich (St. Louis, MO, USA). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). HPLC-grade water reagent and other reagents and solvents were purchased from Merck KGaA (Darmstadt, Germany).

Orange by-products (var. *Navelina*) were obtained after juice production. The resulting by-product was composed of the albedo, flavedo and the remains of the pulp of the orange with a moisture content of  $70 \pm 1.5\%$ . The samples were dried, milled, and sieved to 100  $\mu\text{m}$ , and kept at  $-18\text{ }^{\circ}\text{C}$  until the analyses.

## 2.2. Drying Process and Kinetics

Samples of orange by-product were dried by convection drying using an oven (Memmert, Schwabach, Germany). The loss of weight was measured with a precision balance (Sartorius, Göttingen, Germany) for less than 15 s, until the moisture content was lower than 10% [137].

The temperatures tested were 40, 60 and 80 °C , in concordance to other authors [220–225]. In addition, three different air flows were tried, according to the oven possibilities: 0, 0.8 and 1.6 m/s.

At each time the moisture content was expressed as moisture ratio (MR,) according to its weight loss and remaining solid content; this is a dimensionless term calculated from Equation (1):

$$MR = \frac{M_t - M_e}{M_0 - M_e} \quad (1)$$

where  $M_0$ ,  $M_t$  and  $M_e$  are the moisture content (g/g d.w.) at initial time, time  $t$ , and equilibrium, respectively.

The drying rate (DR) was calculated to express the moisture loss per unit of time, which can be calculated according to Equation (2):

$$DR = \frac{M_{t1} - M_{t2}}{t_2 - t_1} \quad (2)$$

where  $M_{t1}$  and  $M_{t2}$  are the moisture content (g/g d.w.) at the time points  $t_1$  and  $t_2$ , respectively.

The effective moisture diffusivity ( $D_{eff}$ ) was calculated according to Fick's second law, assuming that the sample shrinkage is negligible, so the initial moisture distribution is uniform, and the moisture diffusivity is constant, as shown in Equation (3):

$$MR = \left(\frac{8}{\pi^2}\right) e^{\left(-\frac{\pi^2 D_{eff}}{4L^2} t\right)} \quad (3)$$

where  $MR$ ,  $D_{eff}$ ,  $L$  and  $t$  are the moisture ratio, effective moisture diffusivity, the half-thickness of the sample (m) and the time (min), respectively.

Additionally, the Arrhenius equation was used to calculate the activation energy ( $E_a$ ) described in Equation (4):

$$D_{eff} = Ae^{(-E_a/RT)} \quad (4)$$

where  $A$ ,  $E_a$ ,  $R$  and  $T$  are the pre-exponential factor of the Arrhenius equation, the activation energy (KJ/mol), the universal gas constant (8.314 J/mol K) and the temperature (K), respectively.

To predict accurately the drying kinetics of orange by-product is of huge importance in finding the model which fits the best to its drying behavior. Therefore, for the three different temperatures and the three different air flows, the experimental drying data was fitted with semi-theoretical mathematical drying-models, presented in **Table 1.1**.

**Table 1.1.** Non-linear regression models for predicting moisture ratio of orange by-product samples.

| Model Name                 | Model Equation             | References |
|----------------------------|----------------------------|------------|
| <b>Lewis</b>               | $MR = e^{(-kt)}$ (5)       | [226–229]  |
| <b>Henderson and Pabis</b> | $MR = ae^{(-kt)}$ (6)      | [230–234]  |
| <b>Page</b>                | $MR = e^{(-kt^N)}$ (7)     | [235–238]  |
| <b>Logarithmic</b>         | $MR = a e^{-kt} + c$ (8)   | [239–242]  |
| <b>Weibull</b>             | $MR = a - b e^{-kt^N}$ (9) | [137]      |

MR: Moisture Ratio; t: time; a, b, c, N, and k are the model constants.

### 2.3. Statistical Parameters

Various statistical parameters were used to select the best mathematical model that predicts the drying kinetics of orange by-product, such as the coefficient of determination ( $R^2$ ), defined by Taylor [243]. Furthermore, other statistical parameters previously used by other authors [214,244] were also used to this purpose, such as square error ( $X^2$ ), the square error of estimation (SEE), root-mean-square error (RMSE), the mean absolute error (MAE) and the relative mean error ( $P_0$ ). Overall, these terms indicate how close the experimental and the prediction data are. The model is considered to have a high-quality fit, as the  $R^2$  value is closer to 1 and the  $X^2$ , SEE, RMSE and MAE values are closer to 0. Moreover, the model is acceptable or with a good fit when  $P_0 < 0.5$  [136]. The named statistical parameters were calculated according to Equations 10–15, where the experimental and predicted dimensionless-moisture-ratios are expressed as  $MR_{exp,i}$  and  $MR_{pred,i}$ , respectively. N refers to the number of observations and i to the number of constants in the model.

$$R^2 = \frac{\sum_{i=1}^N (MR_{exp,i} - \overline{MR}_{pred,i}) * (MR_{pred,i} - \overline{MR}_{exp,i})}{\sqrt{\sum_{i=1}^N (MR_{exp,i} - \overline{MR}_{pred,i})^2 * \sum_{i=1}^N (MR_{pred,i} - \overline{MR}_{exp,i})^2}} \quad (10)$$

$$X^2 = \frac{\sum_{i=1}^N (MR_{exp,i} - MR_{pred,i})^2}{N - z} \quad (11)$$

$$SEE = \sqrt{\frac{\sum_{i=1}^N (MR_{exp,i} - MR_{pred,i})^2}{N - n_i}} \quad (12)$$

$$RMSE = \left[ \frac{1}{N} \sum_{i=1}^N (MR_{exp,i} - MR_{pred,i})^2 \right]^{1/2} \quad (13)$$

$$MAE = \frac{1}{N} \sum_{i=1}^N (MR_{pred,i} - MR_{exp,i}) \quad (14)$$

$$P_0 = \frac{1}{N} \sum_{i=1}^N \left| \frac{(MR_{exp,i} - MR_{pred,i})}{MR_{exp,i}} \right| \quad (15)$$

## 2.4. Ultrasound Extraction of Phenolic Compounds

Briefly, 1 g of orange by-product powder was added to 15 mL solution of ethanol/water 80:20 v/v. The mixture was placed in an ultrasonic bath for 15 min and then it was centrifuged for 10 min at 9000 rpm. The supernatant was collected, and the extraction was repeated twice more. Finally, the supernatants were evaporated, and reconstituted in 1 mL of methanol/water (50:50, v/v). The final extracts were filtered with regenerated cellulose filters 0.2 µm (Millipore, Bedford, MA, USA) and stored at −18 °C until the analyses.

## 2.5. HPLC-MS Analysis of Phenolic Compounds

The analyses of the orange by-products drying under the different conditions were carried out in duplicate on an ACQUITY Ultra Performance LC system (Waters Corporation, Milford, MA, USA) coupled to an electrospray ionization (ESI) source operating in the negative mode, and a time-of-flight (TOF) mass detector (Waters Corporation, Milford, MA, USA). The compounds of interest were separated on an ACQUITY UPLC BEH Shield RP18 column (1.7 µm, 2.1 mm × 100 mm; Waters Corporation, Milford, MA, USA) at 40 °C, using a gradient previously stated by Verni et al. [245], using water containing 1% acetic acid as mobile phase A and acetonitrile as mobile phase B. Standard curves of chlorogenic acid, vanillic acid, ferulic acid, rutin, quercetin and catechin were performed, and the results are expressed as µg/g d.w. The data were elaborated using MassLynx 4.1 software (Waters Corporation, Milford, MA, USA).

## 2.6. HPLC-UV/VIS Analysis of Vitamin C

The determination of vitamin C was carried out using the method proposed by Mesías-García et al. [246]. To extract the ascorbic acid (AA), 0.5 mL of extract was mixed with 2.5 mL of a 10% (w/v) metaphosphoric acid solution and then diluted to a final volume of 25 mL in a glass volumetric flask with demineralized water. The mixture was homogenized and centrifuged at 9000 rpm for 15 min (room temperature). The supernatant was filtered through 0.20 µm Millex filters (Millipore, Bedford, MA, USA) and the samples were injected into the high-performance-liquid-chromatography (HPLC) system. To evaluate the total content of vitamin C (DHAA + AA)



present in the samples, the reduction of DHAA to AA must be carried out. For this, a dithiothreitol (DTT) solution (1 mg/mL diluted in 45%  $\text{K}_2\text{HPO}_4$ ) was prepared as a reducing agent, and an aliquot of 0.2 mL was added to 1 mL of the filtered sample, obtained in the AA analysis. The mixtures were kept in the dark for 30 min at room temperature, then the reduction was stopped by the addition of 0.2 mL of 2M  $\text{H}_3\text{PO}_4$  and the samples were injected into the HPLC system. DHAA content was calculated by the difference between the total vitamin C (after DHAA reduction) and initial AA (before reduction). Both determinations (AA and vitamin C) were performed in triplicate. AA and vitamin C were analyzed using HPLC with an UV/VIS detector at 25 °C. Separations were performed on a Gemini 5  $\mu\text{m}$  C18 110 Å (150 × 4.6 mm) Phenomenex column. The measurement was carried out under isocratic conditions, using as the mobile phase demineralized water acidified with sulfuric acid at a pH of 2.2, flow rate of 0.6 mL/min, and a wavelength of 245 nm. The standard curve of AA (from 2.5 to 100 ppm) was created, and the results are expressed as  $\mu\text{g}$  AA/g d.w.

## 2.7. Antioxidant Assays

The antioxidant activity was evaluated in all the samples, using three different methods in duplicate. The DPPH assay was carried out according to a method proposed by several authors [247,248]. A total of 100  $\mu\text{L}$  of each extract was added to 2.9 mL of DPPH, and after rapid stirring the bleaching power of the extract was observed in a time interval of 0 to 30 min, at 517 nm. The ABTS method was carried out according to Re et al. (1999) [249]. The monocation  $\text{ABTS}^{+\bullet}$  was generated by oxidation of the ABTS with potassium persulfate in the dark at room temperature for 12–24 h. For each extract, 1 mL of the ABTS solution was added to 0.01 mL of extract and the detriment of absorbance was measured for 30 min at 734 nm. The FRAP assay was carried out following the procedure developed by Pulido et al. [250]. It is based in the reduction of  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  by the antioxidant substances. A total of 30  $\mu\text{L}$  of each extract was added to 90  $\mu\text{L}$  of distilled water and 900  $\mu\text{L}$  of the FRAP reagent. It was kept for 30 min at 37 °C and measured in the spectrophotometer at 595 nm. Standard curves of TE (1, 5, 10, 20, 50, 80, 100, 150, 200 ppm) were elaborated for each assay. Results are expressed as mg TE/g d.w.

## 3. Results and Discussion

### 3.1. Drying Kinetics and Modelling

The drying curve of orange by-product is shown in

**Figure 1.1;** the changes of the moisture ratio of the orange peel through the drying time at different temperatures (40, 60 and 80 °C) and air flows (0, 0.8 and 1.2 m/s) are reported.

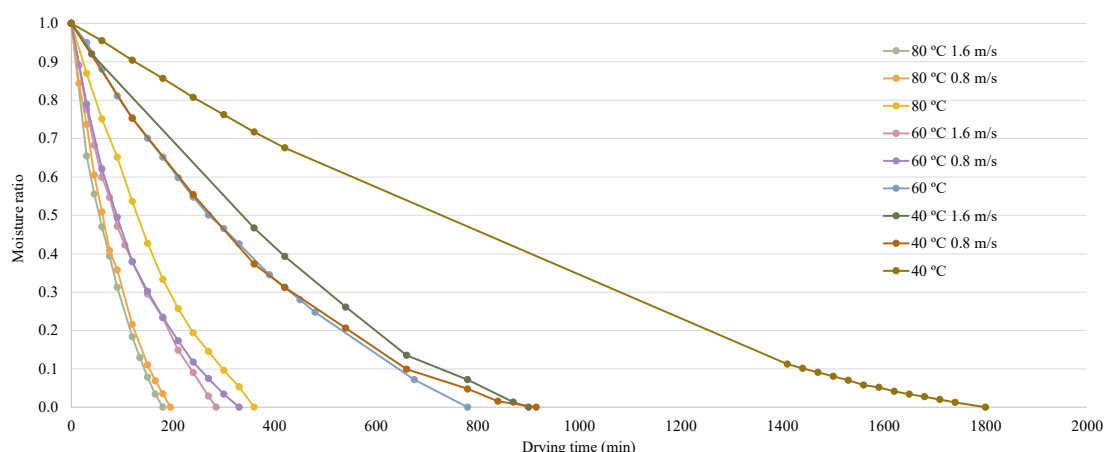


**Figure 1.1.** Drying-kinetics curves of orange peel under different temperatures and air flows.

Without air flow, the drying time required to achieve the final moisture was 360, 720 and 1800 min, at 80, 60 and 40 °C, respectively. The temperature had a high influence on the drying of orange by-product. As expected, using higher temperature for less time was needed to reach the lower moisture-ratio. In the same way, the air flow was demonstrated to affect the drying. Using an air flow of 1.6 m/s, the time reduction was of 50% at all the temperatures tested, compared to not using air flow. There were not significative ( $p < 0.05$ ) differences in time reduction with an air flow of 0.8 or 1.6 m/s. As a combination of temperature and air flow, drying at 80 °C with an air flow of 1.6 m/s was the quickest, needing only 180 min. However, to evaluate and select the best drying conditions, other parameters should be measured.

**Figure 1.2** shows the average drying rates of the orange peels dried at three different temperatures and airflows, against time. Depending on the drying conditions, the initial drying rate reached the maximum value at 30–45 min. The drying rate declined rapidly after this time. These facts can be due to the rapid evaporation of the surface moisture occurring in the first hour and, after that, to the evaporation of the internal moisture. This tendency has been previously reported by other authors in other matrices such as mentha [251], rosemary [252] and thyme [253]. The increase in temperature enhanced the drying rate considerably. In fact, without airflow, the drying rate increased to 78.6% when drying at 80 °C, compared to drying at 40 °C. Furthermore, a consistent effect was found with the airflow. The highest impact on the drying rate with the airflow was at 80 °C (an increase of 61.1% when using 1.6 m/s of airflow). However, higher increases were also found with the airflow when drying at 60 °C (69.6%) and 40 °C (57.2%).

**Figure 1.2** shows that the samples dried at 80 °C using airflow reached the highest drying rate among all the tested conditions.

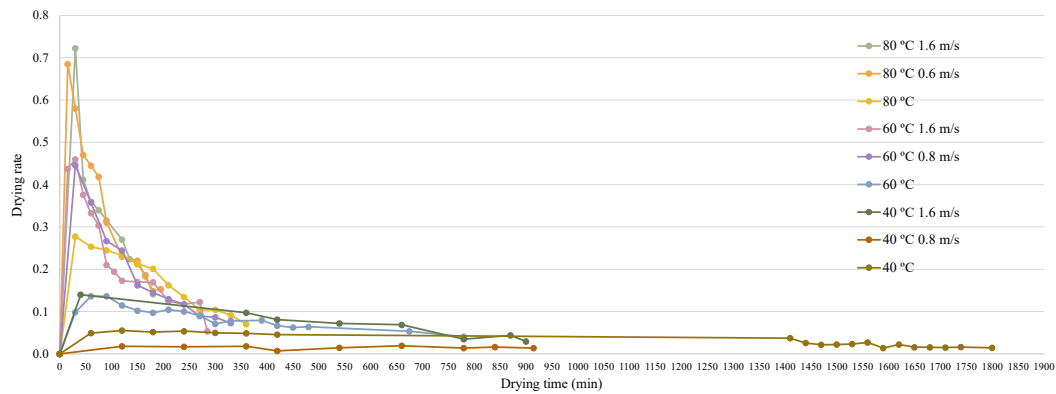


**Figure 1.2.** Drying-rate curves of orange peel under different temperatures and airflows.

As regards the kinetics of drying, in

**Figure 1.2** it can be clearly appreciated that there are two stages of drying. The first one is the most rapid phase and is majorly linear. In this step we appreciate the evaporation of the water of the superficial layers of the orange by-product. The second one is an exponential phase that usually takes longer and is when the remaining water content diffuses to the surface. Therefore, the mathematical modelling of the drying curves is important for a better control of the drying process and better quality of the orange by-product.

To evaluate which model predicts the best drying kinetics of the orange by-product, the statistical parameters presented in **Table 1.2** such as  $R^2$ ,  $X^2$ , RMSE and MAE were taken into account. Overall, these terms indicate how the experimental and the prediction data are comparable. They were calculated at all the three temperatures at the three different air flows for the five proposed mathematical models.

**Table 1.2.** Statistical analysis of the selected mathematical models at three different temperatures and air flows.

| Model               | T (°C) | Air Flow 1.6 m/s |                |        |        |        |                |  | Air Flow 0.8 m/s |                |        |        |        |                |  | No Air Flow    |                |        |        |        |                |  |
|---------------------|--------|------------------|----------------|--------|--------|--------|----------------|--|------------------|----------------|--------|--------|--------|----------------|--|----------------|----------------|--------|--------|--------|----------------|--|
|                     |        | R <sup>2</sup>   | X <sup>2</sup> | SEE    | RMSE   | MAE    | P <sub>0</sub> |  | R <sup>2</sup>   | X <sup>2</sup> | SEE    | RSME   | MAE    | P <sub>0</sub> |  | R <sup>2</sup> | X <sup>2</sup> | SEE    | RSME   | MAE    | P <sub>0</sub> |  |
| Lewis               | 40     | 0.9224           | 9.58E-03       | 0.0979 | 0.1494 | 0.0721 | 0.5174         |  | 0.955            | 7.17E-03       | 0.0847 | 0.1696 | 0.0656 | 0.2042         |  | 0.9739         | 9.97E-03       | 0.0998 | 0.1463 | 0.0660 | 0.1696         |  |
|                     | 60     | 0.9624           | 2.88E-03       | 0.0537 | 0.1348 | 0.0476 | 0.9138         |  | 0.9829           | 1.77E-03       | 0.0421 | 0.0837 | 0.0365 | 0.1512         |  | 0.9677         | 2.79E-03       | 0.0529 | 0.1292 | 0.0507 | 0.1060         |  |
|                     | 80     | 0.9984           | 8.50E-02       | 0.2915 | 0.7517 | 0.2267 | 0.1423         |  | 0.9702           | 5.17E-03       | 0.0719 | 0.1552 | 0.0599 | 0.3093         |  | 0.9765         | 5.35E-03       | 0.0732 | 0.2581 | 0.0596 | 0.2993         |  |
| Henderson and Pabis | 40     | 0.8371           | 8.97E-02       | 0.2995 | 0.1750 | 0.1578 | 1.0087         |  | 0.9622           | 8.66E-03       | 0.0930 | 0.0692 | 0.0608 | 0.1670         |  | 0.9397         | 1.50E-02       | 0.1226 | 0.0630 | 0.0685 | 0.1349         |  |
|                     | 60     | 0.9289           | 6.36E-03       | 0.0798 | 0.0718 | 0.0613 | 0.9684         |  | 0.9611           | 5.00E-03       | 0.0707 | 0.0566 | 0.0480 | 0.1373         |  | 0.9468         | 4.32E-03       | 0.0657 | 0.0406 | 0.0527 | 0.1042         |  |
|                     | 80     | 0.9352           | 8.55E-02       | 0.2924 | 0.7337 | 0.2326 | 0.1472         |  | 0.9543           | 1.06E-02       | 0.1030 | 0.0717 | 0.0687 | 0.2004         |  | 0.9603         | 9.04E-03       | 0.0951 | 0.1820 | 0.0642 | 0.2412         |  |
| Page                | 40     | 0.9682           | 4.29E-04       | 0.0207 | 0.0013 | 0.0174 | 0.9524         |  | 0.9784           | 6.54E-04       | 0.0256 | 0.0171 | 0.0213 | 0.1016         |  | 0.9906         | 5.63E-04       | 0.0237 | 0.0001 | 0.0181 | 0.0634         |  |
|                     | 60     | 0.9850           | 8.54E-04       | 0.0292 | 0.0075 | 0.0229 | 1.0105         |  | 0.9888           | 4.65E-04       | 0.0216 | 0.0030 | 0.0176 | 0.0877         |  | 0.995          | 6.05E-04       | 0.0246 | 0.0064 | 0.0158 | 0.0422         |  |
|                     | 80     | 0.9670           | 5.16E-03       | 0.0718 | 0.2194 | 0.0662 | 1.3791         |  | 0.9843           | 8.33E-04       | 0.0289 | 0.0109 | 0.0240 | 0.1291         |  | 0.9903         | 6.80E-04       | 0.0261 | 0.0265 | 0.0194 | 0.1662         |  |
| Logarithmic         | 40     | 0.8959           | 2.13E-01       | 0.4613 | 1.2140 | 0.4292 | 6.7565         |  | 0.9178           | 1.14E-01       | 0.3373 | 1.0278 | 0.3250 | 0.3856         |  | 0.9426         | 7.51E-02       | 0.2741 | 1.0456 | 0.2585 | 0.4213         |  |
|                     | 60     | 0.9289           | 4.64E-02       | 0.2154 | 0.8036 | 0.2075 | 2.2445         |  | 0.9611           | 5.37E-02       | 0.2318 | 0.7804 | 0.2253 | 0.4121         |  | 0.9468         | 3.29E-02       | 0.1814 | 0.7208 | 0.1699 | 0.2477         |  |
|                     | 80     | 0.9194           | 2.12E-02       | 0.1458 | 0.3491 | 0.1072 | 2.0326         |  | 0.9543           | 6.08E-02       | 0.2465 | 0.8178 | 0.2361 | 0.4897         |  | 0.9644         | 8.95E-02       | 0.2991 | 1.1845 | 0.2900 | 0.5417         |  |
| Weibull             | 40     | 0.8969           | 1.07           | 1.0365 | 2.9293 | 1.0357 | 14.6110        |  | 0.9719           | 7.68E-01       | 0.8766 | 2.7529 | 0.8705 | 0.5651         |  | 0.8895         | 6.06E-01       | 0.7785 | 2.1986 | 0.7182 | 0.5254         |  |
|                     | 60     | 0.9834           | 5.10E-01       | 0.7140 | 2.6252 | 0.6778 | 5.2870         |  | 0.9918           | 6.71E-01       | 0.8189 | 2.7735 | 0.8006 | 0.6357         |  | 0.9899         | 3.27E-01       | 0.5717 | 2.1481 | 0.5063 | 0.4372         |  |
|                     | 80     | 0.9651           | 7.08E-01       | 0.8415 | 2.7550 | 0.8307 | 6.6091         |  | 0.9831           | 5.48E-01       | 0.7403 | 2.4149 | 0.6971 | 0.6567         |  | 0.9958         | 4.63E-01       | 0.6805 | 3.2910 | 0.6098 | 0.6493         |  |

The model is considered to have a high-quality fit, as the  $R^2$  value is closer to 1 and the  $X^2$ , SEE, RMSE and MAE values are closer to 0. In general, all the models gave satisfactory results, with high coefficients of determination ( $R^2 > 0.85$ ). Among the five tested mathematical models when not using air flow and with an air flow of 0.8 m/s, the Page model fitted better at all the three temperatures, giving  $R^2$  values from 0.9784 to 0.9950. When using an air flow of 0.8, the best model adjustment changed, depending on the temperature. At 40 and 60 °C, the Page model reported the highest values of  $R^2$ , keeping  $X^2$ , SEE, RMSE and MAE the lowest. However, at 80 °C the Lewis model was found to have a better fitting with the experimental data. These results demonstrated that, depending on the temperature and air flow used when drying the orange by-product by convective drying, the drying kinetics vary. Additionally, the values obtained for all the constants of the five methods are exposed in **Table 1.3**, such as the drying-rate constants (k), the a for the Henderson and Pabis model, the N for the Page model, a and c for the logarithmic model, and a, b and n for the Weibull model. None of these parameters were constant, and exhibited temperature and air-flow dependence in concordance with other authors [217,244].

**Table 1.3.** Coefficients of Lewis, Henderson and Pabis, Page, logarithmic and Weibull models.

| Air Flow |    | Model |       |       |                     |       |       |       |             |       |       |         |       |   |   |
|----------|----|-------|-------|-------|---------------------|-------|-------|-------|-------------|-------|-------|---------|-------|---|---|
|          |    | Lewis |       |       | Henderson and Pabis |       | Page  |       | Logarithmic |       |       | Weibull |       |   |   |
|          |    | k     |       |       | k                   | a     | k     | N     | k           | a     | c     | k       | a     | b | n |
| 1.6 m/s  | 40 | 0.004 | 0.005 | 1.824 | 0.000               | 1.850 | 0.005 | 1.368 | 0.456       | 0.000 | 1.000 | −0.933  | 1.850 |   |   |
|          | 60 | 0.010 | 0.011 | 1.212 | 0.006               | 1.091 | 0.011 | 0.909 | 0.303       | 0.006 | 1.007 | −0.259  | 1.091 |   |   |
|          | 80 | 0.057 | 0.058 | 1.062 | 0.005               | 1.179 | 0.019 | 0.742 | 0.247       | 0.007 | 1.001 | −0.552  | 1.179 |   |   |
| 0.8 m/s  | 40 | 0.004 | 0.004 | 1.267 | 0.000               | 1.329 | 0.005 | 1.138 | 0.379       | 0.001 | 1.001 | −0.691  | 1.329 |   |   |
|          | 60 | 0.009 | 0.010 | 1.204 | 0.005               | 1.107 | 0.010 | 0.903 | 0.301       | 0.005 | 1.006 | −0.438  | 1.107 |   |   |
|          | 80 | 0.015 | 0.017 | 1.290 | 0.006               | 1.187 | 0.017 | 0.967 | 0.322       | 0.005 | 1.007 | −0.234  | 1.187 |   |   |
| No       | 40 | 0.002 | 0.002 | 1.408 | 0.000               | 1.343 | 0.002 | 0.998 | 0.333       | 0.000 | 1.000 | −0.229  | 1.343 |   |   |
|          | 60 | 0.003 | 0.004 | 1.181 | 0.001               | 1.188 | 0.004 | 0.886 | 0.295       | 0.001 | 1.001 | −0.062  | 1.188 |   |   |
|          | 80 | 0.007 | 0.008 | 1.284 | 0.002               | 1.263 | 0.009 | 1.069 | 0.356       | 0.002 | 1.002 | −0.042  | 1.263 |   |   |

T: Temperature.

The effective moisture diffusivity ( $D_{\text{eff}}$ ) was calculated according to the Fick's second law of diffusion for the three different drying temperatures and air flows for the five mathematical models tested. The obtained values ranged between  $10^{-5}$  and  $10^{-9}$  m<sup>2</sup>/s in concordance with the ranges reported by other authors [217,219,225,254–256].

The activation energy ( $E_a$ ) is the required energy for the initialization of the moisture diffusion from inside to the outside of the sample. The activation energy and the pre-exponential factors for the five studied models at the three different air flows were calculated using the Arrhenius

equation from the plot of  $\ln(k)$  versus  $1/T$ , and the results are exposed in **Table 1.4**. In all cases, the  $E_a$  was calculated in the temperature range from 313 to 353 K.

**Table 1.4.** Estimated energy of activation ( $E_a$ ) and pre-exponential factor ( $A$ ) for orange peels dried with different air flows in the temperature range 313–353 K.

| Model               | Air Flow 1.6 m/s |          | Air Flow 0.8 m/s |         | No Air Flow    |       |
|---------------------|------------------|----------|------------------|---------|----------------|-------|
|                     | $E_a$ (kJ/mol)   | $A$      | $E_a$ (kJ/mol)   | $A$     | $E_a$ (kJ/mol) | $A$   |
| Lewis               | 63.49            | 30576.90 | 32.14            | 4.26    | 30.68          | 17.10 |
| Henderson and Pabis | 58.37            | 5450.70  | 35.12            | 1.35    | 31.63          | 10.61 |
| Page                | 139.57           | 1.62E+15 | 60.60            | 1972.78 | 55.82          | 92.13 |
| Logarithmic         | 33.32            | 2.31     | 31.16            | 5.34    | 32.68          | 7.22  |
| Weibull             | 41.98            | 6.66     | 55.67            | 351.53  | 49.38          | 9.54  |

For the orange by-product dried with an air flow of 1.6 m/s, 0.8 m/s or without air flow, the  $E_a$  value ranges were 41.98–139.57, 31.16–60.60 and 30.68–55.82, respectively. Those results were in concordance with the data reported in other matrices [217,219,244]. In addition, Afrin et al. [213] reported for a modified Page model an activation energy of 53.07 kJ/mol when drying orange pomace without air flow in the range of 323–343 K, a value very similar to the one reported here. It is clear that with an air flow of 1.6 m/s, higher activation-energy is necessary, while without airflow, the  $E_a$  needed to start the moisture diffusion is lower. In all cases, the Page model gave the highest values of  $E_a$ . Comparing the five models, the Lewis and logarithmic models predicted the lowest energy of activation without air flow.

### 3.2. Effect of Drying on the Phenolic Content measured by HPLC-MS

The effect of drying orange by-products on the phenolic content has been evaluated. Phenolic compounds were all identified and quantified according to previous research [257]. The results obtained for the phenolic compounds in the orange by-product treated at the three different temperatures and air-flows are reported in **Table 1.5**.

**Table 1.5.** Phenolic compounds in orange by-product dried at three different temperatures and air flows expressed as µg/g d.w. with the average ± standard deviation.

|                                                                                  | 40 °C           |                  |                  | 60 °C           |                  |                  | 80 °C           |                  |                  |
|----------------------------------------------------------------------------------|-----------------|------------------|------------------|-----------------|------------------|------------------|-----------------|------------------|------------------|
|                                                                                  | No Air Flow     | Air Flow 0.8 m/s | Air Flow 1.6 m/s | No Air Flow     | Air Flow 0.8 m/s | Air Flow 1.6 m/s | No Air Flow     | Air Flow 0.8 m/s | Air Flow 1.6 m/s |
| <b>Total phenolic compounds</b>                                                  | 5456.16 ± 26.04 | 5527.02 ± 25.73  | 5682.05 ± 24.84  | 5629.07 ± 20.61 | 5685.66 ± 25.27  | 6051.14 ± 29.42  | 4819.98 ± 21.81 | 5009.58 ± 28.24  | 5209.04 ± 20.51  |
| <b>Total phenolic acids</b>                                                      | 4455.51 ± 12.66 | 4468.87 ± 12.06  | 4614.93 ± 10.75  | 4606.92 ± 7.04  | 4644.09 ± 11.63  | 4941.70 ± 15.87  | 3864.45 ± 8.63  | 4032.31 ± 15.04  | 4186.94 ± 7.57   |
| Feruloyl galactaric acid isomer a                                                | 2404.84 ± 1.36  | 2509.04 ± 3.68   | 2683.77 ± 6.18   | 2674.52 ± 0.65  | 2740.43 ± 1.55   | 2664.82 ± 2.67   | 2296.02 ± 2.61  | 2180.15 ± 4.10   | 2376.07 ± 1.39   |
| Feruloyl galactaric acid isomer b                                                | 1017.20 ± 3.48  | 1062.22 ± 1.59   | 1112.53 ± 1.78   | 1096.90 ± 1.10  | 994.47 ± 4.83    | 1158.10 ± 5.79   | 907.51 ± 1.77   | 923.03 ± 0.55    | 943.40 ± 0.96    |
| Sinapinic acid-O-glucuronide                                                     | 632.81 ± 4.69   | 660.80 ± 0.99    | 609.71 ± 1.81    | 583.90 ± 1.82   | 664.99 ± 3.82    | 739.48 ± 2.63    | 373.37 ± 2.59   | 586.58 ± 7.54    | 533.36 ± 2.51    |
| Feruloyl isocitric acid isomer a                                                 | 148.99 ± 1.64   | 107.74 ± 1.46    | 93.88 ± 0.49     | 122.21 ± 2.00   | 110.17 ± 0.57    | 150.14 ± 1.41    | 82.42 ± 1.20    | 101.45 ± 0.67    | 114.19 ± 0.60    |
| Feruloyl isocitric acid isomer b                                                 | 67.76 ± 0.75    | 28.36 ± 1.39     | 24.14 ± 0.15     | 21.07 ± 0.22    | 31.18 ± 0.48     | 51.66 ± 0.72     | 50.10 ± 0.03    | 66.44 ± 0.58     | 51.94 ± 0.68     |
| Feruloyl isocitric acid isomer c                                                 | 147.59 ± 0.70   | 77.32 ± 2.48     | 69.85 ± 0.26     | 85.65 ± 1.12    | 78.88 ± 0.32     | 142.24 ± 2.24    | 118.75 ± 0.42   | 151.24 ± 1.15    | 132.76 ± 0.87    |
| Caffeoylmalic acid                                                               | 36.32 ± 0.05    | 23.40 ± 0.46     | 21.04 ± 0.08     | 22.66 ± 0.11    | 23.97 ± 0.06     | 35.25 ± 0.40     | 36.27 ± 0.02    | 23.41 ± 0.45     | 35.22 ± 0.56     |
| <b>Total flavonoids</b>                                                          | 1000.65 ± 13.38 | 1058.15 ± 13.67  | 1067.12 ± 14.09  | 1022.15 ± 13.57 | 1041.57 ± 13.64  | 1109.43 ± 13.55  | 955.53 ± 12.95  | 977.27 ± 13.20   | 1022.09 ± 13.19  |
| Isorhamnetin-3-O-rutinoside isomer a                                             | 18.55 ± 0.52    | 17.67 ± 0.50     | 16.35 ± 0.46     | 18.10 ± 0.50    | 18.15 ± 0.49     | 19.61 ± 0.53     | 15.96 ± 0.44    | 16.86 ± 0.48     | 16.22 ± 0.46     |
| Isorhamnetin-3-O-rutinoside isomer b                                             | 3.88 ± 0.14     | 4.10 ± 0.15      | 3.87 ± 0.14      | 4.21 ± 0.15     | 4.02 ± 0.15      | 4.09 ± 0.14      | 3.29 ± 0.13     | 3.38 ± 0.13      | 4.02 ± 0.14      |
| Isorhamnetin-3-O-rutinoside isomer c                                             | 4.39 ± 0.16     | 3.15 ± 0.12      | 4.33 ± 0.16      | 4.79 ± 0.17     | 4.19 ± 0.15      | 3.66 ± 0.13      | 4.07 ± 0.15     | 4.33 ± 0.16      | 5.47 ± 0.18      |
| Prunin                                                                           | 18.71 ± 0.55    | 24.36 ± 0.68     | 23.75 ± 0.69     | 22.82 ± 0.66    | 11.75 ± 0.36     | 24.17 ± 0.64     | 18.81 ± 0.55    | 20.58 ± 0.60     | 18.79 ± 0.52     |
| Didymin                                                                          | 9.87 ± 0.31     | 10.32 ± 0.31     | 10.21 ± 0.32     | 9.15 ± 0.29     | 11.82 ± 0.36     | 10.45 ± 0.30     | 9.60 ± 0.30     | 10.87 ± 0.33     | 9.74 ± 0.29      |
| Pectolarigenin                                                                   | 644.28 ± 4.60   | 675.57 ± 4.66    | 687.33 ± 4.88    | 667.32 ± 4.76   | 678.64 ± 4.86    | 715.95 ± 4.66    | 604.36 ± 4.35   | 615.58 ± 4.43    | 639.10 ± 4.34    |
| Hesperidin                                                                       | 56.65 ± 1.07    | 58.25 ± 1.06     | 68.40 ± 1.26     | 66.58 ± 1.23    | 71.55 ± 1.32     | 71.99 ± 1.22     | 57.38 ± 1.08    | 57.23 ± 1.08     | 56.01 ± 1.01     |
| Narirutin                                                                        | 40.17 ± 0.80    | 47.79 ± 0.90     | 49.02 ± 0.94     | 42.80 ± 0.85    | 47.16 ± 0.92     | 47.19 ± 0.85     | 46.93 ± 0.91    | 45.01 ± 0.88     | 45.21 ± 0.84     |
| Naringenin                                                                       | 2.59 ± 0.11     | 0.96 ± 0.06      | 0.32 ± 0.05      | 1.99 ± 0.09     | 0.54 ± 0.05      | 3.48 ± 0.13      | 0.67 ± 0.06     | 0.61 ± 0.05      | 2.44 ± 0.10      |
| Naringin hydrate                                                                 | 10.41 ± 0.32    | 13.54 ± 0.39     | 12.23 ± 0.37     | 11.43 ± 0.35    | 12.71 ± 0.39     | 13.98 ± 0.39     | 11.71 ± 0.36    | 11.05 ± 0.34     | 12.86 ± 0.37     |
| Rutin isomer a                                                                   | 15.18 ± 0.45    | 16.22 ± 0.47     | 14.83 ± 0.44     | 15.04 ± 0.45    | 14.92 ± 0.45     | 15.41 ± 0.42     | 13.95 ± 0.42    | 15.86 ± 0.47     | 15.67 ± 0.44     |
| Rutin isomer b                                                                   | 11.97 ± 0.36    | 10.53 ± 0.32     | 10.72 ± 0.33     | 10.91 ± 0.34    | 8.19 ± 0.26      | 8.32 ± 0.25      | 9.80 ± 0.31     | 11.18 ± 0.34     | 11.96 ± 0.35     |
| 3',4'-Didemethylnobiletin                                                        | 2.76 ± 0.12     | 2.20 ± 0.10      | 1.85 ± 0.09      | 0.80 ± 0.06     | 0.10 ± 0.04      | 2.79 ± 0.11      | 1.24 ± 0.07     | 0.48 ± 0.05      | 0.61 ± 0.05      |
| Quercetin-3-O-rutinoside-7-O-Glucoside                                           | 1.95 ± 0.09     | 2.29 ± 0.10      | 2.45 ± 0.10      | 2.34 ± 0.10     | 2.84 ± 0.12      | 2.41 ± 0.10      | 2.59 ± 0.11     | 2.42 ± 0.10      | 2.32 ± 0.10      |
| Apigenin 6-C-glucoside 8-C-arabinoside/Vitexin-O-pentoside isomer a              | 10.46 ± 0.32    | 10.80 ± 0.32     | 11.63 ± 0.36     | 9.88 ± 0.31     | 11.11 ± 0.34     | 12.23 ± 0.34     | 10.12 ± 0.31    | 9.94 ± 0.31      | 11.63 ± 0.34     |
| Apigenin 6-C-glucoside 8-C-arabinoside/Vitexin-O-pentoside isomer b              | 13.04 ± 0.39    | 12.60 ± 0.37     | 13.08 ± 0.39     | 11.77 ± 0.36    | 12.62 ± 0.38     | 14.47 ± 0.40     | 12.28 ± 0.37    | 11.46 ± 0.35     | 14.33 ± 0.41     |
| Apigenin-di-C-hexoside (Vicenin-2)                                               | 63.75 ± 1.18    | 70.19 ± 1.25     | 71.60 ± 1.31     | 66.79 ± 1.24    | 69.71 ± 1.29     | 78.36 ± 1.31     | 60.91 ± 1.14    | 65.73 ± 1.22     | 71.78 ± 1.25     |
| Apigenin 7-O-neohesperidoside                                                    | 4.54 ± 0.16     | 5.97 ± 0.20      | 5.27 ± 0.18      | 3.75 ± 0.14     | 5.28 ± 0.18      | 4.96 ± 0.16      | 4.77 ± 0.17     | 5.42 ± 0.19      | 6.24 ± 0.20      |
| Kaempferol 3-apiosyl-(1->2)-galactoside/Luteolin-C-hexoside-C-pentoside isomer a | 3.02 ± 0.12     | 3.86 ± 0.14      | 2.45 ± 0.11      | 3.04 ± 0.12     | 2.32 ± 0.10      | 3.30 ± 0.12      | 2.59 ± 0.11     | 2.38 ± 0.10      | 2.74 ± 0.11      |
| Kaempferol 3-apiosyl-(1->2)-galactoside/Luteolin-C-hexoside-C-pentoside isomer b | < LOQ           | < LOQ            | 2.58 ± 0.11      | 4.52 ± 0.16     | 1.94 ± 0.09      | 4.57 ± 0.15      | 1.53 ± 0.08     | 1.29 ± 0.07      | 0.03 ± 0.04      |
| Kaempferol 3-[2"-glucosyl-6"-acetyl-galactoside] 7-glucoside isomer a            | 5.09 ± 0.18     | 3.69 ± 0.14      | 4.55 ± 0.16      | 3.31 ± 0.13     | 2.42 ± 0.10      | 3.30 ± 0.12      | 3.93 ± 0.15     | 2.34 ± 0.10      | 5.02 ± 0.17      |
| Kaempferol 3-[2"-glucosyl-6"-acetyl-galactoside] 7-glucoside isomer b            | 5.17 ± 0.18     | 4.39 ± 0.15      | 2.90 ± 0.12      | 4.42 ± 0.16     | 3.39 ± 0.13      | 4.82 ± 0.16      | 4.84 ± 0.17     | 3.27 ± 0.13      | 5.60 ± 0.18      |
| Kaempferol-dihexosyl acetate                                                     | 50.66 ± 0.97    | 58.54 ± 1.07     | 43.42 ± 0.85     | 31.70 ± 0.66    | 43.49 ± 0.86     | 34.87 ± 0.66     | 51.92 ± 0.99    | 58.51 ± 1.10     | 61.64 ± 1.09     |
| kaempferol 3-O-[3",6"-di-O-(E)-cinnamoyl]-beta-D-glucopyranoside                 | < LOQ           | < LOQ            | 0.99 ± 0.07      | 1.51 ± 0.08     | 0.78 ± 0.06      | 1.24 ± 0.07      | 0.06 ± 0.04     | 0.36 ± 0.05      | < LOQ            |
| Kaempferol 3-O-(6"-O-acetyl)glucoside-7-O-rhamnoside                             | 0.63 ± 0.06     | < LOQ            | 0.17 ± 0.04      | < LOQ           | < LOQ            | < LOQ            | < LOQ           | < LOQ            | 0.11 ± 0.04      |
| Kaempferol 3-O-sinapoyl-caffeoyl-sophoroside 7-O-glucoside isomer a              | 1.84 ± 0.09     | 1.30 ± 0.07      | 1.11 ± 0.07      | 1.26 ± 0.07     | 1.20 ± 0.07      | 1.99 ± 0.09      | 0.97 ± 0.06     | 0.78 ± 0.06      | 0.76 ± 0.06      |
| Kaempferol 3-O-sinapoyl-caffeoyl-sophoroside 7-O-glucoside isomer b              | 0.78 ± 0.06     | 0.46 ± 0.05      | 0.73 ± 0.06      | 0.56 ± 0.05     | 0.15 ± 0.04      | 0.89 ± 0.06      | 0.33 ± 0.05     | < LOQ            | 0.67 ± 0.06      |
| Kaempferol 3-apiosyl-(1->4)-rhamnoside-7-rhamnoside                              | 1.11 ± 0.07     | 0.84 ± 0.06      | 0.98 ± 0.06      | 1.37 ± 0.08     | 0.55 ± 0.05      | 0.94 ± 0.06      | 0.93 ± 0.06     | 0.36 ± 0.05      | 1.13 ± 0.07      |

Regarding the phenolic acids, it can be appreciated that total feruloyl-isocitric acid and caffeoylmalic acid are at the highest concentration without air flow and at 40 °C, demonstrating them to be highly sensitive to high temperatures and oxygen exposure. In contrast, total feruloyl-galactaric-acid content increased when air flow was used. In general, at the three temperatures, the content of ferulic-acid derivatives was highest at air flow 1.6 m/s and lowest at air flow 0.8 m/s. In contrast, sinapic acid-O-glucuronide was higher at 0.8 m/s when drying the orange by-products at 40 and 80 °C. However, the highest concentration of this compound was found when drying at 60 °C with 1.6 m/s air flow. Therefore, the total content of phenolic acids was 3.5, 6.8 and 7.7% higher at 40, 60 and 80 °C, respectively, when drying at 1.6 m/s compared with without air flow. Pectolinarigenin is the major flavonoid found in these samples of orange by-product, and it is a flavone aglycone known for a series of biological properties including anti-inflammatory, antidiabetic and especially anticancer, against breast cancer [258]. Therefore, for pectolinarigenin and for total apigenin derivatives it has been seen that at 40 and 60 °C the content increased when increasing the air flow but at 80 °C it was the opposite, and the content decreased when increasing the air flow. It is due to the fact that at moderate temperature when introducing air, the time needed for drying is reduced, so the exposure to temperature is lower. However, in contrast, when increasing the temperature up to 80 °C and including air flow, although the time is even lower, the content degrades the most. However, a different tendency was appreciated for total rutin and total isorhamnetin-3-O-rutinoside. In this case the concentrations were higher in orange by-product treated without air circulation, demonstrating them to be more oxygen-sensitive. Regarding flavanones, the highest recovery was found when drying at 60 °C and 1.6 m/s of air flow and, in fact, the highest concentration of hesperidin, naringenin and naringin hydrate was achieved. Those compounds which have especially been extensively demonstrated to have several bioactivities in human health are those such as anti-cancer in lung and breast cancer [78,79,81], antidiabetic [106], anti-inflammatory [84], cardioprotective and anti-cholesterolemic [85,100–105,259], among others. In contrast, there were no significative changes in the content of hesperidin and narirutin at 80 °C, despite the different air flows. Regarding the total content of flavonoids, it was appreciated that there was a content of 6.2, 7.9 and 6.5% higher when drying at 40, 60 and 80 °C, respectively, at 1.6 m/s in compared with without air flow. Therefore, in general, at all the temperatures tested it can be appreciated that the phenolic-compound content is higher when increasing the air flow, demonstrating that it has a huge impact on the phenolic-compound content. This is because when the air circulation is introduced, the time needed to achieve the final moisture is also reduced and, in consequence, the time of thermal exposure is lower. All these results are also statistically confirmed. A significant moderately strong negative-correlation has been found between the total phenolic acids, total flavonoids, and total phenolic compounds with temperature ( $r = -0.6222$ ,  $-0.5219$  and  $-0.6172$ , respectively). In contrast, a moderately strong positive-correlation of the



phenolic compounds was found with the air flow being strongest, in the case of total flavonoids ( $r = 0.6679$ ) and specifically total flavanones ( $r = 0.5832$ ) and narirutin ( $r = 0.7190$ ). There is a clear tendency of an increment in the total phenolic-compound content when increasing the air flow. However, although this tendency is still taking place when drying at 80 °C, there is a higher reduction in the phenolic content, mainly attributable to the case-hardening process previously reported by other authors in other matrices. In contrast to us, Chen et al. (2011) [260] reported achieving higher amounts of phenolic compounds such as naringin, hesperidin, kaempferol and rutin in a linear way, when increasing the temperature from 50 to 100 °C for drying orange-peel extracts. Despite this, the results obtained here for these compounds are in the same range of magnitude.

### 3.3. Effect of Drying on the Vitamin C Content

The content of ascorbic acid in the orange by-products dried at the three different temperatures and air flows was measured using HPLC-UV/VIS, and the results are shown in **Table 1.6**. Briefly, the high correlation between ascorbic-acid content and the total content of vitamin C ( $r = 0.9656$ ), confirmed that AA was the main compound that constitutes vitamin C.

**Table 1.6.** Vitamin C content in orange by-product dried at three different temperatures and air flows expressed as  $\mu\text{g AA/g d.w.}$  with average  $\pm$  standard deviation.

|               | Ascorbic Acid      | Dehydroascorbic Acid | Ascorbic Acid/<br>Dehydroascorbic<br>Acid Ratio | Total Vitamin C    |
|---------------|--------------------|----------------------|-------------------------------------------------|--------------------|
| 40 °C         | 522.41 $\pm$ 0.00  | 435.51 $\pm$ 0.00    | 1.20                                            | 957.92 $\pm$ 0.00  |
| 40 °C 0.8 m/s | 1007.67 $\pm$ 3.05 | 457.49 $\pm$ 1.39    | 2.20                                            | 1465.17 $\pm$ 4.44 |
| 40 °C 1.6 m/s | 1029.62 $\pm$ 5.38 | 503.60 $\pm$ 2.63    | 2.04                                            | 1533.21 $\pm$ 8.00 |
| 60 °C         | 539.26 $\pm$ 0.04  | 342.30 $\pm$ 0.02    | 1.58                                            | 881.57 $\pm$ 0.06  |
| 60 °C 0.8 m/s | 707.22 $\pm$ 1.81  | 339.75 $\pm$ 0.87    | 2.08                                            | 1046.97 $\pm$ 2.68 |
| 60 °C 1.6 m/s | 715.01 $\pm$ 2.25  | 323.58 $\pm$ 1.02    | 2.21                                            | 1038.58 $\pm$ 3.27 |
| 80 °C         | 478.65 $\pm$ 2.21  | 349.71 $\pm$ 1.62    | 1.37                                            | 828.36 $\pm$ 3.83  |
| 80 °C 0.8 m/s | 615.38 $\pm$ 3.85  | 347.15 $\pm$ 2.17    | 1.77                                            | 962.53 $\pm$ 6.02  |
| 80 °C 1.6 m/s | 507.13 $\pm$ 3.52  | 487.84 $\pm$ 3.39    | 1.04                                            | 994.96 $\pm$ 6.91  |

The highest recovery was obtained when orange by-products were dried at 40 °C and with an air flow of 1.6 m/s. Indeed, a significant ( $p < 0.05$ ) negative, strong correlation between the content of vitamin C and the temperature ( $r = -0.6790$ ) was detected. In contrast, a significative ( $p < 0.1$ ) positive, moderate correlation was found between the total vitamin C and the air flow ( $r = 0.5215$ ). Therefore, it is revealed that there is a lower reduction of the content of vitamin C when increasing the air flow, but at the lower temperature. The vitamin C content of the orange by-product, and

especially the ascorbic acid as the major form, has been demonstrated to be highly sensitive to the exposure to temperature, with the time that can be reduced by using air circulation being the factor that determines the avoidance of the biggest degradations. Within this context, there was a reduction of approximately 13.5% when drying at 80 °C, or 6% when drying at 60 °C compared with drying at 40 °C. Similar results were reported by Afrin et al. [213], who also had an ascorbic-acid reduction of 7.6% when drying orange pomace at 70 °C, compared with drying at 50 °C. This tendency has been also seen in other fruit and vegetables such as tomatoes, where a reduction of around 30% in the ascorbic acid content was reported when drying at 70 °C, compared with drying at 50 °C [261]. In addition, when a temperature of 60 °C with air flow of 1.6 m/s was applied, the highest ascorbic/dehydroascorbic ratio was observed, with no significative differences with the ratio observed at 40 °C and 0.8 m/s air flow. Through these trials, it can be confirmed that the oxygen exposure with the air flow of the samples during drying entails a higher oxidation of ascorbic acid to dehydroascorbic acid but no decrease in its total vitamin C content.

### 3.4. Effect of Drying on the Antioxidant Activity

The antioxidant activity of the orange by-products dried at the three different temperatures and air flows was measured using three different methods, DPPH, ABTS and FRAP, and the results are shown in

**Figure 1.3.** The result ranges were 7.97–10.99, 8.27–14.13 and 7.70–16.69 mg TE/g d.w. for the DPPH, ABTS and FRAP assays, respectively. All methods showed a significant ( $p < 0.05$ ) strong, positive correlation between each other (DPPH vs. ABTS  $r = 0.8357$ ; DPPH vs. FRAP  $r = 0.8643$ ; ABTS vs. FRAP  $r = 0.9512$ ).

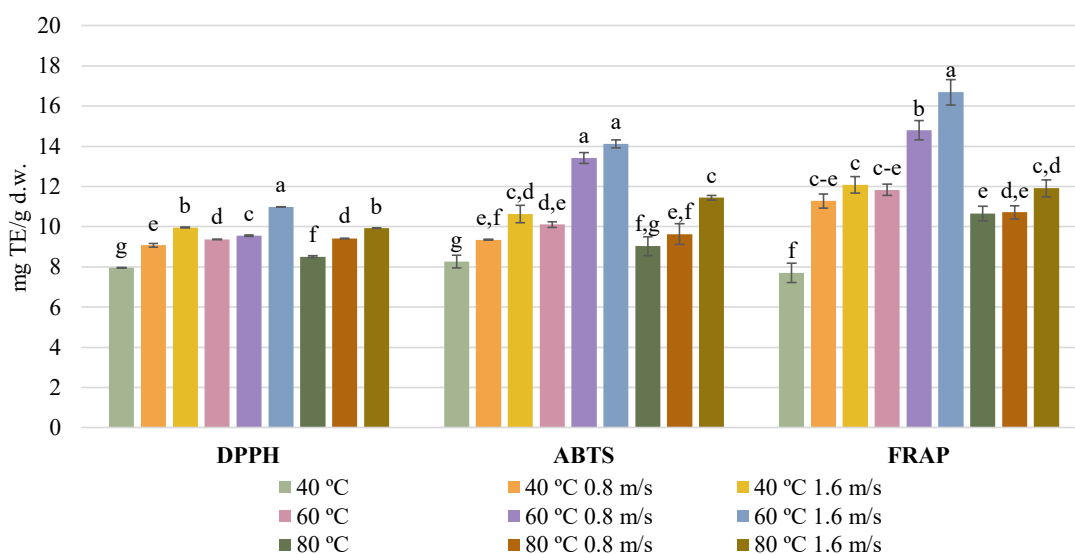


**Figure 1.3.** Antioxidant activity measured using DPPH, ABTS and FRAP of the orange by-product dried by convective drying at different temperatures and air flows. The results are

expressed as mg TE (Trolox equivalents)/g d.w. Different letters (a-f) indicate significant differences.

All the three methods had a strong positive correlation ( $p < 0.05$ ), with the total content of flavonoids being the strongest in the case of DPPH. Going further, this positive correlation is the highest for the total flavanones, and especially for hesperidin and narirutin. Moreover, when fixing a significance level of  $p < 0.1$ , a moderately strong, positive correlation between the total phenolic-acid content and the antioxidant-activity results for ABTS and FRAP was found. For the three tested methods at the three temperatures, it can be appreciated that the highest antioxidant activity was obtained for the orange by-product dried with an air flow of 1.6 m/s. This is statistically confirmed by a correlation between the antioxidant activity measured by the three methods and the air flow ( $r > 0.5921$ ). Moreover, a significant ( $p < 0.1$ ) moderately negative correlation with time was found for the DPPH ( $r = -0.5958$ ), ABTS ( $r = -0.5539$ ) and FRAP ( $r = -0.6156$ ) assays. These data agreed with those of Deng et al. [137], which stated that drying the orange peel at a temperature between 50 and 70 °C achieved the highest antioxidant recoveries, measured by DPPH and FRAP at 65 °C. In addition, Chen et al. [53] reported the highest radical scavenging activity when orange peel extracts were dried at 100 °C.

#### 4. Conclusions

The drying step is essential for the storage of orange by-products at an industrial level. To this aim, the convective drying of the orange by-product was studied at three different temperatures (40, 60 and 80 °C) and air flows (0, 0.8 and 1.6 m/s). All these parameters had a direct effect on the time needed to achieve a final moisture-content lower than 10%. In addition, the five tested mathematical-models for fitting the drying kinetics of the orange by-product demonstrated satisfactory results. However, the Lewis and Page models gave better adjustment, reporting higher coefficient of regressions and lower squared and absolute errors for the major temperatures and air flows tested. The application of the Page equation will allow us to maximize the exploitation of orange by-products, reducing economic and energy costs. Moreover, to select the best drying conditions, the phenolic-compound content was evaluated by HPLC-MS, the antioxidant activity by DPPH, ABTS and FRAP and the vitamin C content by HPLC-UV/VIS. In all cases, both the temperature and the air flow were demonstrated to have a huge influence. When increasing the air flow, the time needed to reach the final moisture content is reduced, so the exposure to temperature is reduced and, in consequence, the phenol, ascorbic acid and antioxidant-compound reduction is lower. However, at 80 °C, the case-hardening phenomenon was increased when increasing the air flow, reducing the bioactive content. Regarding the vitamin C content, this was better with the lowest temperature tested. However, reaching a compromise among all the parameters evaluated, the drying conditions selected were 60 °C, with an air flow of 1.6 m/s that

takes 315 min and allows a lower reduction of phenolic compounds and antioxidant compounds, especially flavanones such as hesperidin, narirutin and pectolinarigenin, keeping an acceptable ascorbic acid content. For future research, it would be interesting to evaluate the use of innovative extraction-techniques for increasing the extraction of phenolic compounds in this dry by-product, with improved management and storage at an industrial level for potential activities, and its application in functional foods and nutraceuticals.

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## **CHAPTER 2. Optimization of ultrasound-assisted extraction via sonotrode of phenolic compounds from orange by-products**

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Article

# Optimization of Ultrasound-Assisted Extraction via Sonotrode of Phenolic Compounds from Orange By-Products

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## Abstract

Orange peel is the main by-product from orange juice industry. It is a known source of bioactive compounds, mostly phenolic compounds, and it has been widely studied for its healthy activities. Thus, this research focuses on the establishment of ultrasound-assisted extraction of phenolic compounds in orange peel using a sonotrode. For this purpose, a Box–Behnken design of 27 experiments was carried out with four independent factors—ratio ethanol/water (v/v), time (min), amplitude (%), and pulse (%). Quantitative analyses of phenolic compounds were performed, and the antioxidant activity was measured by ABTS and DPPH methods. The validity of the experimental design was confirmed by ANOVA and the optimal sonotrode extraction conditions were obtained by response surface methodology (RSM). The extracts obtained in the established conditions were analyzed by High Performance Liquid Chromatography (HPLC) coupled to mass spectrometer detector and 74 polar compounds were identified. The highest phenolic content and antioxidant activity were obtained using 45/55 ethanol/water (v/v), 35 min, amplitude 90% (110 W), and pulse 100%. The established method allows an increment of phenolics recovery up to 60% higher than a conventional extraction. Moreover, the effect of drying on phenolic content was also evaluated.

**Keywords:** Box–Behnken design; phenolic compounds; antioxidant activity; orange peel; sonotrode ultrasound-assisted extraction; HPLC-MS

## 1. Introduction

Orange is the second most produced fruit in the European Union, mainly in the countries of the Mediterranean basin where it supposes 6 million tons, almost 10% of the world production. Increasingly, citrus juices have gained great popularity, representing more than 50% of the juices available in international trade. From them, orange juice is the main product processed by the beverage industry and consumed throughout the world due to its high nutritional value and desirable sensory characteristics. Orange fruit processing to obtain juices or citrus-based beverages generates large amounts of waste by-products, such as peels, which are a rich source of soluble sugars, phenolic compounds, flavonoids, dietary fibers (cellulose, hemicelluloses, and pectin), vitamins, enzymes, and essential oils [58–62], which can be used for the production of nutritional dietary supplements [262]. Recently, several authors successfully used the orange by-products as ingredients with rheological and functional properties for the formulation of biscuits [68] and jams [69], and as fat replacer [71]. The orange peels have been reported to have antioxidant [92], anti-inflammatory [84], anti-cancer [79], anti-rheumatic [263], anti-diabetic [106], and cardioprotective activities [100], mainly attributed to its content in phenolic compounds. However, orange by-product, due to its high moisture content, is easily spoiled. By this way, the drying step is essential for decreasing the moisture content up to a level that inactivate the oxidant, enzymatic, and microbial degradation, ensuring a longer preservation among the time. It also could let to reduce the transport costs in an industrial level. Several drying techniques have been applied to the dehydration of citrus by-products, such as sun drying, hot air drying, freezing drying, microwave drying, etc. Between them, the best one referred to costs and yields and that can be scaled has been demonstrated to be oven-controlled drying [137]. After them, traditionally methods based on maceration and thermal extraction with different solvents such as hexane, acetone, methanol, etc., have been used to recover nutritionally valuable compounds from this type of fruit wastes [264]. According to this approach, the aim of this work is to establish the best ultrasound-assisted extraction via sonotrode to recover the phenolic compounds from orange by-products using food-grade solvents. The antioxidant activity of the extracts was also evaluated by DPPH (2,2-difenil-1-picrylhydrazyl) and ABTS (2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) assays. Moreover, the effect of drying on the phenolic composition was also evaluated.



## 2. Materials and Methods

### 2.1. Chemicals and Samples

Gallic acid, DPPH and ABTS were purchased from Sigma-Aldrich (St. Louis, MO, USA).  $\text{Na}_2\text{CO}_3$  was purchased from BDH AnalR (Poole, England). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Vanillic acid, chlorogenic acid, ferulic acid, quercetin, and rutin were also acquired from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade water, Folin–Ciocalteu reagent and other reagents were purchased from Merck KGaA (Darmstadt, Germany).

Orange by-products (var. *Navelina*) were obtained after juice production. The resulting by-product was composed by the albedo, flavedo and rests of pulp of the orange with a humidity of  $70 \pm 1.5\%$ . For the model the samples were dried at  $60^\circ\text{C}$  according to Garau et al. [211]. Dried and fresh samples of by-products were grinded and frozen at  $-18^\circ\text{C}$  until the analyses.

### 2.2. Experimental Design

The conditions for obtaining the highest recovery of phenolic compounds and antioxidant activity from the orange by-products were optimized by using a Box–Behnken design and response surface methodology (RSM). The design was composed by 27 experiments structured in three blocks with three levels ( $-1$ ,  $0$ ,  $+1$ ). Each experiment was carried out in duplicate. The independent variables were ratio ethanol/water ( $0:100$ ,  $50:50$ ,  $100:0$  v/v), time ( $5$ ,  $25$ ,  $45$  min), amplitude ( $20\%$ ,  $60\%$ ,  $100\%$ ), and pulse ( $10\%$ ,  $50\%$ ,  $100\%$ ). The dependent variables were adjusted to a second order polynomial model equation (Equation (16)), where  $Y$  represents the response variable, the total phenolic compounds (TPC) or the antioxidant assays ABTS or DPPH,  $X_i$  and  $X_j$  are the independent factors that affect the response, and  $\beta_0$ ,  $\beta_i$ ,  $\beta_{ii}$  and  $\beta_{ij}$  are the regression coefficients of the model (interception, linear, quadratic and interaction terms). Statistica 7.0 package (StatSoft, Tulsa, OK, USA) was used for the mathematical operations and simulations.

Equation (16). Second order polynomial equation.

$$Y = \beta_0 + \sum_{i=0}^4 \beta_i X_i + \sum_{i=0}^4 \beta_{ii} X_{ii}^2 + \sum_{i=0}^4 \sum_{j=0}^4 \beta_{ij} X_i X_j \quad (16)$$

ANOVA assay was performed in order to evaluate the adjustment of the models having into account the regression coefficients, the  $p$ -values of the regressions and lack of fit. The optimum conditions were established using RSM.

### 2.3. Extraction of Phenolic Compounds from Orange By-Products by Sonotrode Ultrasonic Extraction

Orange by-products (0.5 g) were extracted with an ethanol/water solution (v/v) (100 mL) by a sonotrode (UP400St ultrasonic processor, Hielscher, Germany) according to the parameters showed in **Table 2.1**. After the extraction, the samples were centrifuged at 3500 rpm for 15 min and the supernatant was stored at  $-18^{\circ}\text{C}$  until the analyses.

**Table 2.1.** Box–Behnken design with natural and coded values (parenthesis) of the conditions of extraction and the experimental results obtained for TPC, and antioxidant assays (ABTS and DPPH) expressed with the average and the standard deviation.

|    | Independent Factors |        |         |         | Dependent Factors      |                        |                        |
|----|---------------------|--------|---------|---------|------------------------|------------------------|------------------------|
|    | X1                  | X2     | X3      | X4      | TPC (mg GAE/g d.w.)    | ABTS (mg TE/g d.w.)    | DPPH (mg TE/g d.w.)    |
| 1  | 0 (−1)              | 5 (−1) | 60 (0)  | 50 (0)  | $20.46 \pm 0.18^{e-g}$ | $26.67 \pm 0.39^{e,f}$ | $10.55 \pm 0.03^b$     |
| 2  | 100 (1)             | 5 (−1) | 60 (0)  | 50 (0)  | $10.33 \pm 0.10^b$     | $11.56 \pm 0.26^a$     | $10.94 \pm 0.21^c$     |
| 3  | 0 (−1)              | 45 (1) | 60 (0)  | 50 (0)  | $21.64 \pm 0.21^{f-i}$ | $24.92 \pm 0.06^{d,e}$ | $19.79 \pm 0.04^i$     |
| 4  | 100 (1)             | 45 (1) | 60 (0)  | 50 (0)  | $19.55 \pm 0.35^e$     | $18.28 \pm 0.38^b$     | $11.07 \pm 0.06^c$     |
| 5  | 50 (0)              | 25 (0) | 20 (−1) | 10 (−1) | $25.33 \pm 0.14^l$     | $25.21 \pm 0.26^{d,e}$ | $20.02 \pm 0.06^i$     |
| 6  | 50 (0)              | 25 (0) | 100 (1) | 10 (−1) | $21.95 \pm 0.16^{g-j}$ | $36.62 \pm 0.50^{n-p}$ | $18.54 \pm 0.05^f$     |
| 7  | 50 (0)              | 25 (0) | 20 (−1) | 100 (1) | $25.12 \pm 0.35^l$     | $39.13 \pm 0.79^{o-q}$ | $21.05 \pm 0.04^{k,l}$ |
| 8  | 50 (0)              | 25 (0) | 100 (1) | 100 (1) | $29.75 \pm 0.36^m$     | $30.14 \pm 0.01^{h,i}$ | $20.95 \pm 0.05^k$     |
| 9  | 50 (0)              | 25 (0) | 60 (0)  | 50 (0)  | $23.59 \pm 0.92^{j,k}$ | $34.97 \pm 0.13^{l,m}$ | $22.43 \pm 0.06^{m,n}$ |
| 10 | 0 (−1)              | 25 (0) | 60 (0)  | 10 (−1) | $20.08 \pm 0.35^{e,f}$ | $36.27 \pm 0.26^{l-n}$ | $21.21 \pm 0.05^{k,l}$ |
| 11 | 100 (1)             | 25 (0) | 60 (0)  | 10 (−1) | $10.11 \pm 0.37^b$     | $22.87 \pm 0.51^c$     | $11.16 \pm 0.04^c$     |
| 12 | 0 (−1)              | 25 (0) | 60 (0)  | 100 (1) | $21.95 \pm 0.42^{h-k}$ | $33.07 \pm 0.78^{j,k}$ | $19.93 \pm 0.05^i$     |
| 13 | 100 (1)             | 25 (0) | 60 (0)  | 100 (1) | $16.27 \pm 0.35^{c,d}$ | $23.60 \pm 0.77^{c,d}$ | $15.44 \pm 0.05^e$     |
| 14 | 50 (0)              | 5 (−1) | 20 (−1) | 50 (0)  | $21.30 \pm 0.03^{f-h}$ | $37.18 \pm 0.77^{m-o}$ | $19.03 \pm 0.05^g$     |
| 15 | 50 (0)              | 45 (1) | 20 (−1) | 50 (0)  | $22.40 \pm 0.14^{h-k}$ | $36.28 \pm 0.64^{m-p}$ | $23.07 \pm 0.05^p$     |
| 16 | 50 (0)              | 5 (−1) | 100 (1) | 50 (0)  | $10.31 \pm 0.04^b$     | $29.71 \pm 0.27^{g,h}$ | $22.69 \pm 0.06^o$     |
| 17 | 50 (0)              | 45 (1) | 100 (1) | 50 (0)  | $23.03 \pm 0.14^{j,k}$ | $40.92 \pm 0.64^q$     | $22.23 \pm 0.05^m$     |
| 18 | 50 (0)              | 25 (0) | 60 (0)  | 50 (0)  | $22.35 \pm 0.07^{h-k}$ | $34.92 \pm 0.59^{k,l}$ | $21.20 \pm 0.08^l$     |
| 19 | 0 (−1)              | 25 (0) | 20 (−1) | 50 (0)  | $20.56 \pm 0.35^{e-g}$ | $32.53 \pm 0.37^{j,k}$ | $23.19 \pm 0.06^p$     |
| 20 | 100 (1)             | 25 (0) | 20 (−1) | 50 (0)  | $8.66 \pm 0.06^a$      | $26.33 \pm 0.12^{e,f}$ | $9.54 \pm 0.04^a$      |
| 21 | 0 (−1)              | 25 (0) | 100 (1) | 50 (0)  | $22.81 \pm 0.26^{j,k}$ | $32.51 \pm 0.03^{j,k}$ | $20.29 \pm 0.05^j$     |
| 22 | 100 (1)             | 25 (0) | 100 (1) | 50 (0)  | $15.98 \pm 0.21^c$     | $27.35 \pm 0.96^{f,g}$ | $12.30 \pm 0.04^d$     |
| 23 | 50 (0)              | 5 (−1) | 60 (0)  | 10 (−1) | $17.30 \pm 0.42^d$     | $35.04 \pm 0.19^{l-n}$ | $19.41 \pm 0.04^h$     |
| 24 | 50 (0)              | 45 (1) | 60 (0)  | 10 (−1) | $20.42 \pm 0.09^{e-g}$ | $38.15 \pm 0.76^{p,q}$ | $21.28 \pm 0.04^l$     |
| 25 | 50 (0)              | 5 (−1) | 60 (0)  | 100 (1) | $23.37 \pm 0.13^k$     | $28.93 \pm 0.15^{g,h}$ | $26.45 \pm 0.04^r$     |
| 26 | 50 (0)              | 45 (1) | 60 (0)  | 100 (1) | $25.30 \pm 0.08^l$     | $31.37 \pm 0.37^{i,j}$ | $25.65 \pm 0.04^q$     |
| 27 | 50 (0)              | 25 (0) | 60 (0)  | 50 (0)  | $22.52 \pm 0.36^{i-k}$ | $32.62 \pm 0.27^{j,k}$ | $22.61 \pm 0.01^{n,o}$ |

X<sub>1-4</sub>: Ethanol/water (v/v), time (min), amplitude (%), pulse (%). TPC: Total phenolic compounds. GAE: Gallic acid equivalents. TE: Trolox equivalents. d.w.: dry weight. Different letters in the same column showed significant differences.

## **2.4. Conventional Extraction of Phenolic Compounds from Orange By-Products**

The conventional extraction was carried out according to Abdurrahman et al. [265] with slightly modifications. Briefly, 0.5 g of orange by-products was added of 100 mL of ethanol/water (45/55 v/v), and it was agitated by a magnetic stirrer during 35 min at 35 °C. The samples were centrifuged at 3500 rpm for 15 min and the supernatant were collected and stored at –18 °C until the analyses.

## **2.5. Determination of Total Phenolic Compounds by Folin–Ciocalteu Assay**

Total phenolic compounds were determined by Folin–Ciocalteu spectrophotometric method [266]. Thus, 100 µL of the extract was added of 500 µL of Folin–Ciocalteu reagent and 6 mL of MilliQ water. The flask was agitated for a minute. After that, 2 mL of 15% (w/v) Na<sub>2</sub>CO<sub>3</sub> was added and made up to 10 mL with MilliQ water and stored at dark conditions. The measures were carried out after 2 h at 750 nm and 25 °C with a UV–Visible spectrophotometer (Spectrophotometer 300 Array, UV–Vis, single beam, Shimadzu, Duisburg, Germany). The results were compared to a standard curve of gallic acid (1, 5, 10, 25, 50, 100, 250 ppm) to calculate the TPC. Results are expressed as mg gallic acid equivalents (GAE)/g dry weight (d.w.).

## **2.6. Determination of Phenolic Compounds from Orange By-Products by HPLC-ESI-TOF-MS**

The analyses of the orange by-products using the optimized conditions obtained with the Box–Behnken design were carried out in duplicate on an ACQUITY Ultra Performance LC system (Waters Corporation, Milford, MA, USA) coupled to an electrospray ionization (ESI) source operating in the negative mode and a time-of-flight (TOF) mass detector (Waters Corporation, Milford, MA, USA). The compounds of interest were separated on an ACQUITY UPLC BEH Shield RP18 column (1.7 µm, 2.1 mm × 100 mm; Waters Corporation, Milford, MA, USA) at 40 °C using a gradient previously stated by Verni et al. [245] using water containing 1% acetic acid as mobile phase A and acetonitrile as mobile phase B. The data were elaborated using MassLynx 4.1 software (Waters Corporation, Milford, MA, USA).

## **2.7. Antioxidant Assays DPPH and ABTS**

The antioxidant capacity was evaluated in the 27 orange by-products extracts by two different methods. The first one was the developed by Re et al. [249] in which the monocation ABTS<sup>•+</sup> is generated by oxidation of the ABTS with potassium persulfate in the dark at room temperature for 12–24 h. For each extract, it was added 1 mL of this ABTS solution to 0.01 mL of extract and it was measured the detriment of absorbance during 10 min at 734 nm. The results were compared with a standard curve of Trolox (1, 10, 20, 50, 80, 100, 150, 200 ppm). DPPH radical scavenging activity was assayed with a method proposed by several authors [247,248]. Then, 100 µL of each

extract was added of 2.9 mL of DPPH, and after rapid stirring, the bleaching power of the extract was observed in a time interval from 0 to 30 min at 517 nm. The results were compared with a standard curve of Trolox in methanol/water (4:1, v/v) (1, 10, 20, 50, 80, 100, 150, 200 ppm). Results for both assays are expressed as mg Trolox equivalents (TE)/g d.w.

### **3. Results and Discussion**

#### **3.1. Determination of Total Phenolic Compounds and Antioxidant Capacity in Orange By-Products**

The Box–Behnken model coupled to response surface methodology was used to optimize the extraction of the extract with the highest TPC and the highest antioxidant capacity (**Table 2.1**).

For TPC, the observed values ranged between 8.7 and 29.7 mg GAE/g d.w. The lowest value corresponds to the conditions 100% ethanol, 25 min, 20% amplitude, and 50% pulse, and the highest one to ethanol/water (50: 50, v/v), 25 min, 100% amplitude, and 100% pulse.

For the antioxidant assays, values ranged from 9.5 to 26.4 mg TE/g d.w. with the DPPH technique, and from 11.5–40.5 mg TE/g d.w. with the ABTS one. The minimum value with both methods was obtained using only ethanol (100%), whereas the highest ones were observed by using a mixture of 50% ethanol and 50% water, similarly to the results described for TPC.

#### **3.2. Fitting the Model**

The experimental data was analyzed adjusting it to a second order polynomial equation, a regression model that, using the least squares method, provides the lowest residual value (Equation (16)). All the regression coefficients are shown in **Table 2.2**.

**Table 2.2.** Estimated regression coefficients of the adjusted second-order polynomial equation and analysis of variance (ANOVA) of the model.

| Regression Coefficients | Response   |                |            |                |           |                |
|-------------------------|------------|----------------|------------|----------------|-----------|----------------|
|                         | TPC        |                | ABTS       |                | DPPH      |                |
|                         | Effect     | <i>p</i> Value | Effect     | <i>p</i> Value | Effect    | <i>p</i> Value |
| $\beta_0$               | 20.9165 *  | 0.0000         | 25.5081 *  | 0.0000         | 16.4511 * | 0.0001         |
| <b>Lineal</b>           |            |                |            |                |           |                |
| $\beta_1$               | -7.39159 * | 0.0000         | -6.9354 *  | 0.0000         | -7.1540 * | 0.0036         |
| $\beta_2$               | 7.14405 *  | 0.0001         | 4.1242 *   | 0.0189         | 3.9375 *  | 0.0343         |
| $\beta_3$               | 0.1177     | 0.8784         | -2.5229    | 0.6391         | 1.2579    | 0.7225         |
| $\beta_4$               | 5.0592 *   | 0.0002         | -6.3625 *  | 0.0227         | 4.2319 *  | 0.0215         |
| <b>Crossed</b>          |            |                |            |                |           |                |
| $\beta_{12}$            | 4.0196 *   | 0.0211         | 4.2385     | 0.1091         | -4.5509 * | 0.0274         |
| $\beta_{13}$            | 2.5322     | 0.0637         | 0.5152     | 0.7383         | 2.8321    | 0.0665         |
| $\beta_{14}$            | 2.1488     | 0.0855         | 1.9652     | 0.2811         | 2.7874    | 0.0684         |
| $\beta_{23}$            | 5.8083 *   | 0.0023         | 6.0598 *   | 0.0182         | -2.2457   | 0.4190         |
| $\beta_{24}$            | -0.5977    | 0.4677         | -0.3305    | 0.8286         | -1.3348   | 0.2248         |
| $\beta_{34}$            | 4.0099 *   | 0.0214         | -10.1977 * | 0.0005         | 0.6917    | 0.4634         |
| <b>Quadratic</b>        |            |                |            |                |           |                |
| $\beta_{11}$            | 1.9745 *   | 0.0001         | 12.6447 *  | 0.0000         | 8.0299 *  | 0.0020         |
| $\beta_{22}$            | -1.0824    | 0.1228         | 7.1362 *   | 0.0000         | 2.1692 *  | 0.03400        |
| $\beta_{33}$            | -1.6717    | 0.0569         | 0.7439     | 0.4660         | 1.1751    | 0.1326         |
| $\beta_{44}$            | -2.2687 *  | 0.0336         | 1.9455     | 0.1091         | 0.4520    | 0.3771         |
| $R^2$                   | 0.9564     |                | 0.9286     |                | 0.8694    |                |
| <i>p</i> Model          | 0.0011     |                | 0.0043     |                | 0.0001    |                |
| <i>p</i> Lack of fit    | 0.1522     |                | 0.2624     |                | 0.1117    |                |

\* Significant at  $\alpha \leq 0.05$ ; 1 Ethanol/water ratio (v/v), 2 time, 3 amplitude, 4 pulse.

The non-significant terms with a significance level of  $p < 0.05$  were discarded, and the model was recalculated only with significant terms. For the three response variables, the individual linear factors ratio ethanol/water ( $\beta_1$ ), time ( $\beta_2$ ) and pulse ( $\beta_4$ ) showed significant effects. For the TPC and DPPH, the crossed interaction  $\beta_{12}$  was significant, and the crossed interactions  $\beta_{23}$  and  $\beta_{34}$  were significant for TPC and ABTS. Moreover, the quadratic term  $\beta_{11}$  was significant for the three response variables; meanwhile,  $\beta_{22}$  was significant for ABTS and DPPH, and  $\beta_{44}$  only for TPC. The amplitude ( $\beta_3$ ) and the rest of interactions did not have significant effect in the meanings chosen. In addition, for the three responses it was revealed a high correlation between the factors and the response variable ( $R^2 = 0.9564$ ,  $0.9286$ , and  $0.8694$  for TPC, ABTS and DPPH, respectively). The validity of the model was tested with ANOVA, which showed that, the models can be statistically accepted because the regression model *p* values are lower than 0.05 and the lacks of fit for the three variables are non-significant ( $p > 0.05$ ).

### 3.3. Confirmation of the Optimal Extraction Parameters by Sonotrode

To establish the optimal conditions of extraction, the obtained response surfaces (Figures 2.1–2.3) were studied.

As can be seen in the figures, the major content of phenolic compounds with the highest antioxidant activity could be obtained with the highest amplitude and pulse values and the intermedium ethanol/water ratio and time values.

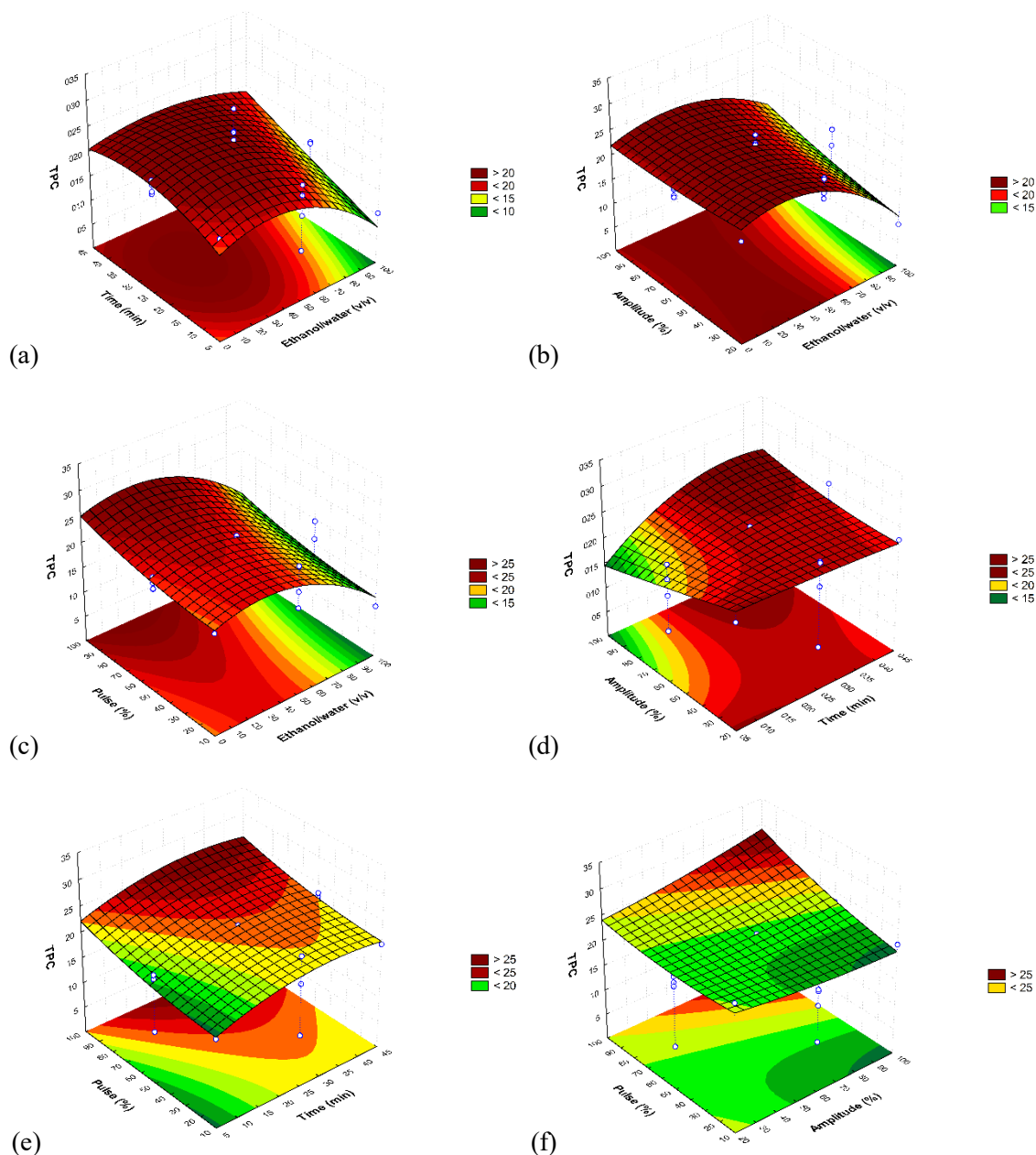


**Figure 2.1.** Response surface graphs (a–f) showing the combined effects of the process variables: Ethanol/water (v/v), time (min), amplitude (%), and pulse (%), for TPC (mg GAE/g d.w.).

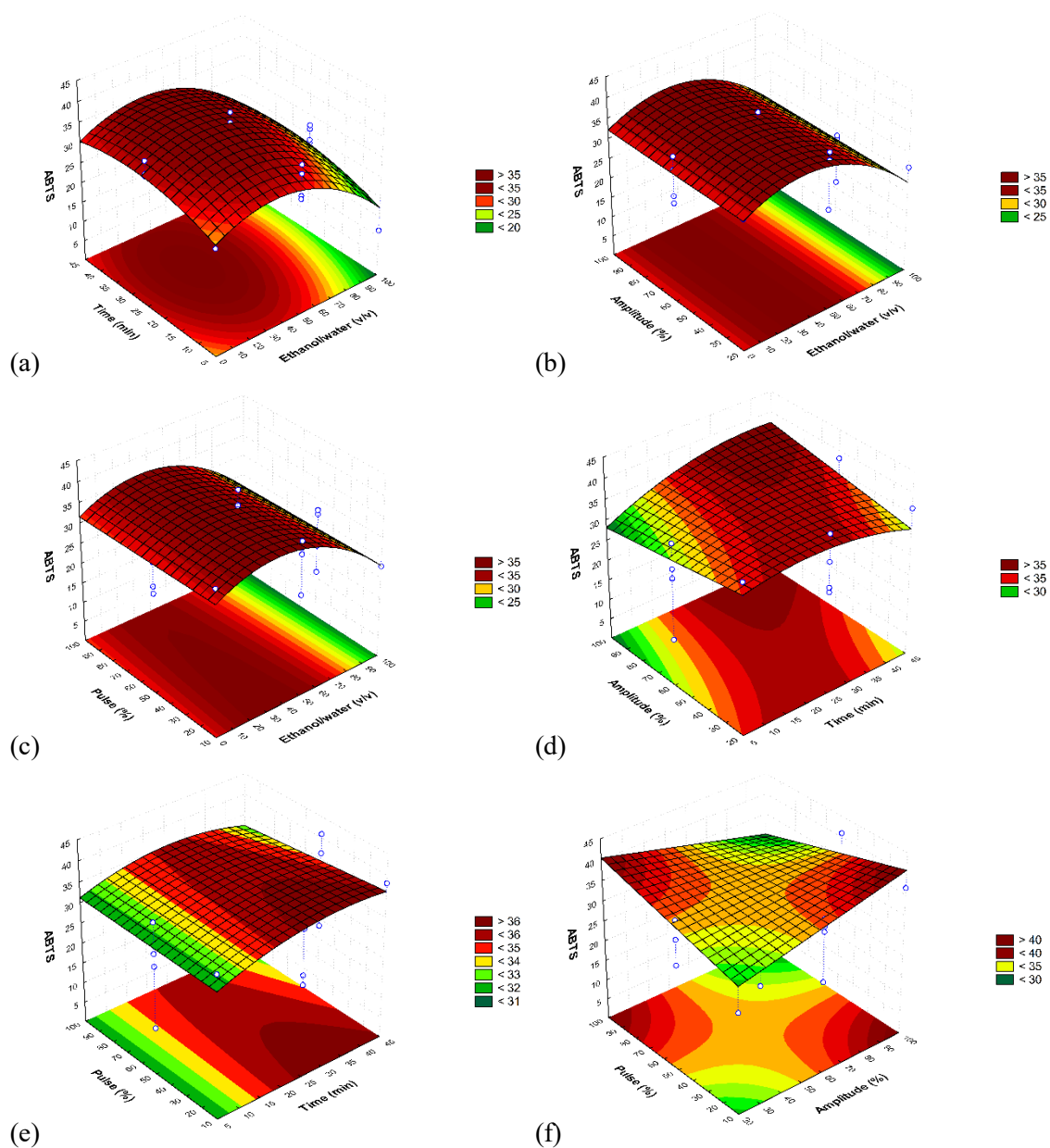


**Figure 2.2.** Response surface graphs (a–f) showing the combined effects of the process variables: Ethanol/water (v/v), time (min), amplitude (%), and pulse (%), for ABTS antioxidant assay (mg TE/g d.w.).

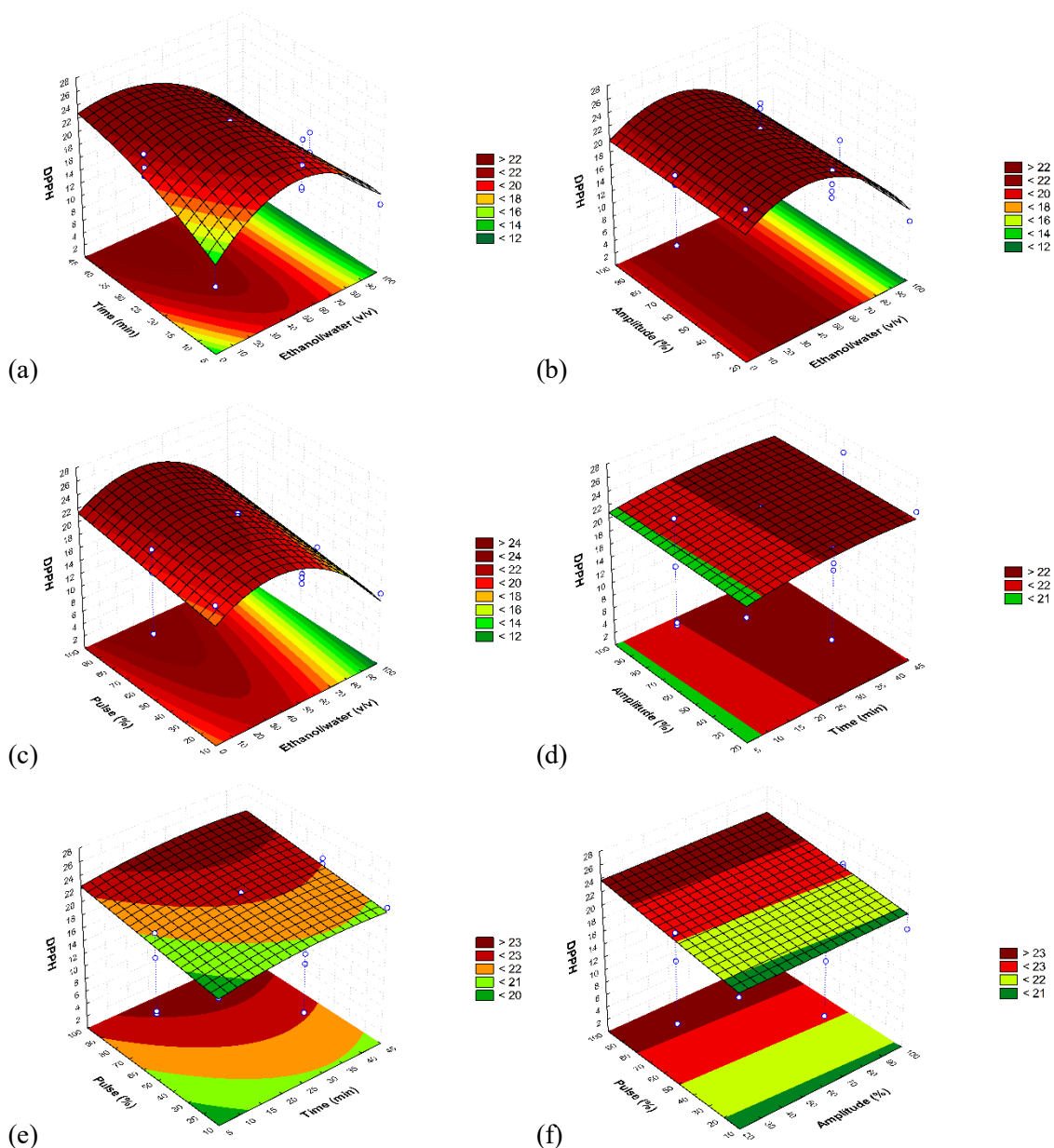


**Figure 2.3.** Response surface graphs (a–f) showing the combined effects of the process variables: Ethanol/water (v/v), time (min), amplitude (%), and pulse (%), for DPPH antioxidant assay (mg

Briefly, optimal sonotrode extraction conditions were 45% ethanol/water (v/v), 35 min, amplitude 90% (110 W), and pulse 100% (Table 2.3). Verifying the accuracy of the mathematical model, the obtained values did not report significant differences with the predicted values, showing coefficients of variation  $<1$  in the case of the total phenolic compounds, and the antioxidant assays (DPPH and ABTS). According to the results, ethanol was not an efficient solvent when used pure, showing better results when it was combined with water in a proportion equal or minor to 50%. This occurs due to the increased solvation provided by the presence of water. Additionally, the optimum time was chosen as lower as possible in order to develop a quick procedure. The highest values of amplitude and pulse are necessary in order to reach higher powers and finally obtain better performances.



**Table 2.3.** Optimal conditions selected and the model predicted values with the obtained values expressed with the mean and the standard deviation.

| Parameter                   | Optimal Conditions |             |             |
|-----------------------------|--------------------|-------------|-------------|
| Ethanol/water (v/v)         | 45                 |             |             |
| Time (min)                  | 35                 |             |             |
| Amplitude (%)               | 90                 |             |             |
| Pulse (%)                   | 100                |             |             |
|                             | TPC                | DPPH        | ABTS        |
| Predicted value (mg/g d.w.) | 29.36 ± 3.5        | 24.44 ± 3.6 | 32.02 ± 7.0 |
| Obtained value (mg/g d.w.)  | 30.42 ± 1.5        | 26.37 ± 1.6 | 35.62 ± 2.1 |
| Coefficient of variation    | 0.025              | 0.053       | 0.075       |

The value of TPC obtained by sonotrode was compared with the same values of the extract obtained by the conventional extraction. The recovery of phenolic compounds by the proposed extraction using sonotrode was 60% higher than obtained with the conventional one (**Figure 2.4**).

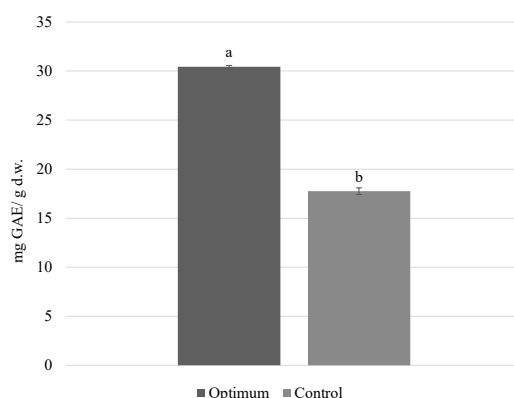


**Figure 2.4.** Comparison of phenolic content in orange by-products extracts obtained by sonotrode and conventional extraction. Different letters reported statistically significant differences.

Victor et al. [267] and Lagha-Benamrouche et al. [268] reached similar results than the reported in this study but using higher times of extraction. Other authors optimized the phenolic extraction from the orange peel by other methods as high voltage electrical discharges [262], microwave-assisted extraction [269], and pressurized liquid extraction [60], but they noticed lower yields compared to our results (**Table 2.4**). The ultrasound-assisted extraction of phenolic compounds from orange peel using ultrasonic bath has been performed by several authors using ethanol [3,28,29] or acetone [269], obtaining extraction times quite similar to our optimum one. In addition, some pre-treatments have been performed by other authors in orange by-products for enhancing the extraction of phenolic compounds. On the one hand, Shahram et al. [270] improved the usual ultrasonic bath extraction combining it with an enzymatic process. However, the TPC seemed to be lower than the previous studies without enzymes. Overall, it required longer times

because of the lower pH. In addition, pectinases are vegetal cells, and the ultrasonic waves can damage them, making them to lose their potential effect. In spite of the fact that they did not achieve a high yield, it could be had into account for future studies making slightly modifications. On the other hand, Luengo et al. [169] studied for the first time the use of pulsed electric fields as pretreatment, obtaining an increment of 23% in the yield in front of the samples directly submitted to pressing extraction. Another interesting pre-treatment was the one carried out in orange peel by El-Kantar et al. [271], who achieved an improvement of extraction about 47% using infrared radiation previously to the conventional water extraction. Although the final results obtained were not satisfactory, these two pre-treatments also ought to be had into account for future studies combining with this optimized sonotrode-assisted extraction.

**Table 2.4.** Previous research about other technologies used for extracting phenolic compounds from the orange by-products with the conditions used and the total phenolic compounds (TPC) obtained.

| Technology Used                                                                  | Conditions                                                                         | TPC (mg GAE/g d.w.) | Ref.  |
|----------------------------------------------------------------------------------|------------------------------------------------------------------------------------|---------------------|-------|
| Solvent extraction                                                               | Water, 60 °C, 12 h                                                                 | 6.89                | [272] |
|                                                                                  | Methanol, 55 °C, 3 h                                                               | 28.00               | [267] |
|                                                                                  | Methanol/water 80:20, 20 °C, 22 h                                                  | 25.60               | [268] |
|                                                                                  | Methanol/sample 20:1 (v/w), 25 °C, 72 h                                            | 18.50               | [273] |
|                                                                                  | Acetone/sample 20:1 (v/w), 25 °C, 72 h                                             | 18.00               | [273] |
|                                                                                  | Acetone 50%/sample 50:1 (v/w), 60 °C, 2 h                                          | 10.21               | [269] |
| High voltage electrical                                                          |                                                                                    |                     |       |
| discharges-assisted extraction                                                   | Water/sample 20:10 (v/w), 50 °C, 200 kJ/kg 0,5 Hz                                  | 7.00                | [262] |
| Ultrasound-assisted extraction (ultrasonic bath)                                 | Ethanol 40%/sample 80:1 (v/w), 40 °C, 30 min, 150 W                                | 2.33                | [274] |
|                                                                                  | Ethanol 50%/sample 57:1 (v/w), 30 °C, 15 min                                       | 5.50                | [60]  |
|                                                                                  | Ethanol 50%/sample 10:1 (v/w), 30 min, 400 W                                       | 1.05                | [275] |
|                                                                                  | Acetone 75.79%/sample 50:1 (v/w), 27 °C, 8.33 min, 65.94% amplitude                | 10.35               | [269] |
| Microwave-assisted extraction                                                    | Acetone 51%/sample 25:1 (v/w), 122 s, 500 W                                        | 12.20               | [269] |
| Pressurized liquid extraction                                                    | Ethanol 50%/sample 47:1 (v/w), 10 MPa, 65 °C, 40 min                               | 10.30               | [60]  |
| Ultrasound-assisted extraction (ultrasonic bath) combined with enzymatic process | Ethanol/sample 10:1 (v/w), 25 °C, 120 min, 500 W, with pectinase 0.50% w/w at pH 4 | 0.88                | [270] |

| Technology Used                                                        | Conditions                                 | TPC (mg GAE/g d.w.) | Ref.  |
|------------------------------------------------------------------------|--------------------------------------------|---------------------|-------|
| Pressing extraction<br>with pulsed electric<br>fields as pre-treatment | 7 kV/cm PEF and 5 bars, 30 min             | 0.35                | [169] |
| Solvent extraction<br>with infrared as pre-<br>treatment               | Ethanol 50%/sample 8:1 (v/w), 50 °C, 1.5 h | 1.5                 | [271] |

Bringing into account the antioxidant assays, the optimal results obtained were  $26.37 \pm 1.6$  and  $35.62 \pm 2.1$  mg TE/g d.w. for the DPPH and ABTS, respectively. DPPH technique has been mostly used in bibliography in this kind of matrix. Comparing results, the optimal value obtained for DPPH is higher than those obtained by Hernández-Carranza et al. [272] (8.94 mg TE/g d.w.) and Barrales et al. [60] (4.30 mg TE/g d.w.). However, Lachos-Pérez et al. [276] got an optimal result slightly higher (26.78 mg TE/g d.w.) but with a time of extraction of 2 h, much higher than the optimized value obtained in this study.

### 3.4. Analytical Parameters of the Method

Five calibration curves were made in order to quantify the phenolic compounds identified in the orange by-product. The curves were made of vanillic acid, chlorogenic acid, ferulic acid, quercetin, and rutin. In **Table 2.5** are contained the standards used, the calibration ranges and curves, the regression coefficients, and the limits of detection (LOD) and of quantification (LOQ).

**Table 2.5.** Standard analytes used for elaborating the calibration curves with the range used, equations,  $R^2$ , LOD, and LOQ of each compound.

| Standards        | Calibration Ranges<br>( $\mu\text{g/mL}$ ) | Calibration Curves<br>( $\mu\text{g/mL}$ ) | $R^2$  | LOD<br>( $\mu\text{g/mL}$ ) | LOQ<br>( $\mu\text{g/mL}$ ) |
|------------------|--------------------------------------------|--------------------------------------------|--------|-----------------------------|-----------------------------|
| Vanillic acid    | 3.7–236.67                                 | $y = 21.069x + 197.15$                     | 0.9979 | 0.47                        | 1.57                        |
| Chlorogenic acid | 3.85–246.67                                | $y = 58.665x - 289.54$                     | 0.9984 | 0.17                        | 0.56                        |
| Ferulic acid     | 3.54–226.67                                | $y = 37.071x + 155.61$                     | 0.9983 | 0.27                        | 0.89                        |
| Quercetin        | 3.54–226.67                                | $y = 154.26x + 1309.1$                     | 0.9988 | 0.06                        | 0.21                        |
| Rutin            | 3.44–220                                   | $y = 239.6x + 690.3$                       | 0.9954 | 0.04                        | 0.14                        |

The calibration curves were elaborated by using the peak areas of each standard measured by HPLC at different concentrations. Calibration ranges were determined previously according to the LOQ values. The regression coefficients were  $>0.99$  in all cases which means that all

calibration curves had good linearity. LOD ranged between 0.04 and 0.47  $\mu\text{g/mL}$ , and LOQ between 0.14 and 1.57  $\mu\text{g/mL}$ .

### 3.5. Identification of Phenolic Compounds by HPLC-ESI-TOF-MS

The optimal conditions of extractions were applied to extract the phenolic compounds from fresh and dried orange by-products in order to evaluate the effect of the drying step. The extracts were characterized by HPLC-MS and a total of seventy-four compounds were identified; forty-four were flavonoids, fourteen phenolic acids, fourteen terpenoids, and two other polar compounds.

All the identified compounds are described in **Table 2.6** with their retention time, molecular formula, experimental and calculated  $m/z$ , score, and error (ppm). For ensuring the mass accuracy, the tolerances chosen had a score higher than 85% and error lower than 5 ppm. To identify the compounds the generated molecular formula and some in source fragments were checked and studied comparing also with different databases such as PUBCHEM, Phenol-Explorer and literature.

**Table 2.6.** Compounds identified by HPLC-ESI-TOF-MS in the optimal extract of orange peel.

| No. | Compound                                    | Retention Time (Min) | Molecular Formula                               | $m/z$ Experimental | $m/z$ Calculated | Score | Error (ppm) |
|-----|---------------------------------------------|----------------------|-------------------------------------------------|--------------------|------------------|-------|-------------|
| 1   | Caffeoylglycolic acid methyl ester isomer a | 3.608                | C <sub>12</sub> H <sub>12</sub> O <sub>6</sub>  | 251.055            | 251.0556         | 86.53 | -2.4        |
| 2   | Norbergenin                                 | 3.806                | C <sub>13</sub> H <sub>14</sub> O <sub>9</sub>  | 313.0563           | 313.056          | 94.08 | 1           |
| 3   | Citric acid                                 | 4.278                | C <sub>6</sub> H <sub>8</sub> O <sub>7</sub>    | 191.0198           | 191.0192         | 94.52 | 3.1         |
| 4   | Cynaroside A                                | 4.774                | C <sub>21</sub> H <sub>32</sub> O <sub>10</sub> | 443.191            | 443.1917         | 99.27 | -1.6        |
| 5   | 2-(E)-O-Feruloyl-D-galactaric acid isomer a | 4.898                | C <sub>16</sub> H <sub>18</sub> O <sub>11</sub> | 385.0769           | 385.0771         | 99.77 | -0.5        |
| 6   | 2-(E)-O-Feruloyl-D-galactaric acid isomer b | 5.113                | C <sub>16</sub> H <sub>18</sub> O <sub>11</sub> | 385.0771           | 385.0771         | 95.25 | 0           |
| 7   | Caffeic acid 3-O-glucuronide                | 5.308                | C <sub>15</sub> H <sub>16</sub> O <sub>10</sub> | 355.0667           | 355.0665         | 99.92 | 0.6         |
| 8   | Citroside                                   | 5.669                | C <sub>19</sub> H <sub>30</sub> O <sub>8</sub>  | 385.1856           | 385.1862         | 87.14 | -1.6        |
| 9   | Ferulic acid O-glucoside                    | 5.854                | C <sub>16</sub> H <sub>20</sub> O <sub>9</sub>  | 355.1024           | 355.1029         | 97.78 | -1.4        |
| 10  | 2-(E)-O-Feruloyl-D-galactaric acid isomer c | 6.007                | C <sub>16</sub> H <sub>18</sub> O <sub>11</sub> | 385.0768           | 385.0771         | 96.52 | -0.8        |
| 11  | Feruloyl isocitric acid isomer a            | 6.131                | C <sub>16</sub> H <sub>16</sub> O <sub>10</sub> | 367.0662           | 367.0665         | 99.75 | -0.8        |
| 12  | Sinapic acid O-glucoside                    | 6.206                | C <sub>17</sub> H <sub>22</sub> O <sub>10</sub> | 385.1133           | 385.1136         | 99.70 | -0.5        |
| 13  | Caffeoylglycolic acid methyl ester isomer b | 6.268                | C <sub>12</sub> H <sub>12</sub> O <sub>6</sub>  | 251.0549           | 251.0556         | 88.53 | -2.8        |
| 14  | Caffeoylmalic acid isomer a                 | 6.45                 | C <sub>13</sub> H <sub>12</sub> O <sub>8</sub>  | 295.0446           | 295.0454         | 98.46 | -2.7        |
| 15  | Sinapinic acid-O-glucuronide                | 6.545                | C <sub>17</sub> H <sub>20</sub> O <sub>11</sub> | 399.0921           | 399.0927         | 89.58 | -1.5        |
| 16  | Feruloyl isocitric acid isomer b            | 6.661                | C <sub>16</sub> H <sub>16</sub> O <sub>10</sub> | 367.0675           | 367.0665         | 90.04 | 2.7         |
| 17  | Rutin isomer a                              | 6.785                | C <sub>27</sub> H <sub>30</sub> O <sub>16</sub> | 609.1467           | 609.1456         | 94.51 | 1.8         |
| 18  | Dihydroisohamnetin 7-rutinoside             | 7.194                | C <sub>28</sub> H <sub>34</sub> O <sub>16</sub> | 625.1765           | 625.1769         | 87.24 | -0.6        |
| 19  | Apigenin-di-C-hexoside (Vicenin-2) isomer a | 7.376                | C <sub>27</sub> H <sub>30</sub> O <sub>15</sub> | 593.1506           | 593.1506         | 99.24 | 0           |
| 20  | Prunin                                      | 7.596                | C <sub>21</sub> H <sub>22</sub> O <sub>10</sub> | 433.1132           | 433.1135         | 100.0 | -0.7        |
| 21  | Isorhamnetin-3-O-rutinoside isomer a        | 7.753                | C <sub>28</sub> H <sub>32</sub> O <sub>16</sub> | 623.1607           | 623.1612         | 96.55 | -0.8        |
| 22  | Isorhamnetin-3-O-rutinoside isomer b        | 7.914                | C <sub>28</sub> H <sub>32</sub> O <sub>16</sub> | 623.1605           | 623.1612         | 95.79 | -1.1        |
| 23  | Caffeoylmalic acid isomer b                 | 8.241                | C <sub>13</sub> H <sub>12</sub> O <sub>8</sub>  | 295.0428           | 295.0454         | 88.92 | -8.8        |
| 24  | Luteolin-C-hexoside-C-pentoside isomer a    | 8.432                | C <sub>26</sub> H <sub>28</sub> O <sub>15</sub> | 579.134            | 579.135          | 86.57 | -1.7        |

| No. | Compound                                                                                                                                                                                     | Retention<br>Time (Min) | Molecular<br>Formula                            | m/z<br>Experimental | m/z<br>Calculated | Score | Error<br>(ppm) |
|-----|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------|-------------------------------------------------|---------------------|-------------------|-------|----------------|
| 25  | Alpha-Glucosyl Hesperidin                                                                                                                                                                    | 8.481                   | C <sub>34</sub> H <sub>44</sub> O <sub>20</sub> | 771.2352            | 771.2348          | 92.05 | 0.5            |
| 26  | Luteolin-C-hexoside-C-pentoside isomer b                                                                                                                                                     | 8.572                   | C <sub>26</sub> H <sub>28</sub> O <sub>15</sub> | 579.1367            | 579.135           | 91.83 | 2.9            |
| 27  | Quercitrin                                                                                                                                                                                   | 8.676                   | C <sub>21</sub> H <sub>20</sub> O <sub>11</sub> | 447.0934            | 447.0927          | 99.92 | 1.6            |
| 28  | Eriocitrin                                                                                                                                                                                   | 8.8                     | C <sub>27</sub> H <sub>32</sub> O <sub>15</sub> | 595.1662            | 595.1663          | 88.90 | -0.2           |
| 29  | Limonin 17-β-D-glucopyranoside                                                                                                                                                               | 8.899                   | C <sub>32</sub> H <sub>42</sub> O <sub>14</sub> | 649.2497            | 649.2496          | 99.94 | 0.2            |
| 30  | Vitexin-O-pentoside isomer a                                                                                                                                                                 | 9.135                   | C <sub>26</sub> H <sub>28</sub> O <sub>14</sub> | 563.1407            | 563.1401          | 99.96 | 1.1            |
| 31  | Apigenin-di-C-hexoside (Vicenin-2) isomer b                                                                                                                                                  | 9.213                   | C <sub>27</sub> H <sub>30</sub> O <sub>15</sub> | 593.1500            | 593.1506          | 96.70 | -1             |
| 32  | Quercetin-O-dihexoside                                                                                                                                                                       | 9.234                   | C <sub>27</sub> H <sub>30</sub> O <sub>17</sub> | 625.1411            | 625.1405          | 98.61 | 1              |
| 33  | Vitexin-O-pentoside isomer b                                                                                                                                                                 | 9.511                   | C <sub>26</sub> H <sub>28</sub> O <sub>14</sub> | 563.14              | 563.1401          | 100.0 | -0.2           |
| 34  | Naringin hydrate                                                                                                                                                                             | 9.615                   | C <sub>27</sub> H <sub>34</sub> O <sub>15</sub> | 597.1816            | 597.1819          | 99.99 | -0.5           |
| 35  | Apigenin-di-C-hexoside (Vicenin-2) isomer c                                                                                                                                                  | 9.697                   | C <sub>27</sub> H <sub>30</sub> O <sub>15</sub> | 593.1506            | 593.1506          | 99.13 | 0              |
| 36  | Apigenin-di-C-hexoside (Vicenin-2) isomer d                                                                                                                                                  | 9.847                   | C <sub>27</sub> H <sub>30</sub> O <sub>15</sub> | 593.1505            | 593.1506          | 98.19 | -0.2           |
| 37  | Narirutin                                                                                                                                                                                    | 10.012                  | C <sub>27</sub> H <sub>32</sub> O <sub>14</sub> | 579.1696            | 579.1714          | 99.80 | 0.3            |
| 38  | Unknown flavonoid                                                                                                                                                                            | 10.074                  | C <sub>26</sub> H <sub>26</sub> O <sub>14</sub> | 561.1246            | 561.1244          | 99.56 | 0.4            |
| 39  | 6-keto-7-β-deacetylномilol 17-O-β-D-glucopyranoside                                                                                                                                          | 10.219                  | C <sub>32</sub> H <sub>44</sub> O <sub>15</sub> | 667.2588            | 667.2602          | 88.13 | -2.1           |
| 40  | Rutin isomer b                                                                                                                                                                               | 10.318                  | C <sub>27</sub> H <sub>30</sub> O <sub>16</sub> | 609.1458            | 609.1456          | 99.99 | 0.3            |
| 41  | Deacetylномilinic acid 17-β-D-glucoside                                                                                                                                                      | 10.359                  | C <sub>32</sub> H <sub>46</sub> O <sub>15</sub> | 669.2781            | 669.2758          | 98.18 | 3.4            |
| 42  | Hesperidin                                                                                                                                                                                   | 10.45                   | C <sub>28</sub> H <sub>34</sub> O <sub>15</sub> | 609.1812            | 609.1819          | 99.98 | -1.1           |
| 43  | Kaempferol 3-O-[3'',6''-di-O-(E)-cinnamoyl]-β-D-glucopyranoside                                                                                                                              | 10.624                  | C <sub>39</sub> H <sub>32</sub> O <sub>13</sub> | 707.1788            | 707.1765          | 98.71 | 3.3            |
| 44  | Isoobacunic acid 17-β-D-glucoside                                                                                                                                                            | 10.827                  | C <sub>32</sub> H <sub>44</sub> O <sub>14</sub> | 651.2661            | 651.2653          | 98.74 | 1.2            |
| 45  | Kaempferol 3-[2''-glucosyl-6''-acetyl-galactoside] 7-glucoside isomer a                                                                                                                      | 10.951                  | C <sub>35</sub> H <sub>42</sub> O <sub>22</sub> | 813.2104            | 813.2089          | 99.92 | 1.8            |
| 46  | Kaempferol 3-[2''-glucosyl-6''-acetyl-galactoside] 7-glucoside isomer b                                                                                                                      | 11.063                  | C <sub>35</sub> H <sub>42</sub> O <sub>22</sub> | 813.2089            | 813.2089          | 97.06 | 0.2            |
| 47  | Apigenin-di-C-hexoside (Vicenin-2) isomer e                                                                                                                                                  | 11.208                  | C <sub>27</sub> H <sub>30</sub> O <sub>15</sub> | 593.1504            | 593.1506          | 99.92 | -0.3           |
| 48  | Isorhamnetin-3-O-rutinoside isomer c                                                                                                                                                         | 11.336                  | C <sub>28</sub> H <sub>32</sub> O <sub>16</sub> | 623.1614            | 623.1612          | 99.20 | 0.3            |
| 49  | Kaempferol-dihexosyl acetate                                                                                                                                                                 | 11.613                  | C <sub>29</sub> H <sub>32</sub> O <sub>17</sub> | 651.1577            | 651.1571          | 86.35 | 2.5            |
| 50  | Nomilin 17-O-β-D-glucopyranoside                                                                                                                                                             | 11.973                  | C <sub>34</sub> H <sub>46</sub> O <sub>15</sub> | 693.2766            | 693.2758          | 99.99 | 1.2            |
| 51  | Neohesperidin                                                                                                                                                                                | 12.184                  | C <sub>28</sub> H <sub>34</sub> O <sub>15</sub> | 609.1824            | 609.1819          | 99.90 | 0.8            |
| 52  | Didymin                                                                                                                                                                                      | 12.41                   | C <sub>28</sub> H <sub>34</sub> O <sub>14</sub> | 593.1885            | 593.187           | 91.36 | 2.5            |
| 53  | Nomilinic acid 17-β-D-glucoside                                                                                                                                                              | 12.602                  | C <sub>34</sub> H <sub>48</sub> O <sub>16</sub> | 711.2878            | 711.2864          | 97.47 | 2              |
| 54  | Apigenin 7-O-neohesperidoside                                                                                                                                                                | 12.693                  | C <sub>27</sub> H <sub>30</sub> O <sub>14</sub> | 577.1564            | 577.1557          | 99.80 | 1.2            |
| 55  | Obacunone 17-β-D-glucoside                                                                                                                                                                   | 12.742                  | C <sub>32</sub> H <sub>42</sub> O <sub>13</sub> | 633.2574            | 633.2547          | 87.20 | 4.3            |
| 56  | Naringin 6''-malonate                                                                                                                                                                        | 13.094                  | C <sub>30</sub> H <sub>34</sub> O <sub>17</sub> | 665.1697            | 665.1718          | 90.41 | -3.2           |
| 57  | Kaempferol 3-O-(6''-O-acetyl) glucoside-7-O-rhamnoside                                                                                                                                       | 13.309                  | C <sub>29</sub> H <sub>32</sub> O <sub>16</sub> | 635.1617            | 635.1612          | 99.28 | 0.8            |
| 58  | Kaempferol 3-apiosyl-(1->4)-rhamnoside-7-rhamnoside                                                                                                                                          | 13.338                  | C <sub>32</sub> H <sub>38</sub> O <sub>18</sub> | 709.1987            | 709.198           | 91.26 | 1              |
| 59  | Quercetin-3-O-rutinoside-7-O-Glucoside                                                                                                                                                       | 13.814                  | C <sub>33</sub> H <sub>40</sub> O <sub>21</sub> | 771.2137            | 771.1984          | 89.33 | 1.8            |
| 60  | Demethylnobiletin                                                                                                                                                                            | 13.888                  | C <sub>20</sub> H <sub>20</sub> O <sub>8</sub>  | 387.1079            | 387.108           | 92.26 | -0.3           |
| 61  | Methyl 2-[(2S,4R,5S,6R)-4-acetyloxy-6-(acetyloxymethyl)-5-[(2R,4R,5S,6R)-4,5-diacetyloxy-6-(acetyloxymethyl) oxan-2-yl]oxyoxan-2-yl]oxy-3,4,5-trihydroxy-6-oxobenzo[7]annulene-8-carboxylate | 14.132                  | C <sub>35</sub> H <sub>40</sub> O <sub>20</sub> | 779.2047            | 779.2035          | 99.98 | 1.5            |

| No. | Compound                                                            | Retention Time (Min) | Molecular Formula                               | $m/z$ Experimental | $m/z$ Calculated | Score | Error (ppm) |
|-----|---------------------------------------------------------------------|----------------------|-------------------------------------------------|--------------------|------------------|-------|-------------|
| 62  | Naringenin                                                          | 14.178               | C <sub>15</sub> H <sub>12</sub> O <sub>5</sub>  | 271.0603           | 271.0606         | 95.40 | -1.1        |
| 63  | Kaempferol 3-O-sinapoyl-caffeoyl-sophoroside 7-O-glucoside isomer a | 14.339               | C <sub>53</sub> H <sub>56</sub> O <sub>28</sub> | 1139.2892          | 1139.2885        | 95.98 | 1.1         |
| 64  | Kaempferol 3-O-sinapoyl-caffeoyl-sophoroside 7-O-glucoside isomer b | 14.476               | C <sub>53</sub> H <sub>56</sub> O <sub>28</sub> | 1139.2933          | 1139.288         | 88.40 | 4.7         |
| 65  | Deacetylnomilin acid                                                | 14.526               | C <sub>26</sub> H <sub>34</sub> O <sub>9</sub>  | 489.2131           | 489.2125         | 90.73 | 1.2         |
| 66  | Limonol                                                             | 14.559               | C <sub>26</sub> H <sub>32</sub> O <sub>8</sub>  | 471.2025           | 471.2019         | 96.48 | 1.3         |
| 67  | Kaempferol 3-O-feruloyl-caffeoyl-sophoroside 7-O-glucoside          | 14.786               | C <sub>52</sub> H <sub>54</sub> O <sub>27</sub> | 1109.2789          | 1109.2774        | 90.94 | 1.4         |
| 68  | Epilimonin                                                          | 15.043               | C <sub>26</sub> H <sub>30</sub> O <sub>8</sub>  | 469.1859           | 469.1862         | 99.98 | -0.6        |
| 69  | Limonin                                                             | 15.708               | C <sub>26</sub> H <sub>30</sub> O <sub>8</sub>  | 469.1849           | 469.1862         | 99.28 | -2.8        |
| 70  | Nomilinic acid                                                      | 15.8                 | C <sub>28</sub> H <sub>36</sub> O <sub>10</sub> | 531.2208           | 531.223          | 91.17 | -4.1        |
| 71  | Pectolinarigenin                                                    | 16.073               | C <sub>17</sub> H <sub>14</sub> O <sub>6</sub>  | 313.0704           | 313.0712         | 98.95 | -2.6        |
| 72  | Deoxylimonin                                                        | 16.102               | C <sub>26</sub> H <sub>30</sub> O <sub>7</sub>  | 453.1881           | 453.1913         | 85.92 | -3.1        |
| 73  | Isosakuranetin                                                      | 16.524               | C <sub>16</sub> H <sub>14</sub> O <sub>5</sub>  | 285.0754           | 285.0763         | 89.61 | -3.2        |
| 74  | 3',4'-Didemethylnobiletin                                           | 16.569               | C <sub>19</sub> H <sub>18</sub> O <sub>8</sub>  | 373.0923           | 373.0923         | 94.17 | 0           |

*Phenolic acids.* Thirteen hydroxycinnamic acids and one hydroxybenzoic acid have been found.

Five caffeic acid derivates (peaks 1, 7, 13, 14, and 23) have been detected in this study. Two of them, at  $m/z$  251 (peaks 1 and 3), have been tentatively identified as caffeoylglycolic acid methyl esters isomers a and b. This compound was first reported in leaves of *Parthenocissus tricuspidate* by Saleem et al. [277]. Another two derivatives at  $m/z$  295 are named as caffeoylmalic acids isomers corresponding to peaks 14 and 23 described previously in plants by Hahn et al. [278], being the major source the lettuce. Moreover, the last derivate of caffeic acid that correspond to the compound found at peak 7 has been assigned to caffeic acid 3-*O*-glucuronide according to Tang et al. [279], who found the compound in hops extracts. To our knowledge, there are not previous references of these compounds found in citrus neither in orange matrixes; however, caffeic acid is a well-known compound found in orange peel by some authors [280,281].

Peaks 5, 6, and 10, with molecular formula C<sub>16</sub>H<sub>18</sub>O<sub>11</sub>, were identified as 2-(*E*)-*O*-feruloyl-*D*-galactaric acids isomers a, b and c first identified in orange peel extracts by Risch et al. [282].

Derivatives of ferulic and sinapic acids have been identified in the peaks 9 and 12, tentatively named as ferulic acid *O*-glucoside and sinapic acid *O*-glucoside, respectively. It seems to be in concordance with Lu et al. [283] who identified in orange pulp a mixture of ferulic acid and sinapic acid glucosides. In addition, both acids have been previously described in orange peel by other authors [269], but not these glucoside derivatives.

At time 6.545 (peak 15) another hydroxycinnamic acid has been tentatively identified as a sinapinic acid derivate according with Anagnostopoulou et al. [284] and Bocco et al. [285] namely as sinapinic acid-*O*-glucuronide described in orange peels.

The compounds found at peaks 11 and 16 with molecular formula  $C_{16}H_{16}O_{10}$ , have been identified as feruloyl isocitric acid isomers, described previously in plants by Masike et al. [286], and never described before in orange peel extracts. However, isocitric acid has been previously found in citrus peel matrices [287]. Furthermore, the ratio citric/isocitric acids usually is used for quality control in the orange juice; in fact, a ratio higher than 130 usually suggests that the beverage has been correcting by adding citric acid [288].

Finally, a hydroxybenzoic acid has been identified at time 3.806 min (peak 2). According with Nazir et al. [289] it was tentatively named as norbergenin. They identified this compound from *Bergenia stracheyi*, but there are no references in citrus matrices.

*Flavonoids.* As a majoritarian group, forty-four flavonoids have been identified in the orange peel.

Peaks 28, 37, 42, 51, 52 and 62 were assigned to flavanone compounds corresponding to eriocitrin, narirutin, hesperidin, neohesperidin, didymin, and naringenin, respectively. Moreover, rutin isomers have been found at  $m/z$  609 (peaks 17 and 40). These compounds are in agreement with several authors who identified them in orange peel [280,290–297].

With a molecular formula  $C_{21}H_{22}O_{10}$  (peak 20), the flavonoid prunin has been identified in concordance with Berhow et al. [298] and Castillo et al. [299], who found it for the first times as a precursor of neohesperidin and naringin in citrus matrices.

According with Oboh et al. [295] and Omoba et al. [297] the compound found at time 8.676 min (peak 27) was identified as quercitrin, found in orange's juices and peels, respectively.

Two flavonoid isomers with  $m/z$  563 and molecular formula  $C_{26}H_{28}O_{14}$  have been found (peaks 30 and 33) and they were assigned to vitexin-*O*-pentoside according to Fayek et al. [300] who found this compound in *Citrus reticulata* peel. It also could be an apigenin derivate called apigenin 6-*C*-glucoside 8-*C*-arabinoside in concordance with Lu et al. [283] who found a similar compound (apigenin-6,8-di-*C*-glucoside) in an orange variety.

At retention time 16.524 min (peak 73), and according with Gattuso et al. [301], it has been identified the flavonoid as isosakuranetin, previously found in orange juice. This compound has been found for the first time in *Citrus reticulata* peel by Fayek et al. [300], and they named it as dihydroxy-methoxyflavanone, so it is the first time this compound is identified in orange by-products.

Two nobiletin derivatives have been found at times 13.88 and 16.57 min (peaks 60 and 74). According with Goh et al. [302], they were tentatively identified as demethylnobiletin and 3',4'-didemethylnobiletin, respectively, described in the orange peel.

A hesperidin derivative, alpha-glucosyl hesperidin, previously found in the orange peel by several authors [303–307] has been identified at time 8.481 min (peak 25). In addition, a naringin derivative found at peak 34 was assigned to naringin hydrate according with Iglesias-Carres et al. [296] and Fayek et al. [300].

Isorhamnetin-3-*O*-rutinoside isomers were identified (peaks 21, 22 and 48) with  $m/z$  623. At 7.194 min (peak 18), the detected signal was identified as dihydroisorhamnetin-7-rutinoside. In addition, the compounds found at times 12.693 and 13.814 min (peaks 54 and 59), were identified as apigenin-7-*O*-neohesperidoside and quercetin-3-*O*-rutinoside-7-*O*-glucoside, respectively. The present data is in agreement with Abad-García et al. [308] who identified fifty-eight new compounds in citrus juices.

Compounds 19, 31, 35, 36, and 47 presented the same mass (593) and molecular formula ( $C_{27}H_{30}O_{15}$ ), and could be identified as apigenin-di-*C*-hexoside (Vicenin-2) according with Fayek et al. [300] who found this compound in *Citrus sinensis* peel and identified it according with Brito et al. [309], who previously detected vicenin-2 in *Citrus aurantiifolia*.

The compounds with molecular formula  $C_{26}H_{28}O_{15}$  (peaks 24 and 26) can be identified as luteolin-*C*-hexoside-*C*-pentoside isomers characterized for the first time by Fayek et al. [300] in *Citrus sinensis* peel according to Roriz et al. [310] who described the compound in *Cymbopogon citratus*.

Six kaempferol derivatives were found. The first one kaempferol derivative with a formula  $C_{39}H_{32}O_{13}$  (peak 43), was tentatively named as kaempferol 3-*O*-[3'',6''-di-*O*-(*E*)-cinnamoyl]- $\beta$ -D-glucopyranoside. The second one with  $m/z$  813 (peaks 45 and 46) was tentatively named as kaempferol-3-[2''-glucosyl-6''-acetyl-galactoside]-7-glucoside. Previously, this compound had been detected only in herbs and spices, but not quantified [311]. The third and fourth ones (peaks 57 and 58) with  $m/z$  635.1617 and 709.1987 are kaempferol 3-*O*-(6''-*O*-acetyl) glucoside-7-*O*-rhamnoside, and kaempferol 3-apiosyl-(1 $\rightarrow$ 4)-rhamnoside-7-rhamnoside, respectively. The fifth derivative is kaempferol-3-*O*-sinapoyl-caffeoyl-sophoroside-7-*O*-glucoside with molecular formula  $C_{53}H_{56}O_{28}$  (peaks 63 and 64 respectively). The sixth one was found at time 14.786 min (peak 67) and is kaempferol-3-*O*-feruloyl-caffeoyl-sophoroside-7-*O*-glucoside. All these compounds have been tentatively identified based on other kaempferol derivatives found in other plant sources [312,313]. Although kaempferol derivatives are known to be present in citrus peels, no previous references have been found, neither in orange peel.



With a mass ( $m/z$ ) 443 (peak 4), this flavonoid has never been described in the orange peel, nor in the citrus family so far. However, according to Zanzer et al. [314], this compound was characterized before in black pepper, so it has been tentatively identified as cynaroside A.

The flavonoid with formula  $C_{27}H_{30}O_{17}$  (peak 32) was tentatively identified as quercetin-*O*-dihexoside as Fayek et al. [300] found it previously in *Citrus reticulata* and *Citrus sinensis* peels.

With a mass of 651, at 11.613 min (peak 49) the flavonoid found can be named as kaempferol-dihexosyl acetate according to Fayek et al. [59], who recently found it from *Citrus aurantiifolia* peel for the first time.

At time 10.074 min and with a mass of 561 (peak 38), a controversial compound was identified. According with Fayek et al. [300], this compound could be an unknown flavonoid, as they found it in *Citrus paradisi* peel. Although no other references have been found in citrus matrixes, for the context this affirmation can be accepted.

The compound naringin 6"-malonate (peak 56) was tentatively identified according with Berhow et al. [315] who found this compound and other malonic acid ester derivatives of naringin for the first time in grapefruit. Kanés et al. [316] found this compound in some *Rutaceae* citrus species, but no references has been found related to orange peel.

With a molecular formula  $C_{17}H_{14}O_6$  (peak 71) this compound has been assigned to pectolinarigenin according with Cheriet et al. [258] who described it in several plants and fruits as oranges. However, this compound has been previously named in *Citrus sinensis* peel by Fayek et al. [300] as dihydroxy-dimethoxyflavone, previously identified in *Citrus reticulata* peel by Yang et al. [317].

*Terpenoids.* Talking about the terpenoids, 13 limonoids have been identified. They have been identified according with Gualdani et al. [318] and Shi et al. [319], who characterized limonoids from several citrus species and matrixes including orange peel. The compounds found are named limonin 17- $\beta$ -D-glucopyranoside, 6-keto-7- $\beta$ -deacetylnomilol 17-*O*- $\beta$ -D-glucopyranoside, deacetylnomilinic acid-17- $\beta$ -D-glucoside, isoobacunoic acid 17- $\beta$ -D-glucoside, nomilin 17-*O*- $\beta$ -D-glucopyranoside, nomilinic acid 17- $\beta$ -D-glucoside, obacunone 17- $\beta$ -D-glucoside, deacetylnomilin acid, limonol, epilimonin, limonin, nomilinic acid, and deoxylimonin (peaks 29, 39, 41, 44, 50, 53, 55 65, 66, 68, 69, 70 and 72, respectively). Another terpene different from those, a sesquiterpene, has been found at 5.7 min (peak 8). According to Umehara et al. [320], it was tentatively identified as citroside. They found this compound from leaves of *Citrus unshiu*.

*Other polar compounds.* Finally, other two polar compounds have been found in the orange by-product. The ion found at  $m/z$  191.0198 (peak 3) at 4.278 min, is well known as citric acid, and according to Liew et al. [321], it has been previously identified in orange peel extracts. Meanwhile

the other compound (peak 61) with the molecular formula  $C_{35}H_{40}O_{20}$  was only found match with the compound methyl 2-[(2S,4R,5S,6R)-4-acetyloxy-6-(acetyloxymethyl)-5-[(2R,4R,5S,6R)-4,5-diacetyloxy-6-(acetyloxymethyl) oxan-2-yl] oxyoxan-2-yl] oxy-3,4,5-trihydroxy-6-oxobenzo [7] annulene-8-carboxylate that no has previous references.

### 3.6. Quantification of Phenolic Compounds in Orange Peel Extracts

A total of 14 phenolic acids and 44 flavonoids were quantified in orange by-product optimal extracts (Table 2.7).

**Table 2.7.** Quantification of phenolic compounds from orange peel dry and fresh by HPLC-MS (expressed as mean  $\pm$  standard deviation  $\mu\text{g/g}$  d.w.).

| Compounds                                   | Fresh By-Product ( $\mu\text{g/g}$ d.w.) | Dry By-Product ( $\mu\text{g/g}$ d.w.) |
|---------------------------------------------|------------------------------------------|----------------------------------------|
| Sum of phenolic compounds                   | 6344.0 $\pm$ 3.6                         | 4737.6 $\pm$ 4.4                       |
| Phenolic Acids                              | 3087.5 $\pm$ 0.2                         | 2891.1 $\pm$ 2.4                       |
| Norbergenin                                 | 383.5 $\pm$ 0.5                          | 340.4 $\pm$ 0.7                        |
| Caffeoylglycolic acid methyl ester isomer a | 201.8 $\pm$ 0.1                          | 284.2 $\pm$ 0.7                        |
| Caffeoylglycolic acid methyl ester isomer b | 133.36 $\pm$ 0.04                        | 178.2 $\pm$ 0.1                        |
| Caffeic acid 3-O-glucuronide                | 218.9 $\pm$ 0.3                          | 128.6 $\pm$ 0.7                        |
| Caffeoylmalic Acid isomer a                 | 127.9 $\pm$ 0.6                          | 99.5 $\pm$ 0.4                         |
| Caffeoylmalic Acid isomer b                 | 109.5 $\pm$ 0.1                          | 77.6 $\pm$ 0.1                         |
| 2-(E)-O-Feruloyl-D-galactaric acid isomer a | 378.8 $\pm$ 0.5                          | 463.0 $\pm$ 0.04                       |
| 2-(E)-O-Feruloyl-D-galactaric acid isomer b | 575.6 $\pm$ 0.3                          | 416.3 $\pm$ 0.4                        |
| 2-(E)-O-Feruloyl-D-galactaric acid isomer c | 313.2 $\pm$ 0.2                          | 564.0 $\pm$ 0.9                        |
| Ferulic acid O-glucoside                    | 235.3 $\pm$ 0.7                          | 48.73 $\pm$ 0.1                        |
| Feruloyl Isocitric acid isomer a            | 195.2 $\pm$ 0.7                          | 170.66 $\pm$ 0.4                       |
| Feruloyl isocitric acid isomer b            | 16.9 $\pm$ 0.03                          | 11.2 $\pm$ 0.2                         |
| Sinapic acid O-glucoside                    | 183.5 $\pm$ 0.4                          | 14.9 $\pm$ 0.1                         |
| Sinapinic acid-O-glucuronide                | 14.0 $\pm$ 0.5                           | 93.7 $\pm$ 0.1                         |
| Flavonoids                                  | 3256.5 $\pm$ 3.4                         | 1846.5 $\pm$ 2.0                       |
| Cynaroside A                                | 13.1 $\pm$ 0.3                           | 6.1 $\pm$ 0.1                          |
| Rutin isomer a                              | 57.8 $\pm$ 0.3                           | 73.6 $\pm$ 0.1                         |
| Rutin isomer b                              | 22.0 $\pm$ 0.1                           | 49.8 $\pm$ 0.05                        |
| Prunin                                      | 122.5 $\pm$ 0.4                          | 35.4 $\pm$ 0.01                        |
| Quercitrin                                  | 1.2 $\pm$ 0.2                            | <LOQ                                   |
| Eriocitrin                                  | 24.6 $\pm$ 0.1                           | 17.5 $\pm$ 0.4                         |
| Narirutin                                   | 799.4 $\pm$ 0.5                          | 319.6 $\pm$ 0.4                        |
| Hesperidin                                  | 894.8 $\pm$ 0.5                          | 320.2 $\pm$ 0.5                        |
| $\alpha$ -glucosyl Hesperidin               | 23.8 $\pm$ 0.1                           | 19.0 $\pm$ 0.1                         |

| Compounds                                                             | Fresh By-Product (µg/g d.w.) | Dry By-Product (µg/g d.w.) |
|-----------------------------------------------------------------------|------------------------------|----------------------------|
| Neohesperidin                                                         | <LOQ                         | <LOQ                       |
| Didymin                                                               | 146.3 ± 0.2                  | 72.8 ± 0.2                 |
| Naringin 6"-malonate                                                  | <LOQ                         | <LOQ                       |
| Naringin hydrate                                                      | 35.1 ± 0.2                   | 51.7 ± 0.2                 |
| Dihydroisorhamnetin 7-rutinoside                                      | 8.4 ± 0.2                    | 10.1 ± 0.06                |
| Isorhamnetin-3-O-rutinoside isomer a                                  | 135.4 ± 0.6                  | 116.6 ± 0.3                |
| Isorhamnetin-3-O-rutinoside isomer b                                  | 10.6 ± 0.1                   | 8.0 ± 0.0005               |
| Isorhamnetin-3-O-rutinoside isomer c                                  | 54.6 ± 0.1                   | 48.9 ± 0.002               |
| Vitexin-O-pentoside isomer a                                          | 72.2 ± 0.5                   | 68.61 ± 0.27               |
| Vitexin-O-pentoside isomer b                                          | 111.5 ± 0.01                 | 94.6 ± 0.2                 |
| Apigenin 7-O-neohesperidoside                                         | 14.8 ± 0.1                   | 9.2 ± 0.5                  |
| Apigenin-di-C-hexoside (Vicenin-2) isomer a                           | 570.7 ± 0.9                  | 318.3 ± 0.4                |
| Apigenin-di-C-hexoside (Vicenin-2) isomer b                           | <LOQ                         | <LOQ                       |
| Apigenin-di-C-hexoside (Vicenin-2) isomer c                           | 7.7 ± 0.2                    | 13.7 ± 0.1                 |
| Apigenin-di-C-hexoside (Vicenin-2) isomer d                           | 22.3 ± 0.1                   | 16.3 ± 0.1                 |
| Apigenin-di-C-hexoside (Vicenin-2) isomer e                           | <LOQ                         | <LOQ                       |
| Luteolin-C-hexoside-C-pentoside isomer a                              | 1.2 ± 0.1                    | 0.2 ± 0.01                 |
| Luteolin-C-hexoside-C-pentoside isomer b                              | 3.8 ± 0.1                    | 2.3 ± 0.02                 |
| Kaempferol 3-[2"-glucosyl-6"-acetyl-galactoside] 7-glucoside isomer a | 24.6 ± 0.3                   | 16.0 ± 0.004               |
| Kaempferol 3-[2"-glucosyl-6"-acetyl-galactoside] 7-glucoside isomer b | 16.9 ± 0.7                   | 12.3 ± 0.04                |
| Kaempferol-dihexosyl acetate                                          | <LOQ                         | <LOQ                       |
| Kaempferol 3-O-(6"-O-acetyl) glucoside-7-O-rhamnoside                 | <LOQ                         | <LOQ                       |
| Kaempferol 3-apiosyl-(1->4)-rhamnoside-7-rhamnoside                   | <LOQ                         | <LOQ                       |
| kaempferol 3-O-[3'',6''-di-O-(E)-cinnamoyl]-β-D-glucopyranoside       | 0.7 ± 0.06                   | 0.3 ± 0.002                |
| Kaempferol 3-O-sinapoyl-caffeoyl-sophoroside 7-O-glucoside isomer a   | <LOQ                         | <LOQ                       |
| Kaempferol 3-O-sinapoyl-caffeoyl-sophoroside 7-O-glucoside isomer b   | <LOQ                         | <LOQ                       |
| Kaempferol 3-O-feruloyl-caffeoyl-sophoroside 7-O-glucoside isomer c   | <LOQ                         | <LOQ                       |
| Quercetin-O-dihexoside                                                | <LOQ                         | <LOQ                       |
| Quercetin-3-O-rutinoside-7-O-Glucoside                                | <LOQ                         | <LOQ                       |
| Demethylnobiletin                                                     | n.d.                         | <LOQ                       |
| 3',4'-Didemethylnobiletin                                             | <LOQ                         | <LOQ                       |

| Compounds         | Fresh By-Product (µg/g d.w.) | Dry By-Product (µg/g d.w.) |
|-------------------|------------------------------|----------------------------|
| Naringenin        | 11.5 ± 0.02                  | 9.4 ± 0.009                |
| Pectolinarigenin  | <LOQ                         | <LOQ                       |
| Isosakuranetin    | n.d.                         | <LOQ                       |
| Unknown flavonoid | 60.3 ± 0.1                   | 125.8 ± 0.5                |

n.d.: non-detected; LOQ: Limit of quantification.

Phenolic acids represented the 61% of the total phenolic compounds found in the dry orange by-product being 48% of those found in the fresh by-product. The most concentrated phenolic acid in fresh and dry by-products were 2-(E)-*O*-feruloyl-D-galactaric acid isomers b and c, accounting for 18.6 and 19.5%, respectively. 2-(E)-*O*-feruloyl-D-galactaric acid isomers were approximately, 40–50% of the phenolic acids present in orange by-products. After those compounds, norbergenin accounted for 12% of the phenolic acids in both by-products. The major caffeic acid derivatives were caffeoylglycolic acid methyl ester isomers a and b, accounting for the 18% and 16% in the fresh and dry by-products, respectively.

The total amount of flavonoids found in the dry and fresh samples represented the 39% and 52% of the total phenolic compounds, respectively. The two most concentrated flavonoids, both, in the fresh and dry by-products were hesperidin and narirutin. However, in the fresh sample, they account for 45% of the present flavonoids, whereas in the dry one they were the 35%. A study which optimized the extraction of these two compounds from the orange peel by subcritical water extraction, obtained an extract in which both compounds were approximately 21% of the total amount of flavonoids, a smaller value than the obtained with this optimized technique [276]. Other studies agree with us about the major compound found, hesperidin. Using an ultrasonic bath, Khan et al. found hesperidin as major compound getting an amount of 146.73 µg/g d.w. [274]. Barrales et al. obtained an extract mainly composed by hesperidin (160 µg/g d.w.) and narirutin (120 µg/g d.w.) [60] with ultrasound bath technology. Despite not being the principal aim of this study, hesperidin and narirutin content was higher than the described in previous studies that used ultrasound technology applied to orange by-products.

The fourth most abundant flavonoid was apigenin-di-*C*-hexoside (Vicenin-2) isomer a, being around 17.5% of the flavonoids in both samples. For the dry by-product, the fifth and sixth most abundant compounds were an unknown flavonoid (6.8%) and vitexin-*O*-pentoside isomer b (5.1%), whereas for the fresh one they are didymin (4.5%), and prunin (3.8%).

Taking into account the antioxidant activity, the fresh orange by-product has been found to have a higher activity than the dry one, with values of 66.39 and 74.32 mg TE/g d.w. for the DPPH and ABTS assays, respectively (**Figure 2.5**).

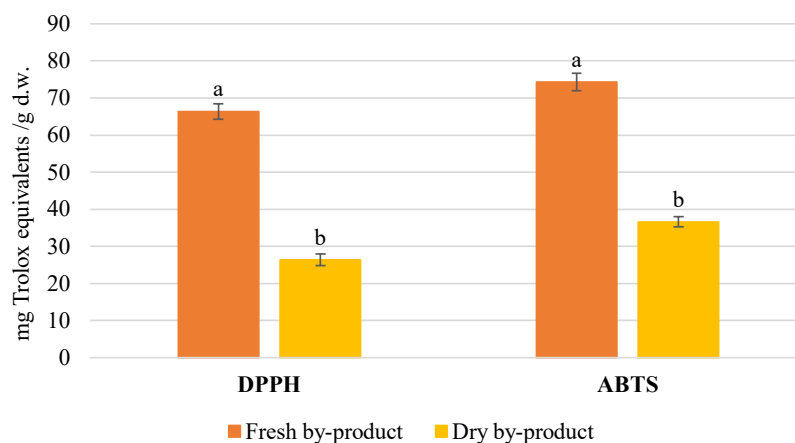


**Figure 2.5.** Comparison of antioxidant activities by two methods (DPPH and ABTS) in fresh and dry orange by-products extracts expressed in mg TE/g d.w. Different letters reported statistically significant differences.

This can be explained because the fresh by-product has a higher amount of total phenolic compounds than the dry one. The loss is around 25% of the total phenolic compounds which means the loss of 43% of the flavonoids, 6% of the phenolic acids and consequently the loss of 50–60% of the antioxidant activity.

#### 4. Conclusions

An extraction based on ultrasound-assisted extraction by sonotrode has been optimized for obtaining the higher amounts of phenolic compounds with high antioxidant activity from the orange by-products. The results of this study have shown that applying the sonotrode extraction is possible to increase 60% more the phenolic recovery compared to a conventional extraction procedure as maceration. Moreover, sonotrode extraction has demonstrated to be faster than the conventional ones. In addition, the extracts have been exhaustively characterized identifying a total of 74 compounds in orange peels being 32 of them tentatively identified in orange by-products for the first time. The distribution of the phenolic compounds is mainly phenolic acids and flavonoids, especially flavonoid glycosides. The effect of drying was also evaluated and, as expected, it caused the loss of 25% of phenolic compounds; perhaps, an optimization of the drying conditions should be carried out in order to limit the phenolic degradation. Finally, the sonotrode ultrasound technology could be scaled-up to pilot and industrial scale in order to obtain orange by-products extracts that could be used as functional ingredients for food, feed, and cosmeceutical scopes. However, to corroborate the potential (bio)-activity of the extracts, further in vitro and in vivo assays should be done.

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### **CHAPTER 3. Combined effect of pulse electric field and probe ultrasound technologies for obtaining phenolic compounds from orange by-product**

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## Combined effect of pulse electric field and probe ultrasound technologies for obtaining phenolic compounds from orange by-product

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### Abstract

Orange peel by-product is a known source of bioactive compounds with several health benefits. This study focuses on optimizing the conditions for pulsed electric field (PEF) technology as a pretreatment to obtain extracts rich in phenolic compounds from the orange peel. A Box-Behnken design of 15 experiments with 3 independent factors (field energy, number of pulses and pulse width) was performed. The phenolic content was extracted by ultrasound technology and analyzed by HPLC-ESI-TOF-MS. Optimum conditions were 1.4 kV/cm and 30 pulses of 110  $\mu$ s achieving a recovery of 40.8 mg/g dry weight of phenolic compounds, 27.5% higher than obtained without PEF. Besides, hesperidin and narirutin content increased by 29.4 and 38.9%, respectively. For the antioxidant assays also significative increments ( $\geq 53\%$ ) were achieved. The combination of optimized PEF pre-treatment with ultrasound extraction has let to obtain orange peel enriched extracts in polyphenols with higher antioxidant activity.

**Keywords:** phenolic compounds; citrus by-products; non-thermal technologies; pulsed electric fields; ultrasound technology

## 1. Introduction

Pulse electric field (PEF) is a novel non-thermal green technology, which is considered to be promising for the food industry. It consists of applying an electric field to a food matrix for a short period of time (nanoseconds, microseconds or milliseconds) resulting in the electroporation of the cells [156]. The transmembrane potential created by the electric field causes the formation of hydrophilic pores in the cell membranes. These pores increase in size and number depending on the length of the treatment or the height of the electric field strength. Into this context, PEF can cause reversible or irreversible electroporation. The latter leads to the leakage of intracellular compounds [158]. Its efficiency depends on the process parameters (electric field strength, number of pulses and pulse width), the food product characteristics and the processing aim [161]. Thus, an adequate knowledge of these factors and their influence on PEF performance is necessary to reach optimum conditions of PEF in food. The potential applications of PEF have been extensively investigated worldwide as a dehydration pre-treatment, for extracting valuable compounds or juice, for preserving food or for food structural modifications among others [322]. Previously, PEF has been applied to orange peel for increasing the efficiency of extracting soluble dietary fiber also improving its physicochemical properties [323]. Also, Carpentieri et al. (2022) used PEF of 5 kV/cm during 3 hours for extracting limonene from orange peels with an increase of 33% in the yield of extraction [324]. PEF was used as a pre-treatment at 1.20 kV/cm and 200–600  $\mu$ s followed by ultrasound technology by Mello et al. (2021) for improving the drying of orange peel, shortening the drying process and preserving bioactive compounds [325]. However, few research have been done using PEF for extracting polyphenols from orange wastes [149,169,326].

The combination of pulse electric field as a pre-treatment with other technologies for extracting bioactive compounds has been recently reported in other matrices such as combined with ultrasound assisted-extraction in leaves of *Aesculus carnea* (Ntourtoglou, et al., 2022) or grape stems (Ntourtoglou, et al., 2022) or combination with subcritical water extraction in *Citrus unshiu* peel [329]. Besides, some authors have used PEF for enhance the extraction of the valuable compounds hesperidin and narirutin from other citrus matrices with promising results [329]. There are no previous references about combining optimized PEF and ultrasound technology for extracting polyphenols, specially hesperidin and narirutin from orange wastes. These specific compounds have been reported to possess several bioactivities demonstrated in humans and animals health such as anti-cancer against lung cancer [77] and breast cancer [78,79], antiatherogenic and anti-inflammatory [84,90–92], antirheumatic [96], cardioprotective [85,100–102,104] and anti-diabetic [106]. In fact, ultrasound assisted extraction by probe was previously optimized in orange wastes with satisfactory results compared to other conventional extraction

procedures [74]. In addition, the use of probe instead of ultrasound bath have demonstrated to be better in terms of reducing the extracting times and the scalability to industrial level [330].

So, the aim of this study was to optimize PEF conditions (electric field strength, pulse wide and number of pulses) as a pre-treatment followed by ultrasound technology with probe in orange wastes for extracting polyphenols, focusing on hesperidin and narirutin, the orange by-product compounds that have been attributed to have the highest bioactive effect. Besides the polyphenol profile measured by HPLC-ESI-TOF-MS and the antioxidant activity measured by DPPH and ABTS were analyzed in the orange peels PEF pre-treated and in a control as reference.

## **2. Materials and Methods**

### **2.1. Reagents and samples**

Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Vanillic acid, chlorogenic acid, ferulic acid, quercetin, and rutin were acquired from Sigma-Aldrich (St. Louis, MO, USA). Other reagents were purchased from Merck KGaA (Darmstadt, Germany).

Orange peels (dried, by-product from juice company) (var. Navelina) were purchased from a local company, as the by-product obtained after juice production. The resulting by-product was composed by the albedo and flavedo.

### **2.2. Experimental design**

To optimize the conditions of the PEF pre-treatment for the orange by-product an experimental design was used to reduce the number of experiments and maintaining the accuracy of the experimental hypothesis. A Box-Behnken design composed by 15 experiments with three levels (-1, 0, +1) was conducted in triplicate. The independent variables were electric field (1.0, 1.5, 1.8 kV/cm), number of pulses (5, 25, 50) and the pulse width (15, 50, 150  $\mu$ s), and the responses like sum of flavonoids, sum of phenolic acids, sum of phenolic compounds, hesperidin and narirutin were taken into account. The dependent variables were adjusted to a second order polynomial model equation. An ANOVA assay to evaluate the adjustment of the model was performed including the regression coefficients, the *p*-values of the regressions and the lack of fit. Statistica 7.0 package (StatSoft, Tulsa, OK, USA) was used for the mathematical operations and simulations. Besides the optimum conditions were established using response surface methodology (RSM).

### 2.3. Pulse electric field treatment

PEF pre-treatments were carried out in a pilot scale system (HVP 5 PEF system, ELEA, Quakenbrück, Germany) that can reach a maximum voltage up to 20 kV. The electric field was applied to the samples in a chamber with distance between electrodes of 2 cm. For each experiment 20 g of orange peels of a size of  $1 \pm 0.2$  cm were added to 60 g of tap water ( $\sim 1$  mS/cm). Previously, it was stated that there is no statistically significant differences between samples cut into different sizes from 1 to 3 cm for PEF treatments [170]. The pulse shape (square wave bipolar pulse) was monitored online using an oscilloscope during PEF treatment. At the end of the process, the peels were filtrated and dried at 60 °C, air flow 1.6 m/s for 315 minutes according to previous research [331].

For the preliminary trials different specific energy inputs (1.5-4.3 kJ/kg), frequencies (1 and 10 Hz), and total treatment times (0-7650  $\mu$ s) were tested.

For the experimental design the frequency was fixed to 1 Hz for all the trials and the experiments were carried out with the conditions (electric field strength, pulse width and number of pulses) plotted in **Table 3.3** with specific energy input in the range of 0.1-1.9 kJ/kg.

### 2.4. Ultrasound assisted extraction

Orange peels PEF pre-treated, or non-PEF pre-treated (control) (0.5 g) were extracted with a 45/55 ethanol/water solution (v/v) (100 mL) by a sonotrode (UP400St ultrasonic processor, Hielscher, Germany) with an amplitude of 90% for 35 minutes, in duplicate. Those parameters were previously optimized [74]. After the extraction, the samples were centrifuged at 3500 rpm for 15 min and the supernatant was stored at  $-18$  °C until the analyses.

### 2.5. Determination of phenolic compounds by HPLC-ESI-TOF-MS

The analyses of the orange by-products were carried out in duplicate on an ACQUITY Ultra Performance LC system (Waters Corporation, Milford, MA, USA) coupled to an electrospray ionization (ESI) source operating in the negative mode and a time-of-flight (TOF) mass detector (MS) (Waters Corporation, Milford, MA, USA). The compounds of interest were separated on an ACQUITY UPLC BEH Shield RP18 column ( $1.7 \mu\text{m}$ ,  $2.1 \text{ mm} \times 100 \text{ mm}$ ; Waters Corporation, Milford, MA, USA) at 40 °C using a gradient previously stated by Verni et al. [245] using water containing 1% acetic acid as mobile phase A and acetonitrile as mobile phase B. Five calibration curves were made in order to quantify the phenolic compounds identified in the orange by-product. Vanillic acid, chlorogenic acid, ferulic acid, quercetin, and rutin were used as standards for quantification and their ranges, calibration curves, coefficients of determination ( $R^2$ ), and limits of detection (LOD) and quantification (LOQ) are shown in **Table 3.1**.

**Table 3.1.** Standard analytes used for elaborating the calibration curves with the range used, equations,  $R^2$ , LOD, and LOQ of each compound.

| Standards        | Calibration Ranges ( $\mu\text{g/mL}$ ) | Calibration Curves ( $\mu\text{g/mL}$ ) | $R^2$  | LOD ( $\mu\text{g/mL}$ ) | LOQ ( $\mu\text{g/mL}$ ) |
|------------------|-----------------------------------------|-----------------------------------------|--------|--------------------------|--------------------------|
| Vanillic acid    | 3.7–236.67                              | $y = 6.1459x + 122.9$                   | 0.9976 | 0.56                     | 1.57                     |
| Chlorogenic acid | 3.85–246.67                             | $y = 63.853x + 135.09$                  | 0.9978 | 0.13                     | 0.56                     |
| Ferulic acid     | 3.54–226.67                             | $y = 12.38x + 92.068$                   | 0.9980 | 0.31                     | 0.89                     |
| Quercetin        | 3.54–226.67                             | $y = 98.589x + 287.32$                  | 0.9957 | 0.12                     | 0.21                     |
| Rutin            | 3.44–220                                | $y = 19.632x + 403.42$                  | 0.9959 | 0.09                     | 0.14                     |

All the compounds detected in the orange by-product samples were identified according to previous work [74] and presented in **Table 3.2**.

**Table 3.2.** Compounds identified by HPLC-ESI-TOF-MS in the optimal extract of orange peel.

| Retention time (min) | m/z experimental | m/z calculated | Error (ppm) | Score (%) | Molecular formula                         | Compound name                                                         |
|----------------------|------------------|----------------|-------------|-----------|-------------------------------------------|-----------------------------------------------------------------------|
| 1.71                 | 313.0563         | 313.056        | 1           | 94.08     | $\text{C}_{13}\text{H}_{14}\text{O}_9$    | Norbergenin                                                           |
| 2.44                 | 385.0769         | 385.0771       | -0.5        | 99.77     | $\text{C}_{16}\text{H}_{18}\text{O}_{11}$ | 2-(E)-O-Feruloyl-D-galactaric acid isomer a                           |
| 3.03                 | 385.0771         | 385.0771       | 0           | 95.25     | $\text{C}_{16}\text{H}_{18}\text{O}_{11}$ | 2-(E)-O-Feruloyl-D-galactaric acid isomer b                           |
| 3.84                 | 385.0768         | 385.0771       | -0.8        | 96.52     | $\text{C}_{16}\text{H}_{18}\text{O}_{11}$ | 2-(E)-O-Feruloyl-D-galactaric acid isomer c                           |
| 4.51                 | 367.0662         | 367.0665       | -0.8        | 99.75     | $\text{C}_{16}\text{H}_{16}\text{O}_{10}$ | Feruloyl isocitric acid isomer a                                      |
| 4.86                 | 385.0753         | 385.0771       | -4.7        | 95.42     | $\text{C}_{16}\text{H}_{18}\text{O}_{11}$ | 2-(E)-O-Feruloyl-D-galactaric acid isomer d                           |
| 5.07                 | 367.0675         | 367.0665       | 2.7         | 90.04     | $\text{C}_{16}\text{H}_{16}\text{O}_{10}$ | Feruloyl isocitric acid isomer b                                      |
| 5.43                 | 399.0921         | 399.0927       | -1.5        | 89.58     | $\text{C}_{17}\text{H}_{20}\text{O}_{11}$ | Sinapic acid-O-glucuronide                                            |
| 5.70                 | 609.1467         | 609.1456       | 1.8         | 94.51     | $\text{C}_{27}\text{H}_{30}\text{O}_{16}$ | Rutin isomer a                                                        |
| 6.20                 | 367.0656         | 367.0665       | -2.5        | 99.1      | $\text{C}_{16}\text{H}_{16}\text{O}_{10}$ | Feruloyl isocitric acid isomer c                                      |
| 6.26                 | 593.1506         | 593.1506       | 0           | 99.24     | $\text{C}_{27}\text{H}_{30}\text{O}_{15}$ | Apigenin-di-C-hexoside isomer a                                       |
| 6.59                 | 433.1132         | 433.1135       | -0.7        | 100       | $\text{C}_{21}\text{H}_{22}\text{O}_{10}$ | Prunin                                                                |
| 6.64                 | 623.1607         | 623.1612       | -0.8        | 96.55     | $\text{C}_{28}\text{H}_{32}\text{O}_{16}$ | Isorhamnetin-3-O-rutinoside a                                         |
| 6.80                 | 623.1605         | 623.1612       | -1.1        | 95.79     | $\text{C}_{28}\text{H}_{32}\text{O}_{16}$ | Isorhamnetin-3-O-rutinoside b                                         |
| 7.07                 | 367.0659         | 367.0665       | -1.6        | 99.81     | $\text{C}_{16}\text{H}_{16}\text{O}_{10}$ | Feruloyl isocitric acid isomer d                                      |
| 7.14                 | 447.0934         | 447.0927       | 1.6         | 99.92     | $\text{C}_{21}\text{H}_{20}\text{O}_{11}$ | Quercitrin isomer a                                                   |
| 7.15                 | 577.1564         | 577.1557       | 1.2         | 99.8      | $\text{C}_{27}\text{H}_{30}\text{O}_{14}$ | Apigenin 7-O-neohesperidose isomer a                                  |
| 7.19                 | 579.134          | 579.135        | -1.7        | 86.57     | $\text{C}_{26}\text{H}_{28}\text{O}_{15}$ | Luteolin-C-hexoside-C-pentoside isomer a                              |
| 7.35                 | 579.1367         | 579.135        | 2.9         | 91.83     | $\text{C}_{26}\text{H}_{28}\text{O}_{15}$ | Luteolin-C-hexoside-C-pentoside isomer b                              |
| 7.38                 | 447.0917         | 447.0927       | -2.2        | 99.55     | $\text{C}_{21}\text{H}_{20}\text{O}_{11}$ | Quercitrin isomer b                                                   |
| 7.49                 | 771.2352         | 771.2348       | 0.5         | 92.05     | $\text{C}_{34}\text{H}_{44}\text{O}_{20}$ | Alpha-glucosyl hesperidin                                             |
| 7.51                 | 367.0661         | 367.0665       | -1.1        | 92.26     | $\text{C}_{16}\text{H}_{16}\text{O}_{10}$ | Feruloyl isocitric acid isomer e                                      |
| 7.57                 | 813.2112         | 813.2089       | 2.8         | 99.9      | $\text{C}_{35}\text{H}_{42}\text{O}_{22}$ | Kaempferol-3-[2"-glucosyl-6"-acetyl-galactoside] 7-glucoside isomer a |

| Retention time (min) | m/z experimental | m/z calculated | Error (ppm) | Score (%) | Molecular formula                               | Compound name                                                         |
|----------------------|------------------|----------------|-------------|-----------|-------------------------------------------------|-----------------------------------------------------------------------|
| 7.67                 | 595.1662         | 595.1663       | -0.2        | 88.90     | C <sub>27</sub> H <sub>32</sub> O <sub>15</sub> | Eriocitrin                                                            |
| 7.79                 | 431.0975         | 431.0978       | -0.7        | 92.32     | C <sub>21</sub> H <sub>20</sub> O <sub>10</sub> | Vitexin                                                               |
| 7.85                 | 577.1567         | 577.1557       | 1.7         | 94.43     | C <sub>27</sub> H <sub>30</sub> O <sub>14</sub> | Apigenin 7-O-neohesperidose isomer b                                  |
| 7.89                 | 563.1407         | 563.1401       | 1.1         | 99.96     | C <sub>26</sub> H <sub>28</sub> O <sub>14</sub> | Vitexin-O-pentoside isomer a                                          |
| 7.95                 | 367.0651         | 367.0665       | -3.8        | 99.97     | C <sub>16</sub> H <sub>16</sub> O <sub>10</sub> | Feruloyl isocitric acid isomer f                                      |
| 8.30                 | 563.14           | 563.1401       | -0.2        | 100       | C <sub>26</sub> H <sub>28</sub> O <sub>14</sub> | Vitexin-O-pentoside isomer b                                          |
| 8.38                 | 597.1816         | 597.1819       | -0.5        | 99.99     | C <sub>27</sub> H <sub>34</sub> O <sub>15</sub> | Naringin hydrate                                                      |
| 8.47                 | 461.1081         | 461.1084       | -0.7        | 92.02     | C <sub>22</sub> H <sub>22</sub> O <sub>11</sub> | Diosmetin 7-O-beta-D-glucopuranoside                                  |
| 8.51                 | 593.15           | 593.1506       | -1          | 96.7      | C <sub>27</sub> H <sub>30</sub> O <sub>15</sub> | Apigenin-di-C-hexoside isomer b                                       |
| 8.64                 | 639.1575         | 639.1561       | 2.2         | 95.42     | C <sub>28</sub> H <sub>32</sub> O <sub>17</sub> | Isorhamnetin 3,4'-diglucoside                                         |
| 8.65                 | 593.1506         | 593.1506       | 0           | 99.13     | C <sub>27</sub> H <sub>30</sub> O <sub>15</sub> | Apigenin-di-C-hexoside isomer c                                       |
| 8.80                 | 387.1079         | 387.108        | -0.3        | 92.26     | C <sub>20</sub> H <sub>20</sub> O <sub>8</sub>  | Demethylnobiletin                                                     |
| 8.90                 | 579.1696         | 579.1714       | 0.3         | 99.80     | C <sub>27</sub> H <sub>32</sub> O <sub>14</sub> | Narirutin                                                             |
| 9.04                 | 491.1182         | 491.119        | -1.6        | 90.74     | C <sub>23</sub> H <sub>24</sub> O <sub>12</sub> | Dimethoxyapigenin 7-glucoside                                         |
| 9.15                 | 609.1458         | 609.1456       | 0.3         | 99.99     | C <sub>27</sub> H <sub>30</sub> O <sub>16</sub> | Rutin isomer b                                                        |
| 9.32                 | 463.0859         | 463.0877       | -3.9        | 97.12     | C <sub>21</sub> H <sub>20</sub> O <sub>12</sub> | Isoquercitrin                                                         |
| 9.40                 | 609.1812         | 609.1819       | -1.1        | 99.98     | C <sub>28</sub> H <sub>34</sub> O <sub>15</sub> | Hesperidin                                                            |
| 9.69                 | 443.191          | 443.1917       | -1.6        | 99.27     | C <sub>21</sub> H <sub>32</sub> O <sub>10</sub> | Cynaroside A                                                          |
| 9.84                 | 813.2099         | 813.2089       | 1.2         | 97.32     | C <sub>35</sub> H <sub>42</sub> O <sub>22</sub> | Kaempferol-3-[2"-glucosyl-6"-acetyl-galactoside] 7-glucoside isomer b |
| 9.98                 | 813.2108         | 813.2089       | 2.3         | 96.21     | C <sub>35</sub> H <sub>42</sub> O <sub>22</sub> | Kaempferol-3-[2"-glucosyl-6"-acetyl-galactoside] 7-glucoside isomer c |
| 10.09                | 593.1505         | 593.1506       | -0.2        | 98.19     | C <sub>27</sub> H <sub>30</sub> O <sub>15</sub> | Apigenin-di-C-hexoside isomer d                                       |
| 10.18                | 607.1296         | 607.1299       | -0.5        | 91.75     | C <sub>27</sub> H <sub>28</sub> O <sub>16</sub> | Kaempferol 3-rhamnoside 7-galacturonide                               |
| 10.23                | 651.1561         | 651.1561       | 0           | 95.91     | C <sub>29</sub> H <sub>32</sub> O <sub>17</sub> | Kaempferol-dihexosyl acetate isomer a                                 |
| 10.55                | 651.1566         | 651.1561       | 0.8         | 91.21     | C <sub>29</sub> H <sub>32</sub> O <sub>17</sub> | Kaempferol-dihexosyl acetate isomer b                                 |
| 10.69                | 681.167          | 681.1667       | 0.4         | 99.52     | C <sub>30</sub> H <sub>34</sub> O <sub>18</sub> | Isorhamnetin 3-(6'''-acetylglucosyl)(1->6)-galactoside isomer a       |
| 10.77                | 651.1561         | 651.1561       | 0           | 99.21     | C <sub>29</sub> H <sub>32</sub> O <sub>17</sub> | Kaempferol-dihexosyl acetate isomer c                                 |
| 11.30                | 593.1885         | 593.187        | 2.5         | 91.36     | C <sub>28</sub> H <sub>34</sub> O <sub>14</sub> | Didymin                                                               |
| 11.35                | 621.1458         | 621.1456       | 0.3         | 98.81     | C <sub>28</sub> H <sub>30</sub> O <sub>16</sub> | Kaempferol 3-(3"-acetyl-alpha-L-arabinopyranosyl)-(1->6)-glucoside    |
| 11.49                | 681.1663         | 681.1667       | -0.6        | 94.55     | C <sub>30</sub> H <sub>34</sub> O <sub>18</sub> | Isorhamnetin 3-(6'''-acetylglucosyl)(1->6)-galactoside isomer b       |
| 12.02                | 665.1697         | 665.1718       | -3.2        | 90.41     | C <sub>30</sub> H <sub>34</sub> O <sub>17</sub> | Naringin 6"-malonate isomer a                                         |
| 12.27                | 665.171          | 665.1718       | -1.2        | 95.16     | C <sub>30</sub> H <sub>34</sub> O <sub>17</sub> | Naringin 6"-malonate isomer b                                         |
| 12.39                | 709.1987         | 709.198        | 1           | 91.26     | C <sub>32</sub> H <sub>38</sub> O <sub>18</sub> | Kaempferol 3-apiosyl-(1->4)-rhamnoside-7-rhamnoside isomer a          |
| 12.65                | 695.1829         | 695.1823       | 0.9         | 91.53     | C <sub>31</sub> H <sub>36</sub> O <sub>18</sub> | Kaempferol 3-apiosyl-(1->2)-alpha-L-arabinofuranoside-7-rhamnoside    |
| 13.48                | 709.1987         | 709.198        | 1           | 96.96     | C <sub>32</sub> H <sub>38</sub> O <sub>18</sub> | Kaempferol 3-apiosyl-(1->4)-rhamnoside-7-rhamnoside isomer b          |

| Retention time (min) | m/z experimental | m/z calculated | Error (ppm) | Score (%) | Molecular formula                              | Compound name             |
|----------------------|------------------|----------------|-------------|-----------|------------------------------------------------|---------------------------|
| 15.42                | 285.0754         | 285.0763       | -3.2        | 91.61     | C <sub>16</sub> H <sub>14</sub> O <sub>5</sub> | Isosakuranetin            |
| 15.57                | 373.0923         | 373.0923       | 0           | 94.17     | C <sub>19</sub> H <sub>18</sub> O <sub>8</sub> | 3',4'-Didemethylnobiletin |

Besides, a representative chromatogram is shown in **Figure 3.1**.

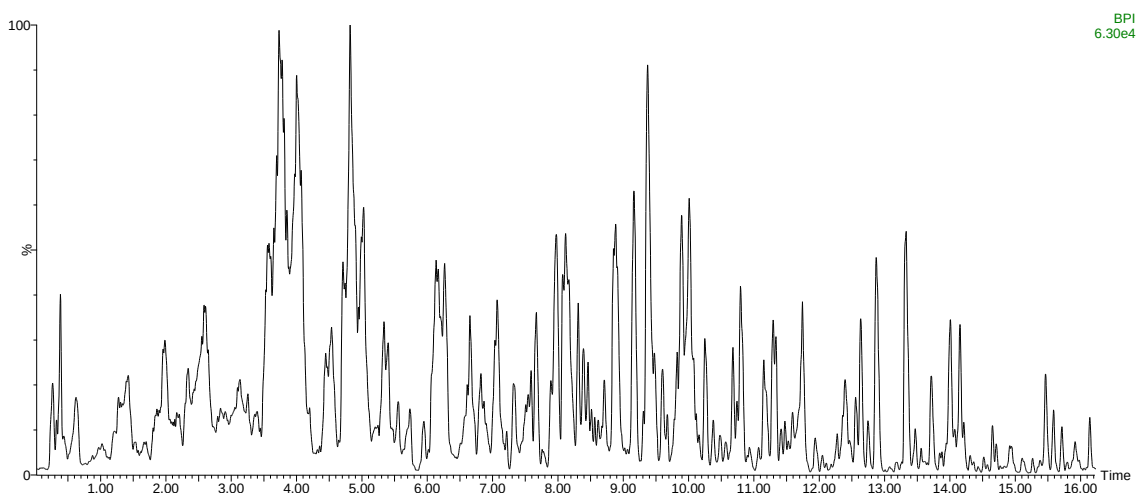


**Figure 3.1.** Base peak chromatogram of the optimal PEF-treated orange peel.

The data were elaborated using MassLynx 4.1 software (Waters Corporation, Milford, MA, USA). Each extract was injected two times.

## 2.6. Antioxidant assays

The antioxidant activity of the orange peel extracts was determined by DPPH and ABTS methods, as described in previous research [74,331]. In all the assays, the calibration curve was made of the standard Trolox, and the results were expressed in mg of Trolox equivalents (TE)/g d.w. Analyses were performed in triplicate and the measurements were carried out using an UV–visible spectrophotometer (Spectrophotometer 300 Array, UV–Vis, single beam, Shimadzu, Duisburg, Germany).

## 3. Results and Discussion

They were carried out different steps. Firstly, they were established the parameter and ranges of the experimental model, and secondly the model was performed, fitted mathematically and the optimal conditions were established.

### 3.1. Establishment of the parameters of the model

Previous experiments were performed in order to establish the range of parameters for the model using as responses the total phenolic content (TPC) measured by Folin-Ciocalteu method and the antioxidant activity by DPPH technique.

In preliminary trials different specific energy (kJ/kg) were tested, and the results are presented in **Figure 3.2**.

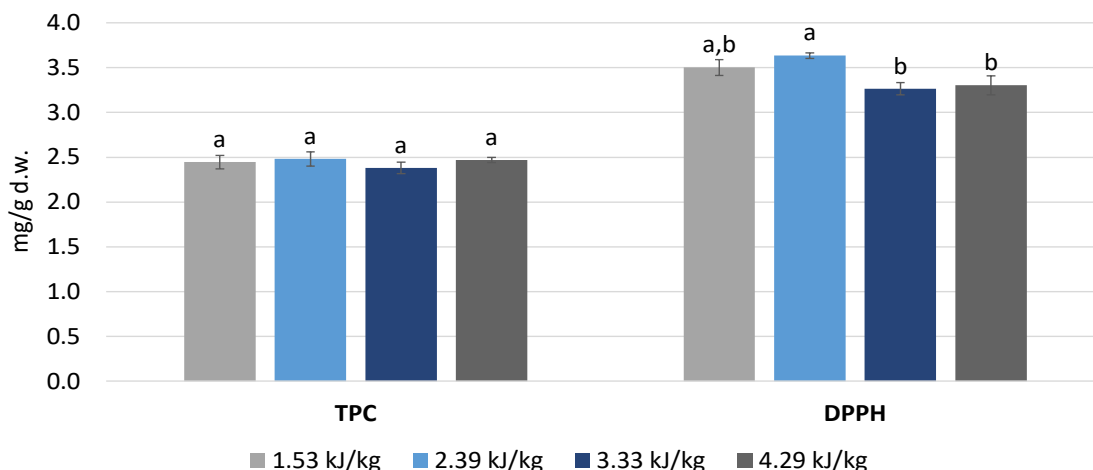


**Figure 3.2.** Preliminary specific energy input test of PEF technology in orange peels. TPC: Total phenolic compounds. Results for TPC in mg gallic acid equivalents, and for DPPH are expressed as mg of Trolox equivalents.

As can be seen from the results for TPC no significative changes were observed in the range tested (1.5-4.3 kJ/kg). In the case of the antioxidant activity, a significative reduction could be shown when using a specific energy of 3.3 kJ/kg. Based on that, it was concluded that probably low specific energies could lead to better results reducing the energy consumption of the process. Thus, the voltage was adjusted to generate electric field strength in the range between 1 and 1.8 kV/cm to obtain specific energy inputs from 0.1 to 1.9 kJ/kg.

Then the specific energy input was fixed to 0.4 kJ/kg and the pulse width to 18  $\mu$ s and another preliminary test were performed. **Figure 3.3** shows the results for TPC and DPPH for different treatments varying the frequency and number of pulses. When the frequency is set to 1 Hz, a noticeable increase in the responses is observed with an increase in the number of pulses. Otherwise, at 10 Hz the opposite effect was observed when increasing the number of pulses from 140 to 240. So, the frequency was fixed at 1 Hz, reducing the number of cycles per second, to not submit to an extreme stress the orange peel cells, as the aim is to permeabilize the membrane, not to break the cells releasing the bioactive compounds directly to the water of treatment. The decision was made based on previous works [170]. Compared to using 10 Hz the treatment time is the same but increasing the operating time. Into this context the maximum operating time



selected was 50 s corresponding with the number of pulses, and the lower 5 seconds, also determining the range of number of pulses (5-50 pulses).

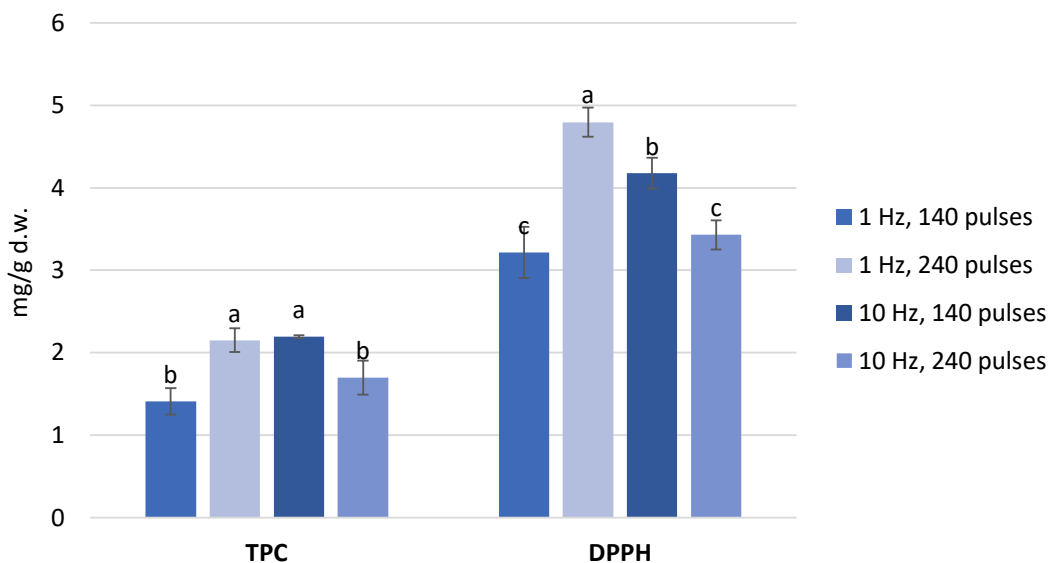


**Figure 3.3.** Preliminary frequency test of PEF technology in orange peels. TPC: Total phenolic compounds. Results for TPC in mg gallic acid equivalents, and for DPPH are expressed as mg of Trolox equivalents.

Thus, another preliminary test was performed in order to select the pulse width range for the model. In **Figure 3.4** it can be seen the results of TPC and DPPH for different total treatment times between 0 to 7650  $\mu$ s. Compared to the non-PEF-treated samples, a significant increase was observed for DPPH, while no significant changes were noted in the case of TPC when using a total treatment time of 4280  $\mu$ s. However, significant ( $p < 0.05$ ) reductions were observed at the highest tested treatment time.

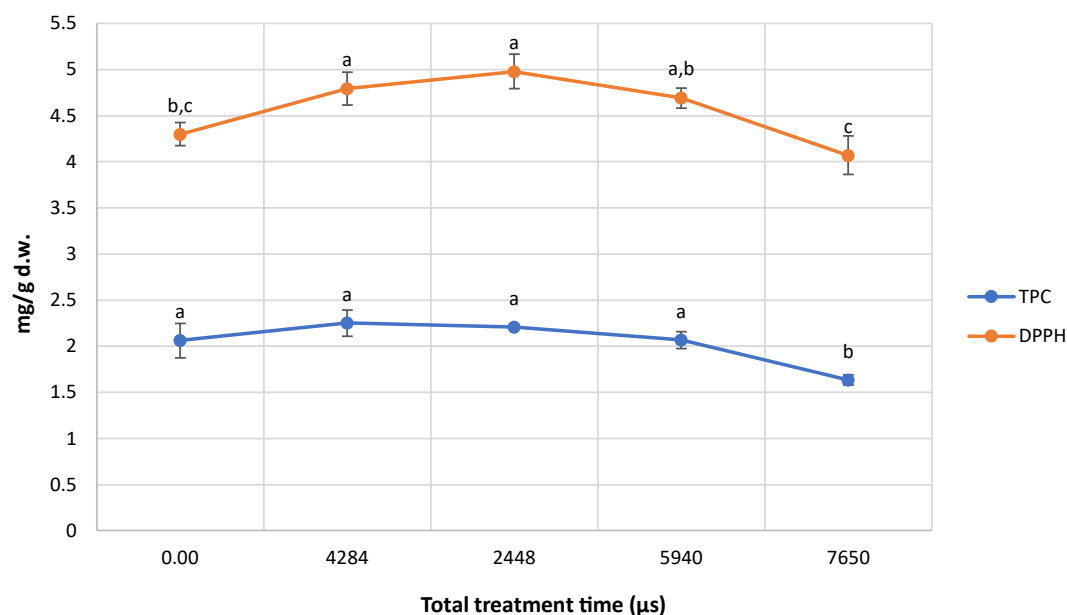


**Figure 3.4.** Preliminary total treatment time test of PEF technology in orange peels. TPC: Total phenolic compounds. Results for TPC in mg gallic acid equivalents, and for DPPH are expressed as mg of Trolox equivalents.

So, it was concluded that total treatment times higher than 7500  $\mu\text{s}$  should be avoided. A compromise between number of pulses and pulse width was done for achieving this goal, so the pulse width was established between 15 and 150  $\mu\text{s}$ , leading to a minimum treatment time of 75  $\mu\text{s}$  and maximum of 7500  $\mu\text{s}$ .

### 3.2. Model fit and optimization of PEF conditions

The established ranges of the parameters electric field strength, number of pulses and pulse width were set in a Box Behnken experimental design of 15 experiments and the obtained results are shown in **Table 3.3**.

**Table 3.3.** Box–Behnken model of the conditions of extraction for the independent factors and the experimental results obtained for the response variables evaluated expressed as mean  $\pm$  standard deviation.

| N° | Electric field (kV/cm) | Number of pulses | Pulse width ( $\mu$ s) | Sum of flavonoids ( $\mu$ g/g d.w.) | Sum of phenolic acids ( $\mu$ g/g d.w.) | Sum of phenolic compounds ( $\mu$ g/g d.w.) | Hesperidin ( $\mu$ g/g d.w.) | Narirutin ( $\mu$ g/g d.w.) |
|----|------------------------|------------------|------------------------|-------------------------------------|-----------------------------------------|---------------------------------------------|------------------------------|-----------------------------|
| 1  | 1                      | 5                | 50                     | 3294.7 $\pm$ 17.5                   | 20019.1 $\pm$ 29.3                      | 23313.8 $\pm$ 46.8                          | 356.9 $\pm$ 1.3              | 378.9 $\pm$ 1.4             |
| 2  | 1.8                    | 5                | 50                     | 3657.4 $\pm$ 18.0                   | 15950.8 $\pm$ 21.8                      | 19608.2 $\pm$ 39.8                          | 552.2 $\pm$ 1.9              | 397.7 $\pm$ 1.4             |
| 3  | 1                      | 50               | 50                     | 6235.2 $\pm$ 26.0                   | 22921.0 $\pm$ 32.6                      | 29156.2 $\pm$ 58.6                          | 925.3 $\pm$ 3.0              | 874.1 $\pm$ 2.9             |
| 4  | 1.8                    | 50               | 50                     | 4704.5 $\pm$ 21.1                   | 20101.9 $\pm$ 27.4                      | 24806.4 $\pm$ 48.5                          | 628.6 $\pm$ 2.1              | 549.6 $\pm$ 1.9             |
| 5  | 1                      | 25               | 15                     | 4728.5 $\pm$ 21.8                   | 20992.7 $\pm$ 30.3                      | 25721.2 $\pm$ 52.1                          | 780.1 $\pm$ 2.6              | 431.9 $\pm$ 1.6             |
| 6  | 1.8                    | 25               | 15                     | 5460.5 $\pm$ 22.9                   | 18292.6 $\pm$ 24.9                      | 23753.1 $\pm$ 47.7                          | 622.7 $\pm$ 2.0              | 629.1 $\pm$ 2.1             |
| 7  | 1                      | 25               | 150                    | 6438.7 $\pm$ 26.1                   | 23951.5 $\pm$ 33.2                      | 30390.2 $\pm$ 59.2                          | 934.3 $\pm$ 3.0              | 826.3 $\pm$ 2.7             |
| 8  | 1.8                    | 25               | 150                    | 6534.5 $\pm$ 26.5                   | 26318.9 $\pm$ 36.9                      | 32853.4 $\pm$ 63.4                          | 902.7 $\pm$ 2.9              | 629.1 $\pm$ 2.1             |
| 9  | 1.5                    | 5                | 15                     | 3991.0 $\pm$ 18.5                   | 20612.7 $\pm$ 27.4                      | 24603.7 $\pm$ 45.9                          | 523.6 $\pm$ 1.7              | 386.0 $\pm$ 1.4             |
| 10 | 1.5                    | 50               | 15                     | 3823.8 $\pm$ 18.3                   | 17690.0 $\pm$ 23.8                      | 21513.8 $\pm$ 42.1                          | 670.0 $\pm$ 2.2              | 460.9 $\pm$ 1.6             |
| 11 | 1.5                    | 5                | 150                    | 6519.1 $\pm$ 27.0                   | 26572.2 $\pm$ 38.2                      | 33091.3 $\pm$ 65.1                          | 975.2 $\pm$ 3.2              | 828.5 $\pm$ 2.7             |
| 12 | 1.5                    | 50               | 150                    | 7774.6 $\pm$ 29.5                   | 27956.4 $\pm$ 37.8                      | 35731.0 $\pm$ 67.3                          | 1042.1 $\pm$ 3.2             | 962.1 $\pm$ 3.0             |
| 13 | 1.5                    | 25               | 50                     | 7361.7 $\pm$ 24.8                   | 28725.1 $\pm$ 35.0                      | 36086.8 $\pm$ 59.7                          | 953.0 $\pm$ 2.1              | 895.1 $\pm$ 2.4             |
| 14 | 1.5                    | 25               | 50                     | 7122.9 $\pm$ 28.0                   | 29164.5 $\pm$ 39.8                      | 36287.4 $\pm$ 67.8                          | 920.0 $\pm$ 2.9              | 923.1 $\pm$ 2.9             |
| 15 | 1.5                    | 25               | 50                     | 7420.5 $\pm$ 22.8                   | 29816.9 $\pm$ 33.0                      | 37237.4 $\pm$ 55.7                          | 944.8 $\pm$ 2.0              | 940.3 $\pm$ 2.5             |

The responses results ranged 3295-7775, 15951-29817, 19608-37237, 357-1042, and 379-962  $\mu$ g/g d.w. for sum of flavonoids, sum of phenolic acids, sum of phenolic compounds, hesperidin and narirutin, respectively, all measured by HPLC-ESI-TOF-MS. The highest results of sum of flavonoids, hesperidin and narirutin were obtained in experiment number 13 where the samples were treated with 1.5 kV/cm and 50 pulses of 150  $\mu$ s. Otherwise for the sum of phenolic acids and the sum of phenolic compounds, the intermedium conditions of all the parameters (1.5 kV/cm, 25 pulses of 50  $\mu$ s) lead to highest recoveries. In contrast, the least favorable outcomes occurred when employing low number of pulses or a narrow pulse width. Therefore, to achieve a high recovery of the targeted compounds (hesperidin and narirutin) it is recommended to use intermediate to high values for both the number of pulses and pulse width, as indicated by these results.

These experimental data was adjusted to a second order polynomial equation and the regression coefficients are shown in **Table 3.4**.

**Table 3.4.** Estimated regression coefficients of the adjusted second-order polynomial equation and analysis of variance (ANOVA) of the model.

| Regression coefficients | Sum of flavonoids<br>( $\mu\text{g/g d.w.}$ ) |                | Sum of phenolic acids<br>( $\mu\text{g/g d.w.}$ ) |                | Sum of phenolic<br>compounds ( $\mu\text{g/g d.w.}$ ) |                | Hesperidin<br>( $\mu\text{g/g d.w.}$ ) |                | Narirutin<br>( $\mu\text{g/g d.w.}$ ) |                |
|-------------------------|-----------------------------------------------|----------------|---------------------------------------------------|----------------|-------------------------------------------------------|----------------|----------------------------------------|----------------|---------------------------------------|----------------|
|                         | Effect                                        | <i>p</i> value | Effect                                            | <i>p</i> value | Effect                                                | <i>p</i> value | Effect                                 | <i>p</i> value | Effect                                | <i>p</i> value |
| $\beta_0$               | 4577.7895                                     | 0.0003*        | 19174.0671                                        | 0.0002*        | 24073.7359                                            | 0.0002*        | 654.6507                               | 0.0002*        | 500.1441                              | 0.0005*        |
| Linear                  |                                               |                |                                                   |                |                                                       |                |                                        |                |                                       |                |
| $\beta_1$               | 1952.2324                                     | 0.0079*        | 7137.7144                                         | 0.0072*        | 9165.0933                                             | 0.0048*        | 243.5578                               | 0.0060*        | 216.1110                              | 0.0134*        |
| $\beta_2$               | 2751.0616                                     | 0.0067*        | 6027.0306                                         | 0.0166*        | 8880.4979                                             | 0.0097*        | 339.2033                               | 0.0052*        | 388.5117                              | 0.0070*        |
| $\beta_3$               | 978.4352                                      | 0.0414*        | 5265.7216                                         | 0.0180*        | 8879.4447                                             | 0.0025*        | 163.7251                               | 0.0182*        | 115.0107                              | 0.0607         |
| Crossed                 |                                               |                |                                                   |                |                                                       |                |                                        |                |                                       |                |
| $\beta_{12}$            | -2191.0431                                    | 0.0105*        | -3766.3866                                        | 0.0413*        | -6146.0778                                            | 0.0200*        | -354.0326                              | 0.0048*        | -324.0986                             | 0.0101*        |
| $\beta_{13}$            | 1160.0955                                     | 0.0338*        | 3931.9391                                         | 0.0356*        | 3267.5693                                             | 0.0295*        | 209.6911                               | 0.0127*        | 59.3293                               | 0.2017         |
| $\beta_{23}$            | 711.3861                                      | 0.0458*        | 2153.4630                                         | 0.0593         | 3048.8536                                             | 0.0381*        | -39.7549                               | 0.1466         | 29.3627                               | 0.3265         |
| Quadratic               |                                               |                |                                                   |                |                                                       |                |                                        |                |                                       |                |
| $\beta_{11}$            | 2340.7786                                     | 0.0036*        | 8652.5437                                         | 0.0032*        | 10294.6142                                            | 0.0026*        | 297.7794                               | 0.0026*        | 387.6758                              | 0.0027*        |
| $\beta_{22}$            | 1764.0324                                     | 0.0025*        | 4564.4974                                         | 0.0045*        | 6125.6880                                             | 0.0027*        | 207.6163                               | 0.0021*        | 199.8211                              | 0.0040*        |
| $\beta_{33}$            | 670.8312                                      | 0.0167*        | 3050.0958                                         | 0.0099*        | 3916.7614                                             | 0.0073*        | 47.7907                                | 0.0378*        | 150.2929                              | 0.0071*        |
| $R^2$                   | 0.9985                                        |                | 0.9694                                            |                | 0.9783                                                |                | 0.9888                                 |                | 0.9946                                |                |
| <i>p</i> model          | 0.0018*                                       |                | 0.0306*                                           |                | 0.0237*                                               |                | 0.0112*                                |                | 0.0051*                               |                |
| <i>p</i> lack of fit    | 0.4654                                        |                | 0.0993                                            |                | 0.0692                                                |                | 0.0874                                 |                | 0.2664                                |                |

\*significant  $p < 0.05$

All considered response variables in the model showed positive significance for the regression ( $\beta_0$ ), linear ( $\beta_1$ ,  $\beta_2$ ,  $\beta_3$ ), and quadratic ( $\beta_{11}$ ,  $\beta_{22}$ ,  $\beta_{33}$ ) terms. This indicates a significant impact of the three chosen independent factors on the modeling process.

Besides, focusing on the crossed terms, for the sum of flavonoids and the sum of phenolic compounds all the crossed terms ( $\beta_{12}$ ,  $\beta_{13}$ ,  $\beta_{23}$ ) were significative. However, for the sum of flavonoids, hesperidin and narirutin the crossed term between the number of pulses and the pulse width ( $\beta_{23}$ ) was not significant. Besides in the case of narirutin also the term  $\beta_{13}$  was found to not have significative effect.

The non-significant terms at  $p < 0.05$  were discarded, and the model was recalculated having into account only the significant terms. The adjusted  $R^2$  values are shown in **Table 3.4** and all the dependent variables revealed high determination coefficients with the independent factors (0.9694-0.9985). Besides, the validity of the model was tested by ANOVA and the regression model *p* values were significant ( $p < 0.05$ ) and the lack of fit non-significant ( $p > 0.05$ ) confirming that the model can be statistically accepted.

Optimal PEF-treatment conditions were established through studying the tri-dimensional graphs presented in **Figure 3.5** with RSM.

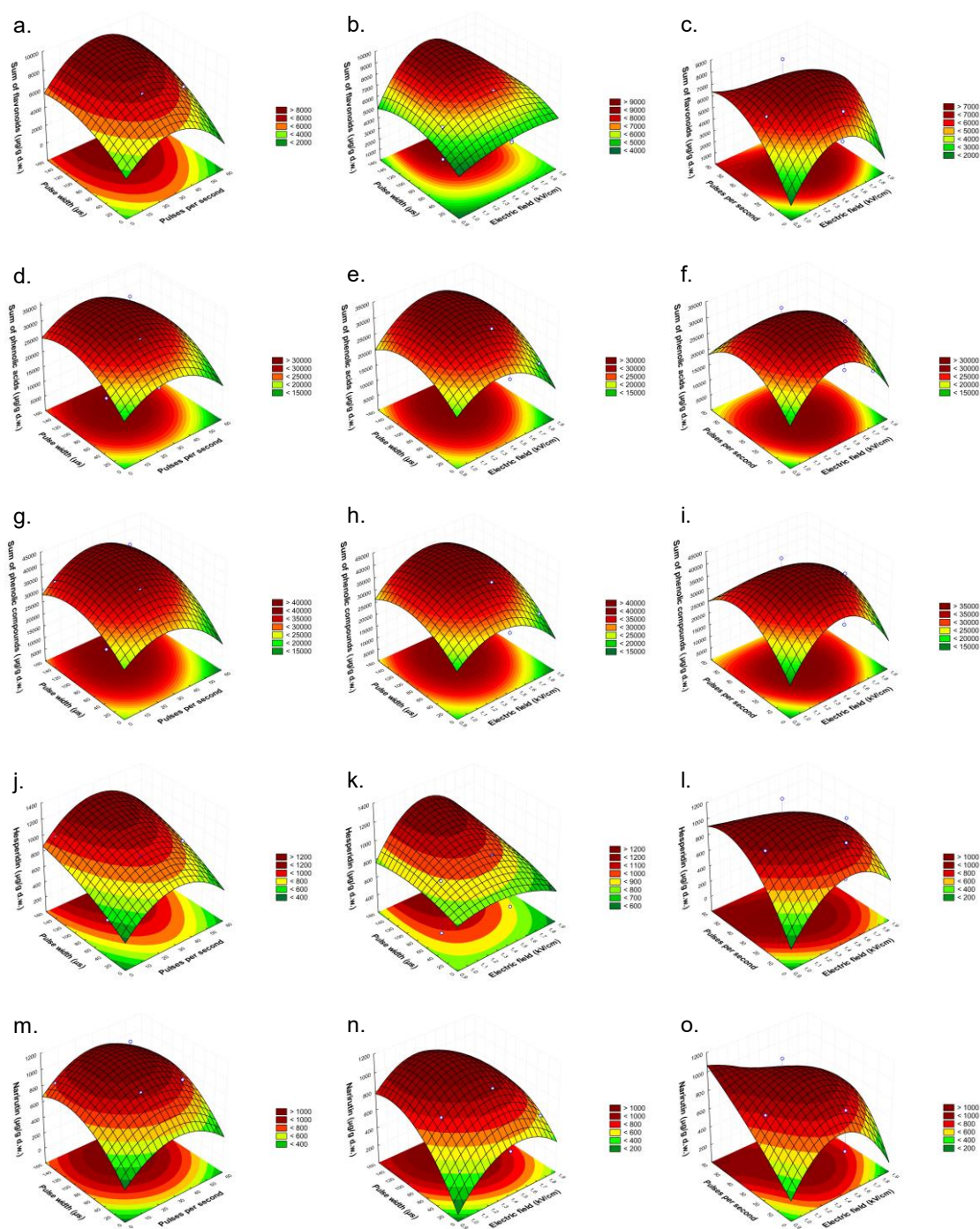


**Figure 3.5.** Response surface plots showing combined effects of process variables for the responses sum of flavonoids (a-c), sum pf phenolic acids (d-f), sum of phenolic compounds (g-i), hesperidin (j-l) and narirutin (m-o).

A compromise between all the independent factors trying to reach the maximum responses for all the dependent variables was made to evaluate the minimum values of the independent factors reducing time and energy costs. Briefly, the optimal pulse electric field conditions were an energy field strength of 1.6 kV/cm, 30 pulses and 110  $\mu$ s per pulse (**Table 3.5**).

**Table 3.5.** Optimal conditions selected by RSM, and the model predicted values with the obtained values expressed with mean  $\pm$  standard deviation.

| Parameter                         | Optimal conditions  |                       |                           |                    |                    |
|-----------------------------------|---------------------|-----------------------|---------------------------|--------------------|--------------------|
| Electric field (kV/cm)            | 1.6                 |                       |                           |                    |                    |
| Number of pulses                  | 30                  |                       |                           |                    |                    |
| Pulse width ( $\mu$ s)            | 110                 |                       |                           |                    |                    |
|                                   | Sum of flavonoids   | Sum of phenolic acids | Sum of phenolic compounds | Hesperidin         | Narirutin          |
| Predicted value ( $\mu$ g/g d.w.) | 8964.1 $\pm$ 1232.3 | 32916.1 $\pm$ 4121.1  | 41528.8 $\pm$ 4611.1      | 1162.8 $\pm$ 134.0 | 1115.5 $\pm$ 171.5 |
| Obtained value ( $\mu$ g/g d.w.)  | 8732.2 $\pm$ 32.4   | 32086.9 $\pm$ 44.3    | 40819.2 $\pm$ 76.7        | 1166.8 $\pm$ 3.5   | 1118.2 $\pm$ 3.6   |
| CV (%)                            | 1.85                | 1.80                  | 1.22                      | 0.25               | 0.17               |

CV: Coefficient of variation

The accuracy of the mathematical model was confirmed by verifying the optimal predicted values, obtaining coefficients of variation consistently lower than 5% in all cases.

Previously Athanasiadis et al. (2022) reported PEF to be a useful pre-treatment for extracting bioactive compounds from orange wastes. They applied a constant PEF treatment with a pulse width of 10  $\mu$ s, 1000 Hz and an electric field of 1.0 kV/cm during 20 minutes of total treatment time [149]. In this study, the total treatment time needed for reaching the highest phenolic content was  $3.3 \times 10^{-3}$  s, a much lower time. As reported previously the electric field needed for generating a permeabilization of the plant tissue membrane range between 0.5-9 kV/cm [332,333], thus, the energy field strength found here as optimum was in the range accordingly with the literature. Other by-products such as pomelo peel, Niu et al. (2021) obtained the best narirutin extraction by using 4 kV/cm and 30 pulses [334].

### 3.3. Comparison of PEF pre-treated and non-pre-treated orange peel

The orange peel PEF pre-treated with the optimized conditions (1.6 kV/cm, 30 pulses and 110  $\mu$ s) and extracted by ultrasound technology has been compared with the orange peel extracted by ultrasound technology without the PEF pre-treatment and all the quantified compounds are shown in **Table 3.6**.

**Table 3.6.** Quantification of phenolic compounds from orange peel pre-treated by PEF with the optimal conditions and no pre-treated (control) by HPLC-MS expressed as mean  $\pm$  standard deviation.

| Compounds    | PEF pre-treated ( $\mu$ g/g d.w.) | Control ( $\mu$ g/g d.w.) |
|--------------|-----------------------------------|---------------------------|
| Norbergenin  | 5303.3 $\pm$ 2.3                  | 4899.1 $\pm$ 2.1          |
| Cynaroside A | < LOQ                             | < LOQ                     |

| Compounds                                        | PEF pre-treated<br>(µg/g d.w.) | Control<br>(µg/g d.w.) |
|--------------------------------------------------|--------------------------------|------------------------|
| 2-(E)-O-Feruloyl-D-galactaric acid isomer a      | 2195.6 ± 3.5                   | 1914.6 ± 3.0           |
| 2-(E)-O-Feruloyl-D-galactaric acid isomer b      | 1939.0 ± 3.1                   | 1267.7 ± 2.0           |
| 2-(E)-O-Feruloyl-D-galactaric acid isomer c      | 8406.2 ± 13.0                  | 7981.7 ± 12.4          |
| 2-(E)-O-Feruloyl-D-galactaric acid isomer d      | 4385.2 ± 6.8                   | 2988.3 ± 4.7           |
| Feruloyl isocitric acid isomer a                 | 1402.2 ± 2.2                   | 949.3 ± 1.5            |
| Feruloyl isocitric acid isomer b                 | 1953.7 ± 3.1                   | 1607.2 ± 2.6           |
| Feruloyl isocitric acid isomer c                 | 1900.0 ± 3.0                   | 1621.0 ± 2.6           |
| Feruloyl isocitric acid isomer d                 | 910.0 ± 1.5                    | 617.3 ± 1.0            |
| Feruloyl isocitric acid isomer e                 | 784.1 ± 1.3                    | 366.3 ± 0.6            |
| Feruloyl isocitric acid isomer f                 | 1147.5 ± 1.8                   | 865.6 ± 1.4            |
| Sinapic acid-O-glucuronide                       | 1760.1 ± 2.8                   | 1297.6 ± 2.1           |
| Apigenin-di-C-hexoside isomer a                  | 680.5 ± 2.2                    | 408.5 ± 1.5            |
| Rutin isomer a                                   | 373.3 ± 1.4                    | 77.5 ± 0.5             |
| Rutin isomer b                                   | 743.6 ± 2.4                    | 476.4 ± 1.7            |
| Apigenin-di-C-hexoside isomer b                  | 46.6 ± 0.4                     | 29.8 ± 0.4             |
| Apigenin-di-C-hexoside isomer c                  | 1.8 ± 0.3                      | < LOQ                  |
| Apigenin-di-C-hexoside isomer d                  | 46.5 ± 0.4                     | 2.0 ± 0.3              |
| Prunin                                           | 176.0 ± 0.8                    | 102.7 ± 0.6            |
| Isorhamnetin-3-O-rutinoside a                    | 260.4 ± 1.0                    | 177.5 ± 0.8            |
| Isorhamnetin-3-O-rutinoside b                    | 159.7 ± 0.7                    | 94.4 ± 0.6             |
| Luteolin-C-hexoside-C-pentoside                  | 18.0 ± 0.3                     | < LOQ                  |
| Alpha-glucosyl hesperidin                        | 0.3 ± 0.3                      | < LOQ                  |
| Quercitrin isomer a                              | 2.1 ± 0.3                      | < LOQ                  |
| Quercitrin isomer b                              | < LOQ                          | < LOQ                  |
| Eriocitrin                                       | 225.2 ± 0.9                    | 205.9 ± 0.9            |
| Vitexin-O-pentoside isomer a                     | 115.9 ± 0.6                    | 94.1 ± 0.6             |
| Vitexin-O-pentoside isomer b                     | 189.2 ± 0.8                    | 152.6 ± 0.7            |
| Naringin hydrate                                 | 166.9 ± 0.8                    | 116.6 ± 0.6            |
| Narirutin                                        | 1118.2 ± 3.6                   | 683.1 ± 2.2            |
| Hesperidin                                       | 1166.8 ± 3.5                   | 823.1 ± 2.6            |
| Kaempferol-3-[2"-glucosyl-6"-acetyl-galactoside] | 79.0 ± 0.5                     | 46.8 ± 0.4             |
| 7-glucoside isomer a                             |                                |                        |
| Kaempferol-3-[2"-glucosyl-6"-acetyl-galactoside] | 430.9 ± 1.5                    | 401.3 ± 1.4            |
| 7-glucoside isomer b                             |                                |                        |
| Kaempferol-3-[2"-glucosyl-6"-acetyl-galactoside] | 512.6 ± 1.8                    | 475.8 ± 1.7            |
| 7-glucoside isomer c                             |                                |                        |
| Kaempferol-dihexosyl acetate isomer a            | 156.6 ± 0.7                    | 96.9 ± 0.6             |
| Kaempferol-dihexosyl acetate isomer b            | 16.5 ± 0.3                     | < LOQ                  |
| Kaempferol-dihexosyl acetate isomer c            | 466.0 ± 1.6                    | 314.4 ± 1.2            |
| Didymin                                          | 244.0 ± 1.0                    | 165.7 ± 0.8            |
| Apigenin 7-O-neohesperidose isomer a             | < LOQ                          | < LOQ                  |
| Apigenin 7-O-neohesperidose isomer b             | < LOQ                          | < LOQ                  |
| Naringin 6"-malonate isomer a                    | < LOQ                          | < LOQ                  |

| Compounds                                                          | PEF pre-treated<br>( $\mu\text{g/g d.w.}$ ) | Control<br>( $\mu\text{g/g d.w.}$ ) |
|--------------------------------------------------------------------|---------------------------------------------|-------------------------------------|
| Naringin 6"-malonate isomer b                                      | $5.9 \pm 0.3$                               | < LOQ                               |
| Kaempferol 3-apiosyl-(1->4)-rhamnoside-7-rhamnoside isomer a       | $235.9 \pm 1.0$                             | $90.1 \pm 0.5$                      |
| Demethylnobiletin                                                  | $7.3 \pm 0.3$                               | < LOQ                               |
| Isosakuranetin                                                     | $4.7 \pm 0.3$                               | < LOQ                               |
| 3',4'-Didemethylnobiletin                                          | $13.1 \pm 0.4$                              | $5.3 \pm 0.3$                       |
| Luteolin-C-hexoside-C-pentoside isomer b                           | $15.6 \pm 0.3$                              | < LOQ                               |
| Kaempferol 3-apiosyl-(1->4)-rhamnoside-7-rhamnoside isomer b       | < LOQ                                       | < LOQ                               |
| Vitexin                                                            | < LOQ                                       | < LOQ                               |
| Diosmetin 7-O-beta-D-glucopuranside                                | $151.6 \pm 0.7$                             | $93.4 \pm 0.6$                      |
| Isorhamnetin 3,4'-diglucoside                                      | $22.9 \pm 0.4$                              | $0.5 \pm 0.1$                       |
| Dimethoxyapigenin 7-glucoside                                      | < LOQ                                       | < LOQ                               |
| Isoquercitrin                                                      | $5.6 \pm 0.3$                               | < LOQ                               |
| Kaempferol 3-rhamnoside 7-galacturonide                            | < LOQ                                       | < LOQ                               |
| Isorhamnetin 3-(6"-acetylglucosyl)(1->6)-galactoside isomer a      | $143.9 \pm 0.7$                             | $105.3 \pm 0.6$                     |
| Isorhamnetin 3-(6"-acetylglucosyl)(1->6)-galactoside isomer b      | $36.1 \pm 0.4$                              | < LOQ                               |
| Kaempferol 3-apiosyl-(1->2)-alpha-L-arabinofuranoside-7-rhamnoside | $554.2 \pm 1.9$                             | $287.8 \pm 1.1$                     |
| Kaempferol 3-(3"-acetyl-alpha-L-arabinopyranosyl)-(1->6)-glucoside | $138.8 \pm 0.7$                             | $104.6 \pm 0.6$                     |
| <b>Sum of flavonoids</b>                                           | $8732.2 \pm 32.4$                           | $5631.9 \pm 23.9$                   |
| <b>Sum of phenolic acids</b>                                       | $32086.9 \pm 44.3$                          | $26375.6 \pm 36.1$                  |
| <b>Sum of phenolic compounds</b>                                   | $40819.2 \pm 76.7$                          | $32007.5 \pm 60.0$                  |

With the established PEF-pre-treatment optimal conditions an increment of 27.5% in the total phenolic compounds compared to only extracting by ultrasound technology could be achieved. Increase in the sum of flavonoids (35.5%) and phenolic acids (17.8%) were also observed. Besides, increments in the hesperidin and narirutin content of 29.4 and 38.9% were obtained, respectively. El Kantar et al. (2018) reported an enhancement of 27.3% in the polyphenol extraction from orange peels using a PEF treatment of 10 kV/cm previously to extraction. However, they did not report increase in the hesperidin content in the albedo, nor in the flavedo of the orange peels [326]. Athanasiadis et al. (2022) reported a comparison between different pre-treatments to improve the extraction of bioactive compounds from orange peels. They also concluded that the combination between PEF+US was better than using one of the technologies alone for the extraction of phenolic compounds. For other by-product, such as grape stems, pomelo peel and onion skins, similar increases were reported. According to Ntourtoglou et al.



(2022) an increase up to 35% in the polyphenol extraction with PEF as pre-treatment followed by US extraction in grape stems was found [328]. Niu et al. (2021) showed a 20% increase in naringin extraction using PEF as a pre-treatment in pomelo peel, and a 70% increase in its antioxidant activity. In onion skins an increment of 33% of the quercetin content was achieved using PEF as a pre-treatment [335]. Niu et al. (2021) increased by 20% the naringin extraction using PEF as a pre-treatment in pomelo peel, and its antioxidant activity by 70% [334].

Moreover, the antioxidant activity of the PEF pre-treated, and the control was performed by DPPH and ABTS assays (**Figure 3.6**).

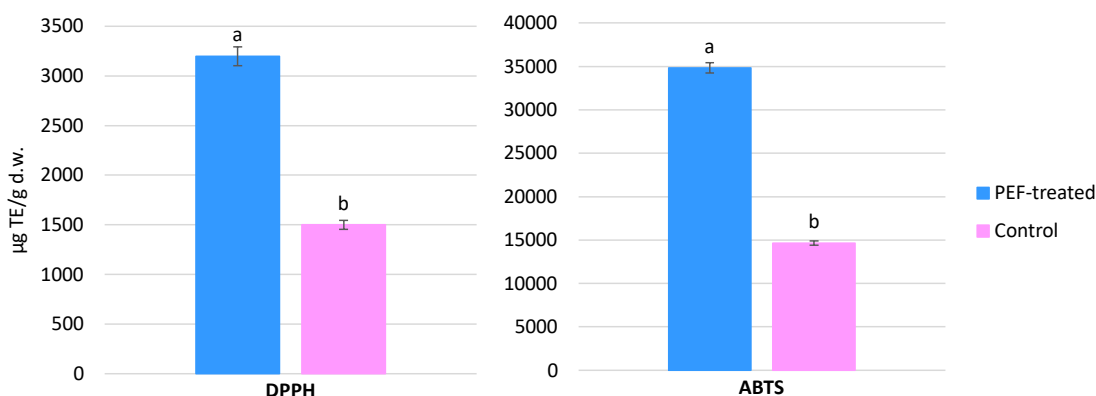


**Figure 3.6.** Antioxidant activity measured by DPPH and ABTS of the optimum PEF-treated orange peel extract and the control non PEF-treated.

Both methods had significant strong correlation with an  $r = 0.9994$ . As it can be seen from the results a significant increase of 53.1 and 57.9% for DPPH and ABTS were achieved in the antioxidant activity when using the optimized PEF conditions. Luengo et al. (2013) reported yields of 51%, 94%, 148% and 192% in the antioxidant compounds in the extracts of orange peel submitted to PEF pre-treatments of 1, 3, 5 and 7 kV/cm followed by pressing extraction, compared to the extracted by pressing without pre-treatment [169]. The increase in the antioxidant activity of PEF-treated orange peel extract is not only due to the permeabilization of the membrane that leads to the leakage of more antioxidant compounds but is also influenced by structural changes of the investigated compounds as reported by Niu et al. (2021). They reported that when submitting naringin to an electric field of 6 kV/cm the microcrystalline structure was more regular and with a crystal size distribution more uniform compared to non-PEF treated and therefore having a higher antioxidant activity. On the other hand a higher electric field strength leads to irregular agglomerates which are decreasing the antioxidant activity [334]. Thus, at the low electric field strength used in this work, the crystal conformation of the polyphenols might be more regular leading to an increasing in the antioxidant activity of the orange peel extracts.

Additional studies are needed to delve into the physiological and structural changes induced by PEF for a deeper understanding of the observed outcomes. Findings are limited to the selected experimental conditions and orange peel samples; generalizing to other conditions or waste materials may need further validation. Moreover, the feasibility of the methods in fresh by-products could be also checked to corroborate the influence of a possible different structure on phenolic release.

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## **CHAPTER 4. Fermentation of orange peels by lactic acid bacteria: impact on phenolic composition and antioxidant activity**

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Article

# Fermentation of Orange Peels by Lactic Acid Bacteria: Impact on Phenolic Composition and Antioxidant Activity

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## Abstract

Orange processing generates peel by-products rich in phenolic compounds, particularly flavanones like hesperidin and narirutin, offering potential health benefits. Utilizing these by-products is of significant interest in supporting Spain's circular bioeconomy. Therefore, the aim of this study was to investigate the fermentation of orange peels by different lactic acid bacteria (LAB) strains and its impact on phenolic composition and antioxidant activity. Three different LAB strains, two *Lactiplantibacillus plantarum*, and one *Levilactobacillus brevis* were utilized. The phenolic compounds were measured by HPLC-ESI-TOF-MS, and antioxidant activity was assessed using DPPH and ABTS methods. The growth of the LAB strains varied, showing initial increases followed by gradual declines, with strain-specific patterns observed. Medium acidification occurred during fermentation. A phenolic analysis revealed an 11% increase in phenolic acids in peels fermented by *La. plantarum* CECT 9567-C4 after 24 h, attributed to glycosylation by LAB enzymes. The flavonoid content exhibited diverse trends, with *Le. brevis* showing an 8% increase. The antioxidant assays demonstrated strain- and time-dependent variations. Positive correlations were found between antioxidant activity and total phenolic compounds. The results underscore the importance of bacterial selection and fermentation time for tailored phenolic composition and antioxidant activity in orange peel extracts. LAB fermentation, particularly with *La. plantarum* CECT 9567 and *Le. brevis*, holds promise for enhancing the recovery of phenolic compounds and augmenting antioxidant activity in orange peels, suggesting potential applications in food and beverage processing.

**Keywords:** pomaces; HPLC-ESI-TOF-MS; submerged fermentation; food by-products

## 1. Introduction

In 2022/2023, the worldwide orange production amounted to 47.77 million metric tons, with orange juice production reaching 1.64 million metric tons [2]. The leading producers of orange juice in the year 2021/2022 were Brazil, the United States, and Mexico, with the European Union occupying the fourth position [2]. This means that around 3.8% of worldwide orange juice production originated from the European Union, amounting to 62,000 metric tons [2]. Moreover, Spain is the main orange producer in the European Union, with oranges being the primary citrus fruit produced in the country [2]. Therefore, leveraging all aspects of orange production is of huge importance for the circular bioeconomy of the country.

During the processing of oranges to obtain their juice, by-products are generated, mainly consisting of the peel composed by the flavedo and albedo, a source of bioactive compounds. These compounds include phenolic acids, flavonoids, lignans, and anthocyanins [74]. Among the flavonoid phenolic family, especially the flavanones hesperidin and narirutin—hesperetin and naringenin metabolites, respectively—naturally present in the orange peel, have been attributed several bioactivities such as anti-inflammatory, antioxidant, anti-cancer, anti-diabetic, anti-rheumatic, and cardio-protective properties [336].

Fermentation is a fundamental biological process that has been harnessed by humans for millennia to transform raw materials into a wide array of valuable products, from food and beverages to pharmaceuticals and biofuels. This process involves the conversion of organic compounds, typically sugars or other carbohydrates, by microorganisms into various metabolites, such as alcohol, acids, or gases, often accompanied by the generation of energy. It plays a pivotal role in a broad range of applications, with profound implications for food production, industrial biotechnology, and environmental sustainability. The scientific understanding of fermentation has evolved over time, and, today, it encompasses a diverse array of microbial and enzymatic activities. Microorganisms, including bacteria, yeasts, and molds, are the primary agents responsible for carrying out fermentative processes [337]. Previously, orange peel has been submitted to solid-state fermentation by different *Aspergillus* species to produce citric acid (*Aspergillus niger*) [338,339], D-galacturonic acid (*Aspergillus oryzae* [340] and *Aspergillus niger* [341]), succinic acid (*Fibrobacter succinogenes*) [342], or other organic acids (*Aspergillus awamori*) [343]. Moreover, protein production from orange peel has been reported with *Aspergillus niger* and *Chaetomium* Spp. [344] and *Trichoderma reesei* and *Trichoderma viride* [64]. The production of industrially important volatile aroma esters from orange peels by yeast has been also reported [345]. Producing pigments using fungal strains (*Monascus purpureus* and *Penicillium purpurogenum*) from oranges' processing waste has been reported by other authors [346]. Submerged fermentation by *Aspergillus fumigatus* for improving the content of ellagic acid

in orange peels was reported by Sepúlveda et al. (2020) [347]. Ahmed et al. (2021) [348] optimized the pectinase of orange peel waste by *Penicillium chrysogenum* MF 318506.

Lactic acid bacteria (LAB) are a diverse and versatile group of Gram-positive microorganisms with a rich history of use in the food industry [349]. These bacteria play a pivotal role in food preservation, with their traditional role being the fermentation of carbohydrates to produce lactic acid at an industrial scale [350]. However, their significance extends far beyond this primary function. LAB have been found to produce a wide array of valuable by-products during their metabolic processes, including bacteriocins, vitamins, amines, short-chain fatty acids, and exopolysaccharides. Furthermore, the influence of LAB goes beyond their direct metabolic activities. When used in food fermentation, LAB have been shown to enhance the nutritional quality of the final products. In animal products such as milk [351] and meat [352], this enhancement is characterized by increased protein digestibility, improved mineral availability, and the release of peptides and amino acids, contributing to the overall nutritional value of the food [353,354]. Additionally, LAB can boost the antimicrobial and antioxidant properties of the fermented foods. In the context of specific food items, such as apple juice, mulberry juice, soy milk, and wheat dough, LAB-driven fermentation has been observed to elevate the levels of antioxidants and phenolic compounds [355,356]. LAB enzymes also play a key role in breaking down cell walls, which not only improves the release of phenolic acids and flavonoids but also converts them into simpler forms, enhancing their bioavailability [357]. This versatility makes LAB a cost-effective and sustainable technology for maintaining or improving the nutritional quality of food while preserving its sensory properties. The application of LAB in fruit and by-product fermentation can occur spontaneously in suitable conditions, thanks to the presence of naturally occurring lactic acid microbiota. Alternatively, it can be controlled and directed using specific lactic acid bacteria starter cultures to achieve various specific objectives, such as improved digestibility [358]. *Lactiplantibacillus plantarum* and *Levilactobacillus brevis* are LAB that share similarities as Gram-positive, facultative anaerobes, but they differ in several aspects [349]. *La. plantarum* exhibits greater acid and alcohol resistance, prefers warmer temperatures, produces a wide range of enzymes, and forms biofilms, whereas *Le. brevis* can grow at lower temperatures, has a more limited enzymatic capacity, and its biofilm-forming ability may vary. Although both species are part of the human intestinal microbiota and may have beneficial effects on health, they can interact differently with the immune system and intestinal epithelial cells. These interactions can influence the inflammatory response and the modulation of the immune system. Furthermore, different strains of *La. plantarum* and *Le. brevis* may vary in their susceptibility to antimicrobial agents. This is important in terms of food safety and the ability of these bacteria to compete with pathogenic microorganisms in fermented environments. Otherwise, genome sequencing has revealed a great genetic diversity within each species, which

can influence their phenotypic characteristics and adaptability to different environments and substrates [359].

Despite their significant role in food science and nutrition, there remain intriguing avenues of research involving LAB, such as their potential to enhance the bioactivities of unique agro-industrial waste products, like orange peels. LAB can offer significant advantages in the valorization of food by-products. One key benefit is their remarkable ability to bioconvert the carbohydrates present in these by-products into valuable metabolites such as lactic acid and organic acids. This process not only stabilizes the pH of the substrate, prolonging its shelf life, but also transforms it into a more valuable resource for further processing. LAB fermentation can enhance the nutritional profile of food by-products by enriching them with bioactive compounds like polyphenols, vitamins, amino acids, and peptides, thereby increasing their overall nutritional value. Additionally, LAB-produced antimicrobial compounds can contribute to the preservation of these by-products, inhibiting the growth of spoilage microorganisms and pathogens. Furthermore, LAB fermentation could enhance the sensory characteristics of food by-products, imparting desirable flavors and aromas, which can enhance their market appeal. Overall, the utilization of LAB in the valorization of food by-products represents a sustainable approach that not only minimizes waste but also adds value to underutilized resources in the food industry [349].

LAB have been previously studied in orange peel with different purposes. Huang et al. (2017) [360] reported that orange peel fiber powder promotes growth, exopolysaccharide production, and antibacterial activity against the spoilage bacteria of *Lactobacillus plantarum* SLC 13, suggesting its potential for industrial applications as an economic prebiotic. In addition, de la Torre et al. (2020) [350] explored the use of *Lactobacillus delbrueckii* sp. *delbrueckii* for D-lactic acid production using orange peel as the substrate. Ricci et al. (2023) also studied the production of lactic acid from orange waste using a combination of *Aspergillus awamori* and *Lactocaseibacillus casei* and *Lactocaseibacillus rhamnosus* [361]. However, as far as we are concerned, LAB have still a wide range of applications in orange peels to be studied.

Therefore, the aim of this study was to develop industrially scalable biotechnological processes to revalorize and reuse orange peels. This was achieved through a combination of fermentation and ultrasound technologies for extracting bioactive compounds, especially phenolic acids, and flavonoids.

## **2. Materials and Methods**

### **2.1. Reagents and Samples**

Gallic acid, DPPH, and ABTS were procured from Sigma-Aldrich (St. Louis, MO, USA). Water underwent purification through a Milli-Q system (Millipore, Bedford, MA, USA). Vanillic acid,



chlorogenic acid, ferulic acid, quercetin, and rutin were likewise sourced from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade water and various other reagents were acquired from Merck KGaA (Darmstadt, Germany).

Orange by-products (variety Navelina) were secured after juice production. These by-products consisted of the albedo, flavedo, and the remaining pulp of the orange, with a moisture content of  $70 \pm 1.5\%$ . For the experiments, the samples were subjected to drying at  $60\text{ }^{\circ}\text{C}$  with an air flow of  $1.6\text{ m/s}$  for 315 min, achieving a moisture  $< 10\%$ , as modeled and optimized previously by Razola-Díaz et al. (2023) [331]. The dried samples were grounded and stored in a frozen state at  $-18\text{ }^{\circ}\text{C}$  until submitted to fermentation. The samples will be referred to as orange peels throughout the manuscript.

## 2.2. Lactic Acid Bacteria Strains and Culture Media

Lactic acid bacteria (LAB) strains were obtained from the Spanish Collection of Type Cultures (CECT): *Levilactobacillus brevis* CECT 5354, *Lactiplantibacillus plantarum* subsp. *plantarum* CECT 748T, and *Lactiplantibacillus plantarum* CECT 9567 (formerly, strain C4). The strains were grown in MRS medium at  $26\text{ }^{\circ}\text{C}$  for 24–48 h and preserved in glycerol stocks at  $-20\text{ }^{\circ}\text{C}$ . *Le. brevis* CECT 5354 and *La. plantarum* CECT 748T were originally isolated from silage and pickled cabbage. In addition, *La. plantarum* CECT 9567 previously isolated from kefir was also used [362]. They all have demonstrated good results in previously published papers on the fermentation of avocado leaves and seeds [188,363].

## 2.3. Fermentation of Orange Peels

Orange peel fermentation with LAB strains was conducted in accordance with the protocols outlined by De Montijo-Prieto et al. (2023) [188]. Briefly, the bacteria were grown in MRS broth for 24 and 48 h at  $26\text{ }^{\circ}\text{C}$ , and the inocula were prepared in sterile saline and adjusted for each strain by turbidimetry, equivalent to a concentration between  $10^7$  and  $10^8$  colony-forming units (CFU)/mL. The viable bacterial counts in the inocula were determined by plating on MRS agar. The fermentation process involved the following steps: 1 g of orange peels was immersed in 8 mL of sterile water at  $90\text{ }^{\circ}\text{C}$  for 10 min to eliminate any microorganism present on the peels. After cooling, microbial counts were performed on Tryptic Soy agar (a nutrient-rich medium for a wide range of bacteria, incubated at  $37\text{ }^{\circ}\text{C}$  for 24–48 h), MacConkey agar (a selective medium for enterobacteria, incubated at  $37\text{ }^{\circ}\text{C}$  for 24 h), and Sabouraud agar (a medium for fungi, incubated at  $30\text{ }^{\circ}\text{C}$  for 7 days). Subsequently, the mixtures were inoculated with the previously prepared inocula to achieve a bacterial concentration ranging between  $10^6$  and  $10^7$  CFU/mL, followed by incubation at  $26\text{ }^{\circ}\text{C}$ . The CFU/mL were counted on MRS agar, and the pH values were measured and recorded after 24 and 48 h of incubation. A control group, prepared without the addition of lactic bacteria, was included in this study. The samples and control were each processed in

duplicate. After incubation, both the samples and the control group were stored at  $-20\text{ }^{\circ}\text{C}$  and subsequently freeze-dried for further analysis.

#### **2.4. Extraction of Phenolic Compounds**

The extraction of phenolic compounds from the fermented orange peels was carried out with an ultrasound sonotrode (UP400St ultrasonic processor, Hielscher, Germany) that works at 400 W and 24 kHz in the conditions previously optimized by Razola-Díaz et al. (2018) [74]. Briefly, 0.5 g of freeze-dried sample was subjected to extraction using 100 mL of ethanol–water at a ratio of 45:55 and sonicated for 35 min at room temperature at an amplitude of 90% and a pulse 100%. Following this, the samples underwent centrifugation at  $9840\times g$  for 15 min. Subsequently, the supernatants were subjected to drying in a rotary evaporator under vacuum at  $40\text{ }^{\circ}\text{C}$ . The dried residue was then dissolved in 1 mL of methanol–water at a ratio of 50:50. The solutions were meticulously filtered through a  $0.45\text{ }\mu\text{m}$  syringe filter and stored at  $-20\text{ }^{\circ}\text{C}$  in amber bottles to prevent degradation until the time of analysis. It is important to note that each extraction was carried out in duplicate.

#### **2.5. Determination of Phenolic Compounds by HPLC-ESI-TOF-MS**

The extraction of the phenolic compounds in the orange peels fermented by selected LAB and a non-fermented control was analyzed in duplicate using the ACQUITY Ultra Performance LC system (Waters Corporation, Milford, MA, USA) linked to an electrospray ionization (ESI) source operating in a negative mode and a time-of-flight (TOF) mass detector (Waters Corporation, Milford, MA, USA). The desired compounds were separated on an ACQUITY UPLC BEH Shield RP18 column ( $1.7\text{ }\mu\text{m}$ ,  $2.1\text{ mm}\times 100\text{ mm}$ ; Waters Corporation, Milford, MA, USA) maintained at  $40\text{ }^{\circ}\text{C}$ , employing a gradient method previously described by Verni et al. [245]. This involved utilizing water with 1% acetic acid as mobile phase A and acetonitrile as mobile phase B. The obtained data were processed using the MassLynx 4.1 software (Waters Corporation, Milford, MA, USA). The compounds were identified by comparing the  $m/z$  and molecular formula with standards and previous research [74].

#### **2.6. Antioxidant Assays: DPPH and ABTS**

Antioxidant activity was assessed in all the samples using two distinct methods, and each method was performed in duplicate. The DPPH assay followed a procedure previously outlined by various researchers [364]. Specifically,  $100\text{ }\mu\text{L}$  of each extract was combined with  $2.9\text{ mL}$  of DPPH. The solution was vigorously stirred, and the fading of the extract's color was monitored over a 30 min interval at  $517\text{ nm}$ . The ABTS method, based on the method described by Re et al. (1999) [249], involved the generation of monocation  $\text{ABTS}^{+\cdot}$  by oxidizing ABTS with potassium persulfate in darkness at room temperature for 12–24 h. For each extract,  $1\text{ mL}$  of the ABTS solution was

mixed with 0.01 mL of the extract, and the reduction in absorbance was recorded for 30 min at 734 nm.

### 3. Results and Discussion

#### 3.1. Growth of LAB Strains in Orange Peels

As previously outlined, orange peels were supplemented with dextrose and yeast extract to encourage the growth of lactic acid bacteria (LAB). Viable microorganism counts were carried out after 24 and 48 h of incubation on MRS agar. The counts on MacConkey, Tryptic Soy, and Sabouraud agar confirmed that the heat treatment applied to the orange peels before lactic acid bacteria inoculation was effective in eliminating the microorganisms present on the peels, which could have interfered with the biotransformation of the phenolic compounds by lactic acid bacteria.

As indicated in **Table 4.1**, the orange peels demonstrated limited support for the growth of the LAB strains used for inoculation. The viable bacterial numbers increased in the first 24 h of incubation, persisting in a gradual decline throughout fermentation, except for *Le. Brevis*, which exhibited increased counts also after 48 h. Both *La. plantarum* strains achieved their peak growth after 24 h ( $8.18 \pm 0.13$  and  $8.60 \pm 0.03$  log CFU/mL for strains CECT 748T and CECT 9567, respectively), while the exponential growth phase of *Le. brevis* was extended over 48 h, reaching comparable counts ( $8.21 \pm 0.03$  log CFU/mL). Our results are comparable to those obtained by Ricci et al. (2019) [365], who analyzed the growth of lactic acid bacteria on orange peel every 24 h for 5 days during a study which aimed to explore the feasibility of using orange peel as a raw material for the production of lactic acid by solid-state fermentation. Their results showed that *Lactiplantibacillus plantarum* strain 285 was able to grow well in the orange peels at different pH values (initial pH values of 5 and 6.5 and initial pH of 6.5 with  $\text{CaCO}_3$ ), increasing their cell number by 2 log cycles. This concentration remained unchanged during the subsequent days, without being affected by the pH. Regarding the pH values during the fermentation of the orange peels in our study, the growth of all the strains led to medium acidification (**Table 4.1**). After 24 h, the pH was reduced from 5.83 to 3.4 with *La. plantarum* CECT 9567 and to 3.7 with *La. plantarum* CECT 748T and *Le. brevis* CECT 5354. After 48 h, both pH values stabilized around 3.5.

**Table 4.1.** Log CFU/mL and pH of lactic acid bacteria in orange peels expressed with average  $\pm$  standard deviation.

|                             | 0 h             |     | 24 h            |     | 48 h            |     |
|-----------------------------|-----------------|-----|-----------------|-----|-----------------|-----|
|                             | Log10 (CFU/mL)  | pH  | Log10 (CFU/mL)  | pH  | Log10 (CFU/mL)  | pH  |
| <i>Le. brevis</i> CECT 5354 | $7.92 \pm 0.03$ | 5.8 | $8.09 \pm 0.09$ | 3.7 | $8.21 \pm 0.03$ | 3.5 |

|                                |             |     |             |     |             |     |
|--------------------------------|-------------|-----|-------------|-----|-------------|-----|
| <i>La. plantarum</i> CECT 748T | 7.94 ± 0.02 | 5.8 | 8.18 ± 0.13 | 3.7 | 8.01 ± 0.02 | 3.5 |
| <i>La. plantarum</i> CECT 9567 | 7.90 ± 0.03 | 5.8 | 8.60 ± 0.03 | 3.4 | 8.51 ± 0.01 | 3.2 |

### 3.2. Quantification of Phenolic Compounds by HPLC-ESI-TOF-MS and Biotransformation during Fermentation in Orange Peels

The orange peels fermented by the three strains for 24 and 48 h were submitted to extraction by sonotrode and analyzed by HPLC-ESI-TOF-MS.

As shown in **Table 4.2**, the major phenolic compounds in the orange peels were phenolic acids (hydroxycinnamic acids). Looking at the phenolic acid profile, there were no detected differences between the samples, and the phenolic acid profile was composed by 68% ferulic acid derivatives, 23% caffeic acid derivatives, and 9% sinapic acid derivatives. Phenolic acids have been previously reported to be the phenolic compounds most found in citrus peels, being ferulic acids the major one followed by caffeic acids [336], in agreement with the proportions found here. The bioactivities of these compounds have been extensively demonstrated [336]. In plants, hydroxycinnamic acids are found both esterified, covalently attached to the cell wall, and in soluble form [366,367]. The antibacterial effect of hydroxycinnamic acids, specifically in their action against lactobacilli, shows a relatively minor dependence on substitutions with hydroxyl or methoxy groups in the aromatic ring. However, it exhibits a pronounced reliance on the presence of a double bond in the side chain [368]. However, in our study, hydroxycinnamic acids, the phenolic group found in major concentrations in orange peels, were not in their aglycone form but in their conjugated form, which is much less toxic for LAB.

**Table 4.2.** Phenolic acids quantified in the fermented orange peels and a control by HPLC-ESI-TOF-MS, expressed as the average  $\pm$  standard deviation in  $\mu\text{g/g}$  d.w.

| Compound                                  | <i>Le. brevis</i> CECT 5354       |                                   | <i>La. plantarum</i> CECT 748T    |                                   | <i>La. plantarum</i> CECT 9567   |                                  | Control                          |
|-------------------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|----------------------------------|----------------------------------|----------------------------------|
|                                           | 24 h                              | 48 h                              | 24 h                              | 48 h                              | 24 h                             | 48 h                             |                                  |
| Caffeic acid 3-O-glucuronide isomer I     | 247.31 $\pm$ 6.16 <sup>b</sup>    | 292.08 $\pm$ 4.35 <sup>c</sup>    | 299.02 $\pm$ 8.16 <sup>b</sup>    | 291.53 $\pm$ 7.42 <sup>b</sup>    | 344.24 $\pm$ 10.58 <sup>a</sup>  | 331.17 $\pm$ 10.82 <sup>a</sup>  | 324.25 $\pm$ 2.11 <sup>a</sup>   |
| Caffeic acid 3-O-glucuronide isomer II    | 1013.41 $\pm$ 9.26 <sup>b</sup>   | 1086.94 $\pm$ 2.52 <sup>a</sup>   | 962.02 $\pm$ 35.99 <sup>c</sup>   | 926.84 $\pm$ 9.88 <sup>c</sup>    | 1136.69 $\pm$ 9.70 <sup>a</sup>  | 835.58 $\pm$ 1.85 <sup>d</sup>   | 1031.70 $\pm$ 26.31              |
| Caffeoylglycolic acid methyl ester        | 566.91 $\pm$ 4.51 <sup>d</sup>    | 586.10 $\pm$ 0.43 <sup>d</sup>    | 699.64 $\pm$ 32.07 <sup>a</sup>   | 609.64 $\pm$ 6.84 <sup>c,d</sup>  | 658.41 $\pm$ 20.12               | 672.53 $\pm$ 11.32               | 643.56 $\pm$ 4.80 <sup>b,c</sup> |
| Caffeoylmalic acid isomer I               | 784.93 $\pm$ 16.56 <sup>c</sup>   | 822.56 $\pm$ 21.66 <sup>b,c</sup> | 913.38 $\pm$ 32.92 <sup>a</sup>   | 816.35 $\pm$ 12.40 <sup>b,c</sup> | 925.70 $\pm$ 33.03 <sup>a</sup>  | 939.33 $\pm$ 12.71 <sup>a</sup>  | 878.32 $\pm$ 20.35               |
| Caffeoylmalic acid isomer II              | 358.13 $\pm$ 10.94 <sup>c</sup>   | 365.61 $\pm$ 11.82 <sup>c</sup>   | 432.31 $\pm$ 20.23 <sup>a,b</sup> | 362.31 $\pm$ 4.25 <sup>c</sup>    | 460.45 $\pm$ 9.25 <sup>a</sup>   | 440.02 $\pm$ 10.83               | 426.22 $\pm$ 4.97 <sup>b</sup>   |
| 2-(E)-O-Feruloyl-D-galactaric acid isomer | 535.18 $\pm$ 25.98 <sup>e</sup>   | 618.73 $\pm$ 3.04 <sup>c</sup>    | 602.65 $\pm$ 25.53 <sup>c,d</sup> | 561.19 $\pm$ 7.69 <sup>d,e</sup>  | 785.52 $\pm$ 19.31 <sup>a</sup>  | 582.45 $\pm$ 27.15               | 682.90 $\pm$ 15.40 <sup>b</sup>  |
| 2-(E)-O-Feruloyl-D-galactaric acid isomer | 959.38 $\pm$ 30.16 <sup>c,d</sup> | 1013.35 $\pm$ 9.56 <sup>b,c</sup> | 923.94 $\pm$ 45.24 <sup>d</sup>   | 907.83 $\pm$ 17.36 <sup>d</sup>   | 1217.10 $\pm$ 37.75              | 890.69 $\pm$ 8.30 <sup>d</sup>   | 1081.95 $\pm$ 11.02              |
| 2-(E)-O-Feruloyl-D-galactaric acid isomer | 1114.06 $\pm$ 12.43               | 1176.40 $\pm$ 48.97               | 1040.48 $\pm$ 50.14               | 993.00 $\pm$ 42.88 <sup>e,f</sup> | 1578.57 $\pm$ 47.40              | 913.48 $\pm$ 24.26 <sup>f</sup>  | 1253.86 $\pm$ 9.81 <sup>b</sup>  |
| 2-(E)-O-Feruloyl-D-galactaric acid isomer | 2660.23 $\pm$ 110.54              | 2834.30 $\pm$ 2.67 <sup>b</sup>   | 2447.51 $\pm$ 88.72               | 2370.97 $\pm$ 22.84               | 3395.69 $\pm$ 25.11              | 2261.85 $\pm$ 46.95              | 2882.53 $\pm$ 33.35              |
| 2-(E)-O-Feruloyl-D-galactaric acid isomer | 1435.76 $\pm$ 67.27               | 1503.91 $\pm$ 32.86               | 1388.00 $\pm$ 49.60               | 1307.67 $\pm$ 31.65               | 1984.38 $\pm$ 90.00              | 1288.17 $\pm$ 11.13              | 1603.01 $\pm$ 65.47              |
| Feruloyl isocitric acid isomer I          | 322.02 $\pm$ 15.04 <sup>c,d</sup> | 309.86 $\pm$ 12.00 <sup>d</sup>   | 342.57 $\pm$ 13.62 <sup>b,c</sup> | 313.79 $\pm$ 8.48 <sup>c,d</sup>  | 403.49 $\pm$ 5.95 <sup>a</sup>   | 391.67 $\pm$ 8.09 <sup>a</sup>   | 354.75 $\pm$ 4.66 <sup>b</sup>   |
| Feruloyl isocitric acid isomer II         | 1122.62 $\pm$ 24.61               | 1112.85 $\pm$ 8.54 <sup>c,d</sup> | 1187.72 $\pm$ 53.48               | 1102.26 $\pm$ 32.63               | 1383.98 $\pm$ 34.79              | 1361.08 $\pm$ 1.97 <sup>a</sup>  | 1232.35 $\pm$ 9.59 <sup>b</sup>  |
| Feruloyl isocitric acid isomer III        | 485.96 $\pm$ 9.92 <sup>b,c</sup>  | 450.20 $\pm$ 2.54 <sup>c</sup>    | 535.49 $\pm$ 19.92 <sup>a</sup>   | 466.59 $\pm$ 15.53 <sup>c</sup>   | 536.72 $\pm$ 16.04 <sup>a</sup>  | 512.50 $\pm$ 10.84               | 507.51 $\pm$ 6.34 <sup>a,b</sup> |
| Ferulic acid O-glucoside                  | 302.87 $\pm$ 5.65 <sup>a</sup>    | 277.48 $\pm$ 1.75 <sup>b</sup>    | 259.58 $\pm$ 2.74 <sup>b,c</sup>  | 253.43 $\pm$ 6.73 <sup>c,d</sup>  | 240.32 $\pm$ 9.06 <sup>d</sup>   | 194.82 $\pm$ 6.28 <sup>c</sup>   | 252.46 $\pm$ 9.04 <sup>c,d</sup> |
| Sinapic acid O-glucoside                  | 241.00 $\pm$ 6.57 <sup>a</sup>    | 224.12 $\pm$ 0.18 <sup>b</sup>    | 215.36 $\pm$ 2.51 <sup>b,c</sup>  | 203.23 $\pm$ 0.98 <sup>c</sup>    | 211.73 $\pm$ 6.59 <sup>b,c</sup> | 142.59 $\pm$ 5.61 <sup>d</sup>   | 211.80 $\pm$ 4.18 <sup>b,c</sup> |
| Sinapinic acid-O-glucuronide isomer I     | 811.12 $\pm$ 27.33 <sup>c</sup>   | 805.08 $\pm$ 18.41 <sup>c</sup>   | 795.88 $\pm$ 34.20 <sup>c</sup>   | 703.32 $\pm$ 23.89 <sup>d</sup>   | 1070.21 $\pm$ 14.86              | 726.92 $\pm$ 17.21 <sup>d</sup>  | 873.06 $\pm$ 0.10 <sup>b</sup>   |
| Sinapinic acid-O-glucuronide isomer II    | 138.55 $\pm$ 5.43 <sup>b,c</sup>  | 130.91 $\pm$ 5.17 <sup>c</sup>    | 158.04 $\pm$ 7.84 <sup>a</sup>    | 129.10 $\pm$ 3.20 <sup>c</sup>    | 154.65 $\pm$ 6.99 <sup>a</sup>   | 145.25 $\pm$ 0.07 <sup>a,b</sup> | 147.95 $\pm$ 1.46 <sup>a,b</sup> |

Different letters (a–f) in the same line indicate significant differences ( $p < 0.05$ ).

As shown in **Figure 4.1**, looking to the sum of phenolic acids, a significant increment of 11% in the peels fermented by *La. plantarum* CECT 9567 can be seen after 24 h, with significant increments of 14.5, 6.8, and 14.4% for ferulic acid, caffeic acid, and sinapic acid derivatives, respectively, compared to the non-fermented orange peel. However, after 48 h of incubation, these hydroxycinnamic acid derivatives were decreased compared to the amounts found in the non-fermented orange peel. These differences when fermenting with *La. plantarum* CECT 9567 between 24 and 48 h of incubation could be related to biotransformation processes, such as the deglycosylation and hydrolysis of phenolic compounds, led by this bacterium during the incubation time. Phenolic compounds can be toxic to lactic acid bacteria. Even though *La. plantarum* has been widely studied for its adaptation to plant habits and capability to metabolize phenolics [369], it can be affected by some phenolic compounds via changes in the membrane's fatty acid composition [370]. Thus, the phenolic compounds released during the incubation time could have had an antibacterial effect on the growth of this bacterium. In addition, the amount of 2-(E)-O-feruloyl-D-galactaric acid decreased significantly during fermentation with *La. plantarum* CECT 748T and *Le. brevis* CECT 5354 after 24 h of incubation.

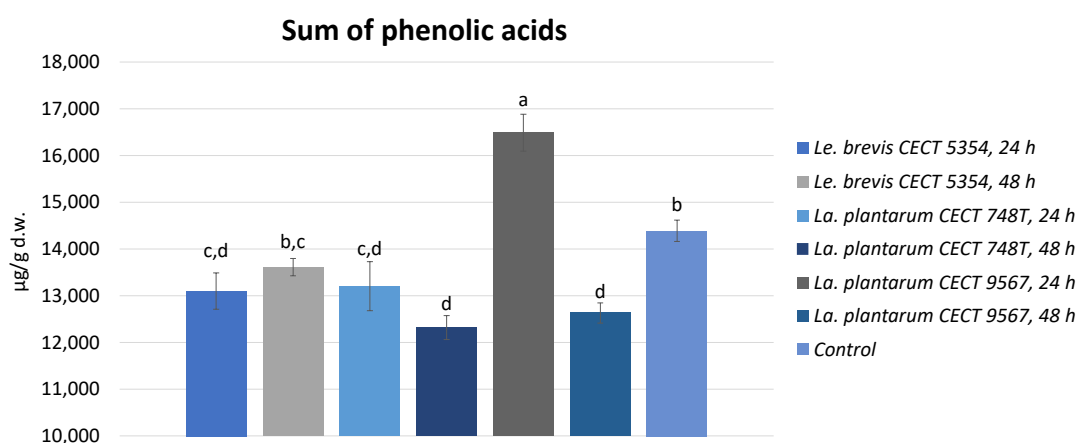


**Figure 4.1.** Sum of phenolic acids in fermented and non-fermented orange peels, expressed as the average and standard deviation. Different letters (a–d) indicate significant differences ( $p < 0.05$ ).

Depending on the chemical structure and concentration of phenolic compounds, the growth and viability of lactic acid bacteria can be affected by affecting the cell wall and membrane [371]. Thus, lactic acid bacteria show several mechanisms for detoxification and tolerance to high concentrations of phenolics in plant niches high in phenolics for optimal growth and survival [371]. The metabolism of phenolic acids by lactobacilli, involving processes such as decarboxylation and/or reduction, is likely a predominant mechanism for detoxifying harmful compounds present in plant substrates encountered by lactobacilli [368]. The mentioned reductions in phenolic acids could be attributed to enzymes such as feruloyl esterases, which have been studied in many microorganisms, including lactic acid bacteria [366]. For example, the

Est\_1092 esterase from *La. plantarum* not only breaks down hydroxycinnamic esters (as a feruloyl esterase) and hydroxybenzoic esters (as a tannase) but is also a broad-range inducible esterase active on numerous esters of other phenolic acids [372]. Feruloyl esterases have also been studied for their activity in removing feruloyl residues that are esterified in pectin [32]. Since the pectin content in orange peel waste is between 20 and 40% [33], this enzyme could also de-esterify some residues as a part of the degradation process of the complex molecular structure of pectin together with polygalacturonase enzymes [34].

In contrast, the increment in some phenolic acid derivatives during fermentation with *La. plantarum* CECT 9567 could also be attributed to the glycosyltransferases that have been reported in lactic acid bacteria for their importance in exopolysaccharide production [373]. Glycosyltransferases (GTs) catalyze the formation of the glycosidic bond by transferring sugar moieties from donor molecules to a specific aglycon, with strict stereo/regioselectivities. The acceptors can be either sugars or other molecules such as polyphenols [374]. Due to the high availability of sugar naturally present in orange peels, it could activate the LAB production of exopolysaccharides. Previously, this action has been reported in some fungal strains [375–377]. Kralj et al. [378] reported, for the first time, the presence of a glucosyltransferase from a *Lactobacillus* strain. Thus, in this study, the increment in sinapic acid glucoside and glucuronide could be attributed to the action of the enzyme sinapate glucosyltransferase from sinapic acid, and the increase in caffeic acid glucuronide could be due to the action of the enzyme uridine-5'-diphosphate-glucuronosyltransferase from caffeic acid. This enzyme has been previously reported in *Arabidopsis* [379] and, a similar one, in *Bacillus subtilis* [380]. Also, some similar reactions have been reported in LAB. For example, a levansucrase from *Leuconostoc mesenteroides* B-512FMC can catalyze glycosyl transfer from sucrose to produce hydroquinone- $\beta$ -fructoside [381]. A  $\beta$ -galactosidase from *Lactobacillus bulgaricus* L3 is able to catalyze galactosyl transfer, resulting in caffeic acid galactoside [382]. The efficacy of phenolic acids' glycosides is contingent upon the type of biological activity, the aglycone's identity, and the specific identity and location of the glycone moiety. Observations from studies examining both glycosides and their corresponding aglycones suggest that phenolic glycosides generally serve as a storage or reserve pool for precursors of more bioactive compounds. Glycosylated compounds are likely to exhibit a higher bioavailability compared to their aglycone forms, attributed to the presence of sugar moieties. In the in vivo environment, hydrolysis of the glycoside would liberate the free aglycone, potentially enhancing its biological activity [383].

The flavonoids identified and quantified in fermented and non-fermented orange peels are presented in **Table 4.3**.

**Table 4.3.** Flavonoids quantified in the fermented orange peels and a control by HPLC-ESI-TOF-MS, expressed as the average  $\pm$  standard deviation in  $\mu\text{g/g}$  d.w.

| Compound                                                             | <i>Le. brevis</i> CECT 5354    |                                 | <i>La. plantarum</i> CECT 748T |                                 | <i>La. plantarum</i> CECT 9567  |                                  | Control                         |
|----------------------------------------------------------------------|--------------------------------|---------------------------------|--------------------------------|---------------------------------|---------------------------------|----------------------------------|---------------------------------|
|                                                                      | 24 h                           | 48 h                            | 24 h                           | 48 h                            | 24 h                            | 48 h                             |                                 |
| Quercetin-3-O-rutinoside-7-O-Glucoside                               | 27.64 $\pm$ 1.17               | 25.46 $\pm$ 0.88 <sup>c,d</sup> | 25.44 $\pm$ 0.77               | 23.84 $\pm$ 0.76 <sup>d</sup>   | 28.64 $\pm$ 0.72 <sup>a</sup>   | 23.97 $\pm$ 0.05 <sup>d</sup>    | 26.23 $\pm$ 0.20 <sup>b,c</sup> |
| Luteolin 7-O-glucoside                                               | 62.27 $\pm$ 0.95               | 61.33 $\pm$ 2.26 <sup>a,b</sup> | 57.68 $\pm$ 1.99               | 52.04 $\pm$ 1.93 <sup>c</sup>   | 62.68 $\pm$ 1.65 <sup>a</sup>   | 57.29 $\pm$ 2.35 <sup>b</sup>    | 59.21 $\pm$ 0.77 <sup>a,b</sup> |
| Prunin                                                               | 149.44 $\pm$ 1.35              | 141.97 $\pm$ 2.65 <sup>b</sup>  | 138.38 $\pm$ 2.75              | 120.88 $\pm$ 1.30 <sup>c</sup>  | 124.48 $\pm$ 1.71 <sup>c</sup>  | 137.31 $\pm$ 0.14 <sup>b</sup>   | 123.74 $\pm$ 2.72 <sup>c</sup>  |
| Isorhamnetin-3-O-rutinoside isomer I                                 | 95.29 $\pm$ 3.24 <sup>a</sup>  | 91.70 $\pm$ 0.63 <sup>a,b</sup> | 85.68 $\pm$ 2.65 <sup>c</sup>  | 88.01 $\pm$ 0.35 <sup>b,c</sup> | 95.80 $\pm$ 1.46 <sup>a</sup>   | 84.86 $\pm$ 1.59 <sup>c</sup>    | 92.54 $\pm$ 1.41 <sup>a,b</sup> |
| Isorhamnetin-3-O-rutinoside isomer II                                | 45.12 $\pm$ 2.14 <sup>a</sup>  | 43.67 $\pm$ 1.40 <sup>a,b</sup> | 40.22 $\pm$ 1.72               | 38.48 $\pm$ 0.79 <sup>c</sup>   | 43.74 $\pm$ 1.51 <sup>a,b</sup> | 38.71 $\pm$ 1.74 <sup>c</sup>    | 42.46 $\pm$ 1.14 <sup>a-c</sup> |
| Vitexin-O-pentoside                                                  | 128.65 $\pm$ 0.81              | 125.94 $\pm$ 0.01               | 121.50 $\pm$ 3.51              | 116.85 $\pm$ 0.21 <sup>c</sup>  | 125.34 $\pm$ 4.05               | 121.79 $\pm$ 0.52 <sup>b,c</sup> | 122.45 $\pm$ 0.45               |
| Naringin hydrate                                                     | 18.83 $\pm$ 0.48               | 19.03 $\pm$ 0.90 <sup>a-c</sup> | 17.68 $\pm$ 0.68               | 16.71 $\pm$ 0.81 <sup>d</sup>   | 20.67 $\pm$ 0.72 <sup>a</sup>   | 16.97 $\pm$ 0.41 <sup>d</sup>    | 19.49 $\pm$ 0.06 <sup>a,b</sup> |
| Apigenin-di-C-hexoside (Vicenin-2) isomer I                          | 490.70 $\pm$ 6.40              | 481.67 $\pm$ 0.88 <sup>a</sup>  | 473.24 $\pm$ 9.59              | 431.92 $\pm$ 3.21 <sup>c</sup>  | 448.12 $\pm$ 15.82              | 470.90 $\pm$ 1.79 <sup>a,b</sup> | 444.48 $\pm$ 19.28              |
| Apigenin-di-C-hexoside (Vicenin-2) isomer II                         | 28.47 $\pm$ 0.89 <sup>b</sup>  | 27.58 $\pm$ 0.02 <sup>b</sup>   | 27.71 $\pm$ 1.17 <sup>b</sup>  | 28.48 $\pm$ 0.84 <sup>b</sup>   | 32.04 $\pm$ 0.79 <sup>a</sup>   | 27.40 $\pm$ 0.91 <sup>b</sup>    | 30.84 $\pm$ 0.35 <sup>a</sup>   |
| Apigenin-di-C-hexoside (Vicenin-2) isomer III                        | 17.27 $\pm$ 0.72 <sup>d</sup>  | 17.88 $\pm$ 0.49 <sup>c,d</sup> | 17.30 $\pm$ 0.02 <sup>d</sup>  | 18.77 $\pm$ 0.48 <sup>b,c</sup> | 21.41 $\pm$ 0.30 <sup>a</sup>   | 17.44 $\pm$ 0.75 <sup>c,d</sup>  | 20.03 $\pm$ 0.49 <sup>a,b</sup> |
| Apigenin 7-O-neohesperidoside                                        | 28.46 $\pm$ 0.58 <sup>a</sup>  | 27.64 $\pm$ 0.78 <sup>a</sup>   | 26.48 $\pm$ 0.96 <sup>a-</sup> | 23.68 $\pm$ 0.67 <sup>d</sup>   | 27.31 $\pm$ 0.99 <sup>a,b</sup> | 24.98 $\pm$ 0.57 <sup>c,d</sup>  | 25.38 $\pm$ 0.10 <sup>b-d</sup> |
| Narirutin                                                            | 621.47 $\pm$ 8.42              | 610.59 $\pm$ 5.64               | 585.25 $\pm$ 2.80              | 558.30 $\pm$ 15.65              | 537.37 $\pm$ 18.90              | 588.76 $\pm$ 12.61               | 560.05 $\pm$ 16.29              |
| Rutin isomer I                                                       | 97.43 $\pm$ 2.67               | 92.69 $\pm$ 2.28 <sup>b,c</sup> | 86.39 $\pm$ 2.88               | 84.59 $\pm$ 3.54 <sup>d</sup>   | 100.77 $\pm$ 0.51 <sup>a</sup>  | 89.37 $\pm$ 0.85 <sup>c,d</sup>  | 91.25 $\pm$ 3.82 <sup>b-d</sup> |
| Rutin isomer II                                                      | 63.38 $\pm$ 2.13               | 63.58 $\pm$ 2.20 <sup>a,b</sup> | 60.96 $\pm$ 2.48 <sup>b</sup>  | 57.69 $\pm$ 1.75 <sup>b</sup>   | 68.30 $\pm$ 3.12 <sup>a</sup>   | 58.31 $\pm$ 1.47 <sup>b</sup>    | 63.48 $\pm$ 1.21 <sup>a,b</sup> |
| Hesperidin                                                           | 960.16 $\pm$                   | 907.31 $\pm$ 23.27              | 924.52 $\pm$ 5.57              | 863.24 $\pm$ 25.80              | 873.10 $\pm$ 26.05              | 833.32 $\pm$ 4.76 <sup>c</sup>   | 868.09 $\pm$ 23.69              |
| Neohesperidin                                                        | 17.48 $\pm$ 0.12               | 18.85 $\pm$ 0.59 <sup>a</sup>   | 17.79 $\pm$ 0.36               | 15.84 $\pm$ 0.03 <sup>d</sup>   | 16.24 $\pm$ 0.12 <sup>d</sup>   | 16.51 $\pm$ 0.48 <sup>c,d</sup>  | 16.09 $\pm$ 0.79 <sup>d</sup>   |
| Alpha-Glucosyl Hesperidin isomer I                                   | <LOQ                           | <LOQ                            | <LOQ                           | <LOQ                            | <LOQ                            | <LOQ                             | <LOQ                            |
| Alpha-Glucosyl Hesperidin isomer II                                  | <LOQ                           | <LOQ                            | <LOQ                           | <LOQ                            | <LOQ                            | <LOQ                             | <LOQ                            |
| Kaempferol 3-O-[3'',6''-di-O-(E)-cinnamoyl]-b-D-glucopyranoside      | 14.98 $\pm$ 0.60               | 16.14 $\pm$ 0.66 <sup>a,b</sup> | 15.62 $\pm$ 0.72               | 14.80 $\pm$ 0.56 <sup>b,c</sup> | 17.41 $\pm$ 0.87 <sup>a</sup>   | 14.41 $\pm$ 0.15 <sup>c</sup>    | 16.00 $\pm$ 0.06 <sup>a-c</sup> |
| Kaempferol 3-O-[3'',6''-di-O-(E)-cinnamoyl]-b-D glucopyranoside      | 19.89 $\pm$ 0.57               | 20.46 $\pm$ 0.52 <sup>a</sup>   | 19.45 $\pm$ 0.83 <sup>a-</sup> | 17.98 $\pm$ 0.70 <sup>c</sup>   | 20.54 $\pm$ 0.90 <sup>a</sup>   | 18.36 $\pm$ 0.52 <sup>b,c</sup>  | 19.82 $\pm$ 0.05 <sup>a,b</sup> |
| Kaempferol 3-[2''-glucosyl-6''-acetyl-galactoside]7-glucoside isomer | 28.58 $\pm$ 1.10               | 29.77 $\pm$ 0.75 <sup>b,c</sup> | 28.32 $\pm$ 0.89               | 27.95 $\pm$ 0.69 <sup>c,d</sup> | 33.89 $\pm$ 1.22 <sup>a</sup>   | 26.92 $\pm$ 0.20 <sup>d</sup>    | 31.60 $\pm$ 0.10 <sup>b</sup>   |
| Kaempferol 3-[2''-glucosyl-6''-acetyl-galactoside]7-glucoside isomer | 31.98 $\pm$ 1.25 <sup>b</sup>  | 30.58 $\pm$ 1.48 <sup>b,c</sup> | 29.27 $\pm$ 0.56 <sup>c</sup>  | 28.92 $\pm$ 0.73 <sup>c</sup>   | 37.23 $\pm$ 0.96 <sup>a</sup>   | 25.95 $\pm$ 0.08 <sup>d</sup>    | 32.48 $\pm$ 0.98 <sup>b</sup>   |
| Kaempferol-dihexosyl acetate                                         | 10.52 $\pm$ 0.50               | 11.33 $\pm$ 0.05 <sup>c</sup>   | 11.04 $\pm$ 0.30               | 10.41 $\pm$ 0.19 <sup>d</sup>   | 15.19 $\pm$ 0.38 <sup>a</sup>   | 8.99 $\pm$ 0.22 <sup>e</sup>     | 12.74 $\pm$ 0.17 <sup>b</sup>   |
| Kaempferol 3-O-(6''-O-acetyl) glucoside-7-O-rhamnoside               | <LOQ                           | <LOQ                            | <LOQ                           | <LOQ                            | <LOQ                            | <LOQ                             | <LOQ                            |
| Kaempferol 3-apiosyl-(1->4)-rhamnoside-7-rhamnoside                  | 2.50 $\pm$ 0.08 <sup>d,e</sup> | 2.81 $\pm$ 0.11 <sup>c</sup>    | 2.67 $\pm$ 0.13 <sup>c,d</sup> | 2.13 $\pm$ 0.10 <sup>f</sup>    | 4.79 $\pm$ 0.15 <sup>a</sup>    | 2.24 $\pm$ 0.08 <sup>e,f</sup>   | 3.15 $\pm$ 0.12 <sup>b</sup>    |
| Kaempferol 3-O-sinapoyl-caffeoyl-sophoroside 7-O-glucoside isomer    | 5.80 $\pm$ 0.06 <sup>b</sup>   | 5.72 $\pm$ 0.21 <sup>b</sup>    | 4.61 $\pm$ 0.20 <sup>c</sup>   | 4.63 $\pm$ 0.18 <sup>c</sup>    | 8.97 $\pm$ 0.23 <sup>a</sup>    | 4.88 $\pm$ 0.12 <sup>c</sup>     | 6.25 $\pm$ 0.28 <sup>b</sup>    |
| Kaempferol 3-O-sinapoyl-caffeoyl-sophoroside 7-O-glucoside isomer    | 4.17 $\pm$ 0.07 <sup>c</sup>   | 3.59 $\pm$ 0.15 <sup>d</sup>    | 3.11 $\pm$ 0.13 <sup>f</sup>   | 3.47 $\pm$ 0.10 <sup>d,e</sup>  | 6.45 $\pm$ 0.09 <sup>a</sup>    | 3.13 $\pm$ 0.10 <sup>e,f</sup>   | 4.71 $\pm$ 0.22 <sup>b</sup>    |
| 3',4'-Didemethylnobiletin                                            | <LOQ                           | <LOQ                            | <LOQ                           | <LOQ                            | <LOQ                            | <LOQ                             | <LOQ                            |

LOQ: Limit of quantification. Different letters (a–f) in the same line indicate significant differences ( $p < 0.05$ ).



Regarding the flavonoid profile, there were no detected significant differences between the samples, and the profile was composed by 33% hesperetin derivatives, 27% naringenin derivatives, 23% apigenin derivatives, and other minor compounds. Regarding the amounts, there were no significant differences in the case of the two *Le. plantarum* bacteria compared to the control, but *Le. brevis* was the most abundant, with a significative increment of 8% (**Figure 4.2**).

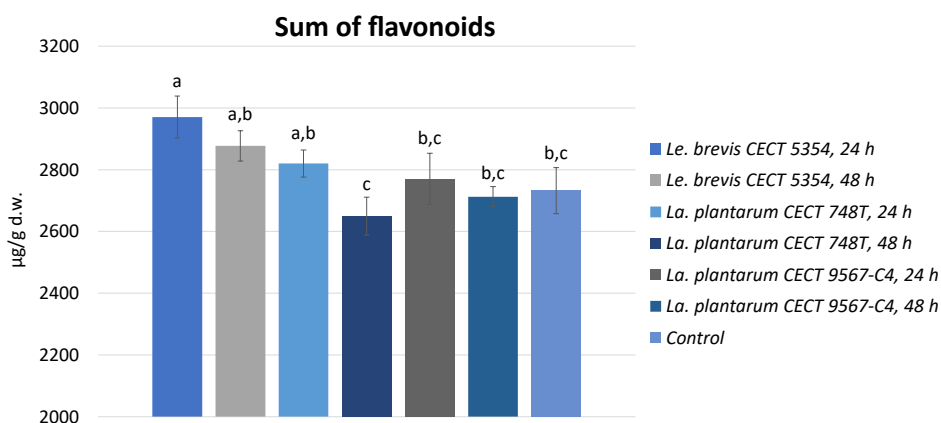


**Figure 4.2.** Sum of phenolic acids of fermented and non-fermented orange peels, expressed as the average with standard deviation. Different letters (a–c) indicate significant differences ( $p < 0.05$ ).

The major flavonoids detected were hesperidin, narirutin, and vicenin-2. Significant increments were observed mostly for the peels fermented by *Le. brevis* compared to the control: these increments were 11% for the sum of naringenin derivatives and 7.3% for the apigenin derivatives. Looking to the minor flavonoids quantified, that were quercetin derivatives, kaempferol derivatives, a luteolin derivative, and an isorhamnetin derivative; the tendency was similar, with the best results for *La. plantarum* C4 and *Le. brevis* measured after 24 h. There was a clear tendency of reducing the flavonoid content after 48 h for all three of the tested bacteria compared to 24 h of fermentation. The significant increase obtained could be attributed to the synergistic effect between fermentation and ultrasound extraction. Moreover, the increments in prunin, narirutin, and hesperidin could be due to the individual or combines action of the enzymes flavanone-7-O-glucosyltransferase and rhamnosyltransferase from the aglycones forms of hesperetin and naringenin [384]. Among the lactobacilli genera, *La. plantarum* and *Le. brevis* have been reported to have the capability to metabolize these two citrus flavanones [385]. Regarding the other minor flavonoids, the mechanisms have also been similarly reported [188,363].

No previous references were found regarding the fermentation of orange peel, focusing on its effect on the peel's phenolic composition. Just recently, Deba-Rementería et al. (2023) [386] proved the acceptability of fermented orange peel-based beverage and snacks, fermented using

*La. plantarum* CECT 749 for 10 days, with the addition of sucrose with 2% NaCl. However, they had a significative reduction of about 50% in the total flavonoid contents but with improvements in the color of the fermented orange peels, which seemed to be more luminous, having a higher color intensity and more yellowness, probably associated with a greater transformation of the peels because of higher microbial activity during the process, with a higher lactic acid content and a less bitter taste than beverages and snacks created from non-fermented orange peels.

Thus, the selection of the fermenting bacteria and fermenting time for orange peels is a determining factor and should be based on the final purpose of the extract.

### 3.3. Antioxidant Activity in Fermented Orange Peel

DPPH and ABTS antioxidant assays were performed on the fermented samples and a control, and the obtained results are presented in **Figure 4.3**.

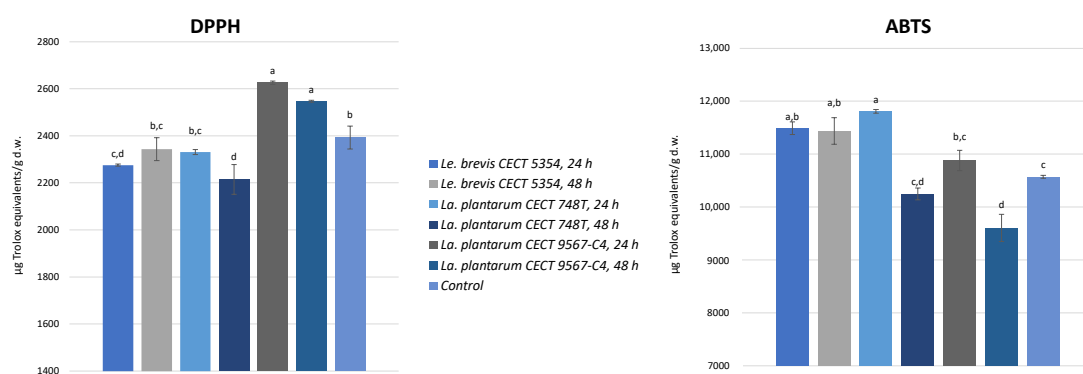


**Figure 4.3.** Antioxidant activity of fermented orange peels, using the three different strains, after 24 and 48 h and a control, expressed as the average with the standard deviation. Different letters (a–d) for each method indicate significant differences ( $p < 0.05$ ) between the samples.

The results obtained ranged from 2214.4 to 2625.0 µg TE/g d.w. for DPPH and from 9604.9 to 11,807.8 µg TE/g d.w. for ABTS. These results are in the same range of magnitude as previous research [331,387]. It is a fact that the polar fractions containing phenolics and flavonoids from lime, lemon, sweet orange, and grapefruit peels have been extensively reported to display a greater antioxidant potential than the volatile fractions containing essential oils from the peels [336].

As it can be seen, different tendencies were obtained, but with significant increments compared to the control for some bacteria. In the two *La. plantarum* strains tested, there were reductions after 48 h compared to 24 h of fermentation, but, for *Le. Brevis*, it seemed to be the opposite. For the DPPH assay, the orange peel fermented using *La. plantarum* CECT 9657, after 24 or 48 h, reported the highest results, with a significant ( $p < 0.05$ ) increment compared to the control. Moreover, the orange peel fermented using *Le. Brevis*, after 48 h, or the other *La. plantarum* strain, after 24 h, did not present significant reductions in terms of its antioxidant activity in

comparison with the non-fermented peel. In the ABTS assay, orange peel fermented using the three tested bacteria exhibited a higher antioxidant activity than the control. Among them, the orange peel fermented using *Le. brevis* (both after 24 and 48 h) and *La. plantarum* CECT 748T (after 24 h) had the highest radical scavenging activity against ABTS cations. Some authors reported increases in antioxidant activity when fermenting avocado seeds [363,388] and apple juice [389] with *La. plantarum*. Also, a higher antioxidant activity in litchi juice [390] and mulberry juice [391] was found when fermenting them with *Le. brevis*.

Furthermore, a Pearson's correlation test was performed (**Figure 4.4**). Both methods of analysis showed a significant positive correlation with the sum of total phenolic compounds. DPPH had a higher correlation with the sum of phenolic acids ( $r = 0.7861$ ,  $p < 0.05$ ), especially caffeic acid derivatives ( $r = 0.8056$ ,  $p < 0.05$ ). As previously noted, caffeic acid-O-glucuronide exhibits antioxidant activity comparable to its parent compound, caffeic acid. The significance of a 4'-hydroxyl group on the aromatic ring appears to be pivotal for its antioxidant activity, particularly in the context of the DPPH assay [392]. ABTS had a higher correlation with the sum of flavonoids ( $r = 0.7725$ ,  $p < 0.05$ ) and especially with hesperidin ( $r = 0.8943$ ,  $p < 0.05$ ). Previously, Abdallah et al. reported a higher correlation between hesperidin and ABTS than with a DPPH antioxidant assay in orange by-products [393].

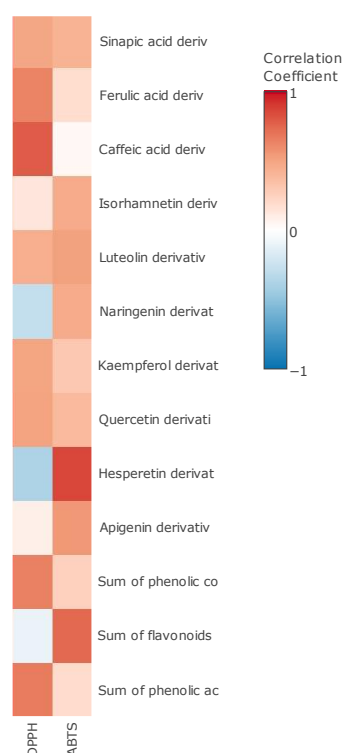


**Figure 4.4.** Pearson's correlation heatmap of antioxidant activity by DPPH and ABTS and phenolic compounds by groups.

#### 4. Conclusions

This study investigated the impact of LAB fermentation on orange peels' phenolic composition and antioxidant properties. While the orange peels showed limited support for LAB growth, the different strains exhibited varied growth patterns and acidification during fermentation. The phenolic acids increased by 11% in the peels fermented using *La. plantarum* CECT 9567 after 24 h, possibly due to glycosylation by LAB enzymes. The flavonoid content remained stable, except for an 8% increase in the peels fermented using *Le. brevis*. The DPPH and ABTS antioxidant assays revealed significant activity increments, particularly with *La. plantarum* CECT 9567 after 24 h and *Le. brevis* after 48 h. Antioxidant activity correlated positively with the total phenolic compounds. These findings underscore the importance of selecting appropriate bacteria and fermentation durations to tailor phenolic composition and antioxidant activity, suggesting that LAB fermentation enhances phenolic compounds' recovery by up to 20%. These results emphasize LAB fermentation's potential to enhance orange peel applications in food and beverage processing.

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**CHAPTER 5. Food grade pilot scale strategy for non-thermal extraction and recovery of phenolic compounds from orange peels**



## **Food grade pilot scale strategy for non-thermal extraction and recovery of phenolic compounds from orange peels**

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### **Abstract**

The orange peels (OP) derived from orange juice factories consist of several polymers and molecules that could be recovered using a biorefinery approach. This study presents a pilot-scale strategy for the extraction and recovery of phenolic compounds from OP, aiming to demonstrate a sustainable, non-thermal process that operates without the use of solvents and complies with food-grade standards. The extraction process was conducted in a food-grade manner on a 1000 L scale, starting with the enzymatic hydrolysis of pectin in the biomass, followed by ultrasound-assisted extraction (UAE) at 54 Ws/gslurry and 20 °C. Solid/liquid separation via decanter centrifuge allowed to recover the solution containing the phenolic compounds, that was further purified via sequential cross-flow microfiltration (MF), ultrafiltration (UF) and nanofiltration (NF). The final product was analyzed by HPLC-ESI-TOF-MS, revealing an interesting phenolic profile with several potential bioactivities and a total polyphenol content (TPC) of 3.3 g/L. The food-grade analysis confirmed the potential application of the final product in food-related processes, such as food packaging or fortification. This work highlights a scalable and environmentally friendly method to convert agro-food waste into valuable bioactive compounds, aligning with green chemistry principles and zero-waste strategies.

**Keywords:** Orange peels; phenolics; downstream processing; pilot scale; food grade.

## 1. Introduction

The global demand for food and energy continues to rise with the growing human population, driving the need for sustainable resource utilization [394]. Agro-food wastes (AFWs) are the by-products generated during the processing and commercialization of raw agricultural materials. These residues, often discarded, contain valuable polymers such as cellulose, hemicellulose, proteins, lipids, lignin, and other compounds of significant biorefinery interest [395]. The renewable nature of AFWs, along with their potential for recyclability and sustainability, positions them as prime candidates for promoting the transition to a bio-based industry [396]. Among these wastes, orange peel (OP) waste, a by-product of orange juice production, represents a considerable portion, approximately 45% of the fruit's total mass [397]. Annually, the production of OP waste is estimated to be between 8 and 20 million tons globally [398]. As the production of oranges continues to rise, so does the volume of waste generated, presenting a growing environmental challenge [399]. Traditionally, OP waste is managed through landfilling and composting [400]. However, these methods can lead to environmental issues, particularly water pollution, necessitating appropriate treatment before disposal [397]. An alternative approach involves using OP waste as feedstock in biorefinery processes, which not only addresses waste disposal issues but contributes to generating bio-based commercial products in line with green chemistry principles and zero-waste strategies.

OP and other citrus peels are valuable source of essential oils, widely used in cosmetics industry, and can be processed to produce biogas, ethanol, or flavoring compounds [401]. Moreover, OP contains several phytochemicals, including pectin, carotenoids, and phenolic compounds, these latter being bioactive compounds recognized to have chemo preventive, anti-inflammatory, antioxidant, and antimicrobial properties [398]. These properties make them suitable for various industrial applications, such as preventing lipid oxidation in meats and extending the shelf life of perishable products when incorporated into food packaging and edible films [402]. OP extracts have been previously reported to be added into poly(vinyl alcohol)-based films [403], or in nanofibers for food packaging [404], among others.

Conventional methods for extracting bioactive compounds from food residues, such as solid-liquid extraction, Soxhlet extraction, liquid-liquid extraction, and maceration, are often time-consuming and require large volumes of solvents. Additionally, these methods typically operate at temperatures above 30 °C, which can degrade thermolabile compounds and reduce extraction efficiency [405]. To overcome these limitations, advanced extraction techniques have been proposed in the last years, like microwave-assisted extraction, supercritical fluid extraction and enzyme assisted extraction [406]. Among them, ultrasound assisted extraction (UAE) has gained increasing attention because this technology can lead to an efficient extraction even at relatively



low temperatures ( $< 20\text{ }^{\circ}\text{C}$ ) compared to other unconventional methods. UAE utilizes cavitation phenomena in the liquid phase, producing high-speed microjets and strong shockwaves that break chemical bonds in the feedstock, releasing the desired molecules into the solution (Luo et al., 2014). Among the ultrasound systems, probe-type sonicators show a higher localized intensity with respect to bath-type sonicators, with the cavitation more controllably distributed [407].

Despite numerous studies on extracting phenolic compounds from orange peels and other type of biomass via UAE at laboratory scale [74,271,274,408,409], mostly involve the use of solvents like ethanol and operate at temperatures above  $30^{\circ}\text{C}$ . Additionally, the extracts often contain various molecules of different sizes, affecting the purity of the final product (e.g. mono- and polysaccharides) [410]. Traditional purification methods, such as precipitation, adsorption on resins, or chromatography, are costly and require solvent changes and temperature adjustments, potentially affecting the bioactivity of the compounds [410]. Pressure-driven membrane processes, such as microfiltration (MF), ultrafiltration (UF) and nanofiltration (NF), offer a more efficient alternative for purification, operating at room temperature and avoiding phase separation or chemical agents [411]. In the last years, membrane filtration technology has been largely investigated for the purification of bioactive compounds from different food matrices [412–415].

This study aims to scale up a non-thermal process for sustainably extracting and recovering bioactive compounds from orange peel waste. By integrating enzymatic hydrolysis and UAE with sequential membrane filtration technologies (MF, UF, and NF), we seek to demonstrate a solvent-free, food-grade process that aligns with green chemistry and zero-waste principles. The process is evaluated at a 1000 L scale, with analysis of the phenolic profile and total polyphenol content (TPC) of the final product. Additionally, the potential application of the phenolic extract in food-related processes, such as packaging and fortification, is assessed.

We hypothesize that this innovative, non-thermal, and solvent-free extraction method will efficiently recover high-purity phenolic compounds from orange peel waste, suitable for food-related applications. The study aims to prove that the scalable process can produce phenolic compounds that meet food-grade standards, highlighting its feasibility and environmental benefits.

A literature search did not highlight any reference to previous similar work at this scale.

## 2. Material and methods

A schematic overview of the process is shown in **Figure 5.1**.

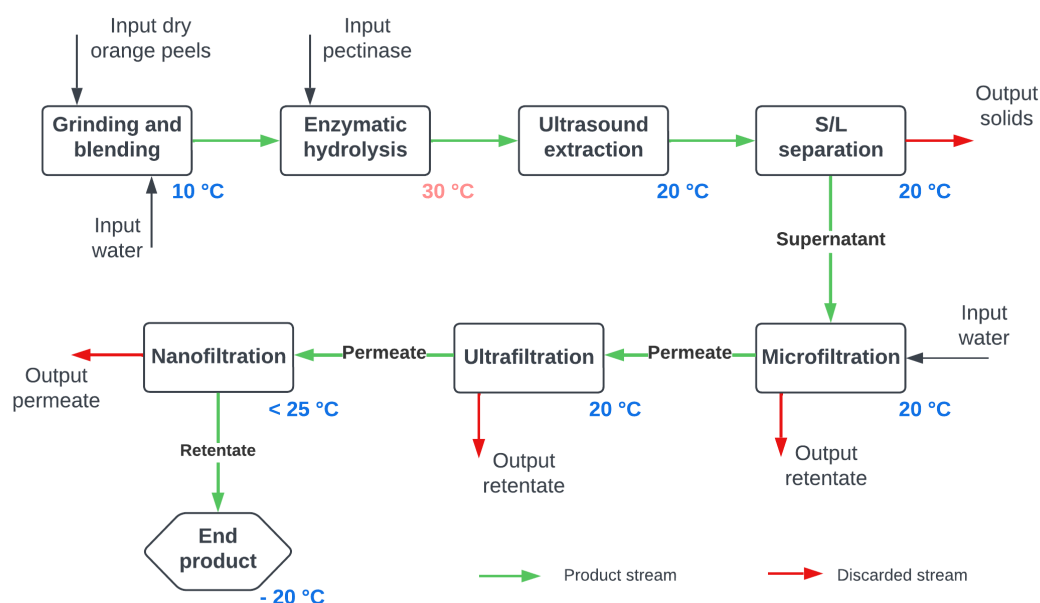


**Figure 5.1.** Schematic overview of the extraction and downstream process.

### 2.1. Raw materials

A batch of 250 kg of dried orange peels from *Citrus sinensis* (Lot: CNE002) was purchased at Bela Vizago Nature S.L. (Murcia, Spain), a Spanish importer and international wholesaler of food materials, in December 2021. The moisture content certified by the supplier was 10.7 % w/w. A total of 70 kg of dried OP was ground in a pilot cutter UM 74E (ProXES, Germany) at 3000 rpm for 5 min. Chilled water was connected to the jacket of the cutter to keep the temperature of the product below 10 °C during the grinding step. The final particle size was < 2 mm. The collected material was stored in closed plastic buckets at – 20 °C until use in the pilot run.

The ground dry OP was mixed with 600 kg reverse osmosis (RO) water in an 8000 L stirred and jacketed stainless-steel tank (Ritter GmbH). The stirrer speed was kept constant at 35 rpm. The temperature of the slurry was kept below 20 °C by recirculating cool water in the jacket of the tank. The slurry was mixed for 1 h.

## **2.2.Pilot scale process**

### **2.2.1.Enzymatic hydrolysis (EH)**

To improve the release of phenolic compounds during UAE, the pectin present in the OP was hydrolyzed with Pectinex Utra SP-L (polygalacturonase), kindly provided by Novozymes (Denmark). The enzyme activity certified by Novozymes was 3300 PGNU/g.

The temperature of the slurry was increased to 30 °C, then the pH adjusted to 5 by adding food grade NaOH 30 % (Brenntag, Belgium) via an automated dosing system. The temperature and pH values were selected as optimal for the enzyme activity, according to the product specifications provided by Novozymes. Based on previous lab tests (data not published yet), the enzyme was added in a concentration of 221 PGNU/g<sub>OP</sub> via manhole of the tank. The hydrolysis was conducted for 24 h at 30 °C, 35 rpm and pH 5. Temperature, stirring, and pH were kept constant during the enzymatic action. At the end of the hydrolysis, the temperature of the slurry was decreased below 20 °C before starting UAE.

### **2.2.2.Ultrasound-assisted extraction (UAE)**

The UAE was performed by recirculating the hydrolyzed slurry over the US system using a peristaltic pump (SPX 25, Bredel). DN25 flexibles and coupling pieces were used to connect tank, pump, and US unit. The US system (UIP2000hdT, Hielscher, Germany) consisted of a generator, transducer (19.5 kHz; max power 2000 W), sonotrode and booster (model CS4d40L3, max amplitude 35 mm) and flow cell with jacket for cooling water. The slurry was recirculated over the US system at 200 L/h for 12 h with measured average net power and amplitude of 500 W and 40 %, respectively. The selection of these parameters was based on previous lab tests (data not published yet) and the maximum delivered power of the system when tested on water. The temperature was kept below 20 °C by recirculating cool water in the jacket of the flow cell and feed tank. Samples were taken and analyzed every 2 hours. At the end of the process the slurry present in the recirculation line was collected with the main bulk by displacing it with RO water. After the process, the specific delivered energy was calculated as the ratio of the total energy provided to the OP slurry divided by the mass of slurry and expressed as Ws/gslurry.

### **2.2.3.Solid/liquid separation on decanter centrifuge**

The OP mixture after UAE was submitted to solid/liquid separation using a Foodec 100 decanter centrifuge (Alfa Laval, Sweden). The slurry was fed to the unit using a peristaltic pump (SPX 25, Bredel) at an average flow rate of 400 L/h. Bowl speed and differential speed of the unit were set at 5100 rpm and 10 rpm, respectively. The operating parameters were selected based on the processing data of similar slurries in previous pilot projects performed at BBEPP. The two outlet

streams were collected separately: the supernatant (liquid stream) was collected in another 8000 L stirred and jacketed stainless-steel tank (Ritter GmbH) and kept at 15 °C, while the solids were collected in a plastic box (120x100x74 cm) positioned under the solid outlet of the unit and discarded after sampling.

#### **2.2.4. Microfiltration**

The solids still present in the supernatant from decanter centrifuge were removed via tangential flow microfiltration (MF). The MF unit (Tami Industries, France) was equipped with three ceramic membranes (After sampling, the concentrated orange peel extract (OPE) was filter sterilized with a 0.2  $\mu$ m PES filter (PESE-020-30-HSF, Masterfilter, Netherlands), then collected in 20 L jerrycans and immediately stored at -20 °C.

The starting operating parameters for the three filtration processes were selected based on the processing of similar streams in previous pilot projects at BBEPP. These parameters were adjusted during the filtrations when needed.

**Table 5.1).** The supernatant from decanter was fed to the filtration unit via a centrifugal pump (Solidc-2 170 SSS, Alfa Laval, Sweden). The optimal pressure in the system was achieved via a fixed 1.1 kW recirculation pump. When the dead volume of the MF setup was almost reached (30 L), diafiltration was performed to recover as much phenolic compounds as possible by adding 60 L RO water in continuous mode to the feed tank at the same flow rate as the filtrate stream. The amount of water added corresponded to two diafiltration volumes. The permeate and diafiltrate streams were collected in an 8000 L stirred and jacketed stainless-steel tank (Ritter GmbH) and kept at 15 °C, while the retentate was displaced in the feed tank and discarded after sampling.

#### **2.2.5. Ultrafiltration**

The permeate solution from MF was further polished via tangential flow ultrafiltration (UF). The UF unit (Uniq Bioflavone) was equipped with one spiral wound membrane (After sampling, the concentrated orange peel extract (OPE) was filter sterilized with a 0.2  $\mu$ m PES filter (PESE-020-30-HSF, Masterfilter, Netherlands), then collected in 20 L jerrycans and immediately stored at -20 °C.

The starting operating parameters for the three filtration processes were selected based on the processing of similar streams in previous pilot projects at BBEPP. These parameters were adjusted during the filtrations when needed.

**Table 5.1).** The MF permeate was fed to the UF unit via a DN25 centrifugal pump (Alflow, UK). The optimal pressure in the system was achieved via a 5-kW recirculation pump. After concentrating the MF permeate until almost dead volume of the UF setup (20 L), diafiltration was performed to recover as much phenolic compounds as possible by adding 40 L RO water in continuous mode to the feed tank at the same flow rate as the filtrate stream. The amount of water added corresponded to two diafiltration volumes. The permeate and diafiltrate streams were collected in an 8000 L stirred and jacketed stainless-steel tank (Ritter GmbH) and kept at about 15 °C, while the retentate was displaced in the feed tank and discarded after sampling.

## 2.2.6. Nanofiltration

The permeate solution from UF was finally concentrated via crossflow nanofiltration (NF). The SW80 NF unit (MMS, Denmark) was equipped with one spiral wound membrane (After sampling, the concentrated orange peel extract (OPE) was filter sterilized with a 0.2 µm PES filter (PESE-020-30-HSF, Masterfilter, Netherlands), then collected in 20 L jerrycans and immediately stored at -20 °C.

The starting operating parameters for the three filtration processes were selected based on the processing of similar streams in previous pilot projects at BBEPP. These parameters were adjusted during the filtrations when needed.

**Table 5.1).** The UF permeate was fed to the NF unit via a fixed DN25 centrifugal pump and recirculated via a fixed 15 kW pump. The permeate was collected in a 8000 L stirred and jacketed stainless steel tank (Ritter GmbH) and discarded after sampling. The retentate (concentrated solution) was displaced in the feed tank and kept at 15 °C. After sampling, the concentrated orange peel extract (OPE) was filter sterilized with a 0.2 µm PES filter (PESE-020-30-HSF, Masterfilter, Netherlands), then collected in 20 L jerrycans and immediately stored at -20 °C.

The starting operating parameters for the three filtration processes were selected based on the processing of similar streams in previous pilot projects at BBEPP. These parameters were adjusted during the filtrations when needed.

**Table 5.1.** Characteristics of the selected filtration membranes.

| Membrane type   | Manufacturer      | Membrane name | Membrane size  | Material                            | Pore size or MWCO | Surface area (m <sup>2</sup> ) /membrane | Total surface area (m <sup>2</sup> ) | Water permeability (LMH/bar) |
|-----------------|-------------------|---------------|----------------|-------------------------------------|-------------------|------------------------------------------|--------------------------------------|------------------------------|
| Microfiltration | Tami Industries   | Inside CéRAM  | ø25 x 1178 mm  | TiO <sub>2</sub> + ZrO <sub>2</sub> | 0.45 µm           | 0.2                                      | 0.6                                  | 1380                         |
| Ultrafiltration | Synder Filtration | ST-2B-3838    | Ø96.5 x 965 mm | PES                                 | 10 kDa            | 6.7                                      | 6.7                                  | 50                           |
| Nanofiltration  | Synder Filtration | NFX-2B-8038   | Ø200 x 965 mm  | PA TFC                              | 0.15-0.30 kDa     | 37.2                                     | 37.2                                 | 4                            |

## **2.3. Analytics**

Homogeneous samples of 40 mL were taken in conical tubes from each process stream and analyzed for dry matter (DM) content, pH, and pellet %Vol or immediately stored at -20 °C to be further analyzed for phenolic content.

### **2.3.1. Determination of dry matter, pH, and pellet vol%**

DM content expressed as DM% was measured on an Infrared moisture analyzer MA35 (Sartorius) by drying about 5 g of sample at 130 °C until constant weight was achieved. The DM content was calculated as ratio of final weight divided the start weight of the sample, expressed in percentage. Online and offline pH was measured with HI 99121 pH probe (Hanna Instruments, USA), calibrated at pH 4.01 and pH 7.01. The pellet %Vol was calculated after spinning down a 40 mL sample in graduated conical tubes in a Haraeus multifuge X3R centrifuge (Thermo Fisher Scientific, USA) at 4100 rpm for 5 min: the obtained pellet volume divided the total volume of sample gave the pellet volume expressed as percentage. Each sample was analyzed in duplicate.

### **2.3.2. Determination of sugars by HPLC-RID**

Galacturonic acid, glucose, and fructose in the final products were quantified by HPLC. A 5 mL sample was centrifuged at 14000 rpm for 5 min in a 5427R tabletop centrifuge (Eppendorf, Germany). The supernatant was filtered through a 0.2 µm PES syringe filter (VWR, USA). 10 µL of PES filtered solution was loaded on an Agilent 1260 Infinity HPLC equipped with a MetaCarb 67H column (300 x 6.5 mm, connected to a varia 5244GC precolumn), and eluted with 2.5 mM H<sub>2</sub>SO<sub>4</sub> at 0.8 mL/min. The column temperature was 40 °C. The sugars were detected by a refractive index detector (RID). Chemstation software (version C.01.05) was used to process the HPLC results (Tönjes et al., 2023). The concentration of sugars was measured with respect to calibration standards. Each sample was analyzed in duplicate. The results are expressed in g/L with average ± standard deviation.

### **2.3.3. Determination of phenolic compounds by HPLC-ESI-TOF-MS**

The final product (concentrated OPE) was analyzed in duplicate using an ACQUITY Ultra Performance LC system (Waters Corporation, Milford, MA, USA) coupled to an electrospray ionization (ESI) source operating in the negative mode and a time-of-flight (TOF) mass detector (Waters Corporation, Milford, MA, USA). The target compounds were separated using an ACQUITY UPLC BEH Shield RP18 column (1.7 µm, 2.1 mm × 100 mm; Waters Corporation, Milford, MA, USA) at a temperature of 40 °C. A gradient previously described [245] utilizing water with 1% acetic acid as mobile phase A and acetonitrile as mobile phase B, was employed.

Data analysis was performed using MassLynx 4.1 software (Waters Corporation, Milford, MA, USA). The identification of the phenolic compounds was done by comparing the  $m/z$  and predicted molecular formula with previous research [257]. For the quantification standard curves of chlorogenic acid, vanillic acid, ferulic acid, rutin, quercetin and catechin were performed, and the results are expressed as  $\mu\text{g/g}$  d.w. The elaboration involved utilizing the peak areas of each standard, measured by HPLC at varying concentrations. Calibration ranges had been predetermined based on the LOQ values. In all cases, the regression coefficients exceeded 0.99, indicating strong linearity in the calibration curves. The LOD ranged from 0.04 to 0.47  $\mu\text{g/mL}$ , while the LOQ ranged between 0.14 and 1.57  $\mu\text{g/mL}$ . The results are expressed in  $\mu\text{g/mL}$  with average  $\pm$  standard deviation.

## **2.4. Food grade requirements and analysis**

The Hazard Analysis of Critical Control Points (HACCP) performed prior the run determined the following Critical Control Points (CCPs) and Operational Pre-requisite Programs (O'PRP) to perform before starting the process:

*CCP:* S/L separation via decanter centrifuge -> dismantling and manual cleaning of all parts before sanitation to avoid growth of pathogens from previous runs.

*O'PRP spiral wound filtration:* HPLC analysis of rinsing water after sanitation to make sure no unwanted chemicals are present from previous runs.

*O'PRP final sterile filtration:* check integrity of 0.2  $\mu\text{m}$  PES filter before installation to avoid introduction of physical hazards due to broken/blocked filter.

Before each step, a sanitation/disinfection process was applied to the involved equipment, consisting of 15 min recirculation of a 0.05 % disinfectant solution (Sopuroxid 15, Sopura, Belgium) at room temperature. After rinsing, the validation of the cleaning was performed via conductivity check (HI 99301, Hanna, USA), protein check (Clean-Trace<sup>TM</sup> Surface Protein Allergen swab, 3M, USA), peroxide check (MQuant<sup>TM</sup>, Merck), ATP check (UltraSnap ATP Rapid Test, Gullimex, Belgium).

Microbial load, allergens, pesticides, and heavy metals in the final OPE were analyzed by an accredited external laboratory (Ecca NV, Belgium) to assess food grade compliance.

### 3. Results and Discussion

The mass balance for each step is reported in **Table 5.2**. The TPC results are reported in **Table 5.3**.

**Table 5.2.** Mass balance during all the steps carried out. Results are expressed as average  $\pm$  standard deviation.

| Process step                         | Process stream           | In/Out | Mass (kg) | DM (% m/m)     | pH            | Pellet Vol%   |
|--------------------------------------|--------------------------|--------|-----------|----------------|---------------|---------------|
| Dry cutting and wet blending         | Dry orange peels         | In     | 70        | 89.3 $\pm$ 0.5 | n.d.          | n.a.          |
|                                      | RO water                 | In     | 600       | 0              | 7.0 $\pm$ 0.1 | 0             |
|                                      | OP slurry                | Out    | 670       | 9.1 $\pm$ 0.2  | 4.1 $\pm$ 0.2 | 46 $\pm$ 2    |
| EH                                   | Enzyme solution          | In     | 0.4       | n.d.           | 4.5 $\pm$ 0.1 | n.d.          |
|                                      | OP slurry after 24 h EH  | Out    | 670       | 9.7 $\pm$ 0.1  | 3.3 $\pm$ 0.1 | 29 $\pm$ 1    |
| UAE                                  | OP slurry after 12 h UAE | Out    | 670       | 9.2 $\pm$ 0.4  | 3.3 $\pm$ 0.2 | 38 $\pm$ 2    |
| S/L separation (decanter centrifuge) | RO water                 | In     | 50        | 0              | 7.0 $\pm$ 0.1 | 0             |
|                                      | Supernatant              | Out    | 624       | 6.3 $\pm$ 0.1  | 3.2 $\pm$ 0.1 | 13 $\pm$ 1    |
|                                      | Solids                   | Out    | 110       | 17.2 $\pm$ 0.5 | 3.2 $\pm$ 0.2 | 74 $\pm$ 2    |
| MF<br>0.45 $\mu$ m                   | RO water                 | In     | 191       | 0              | 7.0 $\pm$ 0.1 | 0             |
|                                      | Retentate                | Out    | 30        | 10 $\pm$ 0.3   | 3.2 $\pm$ 0.2 | 5.9 $\pm$ 0.3 |
|                                      | Permeate+diafiltrate     | Out    | 814       | 3.9 $\pm$ 0.2  | 4.5 $\pm$ 0.2 | 0             |
| UF<br>10 kDa                         | RO water                 | In     | 40        | 0              | 7.0 $\pm$ 0.1 | 0             |
|                                      | Retentate                | Out    | 26.4      | 3.0 $\pm$ 0.2  | 4.5 $\pm$ 0.2 | 0             |
|                                      | Permeate+diafiltrate     | Out    | 621       | 3.7 $\pm$ 0.2  | 4.5 $\pm$ 0.1 | 0             |
| NF<br>0.15-0.30 kDa                  | Permeate                 | Out    | 590       | 1.2 $\pm$ 0.1  | 4.5 $\pm$ 0.1 | 0             |
|                                      | Retentate                | Out    | 76        | 13.1 $\pm$ 0.2 | 4.5 $\pm$ 0.2 | 0             |

n.a. = not analyzed; n.d. = not determined

#### 3.1. Enzymatic hydrolysis

The slurry obtained by mixing the ground dry OP with water was analyzed for dry matter (DM) content, pH, pellet %Vol and total phenolic compounds before and after EH. The results are shown in **Table 5.2**. Then the OP were submitted to enzymatic hydrolysis with polygalacturonase. Polygalacturonase, an enzyme commonly found in plants, fungi, and bacteria, plays a pivotal role in the degradation of pectin, a key component of plant cell walls. When applied to orange peel, polygalacturonase initiates a series of enzymatic reactions that result in significant structural modifications. The enzymatic action of polygalacturonase primarily targets the pectin molecules present in the orange peel. Pectin is a complex polysaccharide composed mainly of  $\alpha$ -(1 $\rightarrow$ 4)-linked D-galacturonic acid residues, with various side chains and branching patterns. Polygalacturonase catalyzes the hydrolysis of the glycosidic bonds between these galacturonic acid residues, leading to the cleavage of the pectin backbone. Therefore, the integrity of the pectin matrix is compromised changing the rheological properties of orange peel extracts, affecting their viscosity and flow behavior. The increased surface area of the powdered form of the orange peel



allows for more efficient enzymatic action, resulting in faster degradation of pectin molecules [416]. According to the lab scale results from previous tests, the hydrolysis of the orange peels with pectinolytic enzymes can improve the phenolic compounds release during UAE due to the degradation of at least part of the pectin structure, which may entrap the molecules of interests (data not published yet). A similar strategy based on pectinase hydrolysis has been applied in several studies to produce fermentable sugars from OP [417–419]. The efficacy of the EH was highlighted by the reduction in pellet Vol% of the end sample (24 h) from 46 to 29 % (after spin test at 4100 rpm; 5 min), indicating that monomers and/or small polymers were released in solution from the pectin structure. The analysis performed at the end of the process showed a 40% decrease in TPC (**Table 5.3**). The unexpected results is probably due to a technical issue during the EH for which the temperature of the reaction tank increased from 30 to 50 °C for about 1 h, causing a possible degradation of the product and pH also decreased of the slurry to 3.3.

**Table 5.3.** TPC for each step.

| Process step   | Process stream                | TPC (g/L) * |
|----------------|-------------------------------|-------------|
|                | Cutting, mixing, and blending | 6.0         |
| EH             | After 24 h                    | 3.6         |
| UAE            | After 12 h UAE                | 3.1         |
| S/L separation | Supernatant                   | 2.0         |
| MF             | RO water                      | 0           |
|                | Retentate                     | 2.1         |
|                | Permeate+diafiltrate          | 2.5         |
| UF             | RO water                      | 0           |
|                | Retentate                     | 0.2         |
|                | Permeate+diafiltrate          | 2.8         |
| NF             | Permeate                      | 0.1         |
|                | Retentate                     | 3.3         |

\*Standard Deviation < 1%

### 3.2. Ultrasound-assisted extraction

The specific energy delivered to the hydrolyzed slurry during UAE (12 h) was calculated in 54 Ws/g<sub>slurry</sub>. **Figure 5.2** shows the TPC at different interval during UAE. The process temperature was kept at 20 ± 3 °C.

Conversely to what reported in literature [257,274,275,409] the application of US to orange peels in these trial did not improve the release of phenolic compounds in solution. This result can be

attributed to several factors. Primarily, most publications in this field typically apply ultrasound (US) to products suspended in an ethanol/water mixture. The presence of ethanol can significantly influence the effectiveness of the ultrasound process, enhancing its impact compared to a purely aqueous suspension. Additionally, these studies often employ higher energy ultrasound but for shorter durations. This approach contrasts with our methodology, where a lower energy ultrasound is applied over a longer period. The combination of these differences in solvent composition and energy application likely accounts for the variation in outcomes observed in our work versus those reported in the literature. By using a mixture of ethanol and water, the cavitation effects induced by ultrasound are more pronounced, leading to more efficient processing. In contrast, our method's extended exposure time with lower energy might result in a different mechanism of action, thus producing the distinct results we observed.

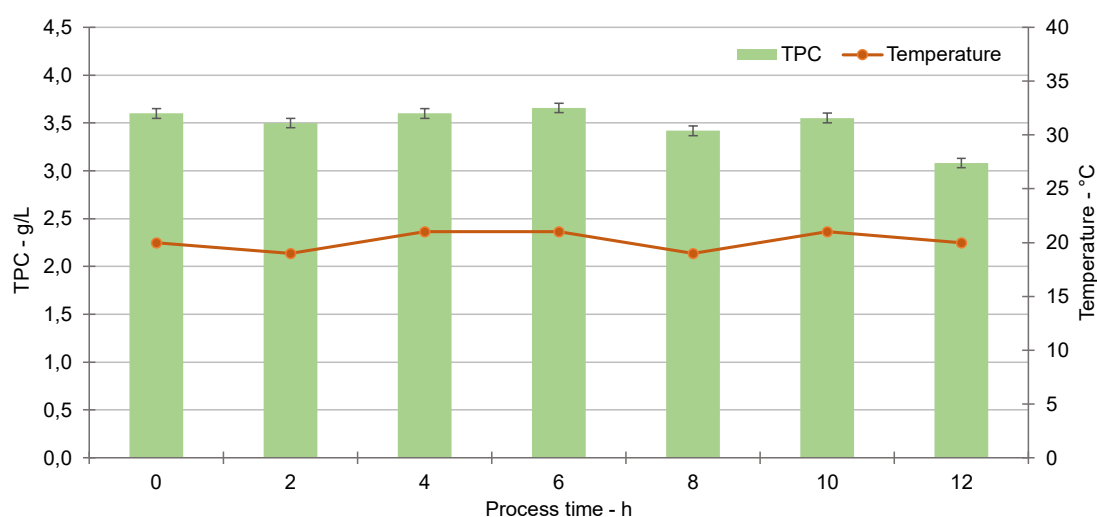


**Figure 5.2.** TPC concentration during UAE.

### 3.3. Solids/liquid separation

In order to recover the bioactive compounds, present in solution, a solids/liquid separation was performed on a decanter centrifuge. After processing the entire batch from UAE, the recovered supernatant still contained some solids (> 20% pellet). A second pass of the supernatant was performed, but no good separation was obtained. Several adjustments of the parameters were tested while recirculating the suspension, but this action did not improve the separation. A swelling of the solids was observed, probably due to the pectin present in solution and the long recirculation time, as already reported in literature for citrus fibers [420]. By applying the optimal process parameters, the pellet %vol in the final supernatant was decreased to 13 %vol. At this point, the process was stopped, and the supernatant was collected in the feed tank for the MF. The phenolic recovery in the supernatant stream was 60 % of the starting solid/liquid mixture. The pellet %Vol for the solids 74 %vol (**Table 5.2**). Even though a reprocessing of the material was

necessary, the S/L separation proved to be successful with the tested slurry. The immediate application of the optimal parameters since the beginning of the process without streams recirculation may have a beneficial effect on improving the separation by reducing the risks of solid swelling.

Considering that many compounds of interest may still be present in the discarded process stream, this material could be used for other applications, like animal feed, preparation of compost, or as feedstock in fermentative processes to produce enzymes and biomolecules [421].

### 3.4. Membrane filtration processes

First, the solids still present in the supernatant from decanter centrifuge were removed via ceramic MF (0.45  $\mu\text{m}$ ). MF is a crucial step following centrifugation in various industrial and laboratory processes to ensure the removal of fine particles and microorganisms that centrifugation alone may not eliminate. While centrifugation effectively separates larger particles based on their density, it often leaves behind smaller contaminants that can affect the clarity and purity of the filtrate. MF, with its fine pore sizes, addresses this by capturing these smaller particles, resulting in a clearer and higher quality product. Additionally, this step is essential for protecting downstream processes from fouling and damage, keeping the consistency and quality of the product, and meeting stringent regulatory standards, particularly in industries like pharmaceuticals, food and beverage, and biotechnology. By providing an added layer of filtration, MF ensures the overall efficiency and safety of the entire processing workflow [422]. The main process parameters are shown in **Figure 5.3**. The presence of solids in the 624 kg feed and the relatively small filtration area (0.6  $\text{m}^2$ ) caused the pressure in the system to be at relatively high value since the beginning of the process. The average trans membrane pressure (TMP) was 4.1 barg, with the maximum achieved permeate flux of 97  $\text{L/h}\cdot\text{m}^2$ . The flux decrease at the beginning of the process required a slightly dilution of the feed in order to improve the filtration process, followed by a recirculation of the retentate in the unit with the permeate outlet closed for about 15 min. This strategy allowed to (partially) unclog the ceramic membranes, which resulted in a permeate flux increase. The average pressure at inlet ( $P_{\text{in}}$ ) and pressure at outlet ( $P_{\text{out}}$ ) of the system throughout the process were 3.0 and 2.6 barg, respectively, corresponding to an average trans membrane pressure (TMP) of 2.6 barg, with the maximum achieved permeate flux of 97 LMH. The retentate and permeate temperature was kept at  $20 \pm 2$   $^{\circ}\text{C}$  by recirculating cold water in the jacket of the feed and receiving tanks. The filtration process run stable, but the relatively small surface area required about 15 h to complete the preconcentration step, followed by the continuous diafiltration with RO water (two diafiltration volumes). Considering these results, the overall MF process could be shortened if a larger filtration area is provided. Despite having similar content in phenolic compounds in the retentate (2.1 g/L) and the permeate+diafiltrate (2.5 g/L), it

is important to note that they were obtained 30 kg of retentate and 814 kg of permeate were obtained. No visual solids (as %Vol) was observed in the permeate stream, indicating that all solid was retained with the retentate. The increase DM% in this stream (10 %m/m) compared to the feed (6.3 %m/m) is an added indication of the increased solid content in the retentate stream. As observed for the solids/liquid separation process, those remaining compounds in the retentate could be used for other applications [421].

The 814 kg of MF permeate was further purified via UF. The effectiveness of UF processes to recover bioactive compounds from food matrices has been discussed in literature in recent years [410,411,423]. UF acts as an essential intermediate purification step that removes larger macromolecules and particulate matter, which MF alone may not eliminate. This pre-processing is crucial for protecting the more delicate NF membranes from fouling and damage, thereby enhancing their efficiency and lifespan. Additionally, UF can selectively retain and concentrate specific molecules, optimizing the feed for the subsequent NF step. While this process may result in the loss of some polyphenols in the UF retentate, it ensures a cleaner, more manageable feed for NF, ultimately improving the overall process efficiency and cost-effectiveness by reducing membrane fouling and maintenance requirements [424,425].

In the first minutes of the process, the TMP was decreased to delay potential flux drop and have a constant flux. A slight decrease in flux was only observed towards the end of the filtration (**Figure 5.3**). The average  $P_{in}$  and  $P_{out}$  throughout the process were 2.0 and 1.0 barg, respectively, corresponding to an average trans membrane pressure (TMP) of 1.5 barg, with the average permeate flux of 37 LMH. The filtration process run stable, also in the successive continuous diafiltration step with RO water (two diafiltration volumes), with the retentate (26.4 kg) and permeate (621 kg) temperature kept at  $20 \pm 2$  °C, and it proved to be successful in purifying the polyphenol stream, with about 90 % product recovery in the filtrate. It is likely that additional diafiltrations can increase the product yield. With this strategy, more water would be required, but then recovered at the end of the process, as described later.

Finally, the UF permeate was concentrated via nanofiltration (NF). The permeate flux decreased from the start to the end of the process, ranging from 9.0 to 3.0 L/h\*m<sup>2</sup>. The TMP was increased after about 2 h processing in order to keep the flux at an acceptable rate to complete the process within the expected time. The average  $P_{in}$  and  $P_{out}$  throughout the process were 11.5 and 10.8 barg, respectively, corresponding to an average trans membrane pressure (TMP) of 11.1 barg. Due to the concentration process and the high shear produced by the recirculating pump, the temperature of the solution increased during the filtration, reaching a maximum value of 20 °C (**Figure 5.3**). At this point, the temperature was decreased by recirculating cold water in the jacket of the retentate tank. A total of 76 kg of concentrated solution was collected corresponding to a volume concentration factor of 8 for this step, with 590 kg of permeate collected.

The TPC values highlighted product breakthrough in the permeate stream, causing the yield in the retentate stream to drop to 15 %. Considering that nanofiltration membranes with molecular weight cut-off similar to this work were already successfully tested in literature for the recovery of phenolic compounds [413], it can be inferred that a molecular weight cut-off smaller than 0.15 kDa should be tested for the phenolic compounds obtained from orange peels.

The TPC values highlighted product breakthrough in the permeate stream, causing the polyphenols concentration in the recovered retentate to drop to 3.3 g/L, which corresponds to a TPC yield in the retentate of 15 %. NF membranes with molecular weight cut-off like those used in this work were already successfully tested in literature for the recovery of phenolic compounds [413]. However, having into account that for example the phenolic acid protocatechuic acid has been reported to have a monoisotopic mass of 154.03 Da, and was retained on a 150 Da membrane [426], as in the orange peel extract the lowest phenolic acids could be ferulic (around 194 Da) or caffeic acid (around 180 Da), the phenolic compounds extracted from orange peels should be tested for retention on membranes with molecular weight cut-off smaller than 150 Da.

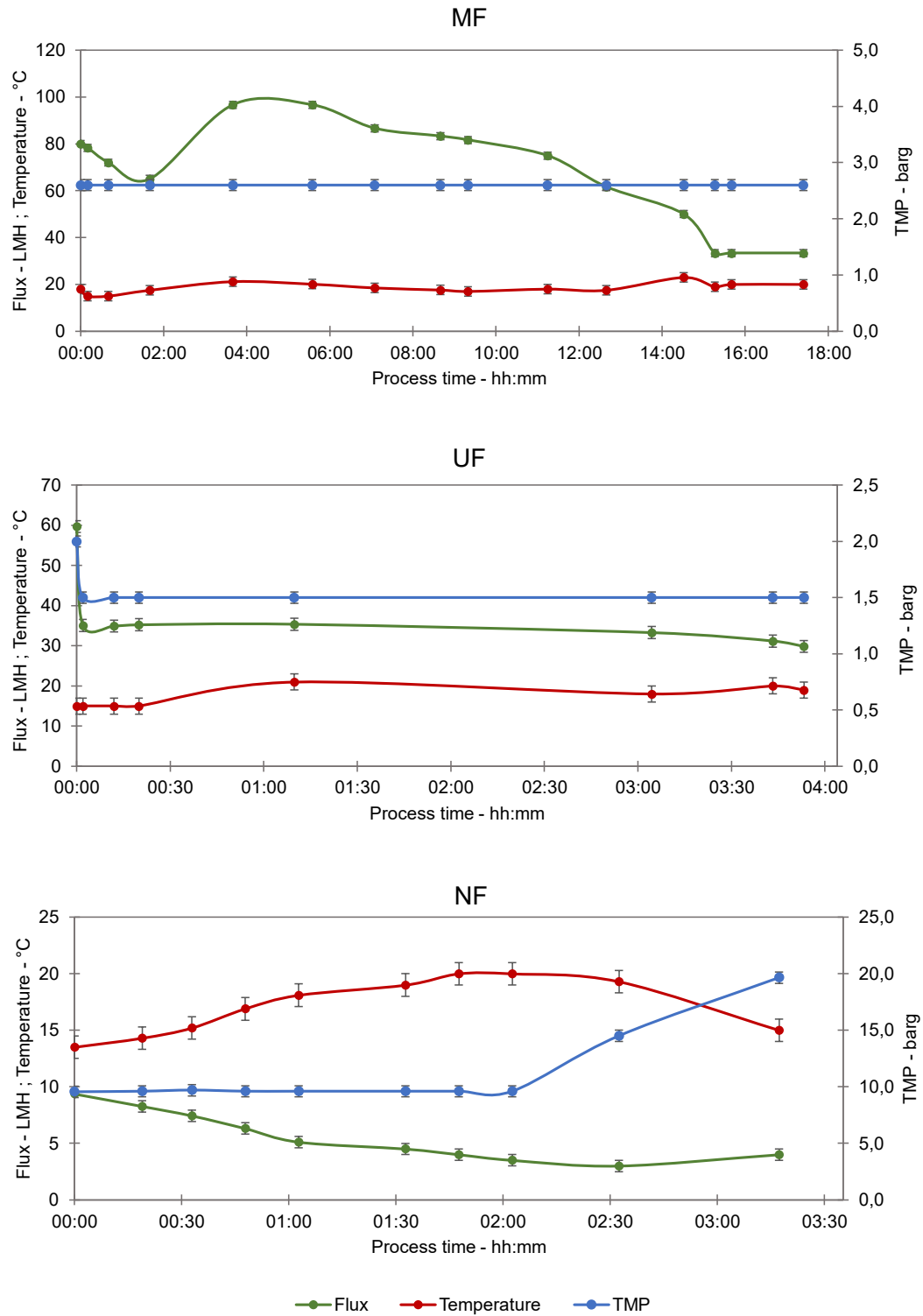


**Figure 5.3.** TMP, Permeate flux and Temperature profile of the sequential membrane filtration processes (MF -> UF -> NF).

### 3.5. Analysis end product

The sugars present in the final product were quantified by HPLC-RID. Galacturonic acid, glucose and fructose were detected as main sugar compounds, with concentrations of  $23.5 \pm 0.1$ ,  $22.5 \pm 0.1$ ,  $30.6 \pm 0.1$  g/L, respectively. The relatively high sugars concentration in the final OPE, compared to the concentration of total reducing sugars found in other fruit peels obtained via saccharification such as mango and pineapple peels [427], can be justified by the heating issue occurred during enzymatic hydrolysis, for which the relatively high temperature may have caused additional degradation of the OP structure, and in particular of the pectin [428], for which released sugars were carried over during the subsequent DSP steps. The concentration of sugars presents in concentrated OPE suggests that a further optimization of the downstream process may be required to improve the purity of the product, according to its application. A further process development including the recovery of these sugars can result in a purer OPE and a sugar-rich stream to exploit in other applications (e.g., fermentative processes), stressing even more the biorefinery concept intrinsic of this work.

The phenolic compounds found in the final product were quantified by HPLC-ESI-TOF-MS and are listed in **Table 5.4**, with their retention time (min),  $m/z$  and molecular formula.

**Table 5.4.** Phenolic compounds identified and quantified in the technical and food grade runs by HPLC-ESI-TOF-MS. Results are expressed as  $\mu\text{g/mL}$  extract with average  $\pm$  standard deviation.

| RT (min) | Molecular formula                         | $m/z$    | Compound                                      | $\mu\text{g/mL}$ extract |
|----------|-------------------------------------------|----------|-----------------------------------------------|--------------------------|
| 2.24     | $\text{C}_{16}\text{H}_{18}\text{O}_{11}$ | 385.0769 | 2-(E)-O-Feruloyl-D-galactaric acid isomer I   | $219.36 \pm 0.29$        |
| 2.65     | $\text{C}_{15}\text{H}_{16}\text{O}_{10}$ | 355.0667 | Caffeic acid 3-O-glucuronide isomer I         | $89.81 \pm 0.13$         |
| 2.75     | $\text{C}_{16}\text{H}_{18}\text{O}_{11}$ | 385.0771 | 2-(E)-O-Feruloyl-D-galactaric acid isomer II  | $457.07 \pm 0.60$        |
| 3.40     | $\text{C}_{16}\text{H}_{18}\text{O}_{11}$ | 385.0768 | 2-(E)-O-Feruloyl-D-galactaric acid isomer III | $494.05 \pm 0.65$        |
| 3.64     | $\text{C}_{15}\text{H}_{16}\text{O}_{10}$ | 355.0680 | Caffeic acid 3-O-glucuronide isomer II        | $134.18 \pm 0.18$        |
| 3.69     | $\text{C}_{16}\text{H}_{18}\text{O}_{11}$ | 385.0789 | 2-(E)-O-Feruloyl-D-galactaric acid isomer IV  | $31.26 \pm 0.05$         |
| 3.81     | $\text{C}_{21}\text{H}_{32}\text{O}_{10}$ | 443.1910 | Cynaroside A                                  | $37.16 \pm 0.14$         |
| 4.17     | $\text{C}_{16}\text{H}_{16}\text{O}_{10}$ | 367.0662 | Feruloyl isocitric acid isomer I              | $69.36 \pm 0.10$         |
| 4.53     | $\text{C}_{16}\text{H}_{18}\text{O}_{11}$ | 385.0789 | 2-(E)-O-Feruloyl-D-galactaric acid isomer V   | $317.75 \pm 0.42$        |
| 4.66     | $\text{C}_{16}\text{H}_{16}\text{O}_{10}$ | 367.0675 | Feruloyl isocitric acid isomer II             | $176.13 \pm 0.24$        |
| 4.68     | $\text{C}_{12}\text{H}_{12}\text{O}_6$    | 251.0550 | Caffeoylglycolic acid methyl ester            | $90.25 \pm 0.13$         |
| 4.94     | $\text{C}_{17}\text{H}_{22}\text{O}_{10}$ | 385.1139 | Sinapoyl-D-glucoside                          | $9.72 \pm 0.02$          |
| 5.03     | $\text{C}_{17}\text{H}_{20}\text{O}_{11}$ | 399.0939 | Sinapinic acid-O-glucuronide isomer I         | $267.95 \pm 0.36$        |
| 5.24     | $\text{C}_{17}\text{H}_{20}\text{O}_{11}$ | 399.0939 | Sinapinic acid-O-glucuronide isomer II        | $18.67 \pm 0.03$         |
| 5.47     | $\text{C}_{27}\text{H}_{30}\text{O}_{16}$ | 609.1467 | Rutin isomer I                                | $39.91 \pm 0.14$         |
| 5.84     | $\text{C}_{16}\text{H}_{16}\text{O}_{10}$ | 367.0674 | Feruloyl isocitric acid isomer III            | $99.37 \pm 0.14$         |
| 5.99     | $\text{C}_{27}\text{H}_{30}\text{O}_{15}$ | 593.1506 | Apigenin-di-C-hexoside (Vicenin-2)            | $164.58 \pm 0.41$        |
| 6.39     | $\text{C}_{21}\text{H}_{22}\text{O}_{10}$ | 433.1132 | Prunin                                        | $25.02 \pm 0.11$         |
| 6.41     | $\text{C}_{28}\text{H}_{32}\text{O}_{16}$ | 623.1607 | Isorhamnetin-3-O-rutinoside isomer I          | $38.29 \pm 0.14$         |
| 6.45     | $\text{C}_{13}\text{H}_{12}\text{O}_8$    | 295.0446 | Caffeoylmalic acid isomer I                   | $160.91 \pm 0.22$        |
| 6.57     | $\text{C}_{28}\text{H}_{32}\text{O}_{16}$ | 623.1605 | Isorhamnetin-3-O-rutinoside isomer II         | $7.33 \pm 0.07$          |
| 6.78     | $\text{C}_{21}\text{H}_{20}\text{O}_{11}$ | 447.0934 | Quercitrin                                    | < LOQ                    |
| 7.25     | $\text{C}_{34}\text{H}_{44}\text{O}_{20}$ | 771.2352 | Alpha-Glucosyl Hesperidin                     | < LOQ                    |
| 7.56     | $\text{C}_{26}\text{H}_{28}\text{O}_{14}$ | 563.1400 | Vitexin-O-pentoside isomer I                  | $39.81 \pm 0.14$         |
| 7.94     | $\text{C}_{26}\text{H}_{28}\text{O}_{14}$ | 563.1408 | Vitexin-O-pentoside isomer II                 | < LOQ                    |
| 8.01     | $\text{C}_{27}\text{H}_{34}\text{O}_{15}$ | 597.1816 | Naringin hydrate                              | $9.98 \pm 0.08$          |
| 8.22     | $\text{C}_{15}\text{H}_{12}\text{O}_5$    | 271.0603 | Naringenin                                    | < LOQ                    |

| RT (min)                  | Molecular formula                               | m/z      | Compound                                                    | µg/mL extract  |
|---------------------------|-------------------------------------------------|----------|-------------------------------------------------------------|----------------|
| 8.24                      | C <sub>13</sub> H <sub>12</sub> O <sub>8</sub>  | 295.0428 | Caffeoylmalic acid isomer II                                | 100.43 ± 0.14  |
| 8.49                      | C <sub>27</sub> H <sub>32</sub> O <sub>14</sub> | 579.1696 | Narirutin isomer I                                          | 129.35 ± 0.34  |
| 8.77                      | C <sub>27</sub> H <sub>30</sub> O <sub>16</sub> | 609.1465 | Rutin isomer II                                             | 3.26 ± 0.07    |
| 8.80                      | C <sub>27</sub> H <sub>32</sub> O <sub>15</sub> | 595.1662 | Eriocitrin                                                  | < LOQ          |
| 8.85                      | C <sub>27</sub> H <sub>32</sub> O <sub>14</sub> | 579.1733 | Narirutin isomer II                                         | < LOQ          |
| 9.01                      | C <sub>16</sub> H <sub>14</sub> O <sub>6</sub>  | 301.0723 | Hesperetin                                                  | < LOQ          |
| 9.14                      | C <sub>28</sub> H <sub>34</sub> O <sub>15</sub> | 609.1842 | Hesperidin                                                  | 95.98 ± 0.26   |
| 9.67                      | C <sub>35</sub> H <sub>42</sub> O <sub>22</sub> | 813.2089 | Kaempferol 3-[2"-glucosyl-6"-acetyl-galactoside]7-glucoside | < LOQ          |
| 9.74                      | C <sub>28</sub> H <sub>34</sub> O <sub>15</sub> | 609.1842 | Neohesperidin                                               | < LOQ          |
| 9.98                      | C <sub>28</sub> H <sub>32</sub> O <sub>16</sub> | 623.1614 | Isorhamnetin-3-O-rutinoside isomer III                      | < LOQ          |
| 11.21                     | C <sub>20</sub> H <sub>30</sub> O <sub>14</sub> | 577.1507 | Apigenin 7-O-neohesperidoside                               | 4.13 ± 0.07    |
| 11.26                     | C <sub>16</sub> H <sub>14</sub> O <sub>5</sub>  | 285.0773 | Isosakuranetin                                              | < LOQ          |
| 12.41                     | C <sub>28</sub> H <sub>34</sub> O <sub>14</sub> | 593.1885 | Didymin                                                     | < LOQ          |
| 15.37                     | C <sub>19</sub> H <sub>18</sub> O <sub>8</sub>  | 373.0923 | 3',4'-Didemethylnobiletin                                   | < LOQ          |
| Sum of phenolic acids     |                                                 |          |                                                             | 2736.28 ± 3.70 |
| Sum of flavonoids         |                                                 |          |                                                             | 568.37 ± 1.98  |
| Sum of phenolic compounds |                                                 |          |                                                             | 3304.65 ± 5.68 |

They were quantified a total of 41 phenolic compounds, being 16 phenolic acids derivatives and 25 flavonoids. The final obtained had a content of phenolic compounds of 3304.65 µg/mL extract. Phenolic acids derivatives accounted for 83 %. The major quantified compound was 2-(E)-O-Feruloyl-D-galactaric acid, a ferulic acid derivative reported for the first time in citrus in 1987 [282]. Ferulic acid and its derivatives have been previously attributed several bioactivities such as antioxidant, anti-inflammatory and antibacterial properties [429]. In this final product account for 46 % of the total phenolic compounds. The second major compounds were caffeic acid derivatives accounting for 575.58 ± 0.80 µg/mL. They were mainly caffeic acid 3-O-glucuronide, caffeoylglycolic acid methyl ester and caffeoylmalic acid, also with demonstrated bioactivities in human health [430,431]. Besides, sinapic acid derivatives were found too. Sinapic acid have been reported to exhibit antioxidative, antimicrobial, anti-inflammatory, anticancer, and anxiolytic properties [432]. Extensively, also the sinapic acid derivatives found in this product, sinapoyl-D-glucoside and sinapinic acid-O-glucuronide [432]. No previous references of isolating or purifying phenolic acids or orange peel extract were found.

Regarding flavonoids, despite being the minor groups of compounds, the importance and relevance of them is widely spread in the research. They accounted for 17 %. Naringenin, known as 5,7,40-trihydroxyflavanone, is highly prevalent in Citrus fruits, and it can be found in its aglycone form or in glycosides [293]. In this final product this aglycone form (naringenin) has been detected in a minor amount. The glycoside naringin has been detected also in a minor amount as naringin hydrate, being one of the principal phenolic responsible for the bitter taste [293]. Narirutin, another significant naringenin glycoside also has been found in this final product, being the most dominant form of naringenin. Also, the glycoside prunin has been quantified. In overall naringenin and derivatives were 29 % of the flavonoids quantified too. They have been reported for displaying strong anti-inflammatory and antioxidant activities with impact in several



pathologies [433–435]. In other studies optimizing the extraction [257] these compounds accounted for 20–26% of the total flavonoids, so there is an improvement here in the extraction yield.

Besides, naringenin is the key intermediate of the flavone/anthocyanin pathway, serving as the common precursors for a large number of downstream flavonoids such as apigenin. In fact, three apigenin derivatives have been found in this final orange product such as apigenin-di-C-hexoside, vitexin-O-pentoside and apigenin 7-O-neohesperidoside. These apigenin derivatives suppose 37 % of the total flavonoids.

Like naringenin, hesperetin and their glycosides are prevalent components in Citrus fruits. The glycosides of hesperetin, namely hesperidin and neohesperidin, are more widespread compared to the aglycone form [293]. In this final product there are low amounts of hesperetin and neohesperidin as well as alpha-glucosyl hesperidin but higher of the hesperidin form accounting for 15% of the total flavonoids [436]. Other authors reported this compound as the main phenolic present in orange peel extract used to create films [437].

Moreover, the flavonoid profile of the end product was composed by 8% of isorhamnetin-3-O-rutinoside, demonstrated to exhibit protective effects on the cardiovascular and cerebrovascular systems, acts as an anti-tumor, anti-inflammatory, and antioxidant agent, provides organ protection, and aids in preventing obesity [438].

The eriodictyol glycoside eriocitrin, and isokuratanetin and its glycoside didymin, didemethylnobiletin, a kaempferol derivative and cynaroside A (a luteolin derivative) were also detected in minor amounts. Some authors have reported to isolate polymethoxyflavones such as nobiletin derivatives from orange peel extract by using supercritical chromatography, a technique that seem mor promising for this kind of compounds, however the scalability of this methodology is more expensive [439].

Thus, in terms of polyphenols the final product obtained have an interesting profile with potential bioactivities with benefits in human health. Membrane filtration was previously applied to orange peel extract for different purposes such isolating pectin-derived oligosaccharides [440] and fatty acids [441].

Besides, the phenolic compounds profile could have potential for food applications such as packaging. Previous published paper relative to creating new food packaging using orange peel extracts did not characterize the phenolic profile [403,404,442–447]. However, they extensively reported the phenolic compounds present in orange peel extract exhibited high antioxidant and antimicrobial activities in the incorporated films that can be successfully used to extend shelf life of food. Phenolic compounds extracted from orange peel have been demonstrated to play a

significant role in enhancing food packaging materials due to their diverse bioactive properties. Flavonoids and phenolic acids, help prevent the oxidation of lipids in food products, thereby extending the shelf life of packaged foods. By slowing down oxidative degradation processes, they maintain the quality and freshness of food over time. Moreover, phenolic compounds exhibit robust antimicrobial activity against a variety of foodborne pathogens. Incorporating these compounds into packaging materials can inhibit the growth of bacteria, fungi, and other microorganisms, significantly reducing the risk of food spoilage and contamination. This antimicrobial effect enhances food safety, making the packaging more effective in preserving the integrity of its contents. In addition to their bioactive properties, phenolic compounds can improve the physical properties of packaging materials as well. They can interact with polymer matrices to enhance mechanical strength, barrier properties (against oxygen, moisture, and UV light), and thermal stability. Improved barrier properties are essential for maintaining the packaged food's integrity and extending its shelf life.

This characterization of the orange peel extract obtained by green methodology would be as a first overview for further studies. It is just a preliminary approach study which requires a further evaluation of this by-product and its phenolic fraction in a protective action against oxidative and microbiological degradation of foods.

### 3.6. Food grade analysis

The well-known preservative properties of polyphenols make them suitable to be added in active food packaging to retard the microbial invasion and putrefaction of many fruits and vegetables [402]. Taking into account this application, the OPE was produced following the food grade legislation provided by the European Union [448].

The food grade analysis of dried orange peels (OP) and final extract (OPE) are reported in **Table 5.5**. Since the accuracy of measurement on the pesticides screening is 50 %, the results on final product are considered conform the EU specifications. The positive results allowed to release the produced OPE as food ingredient, making possible to add these bioactive compounds in food packaging.

**Table 5.5.** Food grade analysis of dried orange peels (OP) and extract (OPE)

| Parameter                          | Method              | Results OP | Results OPE | Unit      |
|------------------------------------|---------------------|------------|-------------|-----------|
| <b>Microbial load</b>              |                     |            |             |           |
| Total aerobic plate count at 30 °C | Colony-count method | 800        | 60          | cfu/g  mL |
| Coliforms at 30 °C                 | Colony-count method | n.a.       | < 1         | cfu/g  mL |
| <i>E. coli</i>                     | Colony-count method | < 10       | < 1         | cfu/g  mL |
| <i>Enterobacteriaceae</i> at 30 °C | Colony-count method | n.a.       | < 1         | cfu/g  mL |
| Yeast                              | Colony-count method | 300        | < 10        | cfu/g  mL |

| Parameter                               | Method                                              | Results OP         | Results OPE          | Unit                      |
|-----------------------------------------|-----------------------------------------------------|--------------------|----------------------|---------------------------|
| Molds                                   | Colony-count method                                 | < 100              | < 10                 | cfu/g mL                  |
| <i>Listeria monocytogenes</i>           | detection                                           | n.d.               | n.d.                 | cfu/25g                   |
| <i>Pseudomonas aeruginosa</i>           | Colony-count method                                 | n.a.               | < 1                  | cfu/g mL                  |
| <i>Salmonella</i>                       | detection                                           | n.d.               | n.d.                 | cfu/25g                   |
| <i>Staphylococcus aureus</i>            | detection                                           | < 10               | n.d.                 | cfu/g                     |
| Allergens                               |                                                     |                    |                      |                           |
| Allergen egg                            | ELISA                                               | n.a.               | n.d. (< 30)          | mg allergenic protein /kg |
| Allergen gluten                         | R5-sandwich ELISA                                   | < 5.0              | < 5.0                | mg allergenic protein /kg |
| Allergen soy                            | ELISA                                               | n.d. (< 4)         | n.d. (< 4)           | mg allergenic protein /kg |
| Allergen hazelnut                       | ELISA                                               | n.d. (< 1.0)       | < 0.4                | mg allergenic protein /kg |
| Allergen walnut                         | outsourcing                                         | n.a.               | n.d. (< 2.0)         | mg/kg                     |
| Allergen sulphite (as SO <sub>2</sub> ) | outsourcing                                         | < 10               | < 10                 | mg/kg                     |
| Beta-lactoglobulin                      | ELISA                                               | n.d. (< 1.5)       | n.d. (< 1.5)         | mg allergenic protein /kg |
| Allergen casein                         | ELISA                                               | n.d. (< 1.5)       | n.d. (< 1.5)         | mg allergenic protein /kg |
| Allergen lactose                        | GCFID-determination after derivatization with TMSIM | < 0.010            | < 0.010              | %                         |
| Pesticides                              |                                                     |                    |                      |                           |
| Perchlorate                             | outsourcing                                         | 0.013              | < 0.010              | mg/kg                     |
| Pesticides                              | outsourcing GCMS                                    | No compounds > LOQ | No compounds > LOQ   |                           |
| Pesticides                              | outsourcing LCMS                                    | No compounds > LOQ | See <b>Table 5.6</b> |                           |
| Heavy metals                            |                                                     |                    |                      |                           |
| Arsenic (As)                            | ICPMS                                               | < 0.050            | < 0.050              | mg/kg                     |
| Cadmium (Cd)                            | ICPMS                                               | < 0.0050           | < 0.0050             | mg/kg                     |
| Lead (Pb)                               | ICPMS                                               | 0.027 ± 0.0051     | < 0.020              | mg/kg                     |
| Mercury (Hg)                            | Cold vapor technique and atomic absorption          | < 0.0050           | < 0.0050             | mg/kg                     |
| Mycotoxins                              |                                                     |                    |                      |                           |
| Deoxynivalenol (DON)                    | LCMSMS                                              | < 75               | < 75                 | mg/kg                     |
| Zearalenon                              | LCMSMS                                              | < 10               | < 10                 | mg/kg                     |
| Fumosin B1                              | LCMSMS                                              | < 125              | < 125                | mg/kg                     |
| Fumosin B2                              | LCMSMS                                              | < 50.0             | < 50.0               | mg/kg                     |
| T2                                      | LCMSMS                                              | < 4.00             | < 4.00               | mg/kg                     |
| HT2                                     | LCMSMS                                              | < 4.00             | < 4.00               | mg/kg                     |
| Ocratoxin A                             | LCMSMS                                              | < 1.00             | < 1.00               | mg/kg                     |
| Aflatoxin – B1                          | LCMSMS                                              | < 0.500            | < 0.500              | mg/kg                     |
| Aflatoxin – B2                          | LCMSMS                                              | < 0.500            | < 0.500              | mg/kg                     |
| Aflatoxin – G1                          | LCMSMS                                              | < 0.500            | < 0.500              | mg/kg                     |

| Parameter                       | Method        | Results OP                                   | Results OPE                                  | Unit  |
|---------------------------------|---------------|----------------------------------------------|----------------------------------------------|-------|
| Aflatoxin – G2                  | LCMSMS        | < 0.500                                      | < 0.500                                      | mg/kg |
| Aflatoxin – total               | LCMSMS        | < 0.500                                      | < 0.500                                      | mg/kg |
| DNA                             |               |                                              |                                              |       |
| DNA detection                   | Real time PCR | DNA detected                                 | GMO n.d.                                     | -     |
| P35S QL rt (NG)                 | Real time PCR | Not demonstrated<br>at level of 0.9 %<br>GMO | Not demonstrated<br>at level of 0.1 %<br>GMO | -     |
| tNOS QL rt (NG)                 | Real time PCR | Not demonstrated<br>at level of 0.9 %<br>GMO | Not demonstrated<br>at level of 0.1 %<br>GMO | -     |
| pFMV QL rt<br>screening (CRA-W) | Real time PCR | Not demonstrated<br>at level of 0.9 %<br>GMO | Not demonstrated<br>at level of 0.1 %<br>GMO | -     |

n.a. = not analyzed, n.d. = not detected

**Table 5.6.** Pesticides screening of OPE.

| Parameter                  | Method | Unit  | Test result         | Specification        |
|----------------------------|--------|-------|---------------------|----------------------|
| <i>Pesticide screening</i> | GCMS   | mg/kg | No compounds > LOQ  | < EU MRL*            |
|                            | LCMS   | mg/kg | Compounds > LOQ:    | EU food legislation: |
|                            |        |       | Trinexapac: 0.012   | MRL max 0.01         |
|                            |        |       | Isoxaflutole: 0.025 | MRL max 0.02         |
|                            |        |       | Tembrotion: 0.022   | MRL max 0.02         |

\*MRL = Maximum Residue Level

### 3.7. Process industrialization

Even though losses of polyphenols were observed during the process, this work still highlights how bioactive compounds can be recovered via non-thermal extraction (and recovery) systems that can be potentially scaled up once the optimized process is established. Processing at room temperature (or below) and reduced amount of chemicals (e.g. solvents) are some of the advantages of this technology compared to classical (thermal) extraction system, such as maceration, decoction, and solvent extraction [405], for which higher costs in terms of energy consumption and process sustainability should be taken into account.

The OP tested in this study was bought as dry product due to logistic issues related to the cold storage of large amount of fresh OP at BBEPP, for which the product quality might have not been guaranteed. At industrial scale, the processing of non-dried OP would reduce the amount of water requested for the slurry preparation since water is already present in the product. A cutting/milling step would be required to produce a pumpable, hence processable slurry, and to reduce the particle size in order to improve the efficiency of the enzymatic hydrolysis. If the Considering the UAE results discussed previously, if this step were removed from the process, the scale up of the other equipment, from tanks to decanter to filtration units, would be less challenging since their technology is already well-developed at large scale. The capital expenses to build and run a plant for OPE production may be reduced by applying several accessible strategies: I. by building the plant close to an orange processing facility (juices/smoothies producers) and processing in

continuous mode would reduce the costs of transportation and (partly) solve the storage issues of the fruit residues, if the biomass is immediately processed; II. the water used in the filtration steps is collected at the end of the process as NF permeate. The re-use of this water (e.g., making the starting OP slurry) can significantly reduce the environmental impact of the process. After several cycles of reuse, a treatment process (e.g., RO filtration) can be applied to remove the accumulated by-products and obtain clean water to use again in the process or for other applications [449]; III. the valorization of the waste streams, such as solids from decanter or retentate from MF [421]. The suggested strategies are an example of a possible industrialization of the process described in this work, which should be then supported by in-depth studies on financial aspects (CAPEX and OPEX), together with evaluation of life cycle assessment (LCA) and life cycle costing (LCC).

#### **4. Conclusions**

Dry orange peels (OP) were processed in a pilot scale run to extract and recover bioactive compounds. This paper gives the first positive indication on the scale-up of phenolic compounds extraction and recovery from a by-product (orange peels) with non-thermal technologies. The proposed process demonstrated successful scalability, producing phenolic compounds at a 1000 L scale in a food-grade manner. The enzymatic hydrolysis of pectin followed by ultrasound-assisted extraction did not significantly enhance yield, suggesting the potential elimination of ultrasound to reduce energy consumption. The sequential purification using MF, UF and NF yielded a final product with a rich phenolic profile and notable bioactivities. While the process showed promise, future research should focus on optimizing the enzymatic hydrolysis to maximize phenolic compound extraction efficiency and energy savings. The use of fresh OP, combined with the possible treatment and re-use of processing water, as well as the utilization of waste streams in other application (e.g., fertilizer and/or animal feed) make this system compatible with the biorefinery and sustainability concepts. Detailed LCA and LCC analyses are essential to fully understand the environmental and economic impacts of the proposed process. Additionally, exploring the potential of integrating this process into existing biorefinery frameworks could enhance sustainability and economic viability. Besides, the final product has been demonstrated to be food grade in microbial, mycotoxins, allergens, pesticides, and heavy metal terms. Expanding the scale of production and exploring other by-products for phenolic compound extraction could broaden the application of this technology, offering new opportunities for the valorization of agricultural waste, and contributing to the development of a sustainable waste management. The further exploration, from various aspects, of non-thermal technologies to recover polyphenols from OP (and other AFWs) can open avenues for utilizing this biomass in high-value applications, aligning with the principles of a circular economy.

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## SECTION 2. AVOCADO BY- PRODUCTS





## **CHAPTER 6. Mathematical modelling of drying kinetics of avocado peels and its influence on flavan-3-ols content and antioxidant activity**

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## Mathematical modelling of drying kinetics of avocado peels and its influence on flavan-3-ols content and antioxidant activity

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### Abstract

Avocado peel is one of the main by-products of avocado processing and is considered a promising source of phenolic compounds with various bioactivities. The drying step is essential for its storage at the industrial level, and it is the first step in the strategy of transforming by-products into functional ingredients. Therefore, this research evaluates the effect of the convective air-drying of avocado peels at three different temperatures (40, 60 and 80 °C) and airflows (0, 0.8 and 1.6 m/s) on the flavan-3-ols content and antioxidant activity. Moreover, the mathematical modelling of its drying kinetic was developed. A decrease in the flavan-3-ol and antioxidant content was found with increasing temperatures. However, a high impact of the airflow reducing the drying time and limiting the decrease in interesting compounds was found. Among the tested mathematical models, the Page model reported the highest values of  $R^2$  (from 0.9907 to 0.9973) and the lowest errors for most of the temperatures and airflows tested. However, at 80 °C with airflow, the Lewis model seemed to fit better ( $R^2 = 0.9982$ ). Finally, the drying conditions that showed the lowest decrease in procyanidin and antioxidants were 40 °C and an airflow of 1.6 m/s for 105 min.

**Keywords:** HPLC-FLD; avocado by-product; avocado wastes; air-drying; polyphenols.

## 1. Introduction

Worldwide avocado (*Persea americana*) production has been growing, reaching a volume of 8.02 million tons in 2020. The largest producing countries of avocado are those with subtropical climate conditions, such as México, Dominican Republic and Perú. In 2019 the avocado industry had a worldwide value of 12.82 billion dollars, which is expected to increase over the next five years. In this context, avocado production in Spain is also constantly growing. In the last three years, it has increased from 89.7 thousand tons in 2018 to 95.5 thousand tons in 2019 and to 107.9 thousand tons in 2020. Moreover, in 2020 Spain was the third and fourth worldwide country in exporting and importing avocados, with values of 442.89 and 389.3 million dollars, respectively [450]. However, all these movements and avocado production are increasing waste generation, which also has economic and environmental costs.

Avocado by-products are industrial waste generated during the processing of avocado fruit, principally composed of peels and seeds [111]. These by-products are a potential source of bioactive compounds with various bioactivities, such as antimicrobial, anti-inflammatory, anticancer, antidiabetic and antihypertensive [110]. Special attention has been paid to agro-industrial waste because of its potential use in developing high-value-added products such as nutraceuticals or cosmeceuticals while reducing its environmental impact [57]. In this context, finding the best procedure to take advantage of these by-products should be an important research focus. Especially avocado peel is a well-known source of polyphenols such as phenolic acids and flavonoids such as (epi)catechin and its derivatives procyanidins at different degrees of polymerization. However, the concentration and stability of these compounds are affected by several factors, such as the avocado variety, light and oxygen exposure, pH, storage temperature and extraction solvents and procedure.

Drying is one of the most common and extended processes used to preserve quality and stability and to extend the shelf-life of fruits, vegetables, and their by-products. It reduces the moisture content and water activity avoiding spoilage during management and storage [130]. Among all the different techniques used in this scope, convective drying has been demonstrated to be the most scalable and cheapest at the industrial scale [138]. Finding the best conditions to dry avocado peels is important, and some aspects should be considered. Temperature is important because the bioactive and antioxidant compounds are thermolabile, and high temperature could lead to their destruction. Moreover, in economic and industrial terms, the drying process should be short and low energy consuming. Furthermore, drying involves a simultaneous transference of heat and mass, turning it into a complex phenomenon. Therefore, using mathematical models to simulate its kinetic and explain the mechanism of the water transference during this operation is a useful tool to control the process [136].

Some authors have evaluated the effect of drying on some parameters of the avocado peel [451,452]; however, they did not discuss its drying kinetic. Avhad & Marchetti (2016) described the drying behavior of 'Hass' avocado seeds using four mathematical models [244]. Saavedra et al. (2017) analyzed the avocado by-product drying kinetics using the Lewis model, but no other more complex models were considered [224]. Moreover, they optimized the convective drying process in avocado peel by focusing on the total phenolic content measured using Folin-Ciocalteu reagent. Alkaltham et al. (2021) evaluated the effect of different drying techniques on the polyphenol content in different parts of avocado [453]. They reported catechin concentrations of 99.11, 180.37 and 62.52 mg/100g in ripe pulp, peel, and seeds, respectively, of avocado oven-dried at 60 °C measured using high-performance liquid chromatography (HPLC) with photodiode array detector (PDA). According to these authors, the avocado peel is the richest avocado part in catechins and, by extension, in flavan-3-ols. However, its drying kinetics have not been completely studied to date. Moreover, according to Vidal-Casanella et al. (2022) [454], the HPLC with a fluorometric detector (FLD) showed better selectivity and sensitivity for measuring flavan-3-ols compositions of fruit samples being simpler, cheaper, and more robust alternative than other sophisticated methods, such as those based on HPLC-mass spectrometry (MS). To our knowledge, the influence of air convective drying on the procyanidin content of the avocado by-products has never been reported before.

Considering the above, the aim of the present research is to evaluate the effect of convective air-drying of avocado peels targeting their procyanidin content and antioxidant activity. It is the first step in transforming these by-products into functional ingredients or nutraceuticals. Moreover, the mathematical modelling of its drying kinetic was developed using fourteen different models to investigate the avocado peel behavior and to find the model that fits the best.

## **2. Materials and methods**

### **2.1. Chemicals and samples**

Catechin, Trolox, DPPH (1,1-diphenyl-2-(2,4,6-trinitrophenyl) hydrazine), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) and FRAP (ferric reducing antioxidant power) reagents with purity  $\geq 98\%$  in all cases were acquired from Sigma-Aldrich (St. Louis, MO, USA). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). HPLC grade water reagent and other reagents and solvents were purchased from Merck KGaA (Darmstadt, Germany).

The avocado by-product from the variety "Hass" was obtained from a company located in Motril, on the subtropical coast of Granada province, Spain, in October 2020. The by-product was obtained directly after the industrial processing for obtaining guacamole. It was maintained at 5



°C and then the samples were dried, milled and sieved to 100 µm and remained at -18 °C till analysis.

## 2.2. Drying process and kinetic

Samples of avocado peels were dried by convective drying using an oven (Mettler, Schwabach, Germany). The weight loss was measured manually with a precision manual balance (Sartorius, Göttingen, Germany) in less than 15 seconds until the moisture content was below 10% [137].

Similarly to other authors [220–225], the temperatures tested were 40, 60 and 80 °C. In addition, three different airflows were tested according to the oven possibilities: 0, 0.8 and 1.6 m/s.

At each time point, the moisture content was expressed as moisture ratio (MR) according to the weight loss and solid content remaining, a dimensionless term calculated from equation (1):

$$MR = \frac{M_t - M_e}{M_0 - M_e} \quad (1)$$

where  $M_0$ ,  $M_t$  and  $M_e$  are the moisture content (g/g dry weight (d.w.)) at the initial time, time  $t$ , and equilibrium, respectively.

The drying rate (DR) was calculated to express the moisture loss per unit of time, which can be calculated according to equation (2):

$$DR = \frac{M_{t1} - M_{t2}}{t_2 - t_1} \quad (2)$$

where  $M_{t1}$  and  $M_{t2}$  are the moisture content (g/g d.w.) at the time points  $t_1$  and  $t_2$ , respectively.

The effective moisture diffusivity ( $D_{eff}$ ) was calculated according to Fick's second law when assuming that the sample shrinkage is negligible. Therefore, the initial moisture distribution is uniform, and the moisture diffusivity is constant, as shown in equation (3):

$$MR = \left(\frac{8}{\pi^2}\right) e^{\left(-\frac{\pi^2 D_{eff}}{4L^2} t\right)} \quad (3)$$

Where MR,  $D_{eff}$ ,  $L$  and  $t$  are the moisture ratio, effective moisture diffusivity, the half-thickness of the sample (m) and the time (min), respectively.

Additionally, the Arrhenius equation was used to calculate the activation energy ( $E_a$ ) described in equation (4):

$$D_{eff} = Ae^{(-E_a/RT)} \quad (4)$$

Where  $D_{\text{eff}}$ ,  $A$ ,  $E_a$ ,  $R$  and  $T$  are the effective moisture diffusivity, the pre-exponential factor of the Arrhenius equation, the activation energy (KJ/mol), the universal gas constant (8.314 J/mol K) and the temperature (K), respectively.

Finding the best-fitting model for avocado by-product drying behavior is extremely important to predict its drying kinetics accurately. The drying mathematical modelling has been studied recently in other fruits and vegetables such as asparagus root [214], dragon fruit [215], edamame [216], pomelo albedo [217], uvaia fruit (Gomes et al., 2021), yam (Sahoo et al., 2022), *Ascophyllum Nodosum* [455] or black gram papad [255].

Therefore, the experimental drying data was fitted with fourteen semi-theoretical drying mathematical models for the three different temperatures and the three different airflows, and the mathematical equations (5-9 and 17-25) are shown in **Table 6.1**, where MR is the moisture ratio,  $a$ ,  $b$ ,  $c$ ,  $n$ ,  $N$  and  $k$  are model constants and  $t$  is the time.

**Table 6.1.** Mathematical drying models fitted to the drying curves of avocado peels.

| Model               | Equation                                         | References    |
|---------------------|--------------------------------------------------|---------------|
| Lewis               | $MR = e^{(-kt)}$ (5)                             | [226–229]     |
| Henderson and Pabis | $MR = ae^{(-kt)}$ (6)                            | [230–234]     |
| Page                | $MR = e^{(-kt^N)}$ (7)                           | [235–238,242] |
| Logarithmic         | $MR = a e^{-kt} + c$ (8)                         | [239–242]     |
| Avhad and Marchetti | $MR = a e^{(-kt^n)}$ (17)                        | [244]         |
| Wang and Singh      | $MR = 1 + at + bt^2$ (18)                        | [456,457]     |
| Parabolic           | $MR = a + bt + ct^2$ (19)                        | [214]         |
| Polynomial          | $MR = a + bt + ct^2 + dt^3$ (20)                 | [458]         |
| Thompson            | $t = a \ln MR + b (\ln MR)^2$ (21)               | [240,255]     |
| Geometric           | $MR = a t^{-n}$ (22)                             | [459]         |
| Verma               | $MR = a e^{(-k_1 t)} + (1 - a)e^{(-k_2 t)}$ (23) | [216,255]     |
| Two-factor          | $MR = a e^{(-k_1 t)} + b e^{(-k_2 t)}$ (24)      | [219,255]     |
| Midilli             | $MR = a e^{(-kt^n)} + b t$ (25)                  | [225,255]     |
| Weibull             | $MR = a - b e^{(-kt^n)}$ (9)                     | [233,255,460] |

### 2.3. Statistical parameters

Various statistical parameters were used to select the best mathematical model that predicts the drying kinetics of avocado peels, such as the coefficient of determination ( $R^2$ ), defined by Taylor (1997). Furthermore, other statistical parameters previously used by other authors [214,244,255] were also used for this purpose, such as chi-square error ( $X^2$ ), the standard error of estimation (SEE), root mean square error (RMSE), the mean absolute error (MAE) and the relative mean

error ( $P_0$ ). Overall, these terms indicate how close the experimental data and the prediction are. They were calculated for all three temperatures at the three different airflows for all the proposed mathematical models. A model is considered to have a high-quality fit when the  $R^2$  value is close to 1 and the  $X^2$ , SEE, RMSE and MAE values are close to 0. Moreover, the model is acceptable or fits well when  $P_0 < 0.5$  [136]. The mentioned statistical parameters were calculated according to equations (10-15), where the experimental and predicted dimensionless moisture ratios are expressed as  $MR_{exp,i}$  and  $MR_{pred,i}$ , respectively.  $N$  represents the number of observations, and  $i$  denotes the number of constants in the model.

$$R^2 = \frac{\sum_{i=1}^N (MR_{exp,i} - \overline{MR}_{pred,i}) * (MR_{pred,i} - \overline{MR}_{exp,i})}{\sqrt{\sum_{i=1}^N (MR_{exp,i} - \overline{MR}_{pred,i})^2 * \sum_{i=1}^N (MR_{pred,i} - \overline{MR}_{exp,i})^2}} \quad (10)$$

$$X^2 = \frac{\sum_{i=1}^N (MR_{exp,i} - MR_{pred,i})^2}{N - z} \quad (11)$$

$$SEE = \sqrt{\frac{\sum_{i=1}^N (MR_{exp,i} - MR_{pred,i})^2}{N - n_i}} \quad (12)$$

$$RMSE = \left[ \frac{1}{N} \sum_{i=1}^N (MR_{exp,i} - MR_{pred,i})^2 \right]^{1/2} \quad (13)$$

$$MAE = \frac{1}{N} \sum_{i=1}^N (MR_{pred,i} - MR_{exp,i}) \quad (14)$$

$$P_0 = \frac{1}{N} \sum_{i=1}^N \left| \frac{(MR_{exp,i} - MR_{pred,i})}{MR_{exp,i}} \right| \quad (15)$$

## 2.4. Ultrasound extraction

Briefly, 2 g of avocado by-product powder was added to 15 mL of ethanol/water solution (80:20 v/v). The mixture was placed in an ultrasonic bath (Bandelin, Sonorex, RK52, Berlin, Germany) which worked at a frequency of 35 kHz and 60 W for 15 min; then, it was centrifuged for 10 min at  $8603 \times g$ . The supernatant was collected, and the extraction was repeated twice more. Finally, the supernatants were evaporated and reconstituted in 1 mL of methanol/water (50:50, v/v). The final extracts were filtered with regenerated cellulose filters 0.2  $\mu m$  (Millipore, Bedford, MA, USA) and stored at  $-18^\circ C$  until the analyses.

## 2.5. Antioxidant assays

The antioxidant activity was evaluated in all the samples by three different methods in duplicate. The DPPH assay was performed according to a method proposed by several authors [247,248]. Briefly, 100  $\mu$ L of each extract was added to 2.9 mL of DPPH<sup>•</sup> solution, and after rapid stirring, the bleaching power of the extract was observed in a time interval from 0 to 30 minutes at 517 nm. The ABTS method was performed according to the protocol of Re et al. (1999). The monocation ABTS<sup>•+</sup> is generated by oxidation of the ABTS with potassium persulfate in the dark at room temperature for 12-24 hours. For each extract, 1 mL of this ABTS<sup>•+</sup> solution was added to 0.01 mL. Then, the decrease in absorbance was measured for 30 minutes at 734 nm. The FRAP assay was conducted following the procedure developed by Pulido et al. (2000). It is based on the reduction of Fe<sup>3+</sup> to Fe<sup>2+</sup> by the antioxidant substances. Briefly, 30  $\mu$ L of each extract were added to 90  $\mu$ L of distilled water and 900  $\mu$ L of the FRAP reagent. The resulting solution was kept for 30 min at 37 °C; then, absorbance was measured in the spectrophotometer at 595 nm. Standard curves of Trolox equivalents (TE) (1, 5, 10, 20, 50, 80, 100, 150, 200  $\mu$ g/g) were generated for each assay. Results are expressed as mg TE/g d.w.

## 2.6. HPLC-FLD Analysis

The methodology used to determine flavan-3-ols was previously reported by López-Cobo et al. (2016). An Agilent 1200 Series (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump delivery system, a degasser, an autosampler and a FLD was used for the analyses. A Develosil Diol 100 Å column 5 mm, 250 x 4.6 mm ID (Phenomenex, Torrance, CA, USA) was used. Mobile phase A and B consisted of an acidic acetonitrile ((A), CH<sub>3</sub>CN:CH<sub>3</sub>COOH, 98:2; v/v) and acidic aqueous methanol ((B), CH<sub>3</sub>OH:H<sub>2</sub>O:CH<sub>3</sub>COOH, 95:3:2; v/v/v). The gradient elution was: 3% B for 50 min, 38% B for 3 min, 100% B for 13 min and 100% B for 10 min. Then the initial conditions were set, 0% B for 10 min. Fluorescence detection was performed with an excitation wavelength of 230 nm and an emission wavelength of 321 nm. The injection volume was 10  $\mu$ L. All the analyses were conducted at 35 °C. The identification of flavan-3-ols was performed according to the previously described [461], as they are eluted according to their degree of polymerization, firstly eluting the monomers and then the different oligomers [462]. A standard curve of catechin at 6 concentrations from 10 to 650  $\mu$ g/g was generated to quantify flavan-3-ols. In addition, the correction factors suggested by Robbins et al. (2009) were used. The results are expressed as mg catechin equivalents (CE)/g d.w.

## 3. Results and discussion

### 3.1. Drying kinetics and modelling

**Figure 6.1** shows the drying curve of avocado peel, where the changes in the moisture ratio of the avocado peel over the time at different temperatures (40, 60 and 80 °C) and airflows (0, 0.8 and 1.6 m/s) are represented.

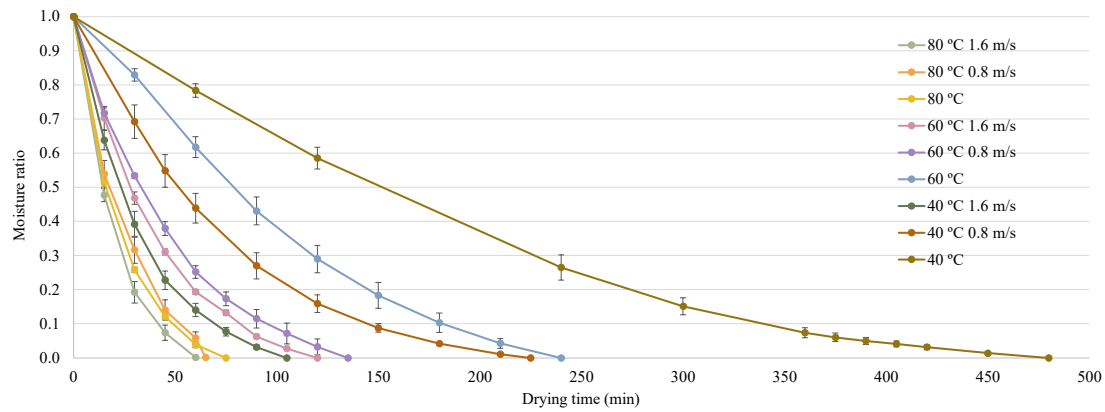


**Figure 6.1.** Drying kinetics curves of avocado peel under different temperatures and air flows.

Without airflow, the drying time required to achieve the final moisture content of the avocado by-product was 75, 240 and 480 min at 80, 60 and 40 °C, respectively. Özbek et al. (2022) reported a drying time of 180 min when using hot air-drying at 50 °C in avocado pulp, demonstrating the differences between various parts of the avocado. The temperature has a high influence on the drying of the avocado peel because less time was needed to reach the lowest moisture ratio when a higher temperature was used. Similarly, the air has demonstrated its effect on drying. In this case, the decrease in the drying time with the airflow was more remarkable at lower temperatures. At 40 °C, the drying time decreased 53% when using an airflow of 0.8 m/s, and the drying time decreased 78% when the airflow was 1.6 m/s. As a combination of temperature and airflow, drying at 80 °C with an airflow of 1.6 m/s was the quickest, requiring only 60 min.

However, other parameters should be measured to evaluate and select the best drying conditions.

**Figure 6.2** shows the average drying rates of the avocado peels dried at three different temperatures and airflows against time.

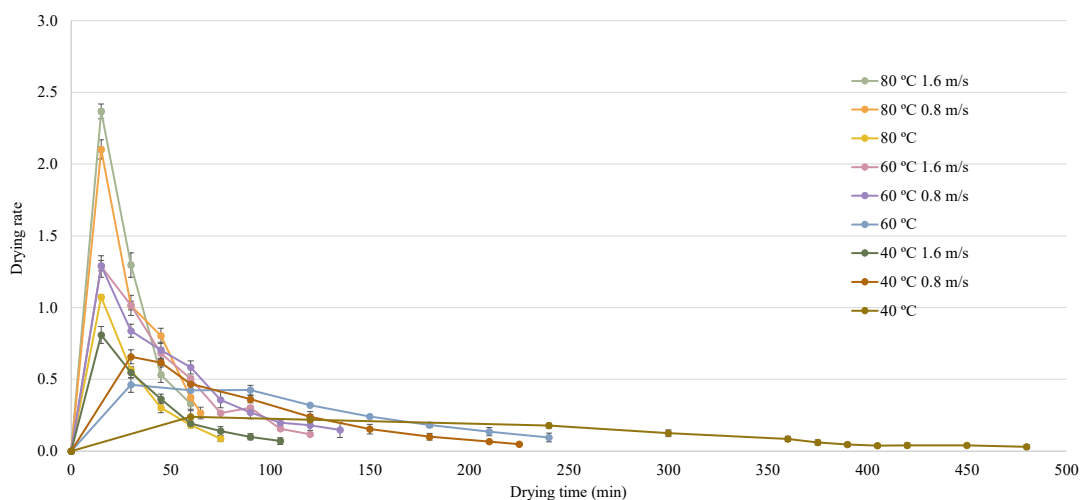


**Figure 6.2.** Drying rate curves of avocado peel under different temperatures and air flows.

Depending on the drying conditions, the initial drying rate reached the maximum value at 15-30 minutes. The drying rate declined rapidly after this time. These facts can be due to the rapid evaporation of the surface moisture occurring in the first half hour and, after that, to the evaporation of the internal moisture. The increase in temperature enhanced the drying rate considerably. In fact, without airflow, the drying rate increased by 77.6% when drying at 80 °C compared to drying at 40 °C. Furthermore, a consistent effect was found with the airflow. The highest impact in the drying rate with the airflow was at 40 °C (an increase of 70.4% when using 1.6 m/s of airflow). However, higher increases were also found with the airflow when drying at 60 °C (64.3%) and 80 °C (54.9%). **Figure 6.2** shows that the samples dried at 80 °C with airflow reached the highest drying rate among all the tested conditions.

Regarding the drying kinetics, **Figure 6.1** clearly shows two stages of drying. The first one is the most rapid phase and is mostly linear. In this case, the water of the most superficial layers of the avocado by-product is evaporated. The second one is an exponential phase that usually takes longer and occurs when the remaining water content diffuses to the surface. Therefore, the mathematical modelling of the drying curves is important for better control of the drying process and better quality of the avocado peels.

The statistical parameters presented in **Table 6.2**, such as  $R^2$ ,  $X^2$ , RMSE, MAE and  $P_0$ , were considered to evaluate which model best predicts the drying kinetics of the avocado peel.

**Table 6.2.** Results obtained for the statistical analysis for all the mathematical models at three different temperatures and airflows for avocado peels.

| Model               | T (°C) | Air flow 1.6 m/s |                |        |          |        |                | Air flow 0.8 m/s |                |        |          |        |                | no air flow    |                |        |        |        |                |
|---------------------|--------|------------------|----------------|--------|----------|--------|----------------|------------------|----------------|--------|----------|--------|----------------|----------------|----------------|--------|--------|--------|----------------|
|                     |        | R <sup>2</sup>   | X <sup>2</sup> | SEE    | RMSE     | MAE    | P <sub>0</sub> | R <sup>2</sup>   | X <sup>2</sup> | SEE    | RMSE     | MAE    | P <sub>0</sub> | R <sup>2</sup> | X <sup>2</sup> | SEE    | RMSE   | MAE    | P <sub>0</sub> |
| Lewis               | 40     | 0.9947           | 7.61E-04       | 0.0276 | 0.0385   | 0.0222 | 0.1032         | 0.9779           | 0.0040         | 0.0635 | 0.1331   | 0.0479 | 0.2477         | 0.9801         | 0.0066         | 0.0810 | 0.1417 | 0.0488 | 0.2531         |
|                     | 60     | 0.9880           | 0.0017         | 0.0416 | 0.0724   | 0.0333 | 0.1525         | 0.9913           | 0.0012         | 0.0353 | 0.0591   | 0.0282 | 0.1223         | 0.9723         | 0.0070         | 0.0840 | 0.1414 | 0.0644 | 0.1950         |
|                     | 80     | 0.9982           | 7.05E-04       | 0.0266 | 0.0088   | 0.0190 | 0.0430         | 0.9951           | 0.0012         | 0.0339 | 0.0115   | 0.0257 | 0.0741         | 0.9937         | 7.21E-04       | 0.0268 | 0.0275 | 0.0221 | 0.1052         |
| Henderson and Pabis | 40     | 0.9882           | 0.0023         | 0.0481 | 0.0388   | 0.0289 | 0.0928         | 0.9895           | 0.0033         | 0.0573 | 0.0435   | 0.0312 | 0.2398         | 0.9499         | 0.0844         | 0.2905 | 0.3454 | 0.1221 | 0.2734         |
|                     | 60     | 0.9749           | 0.0057         | 0.0756 | 0.0574   | 0.0443 | 0.1349         | 0.9809           | 0.0036         | 0.0599 | 0.0494   | 0.0386 | 0.1160         | 0.9558         | 0.0142         | 0.1192 | 0.0716 | 0.0722 | 0.1702         |
|                     | 80     | 0.9967           | 0.0012         | 0.0348 | 0.0256   | 0.0276 | 0.0438         | 0.9894           | 0.0023         | 0.0484 | 0.0422   | 0.0354 | 0.0723         | 0.9863         | 0.0027         | 0.0515 | 0.0382 | 0.0339 | 0.1000         |
| Page                | 40     | 0.9971           | 9.76E-05       | 0.0099 | 0.0043   | 0.0076 | 0.0542         | 0.9937           | 1.27E-04       | 0.0113 | 0.0042   | 0.0097 | 0.1224         | 0.9907         | 3.49E-04       | 0.0187 | 0.0122 | 0.0132 | 0.1316         |
|                     | 60     | 0.9940           | 1.72E-04       | 0.0131 | 3.56E-04 | 0.0104 | 0.0843         | 0.9931           | 2.58E-04       | 0.0161 | 2.46E-04 | 0.0131 | 0.0739         | 0.9973         | 1.93E-04       | 0.0139 | 0.0027 | 0.0103 | 0.0582         |
|                     | 80     | 0.9325           | 9.35E-04       | 0.0306 | 0.0147   | 0.0245 | 0.1659         | 0.9899           | 6.79E-04       | 0.0261 | 0.0166   | 0.0183 | 0.0482         | 0.9940         | 1.45E-04       | 0.0121 | 0.0047 | 0.0105 | 0.0628         |
| Logarithmic         | 40     | 0.9882           | 0.0497         | 0.2230 | 0.6064   | 0.2144 | 1.9407         | 0.9623           | 0.0844         | 0.2905 | 0.8983   | 0.2841 | 4.6860         | 0.9493         | 0.1134         | 0.3368 | 1.1336 | 0.3272 | 5.7422         |
|                     | 60     | 0.9749           | 0.0594         | 0.2438 | 0.7128   | 0.2376 | 2.2767         | 0.9809           | 0.0526         | 0.2293 | 0.7022   | 0.2221 | 1.8379         | 0.9558         | 0.0716         | 0.2675 | 0.7640 | 0.2547 | 1.5872         |
|                     | 80     | 0.9967           | 0.0165         | 0.1285 | 0.2051   | 0.0980 | 0.3598         | 0.9894           | 0.0461         | 0.2147 | 0.4925   | 0.2011 | 1.1770         | 0.9863         | 0.0487         | 0.2207 | 0.5168 | 0.2110 | 1.7013         |
| Avhad and Marchetti | 40     | 0.9839           | 0.1113         | 0.3336 | 0.3597   | 0.1435 | 0.2252         | 0.9900           | 0.0169         | 0.1301 | 0.1676   | 0.0695 | 0.1999         | 0.9535         | 0.1294         | 0.3597 | 0.4134 | 0.1447 | 0.3049         |
|                     | 60     | 0.9754           | 0.0830         | 0.2882 | 0.3230   | 0.1291 | 0.2295         | 0.9718           | 0.0444         | 0.2107 | 0.2414   | 0.0975 | 0.1857         | 0.9735         | 0.0435         | 0.2086 | 0.2441 | 0.1070 | 0.1878         |
|                     | 80     | 0.9972           | 0.0199         | 0.1411 | 0.2588   | 0.1157 | 0.4929         | 0.9720           | 0.0984         | 0.3137 | 0.3405   | 0.1576 | 0.2041         | 0.9830         | 0.2260         | 0.4754 | 0.4955 | 0.2187 | 0.2960         |
| Wang and Singh      | 40     | 0.9894           | 0.0238         | 0.1543 | 0.2850   | 0.1184 | 1.4497         | 0.9942           | 0.0059         | 0.0769 | 0.1795   | 0.0568 | 1.3834         | 0.9998         | 3.04E-05       | 0.0055 | 0.0029 | 0.0047 | 0.0675         |
|                     | 60     | 0.9932           | 8.92E-04       | 0.0299 | 0.0095   | 0.0256 | 0.2032         | 0.9931           | 0.0010         | 0.0318 | 0.0460   | 0.0256 | 0.1082         | 0.9983         | 0.0094         | 0.0971 | 0.2171 | 0.0724 | 0.5945         |
|                     | 80     | 0.9943           | 0.0091         | 0.0955 | 0.1547   | 0.0692 | 0.2137         | 0.9927           | 0.0099         | 0.0994 | 0.1618   | 0.0827 | 0.6273         | 0.9896         | 0.0215         | 0.1468 | 0.2316 | 0.1135 | 1.1474         |
| Parabolic           | 40     | 0.9898           | 0.0100         | 0.1002 | 0.1945   | 0.0814 | 0.9668         | 0.9946           | 0.0165         | 0.1284 | 0.2854   | 0.0969 | 2.5037         | 0.9946         | 2.6585         | 1.6305 | 4.0719 | 1.2909 | 31.8451        |
|                     | 60     | 0.9935           | 0.0016         | 0.0396 | 0.0658   | 0.0348 | 0.4234         | 0.9936           | 0.0011         | 0.0337 | 0.0520   | 0.0293 | 0.2932         | 0.9984         | 0.0153         | 0.1237 | 0.2654 | 0.0929 | 0.7676         |
|                     | 80     | 0.9944           | 0.0060         | 0.0772 | 0.1150   | 0.0624 | 0.3674         | 0.9928           | 0.0043         | 0.0653 | 0.1074   | 0.0589 | 0.4286         | 0.9898         | 0.0097         | 0.0982 | 0.1607 | 0.0815 | 0.7940         |
| Polynomial          | 40     | 0.9998           | 0.0094         | 0.0971 | 0.1859   | 0.0661 | 0.7774         | 0.9998           | 0.0056         | 0.0746 | 0.1731   | 0.0554 | 1.5177         | 0.9998         | 0.0019         | 0.0432 | 0.1202 | 0.0361 | 0.8143         |
|                     | 60     | 0.9997           | 0.0029         | 0.0538 | 0.1154   | 0.0389 | 0.5402         | 0.9996           | 0.1499         | 0.3871 | 0.8918   | 0.2831 | 3.1819         | 0.9986         | 3.28E-04       | 0.0181 | 0.0228 | 0.0161 | 0.1117         |
|                     | 80     | 0.9999           | 0.0019         | 0.0439 | 0.0798   | 0.0357 | 0.1778         | 0.9987           | 7.12E-04       | 0.0267 | 0.0429   | 0.0208 | 0.1495         | 0.9995         | 0.0054         | 0.0735 | 0.1250 | 0.0522 | 0.4952         |

| Model      | T (°C) | Air flow 1.6 m/s |                |        |          |        |                | Air flow 0.8 m/s |                |        |          |        |                | no air flow    |                |        |          |        |                |
|------------|--------|------------------|----------------|--------|----------|--------|----------------|------------------|----------------|--------|----------|--------|----------------|----------------|----------------|--------|----------|--------|----------------|
|            |        | R <sup>2</sup>   | X <sup>2</sup> | SEE    | RMSE     | MAE    | P <sub>0</sub> | R <sup>2</sup>   | X <sup>2</sup> | SEE    | RMSE     | MAE    | P <sub>0</sub> | R <sup>2</sup> | X <sup>2</sup> | SEE    | RMSE     | MAE    | P <sub>0</sub> |
| Thompson   | 40     | 0.9990           | 0.9709         | 0.9854 | 0.2931   | 0.8227 | 0.0170         | 0.9998           | 0.9216         | 0.9600 | 0.2783   | 0.7268 | 0.0114         | 0.9973         | 91.0028        | 9.5395 | 8.8423   | 7.6826 | 0.0474         |
|            | 60     | 0.9988           | 1.4468         | 1.2028 | 0.3100   | 0.8242 | 0.0150         | 0.9991           | 1.4410         | 1.2004 | 0.5896   | 0.9287 | 0.0202         | 0.9976         | 18.2727        | 4.2747 | 4.1951   | 3.3757 | 0.0592         |
|            | 80     | 0.9997           | 0.1011         | 0.3179 | 0.0927   | 0.2011 | 0.0119         | 0.9986           | 0.6375         | 0.7984 | 0.1549   | 0.5863 | 0.0265         | 0.9992         | 0.3765         | 0.6136 | 0.2359   | 0.4972 | 0.0189         |
| Geometric  | 40     | 0.9059           | 0.1195         | 0.3457 | 0.3024   | 0.2064 | 0.7965         | 0.8321           | 0.0367         | 0.1917 | 0.1068   | 0.1042 | 0.4628         | 0.8066         | 0.0458         | 0.2141 | 0.1033   | 0.1048 | 0.4397         |
|            | 60     | 0.8615           | 0.0245         | 0.1565 | 0.0732   | 0.0979 | 0.3662         | 0.8272           | 0.0267         | 0.1634 | 0.0728   | 0.0994 | 0.3352         | 0.8176         | 0.0360         | 0.1898 | 0.0767   | 0.1261 | 0.3528         |
|            | 80     | 0.9867           | 0.0011         | 0.0337 | 0.0294   | 0.0303 | 0.0861         | 0.9161           | 0.0055         | 0.0743 | 0.0426   | 0.0631 | 0.1948         | 0.9431         | 0.0044         | 0.0665 | 0.0358   | 0.0549 | 0.2371         |
| Verma      | 40     | 0.9392           | 8.66E-05       | 0.0093 | 0.0065   | 0.0064 | 0.0504         | 0.9097           | 7.89E-05       | 0.0089 | 0.0092   | 0.0047 | 0.1605         | 0.7899         | 3.66E-04       | 0.0191 | 0.0225   | 0.0170 | 0.2831         |
|            | 60     | 0.9097           | 1.41E-04       | 0.0119 | 0.0094   | 0.0093 | 0.0965         | 0.8770           | 1.96E-04       | 0.0140 | 0.0121   | 0.0102 | 0.0662         | 0.8609         | 2.97E-04       | 0.0172 | 0.0159   | 0.0126 | 0.0817         |
|            | 80     | 0.8916           | 5.42E-04       | 0.0233 | 0.0143   | 0.0184 | 0.0627         | 0.9717           | 2.91E-04       | 0.0171 | 0.0034   | 0.0143 | 0.0649         | 0.9404         | 1.31E-04       | 0.0115 | 0.0072   | 0.0096 | 0.0660         |
| Two-factor | 40     | 0.8391           | 2.85E-04       | 0.0169 | 0.0172   | 0.0140 | 0.1107         | 0.7165           | 6.75E-04       | 0.0260 | 0.0290   | 0.0202 | 0.4270         | 0.8088         | 3.66E-04       | 0.0191 | 0.0205   | 0.0175 | 0.2810         |
|            | 60     | 0.7814           | 5.11E-04       | 0.0226 | 0.0227   | 0.0194 | 0.1926         | 0.7811           | 4.77E-04       | 0.0218 | 0.0215   | 0.0172 | 0.1402         | 0.8788         | 2.94E-04       | 0.0171 | 0.0138   | 0.0129 | 0.0811         |
|            | 80     | 0.8639           | 5.27E-04       | 0.0230 | 0.0180   | 0.0195 | 0.0614         | 0.7696           | 0.0010         | 0.0313 | 0.0273   | 0.0241 | 0.0934         | 0.9361         | 1.28E-04       | 0.0113 | 0.0077   | 0.0096 | 0.0657         |
| Midilli    | 40     | 0.9942           | 1.64E-05       | 0.0040 | 1.31E-04 | 0.0032 | 0.0209         | 0.9864           | 5.06E-06       | 0.0022 | 6.06E-05 | 0.0014 | 0.0105         | 0.9871         | 0.2082         | 0.4563 | 1.4577   | 0.4214 | 8.3175         |
|            | 60     | 0.9896           | 2.32E-05       | 0.0048 | 6.55E-05 | 0.0034 | 0.0255         | 0.9915           | 3.58E-05       | 0.0060 | 4.97E-05 | 0.0049 | 0.0234         | 0.9927         | 0.1818         | 0.4264 | 1.1322   | 0.3774 | 2.7125         |
|            | 80     | 0.9537           | 1.91E-05       | 0.0044 | 7.18E-05 | 0.0035 | 0.0262         | 0.7757           | 1.04E-04       | 0.0102 | 1.31E-05 | 0.0083 | 0.0690         | 0.9965         | 3.60E-06       | 0.0019 | 3.17E-05 | 0.0013 | 0.0191         |
| Weibull    | 40     | 0.8968           | 0.0103         | 0.1015 | 0.2596   | 0.0921 | 0.7335         | 0.8706           | 6.48E-06       | 0.0025 | 7.83E-06 | 0.0017 | 0.0147         | 0.8746         | 3.20E-05       | 0.0057 | 1.60E-05 | 0.0050 | 0.0561         |
|            | 60     | 0.8569           | 2.60E-05       | 0.0051 | 3.73E-06 | 0.0038 | 0.0243         | 0.8360           | 3.41E-05       | 0.0058 | 3.31E-09 | 0.0049 | 0.0256         | 0.8968         | 9.73E-06       | 0.0031 | 9.97E-06 | 0.0026 | 0.0089         |
|            | 80     | 0.7210           | 2.24E-05       | 0.0047 | 9.51E-07 | 0.0038 | 0.0281         | 0.4130           | 1.03E-04       | 0.0101 | 1.97E-06 | 0.0082 | 0.0689         | 0.8984         | 2.74E-06       | 0.0017 | 4.56E-06 | 0.0012 | 0.0164         |

R<sup>2</sup>: coefficient of determination; X<sup>2</sup>: chi-square error; SEE: standard error of estimation; RMSE: root mean square error; MAE: mean absolute error; P<sub>0</sub>: relative mean error.



In general, most of the models provided satisfactory results with a high coefficient of determination ( $R^2 > 0.9$ ). Among the fourteen tested mathematical models, when no airflow was used, the Page, Polynomial and Thompson models fitted the best at all three temperatures showing  $R^2$  values of 0.9907-0.9973, 0.9986-0.9998, and 0.9973-0.9992, respectively. When using airflows of 0.8 or 1.6 m/s, the best model adjustment changed depending on the temperature. At 40 °C and 60 °C, Page, polynomial and Thompson models reported the highest values of  $R^2$ , but only Page model kept  $X^2$ , SEE, RMSE, MAE and  $P_0$  the lowest. However, at 80 °C, the Lewis model was found to better fit the experimental data with polynomial and Thompson models. Other models such as Verma, Two-factor, Midilli and Weibull did not show good statistical results for the temperatures and air flows tested predicting the drying kinetics of the avocado peel. These results show that the drying kinetics vary depending on the temperature and airflow used when drying avocado peel by convective drying. Additionally, **Table 6.3** shows the values obtained for all the constants of all the models. All these parameters were variable and exhibited temperature and airflow dependence in consistency with other authors [217,244].

**Table 6.3.** Results of the nondimensional coefficients for the different mathematical models for the avocado peels.

| Air flow            |   | 1.6 m/s |         |         | 0.8 m/s |         |         | No      |         |           |
|---------------------|---|---------|---------|---------|---------|---------|---------|---------|---------|-----------|
| Temperature (°C)    |   | 40      | 60      | 80      | 40      | 60      | 80      | 40      | 60      | 80        |
| Lewis               | K | 0.0353  | 0.0305  | 0.0565  | 0.0180  | 0.0254  | 0.0448  | 0.0078  | 0.0126  | 0.0504    |
|                     | k | 0.0371  | 0.0331  | 0.0582  | 0.0175  | 0.0272  | 0.0468  | 0.0096  | 0.0145  | 0.0529    |
| Henderson and Pabis | a | 1.1296  | 1.2148  | 1.0625  | 1.1730  | 1.1687  | 1.0952  | 1.9519  | 1.3354  | 1.1192    |
|                     | k | 0.0216  | 0.0145  | 0.0114  | 0.0053  | 0.0153  | 0.0301  | 0.0007  | 0.0014  | 0.0309    |
| Page                | N | 1.1123  | 1.1635  | 1.4974  | 1.2349  | 1.1056  | 1.0987  | 1.3975  | 1.4202  | 1.1226    |
|                     | k | 0.0582  | 0.0331  | 0.0371  | 0.0468  | 0.0272  | 0.0202  | 0.0529  | 0.0145  | 0.0089    |
| Logarithmic         | a | 0.7969  | 0.9111  | 0.8472  | 0.8214  | 0.8765  | 1.0268  | 0.8394  | 1.0015  | 1.1068    |
|                     | c | 0.2656  | 0.3037  | 0.2824  | 0.2738  | 0.2922  | 0.3423  | 0.2798  | 0.3338  | 0.3689    |
| Avhad and Marchetti | k | 0.1140  | 0.0753  | 0.0228  | 0.0126  | 0.0610  | 0.1161  | 0.0147  | 0.0140  | 0.1584    |
|                     | a | 1.9358  | 1.8450  | 0.9008  | 1.3818  | 1.6401  | 1.7620  | 2.1964  | 1.5932  | 2.1615    |
|                     | n | 0.7822  | 0.8472  | 1.4974  | 1.0939  | 0.8509  | 0.8174  | 0.9347  | 1.0219  | 0.7789    |
| Wang and Sigh       | a | -0.0225 | -0.0189 | -0.0374 | -0.0104 | -0.0164 | -0.0304 | -0.004  | -0.0074 | -0.0316   |
|                     | b | 1E-04   | 9E-05   | 4E-04   | 3E-05   | 7E-05   | 2E-04   | 4E-06   | 1E-05   | 2E-04     |
| Parabolic           | a | 0.9585  | 0.9659  | 0.9814  | 0.9669  | 0.9589  | 0.9756  | 0.9669  | 1.0199  | 0.9652    |
|                     | b | -0.0211 | -0.0179 | -0.0363 | -0.0098 | -0.0153 | -0.0291 | -0.0098 | -0.0077 | -0.0299   |
|                     | c | 1E-04   | 8E-05   | 3E-04   | 3E-05   | 6E-05   | 2E-04   | 3E-05   | 1E-05   | 2E-04     |
| Polynomial          | a | 0.9985  | 0.9999  | 1.0070  | 1.0033  | 0.9937  | 0.9973  | 1.0038  | 1.0139  | 0.9965    |
|                     | b | -0.0280 | -0.0228 | -0.0455 | -0.0123 | -0.0195 | -0.038  | -0.004  | -0.0073 | -0.0395   |
|                     | c | 3E-04   | 2E-04   | 8E-04   | 5E-05   | 1E-04   | 6E-04   | 4E-06   | 1E-05   | 6E-04     |
|                     | d | -1E-06  | -6E-07  | -5E-06  | -8E-08  | -4E-07  | -4E-06  | 7E-10   | 1E-08   | -3.00E-06 |
| Thompson            | a | -35.12  | -44.01  | -20.59  | -78.93  | -50.92  | -27.67  | -201.21 | -118.61 | -25.15    |
|                     | b | -2.540  | -4.119  | -1.296  | -7.151  | -4.536  | -2.312  | -23.01  | -16.74  | -2.0261   |

| Air flow         |    | 1.6 m/s |         |         | 0.8 m/s |         |         | No      |          |         |
|------------------|----|---------|---------|---------|---------|---------|---------|---------|----------|---------|
| Temperature (°C) |    | 40      | 60      | 80      | 40      | 60      | 80      | 40      | 60       | 80      |
| Geometric        | a  | 60.66   | 71.58   | 45.31   | 739.52  | 48.25   | 42.68   | 2287.50 | 146.12   | 72.70   |
|                  | n  | 1.554   | 1.537   | 1.658   | 1.879   | 1.373   | 1.541   | 1.798   | 1.385    | 1.750   |
| Verma            | a  | 13.1485 | 10.0958 | 0.8166  | 6.0032  | 10.0963 | 2.4646  | 11.6018 | 9.9967   | -1.2061 |
|                  | k1 | 0.0464  | 0.0402  | 0.0537  | 0.0228  | 0.0332  | 0.0260  | 0.0106  | 0.0196   | 0.0772  |
|                  | k2 | 0.0480  | 0.0424  | 0.0537  | 0.0253  | 0.0348  | 0.0188  | 0.0115  | 0.0216   | 0.0598  |
| Two factor       | a  | 0.8561  | 0.8491  | 0.8165  | 0.8916  | 0.8308  | 0.8998  | -6.0258 | 19.5327  | -3.0924 |
|                  | b  | 0.1563  | 0.1716  | 0.1920  | 0.1386  | 0.1847  | 0.1115  | 7.0192  | -18.5368 | 4.0914  |
|                  | k1 | 0.0328  | 0.0273  | 0.0540  | 0.0149  | 0.0233  | 0.0436  | 0.0119  | 0.0201   | 0.0705  |
|                  | k2 | 0.0328  | 0.0273  | 0.0540  | 0.0149  | 0.0233  | 0.0346  | 0.0104  | 0.0211   | 0.0628  |
| Midilli          | k  | 0.0271  | 0.0202  | 0.0386  | 0.0081  | 0.0206  | 0.0603  | 0.0012  | 0.0021   | 0.0439  |
|                  | a  | 1.0005  | 1.0008  | 1.0002  | 1.0007  | 0.9985  | 0.9999  | 0.9963  | 1.0016   | 1.0000  |
|                  | b  | -0.0003 | -0.0004 | -0.0006 | -0.0001 | -0.0004 | -0.0023 | -0.0001 | -0.0002  | -0.0006 |
|                  | n  | 1.0345  | 1.0548  | 1.0833  | 1.1231  | 1.0021  | 0.8179  | 1.2646  | 1.3165   | 0.9946  |
| Weibull          | k  | 0.0204  | 0.0202  | 0.0390  | 0.0083  | 0.0204  | 0.0494  | 0.0013  | 0.0022   | 0.0442  |
|                  | a  | -0.0824 | -0.0538 | -0.0443 | -0.0326 | -0.0824 | -0.3612 | -0.0717 | -0.0706  | -0.0565 |
|                  | b  | -1.0812 | -1.0547 | -1.0444 | -1.0334 | -1.0812 | -1.3612 | -1.0684 | -1.0724  | -1.0565 |
|                  | n  | 0.9833  | 1.0401  | 1.0655  | 1.1114  | 0.9833  | 0.7831  | 1.2413  | 1.2971   | 0.9732  |

The effective moisture diffusivity ( $D_{\text{eff}}$ ) was calculated according to Fick's second law of diffusion for the three different drying temperatures and airflows at all the mathematical models tested, when possible, as some models such as Wang and Singh, Parabolic, Polynomial, Thompson and Geometric did not have kinetic constant (k). The obtained values ranged between  $10^{-7}$  and  $10^{-9}$   $\text{m}^2/\text{s}$  in consistency with the ranges reported by other authors [217,219,225,254,255].

The  $E_a$  is the required energy for initializing the moisture diffusion from the inside to the outer of the sample. The  $E_a$  and the pre-exponential factors for the studied models at the three different airflows were calculated using the Arrhenius equation from the plot of  $\ln(D_{\text{eff}})$  versus  $1/T$ . **Figure 6.3** shows an example of this plot for the Page model for the three different airflows. The results are represented in **Table 6.4**. The  $E_a$  was calculated in the temperature range from 313 to 353 K for all the cases.

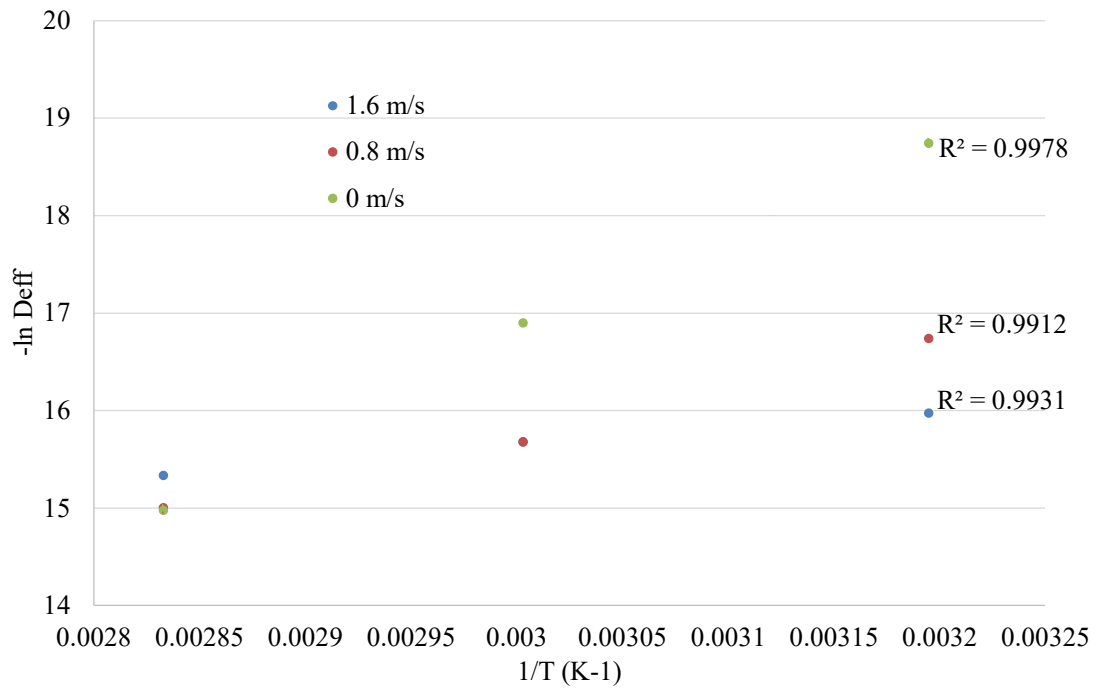


**Figure 6.3.** Relationship between effective moisture diffusivity ( $De_{eff}$ ) and drying temperatures at the three different air flows according to the Page Model.

**Table 6.4.** Estimated activation energy ( $E_a$ ) and pre-exponential factor ( $A$ ) for avocado peels dried with different airflows in the temperature range 313-353 K.

| Model               | Air flow 1.6 m/s |          | Air flow 0.8 m/s |          | No air flow    |          |
|---------------------|------------------|----------|------------------|----------|----------------|----------|
|                     | $E_a$ (kJ/mol)   | $A$      | $E_a$ (kJ/mol)   | $A$      | $E_a$ (kJ/mol) | $A$      |
| Lewis               | 10.44            | 2.29E+05 | 20.81            | 7.70E+03 | 42.38          | 5.02     |
| Henderson and Pabis | 10.02            | 2.53E+05 | 22.52            | 4.04E+03 | 38.74          | 16.42    |
| Page                | 14.69            | 5.28E+09 | 39.95            | 15.31    | 85.29          | 4.82E-06 |
| Logarithmic         | 10.02            | 2.53E+05 | 19.16            | 1.30E+04 | 40.51          | 8.87     |
| Avhad and Marchetti | 36.56            | 3.81E+12 | 51.37            | 13.52    | 53.39          | 20.13    |
| Midilli             | 7.68             | 8.86E+05 | 45.93            | 1.10     | 80.93          | 1.54E-05 |
| Weibull             | 14.51            | 8.16E+04 | 41.01            | 6.94     | 79.61          | 2.39E-05 |
| Verma               | 2.76             | 1.54E+06 | 1.27             | 1.12E+07 | 41.55          | 0.43     |
| Two factor          | 11.06            | 9.90E+04 | 22.09            | 2.79E+03 | 40.75          | 3.11     |

For the avocado peel dried with an airflow of 1.6 m/s, 0.8 m/s or without airflow, the  $E_a$  values were 2.76-36.56, 1.27-51.37 and 38.74-85.29 kJ/mol, respectively. Nguyen & Le (2022) [217] reported  $E_a$  for avocado and mango pulp of 32.06 and 66.03 kJ/mol, respectively, drying by refractance window drying at 80-90 °C. Moreover, the results were consistent with those reported for other matrices [217,219,221,254]. It is clear from the results that higher  $E_a$  is necessary when drying without airflow; in contrast, the  $E_a$  needed to start the moisture diffusion is lower if the airflow is introduced. The highest  $E_a$  values were obtained with Avhad and Marchetti's model

when drying with airflow and with Page's model when no airflow was used. Among all the models, the Verma model predicted the lowest  $E_a$  when using an airflow of 1.6 and 0.8 m/s, and the Henderson and Pabis model then drying without air flow.

### **3.2. Effect of drying on procyanidin content determined using HPLC-FLD**

Procyanidins are valuable and thermolabile compounds of great interest, and they are present in the avocado by-products that could be exploited and revalued. Therefore, the procyanidin contents of the samples dried at 40, 60 and 80 °C at three different airflows were evaluated after the extraction. Ultrasound technology was used, allowing the extraction of the highest possible content of bioactive compounds without using high temperatures [74,464,465]. Procyanidin content was determined using HPLC-FLD. **Table 6.5** shows the procyanidin contents obtained. Procyanidins are ordered according to their polymerization degree (dp2-dp8), including the sum of monomers (catechin+epicatechin) and the rest of polymers with a polymerization degree above 8.

**Table 6.5.** Procyanidin content of the avocado peels dried at different temperatures and airflows measured using high-performance liquid chromatography with a fluorometric detector (HPLC-FLD). Procyanidin content is expressed as  $\mu\text{g}$  Catechin Equivalents (CE)/g dry weight (d.w.).

|                           | 40 °C                 | 40 °C 0.8 m/s         | 40 °C 1.6 m/s          | 60 °C                 | 60 °C 0.8 m/s         | 60 °C 1.6 m/s         | 80 °C                 | 80 °C 0.8 m/s         | 80 °C 1.6 m/s         |
|---------------------------|-----------------------|-----------------------|------------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| <b>Monomers</b>           | 1045.07 $\pm$ 6.68 e  | 1300.15 $\pm$ 8.21 a  | 1274.12 $\pm$ 7.58 a,b | 1098.53 $\pm$ 5.47 d  | 1110.02 $\pm$ 5.11 d  | 1188.64 $\pm$ 8.33 c  | 1255.05 $\pm$ 7.80 b  | 1167.43 $\pm$ 5.47 c  | 1105.03 $\pm$ 5.55 d  |
| <b>Dimers</b>             | 208.97 $\pm$ 5.90 c   | 704.97 $\pm$ 19.99 a  | 752.88 $\pm$ 21.78 a   | 219.39 $\pm$ 6.67 c   | 391.90 $\pm$ 12.15 b  | 464.01 $\pm$ 12.66 b  | 419.35 $\pm$ 11.96 b  | 432.38 $\pm$ 13.35 b  | 201.21 $\pm$ 6.11 c   |
| <b>dp3</b>                | 140.56 $\pm$ 3.16 e   | 703.91 $\pm$ 15.92 a  | 745.11 $\pm$ 17.25 a   | 240.75 $\pm$ 5.91 d   | 389.35 $\pm$ 9.77 c   | 451.96 $\pm$ 9.77 b   | 404.48 $\pm$ 9.21 b,c | 397.81 $\pm$ 9.94 b,c | 190.79 $\pm$ 4.67 d,e |
| <b>dp4</b>                | 153.49 $\pm$ 5.33 c   | 438.36 $\pm$ 15.28 a  | 475.38 $\pm$ 16.86 a   | 172.72 $\pm$ 6.40 c   | 264.85 $\pm$ 9.98 b   | 289.96 $\pm$ 9.78 b   | 262.45 $\pm$ 9.19 b   | 258.32 $\pm$ 9.69 b   | 132.20 $\pm$ 4.89 c   |
| <b>dp5</b>                | 88.94 $\pm$ 3.57 d    | 249.88 $\pm$ 10.07 b  | 289.97 $\pm$ 11.87 a   | 97.72 $\pm$ 4.16 d    | 152.49 $\pm$ 6.60 c   | 160.20 $\pm$ 6.27 c   | 152.59 $\pm$ 6.18 c   | 144.60 $\pm$ 6.24 c   | 73.03 $\pm$ 3.11 d    |
| <b>dp6</b>                | 36.08 $\pm$ 2.61 b,c  | 105.00 $\pm$ 7.60 a   | 131.07 $\pm$ 9.59 a    | 39.48 $\pm$ 2.97 b,c  | 66.27 $\pm$ 5.05 b    | 66.04 $\pm$ 4.68 b    | 65.96 $\pm$ 4.79 b    | 62.77 $\pm$ 4.77 b    | 32.08 $\pm$ 2.41 c    |
| <b>dp7</b>                | 24.28 $\pm$ 0.80 d    | 83.19 $\pm$ 2.76 b    | 110.40 $\pm$ 3.73 a    | 26.53 $\pm$ 0.94 d    | 46.73 $\pm$ 1.68 c    | 47.07 $\pm$ 1.51 c    | 50.80 $\pm$ 1.69 c    | 45.90 $\pm$ 1.64 c    | 20.31 $\pm$ 0.72 d    |
| <b>dp8</b>                | 6.31 $\pm$ 0.33 d     | 36.27 $\pm$ 1.93 b    | 53.78 $\pm$ 2.90 a     | 6.75 $\pm$ 0.38 d     | 15.11 $\pm$ 0.85 c    | 16.53 $\pm$ 0.86 c    | 19.27 $\pm$ 1.03 c    | 15.76 $\pm$ 0.89 c    | 3.69 $\pm$ 0.21 d     |
| <b>Polymers</b>           | 61.84 $\pm$ 4.47 c    | 225.69 $\pm$ 16.34 b  | 349.74 $\pm$ 25.60 a   | 55.30 $\pm$ 4.17 c    | 57.39 $\pm$ 4.37 c    | 60.19 $\pm$ 4.26 c    | 75.37 $\pm$ 5.47 c    | 45.59 $\pm$ 3.47 c    | 26.06 $\pm$ 1.96 c    |
| <b>Total procyanidins</b> | 1765.54 $\pm$ 32.86 c | 3867.07 $\pm$ 98.09 a | 4225.96 $\pm$ 117.15 a | 1957.17 $\pm$ 37.07 c | 2496.46 $\pm$ 55.56 b | 2747.95 $\pm$ 58.11 b | 2708.97 $\pm$ 57.32 b | 2570.55 $\pm$ 55.45 b | 1784.40 $\pm$ 29.62 c |

dp: polymerization degree. Different letters (a-e) in the same row indicate significant differences ( $p < 0.05$ ).

The results show the huge impact of temperature and airflow on total procyanidin content in the avocado by-product, ranging from 1765.54 to 4225.96  $\mu\text{g CE/g d.w.}$  To our knowledge, no previous references about the evaluation of the drying conditions among the procyanidin content of avocado peel are available. However, previously, López-Cobo et al. (2016) [461] have reported the procyanidin content of avocado peels measured using HPLC-FLD, and the profile distribution reported in the present study is consistent with them. Other authors have also reported similar procyanidin profiles measured in hawthorn [466] and cranberries [454] using HPLC-FLD.

The highest procyanidin retention was found at 80 °C when the samples were dried without airflow, mainly due to the reduced time needed to reach the final moisture content. However, differences in this tendency were found when considering the effect of the airflow. When airflow increased, an increase in procyanidin content was found at 40 and 60 °C but occurred the opposite at 80 °C. It is mainly attributable to the case-hardening effect occurring at the highest temperatures, promoted by the airflow, and entailing a decrease in the procyanidin content. The procyanidin content decreased to 1784.40  $\mu\text{g CE/g d.w.}$  when drying at 80 °C and airflow of 1.6 m/s for 60 min, very similar to the result obtained when drying at 40 °C without airflow for 480 min. If comparing the total procyanidin content at 60 °C with airflow of 1.6 m/s (2747.9  $\mu\text{g CE/g d.w.}$ ) and at 80 °C without airflow (2708.97  $\mu\text{g CE/g d.w.}$ ), as no significant differences were found between the results ( $p > 0.05$ ), probably the selected drying conditions would be those at 80 °C because the time needed was 37.5% lower. However, the highest procyanidin content in all cases was obtained when drying at 40 °C with airflow of 1.6 m/s for 105 min. Even though the time required is 43% higher than at 80 °C without airflow, the increase in total procyanidin content is 36%. Wojdyło et al. (2016) [467] reported that total flavan-3-ols content decreased when drying jujube fruits at an airflow of 1 m/s and increasing the temperature from 50 to 70 °C. Szychowski et al. (2018) [468] also reported decreased total procyanidin content with the convective drying temperature in quinces. Similarly, Donlao & Ogawa (2019) [469] reported a decrease in the total procyanidin content of green tea leaves when the convective drying temperature increased from 80 to 160 °C. In the present study, we found similar decreases in the total flavan-3-ol content when increasing the drying temperature from 40 to 60 °C with airflows of 0.8 or 1.6 m/s.

Furthermore, according to Liu et al. (2021) [470], convective drying maintains or increases the procyanidins degree of polymerization in strawberries, apple pomaces, and grapes. In our cases, we found no changes in the degree of polymerization, but it was maintained among the temperatures and airflows tested.

### **3.3. Effect of drying on the antioxidant activity**

The antioxidant activity was measured using three different methods to select the best convective drying conditions in terms of temperature and airflow. **Figure 6.4** shows the results obtained in

the avocado peel by-products dried at 40, 60 and 80 °C with an airflow of 0.8 m/s or 1.6 m/s or without airflow for the three antioxidant assays performed.

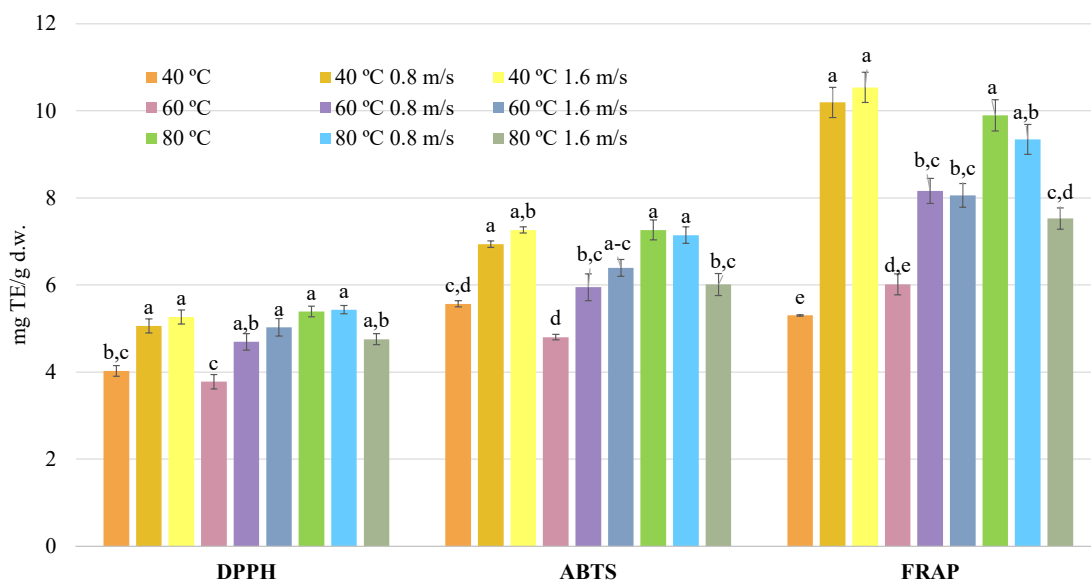


**Figure 6.4.** Antioxidant activity measured by DPPH, ABTS and FRAP of the avocado by-product dried by convective drying at different temperatures and air flows. The results are expressed as mg TE (Trolox equivalents)/g d.w. Different letters (a-e) for the same method indicate significant differences ( $p < 0.05$ ).

The results ranged from  $3.78 \pm 0.17$  to  $5.43 \pm 0.09$  mg TE/g d.w. for the DPPH, from  $4.80 \pm 0.06$  to  $7.26 \pm 0.07$  mg TE/g d.w. for the ABTS method, and from  $5.30 \pm 0.14$  to  $10.54 \pm 0.46$  mg TE/g d.w. for FRAP. Temperature and airflow also have shown an important influence on the antioxidant activity of the avocado by-product. When drying without airflow at high temperatures (80 °C), the samples exhibited higher antioxidant activity than at lower temperatures (60 and 40 °C), in consistency with the reported by Vega-Gálvez et al. (2009) for red peppers [220]. According to these authors, this behavior is explained because the drying process at low temperatures implies longer drying times that clearly promote a decrease in antioxidant activity. Moreover, it could also be explained by the generation and accumulation of Maillard compounds at higher temperatures that could enhance the antioxidant properties. Llavata et al. (2022) reported consistent results, with values of 28, 30 and 35 mg/kg d.w., determined using FRAP when drying cider apple pomace at 40, 60 and 80 °C, respectively [221]. In the same way as the procyanidin content, at 80 °C the antioxidant activity decreases when increasing the airflow. In contrast, at 60 °C with 1.6 m/s of airflow, the samples showed 25% higher antioxidant activity by the three methods than without airflow. For the DPPH and ABTS methods, the results obtained when drying at 40 °C and 1.6 m/s and 80 °C without airflow showed no significant differences ( $p < 0.05$ ).

Previously some authors have evaluated the effect of drying on the antioxidant activities of avocado matrices. Saavedra et al. (2017) [224] reported 5180.81 mg GAE/100 g d.w. by using DPPH in avocado peel dried at its optimal conditions of 72.71 °C and 0.8 m/s of airflow. In seeds drying at 65.52 °C and 1.39 m/s, the result obtained was 4132.7 mg GAE/g d.w. In avocado leaves, Loh & Lim (2018) [222] reported values of 7.17 and 8.47 mg GAE/g d.w. using the FRAP assay when dried in an oven at 50 °C and air dried, respectively, and completely in the same magnitude range as our results. Yamassaki et al. (2017) [223], when drying at 40, 60 and 90 °C, reported IC<sub>50</sub> DPPH values of 53.4, 53.4 and 106.7 µg/mL in avocado leaves, respectively. Our IC<sub>50</sub> DPPH values were 66.10, 64.25 and 86.54 µg/mL in the avocado peel samples dried at 40, 60 and 80 °C without airflow, respectively. Babiker et al. (2021) [451] reported values of 53.89, 79.21 and 76.73 µg/mL in avocado pulp, peel, and seed, respectively, when drying in oven at 60 °C for 19 hours.

#### 4. Conclusions

The drying step is essential for storing this avocado by-product at the industrial level to take advantage of it. Therefore, the convective drying of the avocado peel by-product has been studied at three different temperatures (40, 60 and 80 °C) and airflows (0, 0.8 and 1.6 m/s). All these parameters directly influence the time needed to reach a final moisture content below 10%. In addition, the fourteen tested mathematical models for fitting the drying kinetics of the avocado by-product demonstrated satisfactory results. However, the Page model provided better adjustment reporting higher coefficient of regressions and lower square and absolute errors for most of the temperatures and airflows tested. To understand well the behavior of the avocado peel during drying by using the Page equation will allow us to maximize its exploitation, reducing economic and energy costs. However, this model could be useless for drying the avocado peel using other techniques. Moreover, the procyanidin content determined using HPLC-FLD and the antioxidant activity determined using DPPH, ABTS and FRAP were evaluated to select the better drying conditions. In all cases, both the temperature and the airflow have proven to have a huge influence. The time needed to reach the final moisture content decreased when the airflow increased; therefore, the exposure to temperature is reduced, and consequently, the decrease in procyanidin and antioxidant compounds content is lower. However, the case-hardening phenomenon increased at 80 °C when increasing the airflow, decreasing thus the bioactive content. Finally, taking everything into account, the drying conditions of choice are 40 °C, an airflow of 1.6 m/s for 105 min, which allowed the lowest decrease in flavan-3-ols content and antioxidant activity. The main limitation of these drying conditions is that they could vary slightly depending on the oven size and the by-product itself, which could have different moisture content depending on the avocado growing location and the company processing it. However, convective drying at the optimized conditions would be a beneficial and low-time-consuming drying method for the avocado by-product from guacamole factories in the south of Spain. Finally, this could be



scaled up to pilot and industrial scale to obtain dry avocado by-products that could be used as functional ingredients for food, feed, and cosmeceutical uses.

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## **CHAPTER 7. Response surface methodology for the optimization of flavan-3-ols extraction from avocado by-products via sonotrode ultrasound-assisted extraction**

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## Article

# Response Surface Methodology for the Optimization of Flavan-3-ols Extraction from Avocado By-Products via Sonotrode Ultrasound-Assisted Extraction

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## Abstract

Avocado peel and seed are the main by-products of avocado processing and are considered as promising sources of phenolic compounds with biological activities. Thus, this research focuses on the establishment, for the first time, of ultrasound-assisted extraction of flavan-3-ols with high antioxidant activity from avocado peel and seed using a sonotrode. Indeed, 2 Box–Behnken designs were performed for 15 experiments, with each design having three independent factors (ratio ethanol/water (v/v), time (min) and amplitude (%)). In both models, the responses included total procyanidins (flavan-3-ols) measured via HPLC-FLD and antioxidant activity measured via DPPH, ABTS and FRAP. The results showed that applying the sonotrode extraction method could increase flavan-3-ols recovery by 54% and antioxidant activity by 62–76% compared to ultrasound bath technology. Therefore, this technology was demonstrated to be a non-thermal, low time-consuming and scalable method that allowed the recovery of flavan-3-ols from avocado by-products that could be used as functional ingredients.

**Keywords:** avocado seed; avocado peel; flavan-3-ols; sonotrode; Box–Behnken; procyanidins

## 1. Introduction

Avocado is a tropical and subtropical fruit classified in the family *Lauraceae*, genus *Persea*, and species *P. americana*. There are numerous cultivars of avocado around the world, depending on the climate where they were grown, that confer those different shapes, flavors, textures, colors, and smells. The most well-known and marketed types are ‘Hass’ and ‘Fuerte’ cultivars. Avocado is known for its high nutritional content and health benefits, which are essentially due to the fact that they are a source of fat-soluble nutrients or phytochemicals [110]. Nowadays the consumption of avocado is increasing due to not only its nutritional and organoleptic properties, but also the increased availability of avocado based products as guacamole, oil, jams, juices, ice creams, sauces, etc [111]. In addition, in 2020 it was reported an avocado production of 8.06 million tons worldwide, 35% higher than in 2015, being the main producing countries Mexico, Dominican Republic, Peru and Colombia [450]. From avocado fruit, only up to 65-73% of its weight is consumed (pulp) and the rest is discarded as wastes. These wastes are mainly composed of peel and seed, accounting for 21-39% of the whole avocado [111], which means a by-product generation from 1.24 to 2.5 million tons in 2020. Into this context, the valorization of those by-products is an important environmental problem to be faced. Until now, avocado by-products have been processed with different biorefinery approaches as for obtaining bioenergy, biogas, biofuel, biodiesel or bioethanol, or with the scope of extracting oils, fermentable sugars, starches, proteins, colorants or phenolic compounds [111]. Avocado peel and seed are considered as promising sources of phenolic compounds that have been reported to possess anti-carcinogenic, anti-inflammatory, anti-microbial and antioxidant properties, among others [117,121,471,472]. Specially, flavan-3-ols from avocado by-products have demonstrated to possess anticancer activity against melanoma cells [122], to inhibit *Helicobacter pylori* [123] and to improve the colonic homeostasis in protein rich diets [126]. Besides, avocado procyanidins have also been reported to inhibit the enzyme polyphenol oxidase, an enzyme that plays critical roles in the phenolic degradation, browning, development of insects, and melanogenesis in food [473]. This indicates that procyanidins from avocado by-products could be of huge application in the fields of nutraceuticals and food. Some authors have reported the extraction of total phenolic compounds from avocado peel and seed by traditional methods as maceration and by innovative ones as microwaves, ultrasound bath or pressurized fluid assisted extractions, but with no focus into any compound. So, the aim of this work is to establish the best ultrasound-assisted extraction by sonotrode, a technology that could be scaled up industrially, to obtain enriched extracts in procyanidins (flavan-3-ols) from avocado peel and seed that also present high antioxidant activity.

## 2. Materials and Methods

### 2.1. Chemicals and Reagents

The reagents used for the antioxidant assays and catechin standard were purchased from Sigma-Aldrich (St. Louis, MO, USA). Double-deionized water was from Millipore (Bedford, MA, USA). HPLC-grade water, acetic acid, acetonitrile, and methanol were purchased from Merck KGaA (Darmstadt, Germany).

## 2.2. Samples

Avocado by-products from the variety 'Hass' were obtained from a local company located in the subtropical coast of the province of Málaga (Vélez-Málaga, Spain) in October 2020. We were provided the generated by-products from the production of guacamole, peels (moisture ~75%) and seeds (moisture ~45%), separately. The samples were dried at 50 °C, grinded and stored at -18 °C until analysis.

## 2.3. Experimental Design

The obtention of avocado peel and seed extracts enriched in procyanidins with a high antioxidant activity was optimized using Box–Behnken designs. Each design consisted of 15 experiments with 3 levels (-1, 0, 1), which were carried out in duplicate. In both peel and seed models, the independent factors were the ratio of ethanol/water (30/70, 65/45, 100/0 % v/v), time (5, 25, 45 min) and amplitude (20, 60, 100%). The dependent variables were total procyanidins and FRAP, DPPH and ABTS. The variables were adjusted to fit a second-order polynomial equation (Equation (16)), in which the response variable was represented by  $Y$ , the independent factors were represented by  $X_i$  and  $X_j$ , and the regression coefficients of the model (interception, linear, quadratic and interaction terms) were  $\beta_0$ ,  $\beta_i$ ,  $\beta_{ii}$  and  $\beta_{ij}$ , respectively.

Equation (16). was the second-order polynomial equation.

$$Y = \beta_0 + \sum_{i=1}^4 \beta_i X_i + \sum_{i=1}^4 \beta_{ii} X_i^2 + \sum_{i=1}^4 \sum_{j=0}^4 \beta_{ij} X_i X_j \quad (16)$$

ANOVA analyses were performed in order to evaluate the adjustment of the models. Optimal conditions were established via response surface methodology (RSM).

## 2.4. Sonotrode Extraction

For each trial, 0.5 g of the sample was weighted, and 100 mL of ethanol/water (v/v) were added in 250 mL beakers. After that, the sonotrode (UP400St ultrasonic processor, Hielscher, Teltow, Germany), for which the amplitude was previously configured, was introduced into the beaker and the sample was sonicated during the established time. The pulse was locked at 100% for all analyses. Once the analysis time finished, the content of the beaker was verted to a Falcon tube, and it was centrifuged for 10 min at 8603×  $g$  and 0 °C. The supernatants were collected, evaporated, and reconstituted in 2–4 mL of methanol/water (1:1, v/v). The final extracts were

filtered using cellulose acetate filters of 0.2 mm (Millipore, Bedford, MA, USA) and stored at  $-18^{\circ}\text{C}$  until later analysis.

## **2.5. Ultrasonic Bath Extraction**

The ultrasonic bath extraction was carried out following the protocol previously used by López-Cobo et al. [461], albeit with slight modifications. In brief, 2 g of sample powder was extracted with 15 mL of ethanol/water (80:20, v/v) in 50 mL Falcon tubes. The mixture was placed in an ultrasonic bath (Sonorex RK 52, Bandelin, Berlin, Germany) for 15 min and then centrifuged for 10 min at  $8603\times g$  and  $0^{\circ}\text{C}$ . The supernatant was removed, and the extraction was twice repeated. The supernatants were collected, evaporated, and reconstituted in 3 mL of methanol/water (50:50, v/v). The final extracts were filtered using cellulose filter of 0.2 mm (Millipore, Bedford, MA, USA) and stored at  $-18^{\circ}\text{C}$  until later analysis.

## **2.6. Determination of Procyanidins via HPLC-FLD-MS**

The methodology used in the determination of flavan-3-ols was previously reported by López-Cobo et al. [461]. For the analysis, an Agilent 1200 Series (Agilent Technologies, Palo Alto, CA, USA), which was equipped with a quaternary pump delivery system, a degasser, an autosampler and a fluorometric detector (FLD), was used. It used a Develosil Diol 100 Å column that had the following dimensions: 5  $\mu\text{m}$  and  $250 \times 4.6$  mm ID (Phenomenex, Torrance, CA, USA). Mobile phases A and B consisted of acidic acetonitrile ((A)  $\text{CH}_3\text{CN}:\text{CH}_3\text{COOH}$ , 98:2; v/v) and acidic aqueous methanol ((B)  $\text{CH}_3\text{OH}:\text{H}_2\text{O}:\text{CH}_3\text{COOH}$ , 95:3:2; v/v/v), respectively. The starting mobile phase condition was 0% B, which was held for 10 min. Next, the gradient elution was 0% B for 5 min; B was increased to 38% over 50 min, and then to 100% over 3 min. Next, 100% B was maintained for 10 min and then returned to 0% B over 6 min and maintained for 10 min. An excitation wavelength of 230 nm and an emission wavelength of 321 nm were fixed for the fluorescence detection. The injection volume was 10  $\mu\text{L}$ . The identification of flavan-3-ols was done according to data previously described in literature [461], as they elute according to their degree of polymerization, firstly eluting the monomers and then the different oligomers [462]. A standard curve of catechin at 6 concentration levels from 10 to 650 ppm was carried out for the quantification of flavan-3-ols ( $y = 3137.9x - 9865.6$ ,  $R^2 = 0.999982$ ). In addition correction factors suggested by Robbins et al. [462] were used for quantification. Results are expressed as mg catechin equivalents (CE)/g d.w.

## **2.7. Antioxidant Assays: ABTS, DPPH and FRAP Methods**

The antioxidant capacity was evaluated twice in the all the experiments and by three different methods: ABTS, DPPH and FRAP. ABTS method was carried out by adding 1 mL of the ABTS



solution to 100  $\mu$ L of extract and the detriment of absorbance during 30 minutes at 734 nm was measured [249]. For DPPH, 2.9 mL of DPPH were added to 100  $\mu$ L of each extract and the absorbance was measured for 30 min at 517 nm [247,248]. FRAP assay was done by adding 90  $\mu$ L of distilled water and 900  $\mu$ L of the FRAP reagent to 30  $\mu$ L of each extract, kept in darkness for 30 min at 37 °C and measured at 595 nm [250]. For all the assays the measurements were done in UV–visible spectrophotometer (Spectrophotometer 300 Array, UV–Vis, single beam, Shimadzu, Duisburg, Germany). They were used standard curves of Trolox (1, 5, 10, 20, 50, 80, 100, 150, 200 ppm). Results are expressed as mg Trolox equivalents (TE)/g d.w.

## **2.8. Data Elaboration**

The software used for the simulations and statistical analyses, such as ANOVA and statistical differences (Tukey test), was Statistica 8.0 package (StatSoft, Tulsa, OK, USA). Metaboanalyst 5.0 software (Xia Lab, McGill, Montréal, QC, Canada) was used to create the Pearson correlation heatmap.

## **3. Results and Discussion**

### **3.1. Determination of Procyanidins and Antioxidant Capacity in Avocado By-Products**

To optimize the extraction conditions via sonotrode of avocado by-products, two Box–Behnken designs were created, using the total procyanidin content measured via HPLC-FLD and the antioxidant activities measured via three methods (DPPH, ABTS and FRAP) as response variables (**Table 7.1**).

**Table 7.1.** Avocado peel and seed Box–Behnken designs with natural and coded values (parenthesis) of the extraction conditions and the experimental results obtained for total procyanidins, and antioxidant assays (DPPH, ABTS and FRAP) expressed with the average and the standard deviation.

| Run | Independent factors |            |               | Dependent factors                 |                     |                     |                     |                                   |                     |                     |                     |
|-----|---------------------|------------|---------------|-----------------------------------|---------------------|---------------------|---------------------|-----------------------------------|---------------------|---------------------|---------------------|
|     | Ethanol (%)         | Time (min) | Amplitude (%) | Avocado peel                      |                     |                     |                     | Avocado seed                      |                     |                     |                     |
|     |                     |            |               | Total procyanidins (mg CE/g d.w.) | DPPH (mg TE/g d.w.) | ABTS (mg TE/g d.w.) | FRAP (mg TE/g d.w.) | Total procyanidins (mg CE/g d.w.) | DPPH (mg TE/g d.w.) | ABTS (mg TE/g d.w.) | FRAP (mg TE/g d.w.) |
| 1   | 100 (1)             | 5 (-1)     | 60 (0)        | 1.05 ± 0.02                       | 0.31 ± 0.00         | 2.07 ± 0.03         | 1.15 ± 0.00         | 4.29 ± 0.05                       | 2.22 ± 0.04         | 4.08 ± 0.07         | 2.53 ± 0.12         |
| 2   | 30 (-1)             | 5 (-1)     | 60 (0)        | 6.53 ± 0.01                       | 7.62 ± 0.07         | 5.18 ± 0.01         | 10.11 ± 0.12        | 12.18 ± 0.06                      | 16.88 ± 0.08        | 16.12 ± 0.05        | 17.11 ± 0.20        |
| 3   | 100 (1)             | 45 (1)     | 60 (0)        | 2.93 ± 0.05                       | 0.73 ± 0.01         | 8.44 ± 0.10         | 1.96 ± 0.02         | 8.88 ± 0.07                       | 8.26 ± 0.05         | 6.98 ± 0.14         | 9.39 ± 0.01         |
| 4   | 30 (-1)             | 45 (1)     | 60 (0)        | 8.03 ± 0.03                       | 7.80 ± 0.05         | 5.83 ± 0.03         | 11.09 ± 0.06        | 13.05 ± 0.09                      | 13.95 ± 0.12        | 4.06 ± 0.22         | 11.56 ± 0.09        |
| 5   | 100 (1)             | 25 (0)     | 20 (-1)       | 1.89 ± 0.11                       | 0.37 ± 0.00         | 1.17 ± 0.04         | 1.05 ± 0.02         | 5.13 ± 0.14                       | 1.92 ± 0.02         | 1.93 ± 0.09         | 2.36 ± 0.11         |
| 6   | 30 (-1)             | 25 (0)     | 20 (-1)       | 8.83 ± 0.06                       | 9.23 ± 0.08         | 10.35 ± 0.07        | 11.00 ± 0.01        | 13.81 ± 0.18                      | 16.78 ± 0.09        | 20.68 ± 0.13        | 19.41 ± 0.02        |
| 7   | 100 (1)             | 25 (0)     | 100 (1)       | 3.71 ± 0.01                       | 0.70 ± 0.02         | 1.20 ± 0.00         | 1.67 ± 0.09         | 8.35 ± 0.12                       | 5.87 ± 0.03         | 1.29 ± 0.10         | 6.50 ± 0.10         |
| 8   | 30 (-1)             | 25 (0)     | 100 (1)       | 11.44 ± 0.10                      | 8.68 ± 0.02         | 5.62 ± 0.11         | 11.38 ± 0.02        | 12.52 ± 0.08                      | 13.63 ± 0.03        | 12.29 ± 0.04        | 15.86 ± 0.08        |
| 9   | 65 (0)              | 5 (-1)     | 20 (-1)       | 9.19 ± 0.08                       | 2.58 ± 0.09         | 6.33 ± 0.07         | 8.30 ± 0.04         | 9.95 ± 0.04                       | 10.31 ± 0.10        | 7.51 ± 0.09         | 11.48 ± 0.01        |
| 10  | 65 (0)              | 45 (1)     | 20 (-1)       | 13.32 ± 0.02                      | 10.80 ± 0.04        | 23.12 ± 0.06        | 11.65 ± 0.10        | 12.61 ± 0.08                      | 15.75 ± 0.07        | 15.22 ± 0.02        | 14.27 ± 0.06        |
| 11  | 65 (0)              | 5 (-1)     | 100 (1)       | 13.98 ± 0.11                      | 10.58 ± 0.08        | 16.09 ± 0.12        | 14.04 ± 0.03        | 15.11 ± 0.03                      | 16.89 ± 0.01        | 25.77 ± 0.08        | 20.16 ± 0.20        |
| 12  | 65 (0)              | 45 (1)     | 100 (1)       | 16.64 ± 0.09                      | 12.15 ± 0.10        | 11.03 ± 0.10        | 11.72 ± 0.04        | 16.09 ± 0.11                      | 16.88 ± 0.12        | 21.43 ± 0.15        | 24.82 ± 0.03        |
| 13  | 65 (0)              | 25 (0)     | 60 (0)        | 20.02 ± 0.01                      | 12.29 ± 0.04        | 20.07 ± 0.07        | 14.42 ± 0.10        | 15.86 ± 0.12                      | 17.10 ± 0.08        | 24.19 ± 0.01        | 22.77 ± 0.05        |
| 14  | 65 (0)              | 25 (0)     | 60 (0)        | 19.87 ± 0.03                      | 12.19 ± 0.11        | 19.14 ± 0.04        | 14.79 ± 0.02        | 15.65 ± 0.02                      | 17.11 ± 0.06        | 22.86 ± 0.07        | 22.83 ± 0.01        |
| 15  | 65 (0)              | 25 (0)     | 60 (0)        | 20.02 ± 0.00                      | 12.17 ± 0.07        | 18.80 ± 0.02        | 14.55 ± 0.08        | 15.56 ± 0.04                      | 16.70 ± 0.07        | 23.51 ± 0.10        | 22.21 ± 0.02        |

Regarding the avocado peel model, the results obtained for procyanidins ranged from 1.05 to 20.02 mg CE/g d.w., while for the antioxidant assays, values ranged from 0.31 to 12.29  $\mu$ g TE/g d.w. for DPPH, 1.17–23.12 mg TE/g d.w. for ABTS, and 1.05–14.79 mg TE/g d.w. for FRAP technique. In all cases, the lowest values corresponded to the extracts obtained using 100% ethanol as solvent, increasing the content as the time and amplitude of extraction increased. In contrast, the highest value for all variables was obtained using 65% of ethanol.

The total content of procyanidins obtained in the avocado seed model was between 4.29 and 16.09 mg CE/g d.w., and the ranges observed for DPPH, ABTS and FRAP were 1.92–17.11, 1.93–25.77 and 2.36–24.82 mg TE/g d.w., respectively. In the antioxidant assays, the lowest values were obtained for the same extraction conditions: 100% ethanol, 25 min and 20% amplitude. Similar to the avocado peel model, the highest value for all variables was obtained using ethanol/water in the intermedium value tested and at amplitudes between 60 and 100%.

### 3.2. Fitting the Model

The experimental results of the avocado peel and seed models were adjusted to fit second-order polynomial equations (Equation (16)), and all regression coefficients with the effects and  $p$  values obtained are shown in **Table 7.2**. In all cases, we selected a significance level of  $p < 0.05$ , the non-significant terms were discarded, and the models were recalculated by considering only the significant terms.

**Table 7.2.** Estimated regression coefficients of the adjusted second-order polynomial equations and analysis of variance (ANOVA) of avocado peel and seed sonotrode models.

| Regression Coefficients | Responses      |         |                |         |                |         |                |         |                |         |                |         |                |         |                |         |
|-------------------------|----------------|---------|----------------|---------|----------------|---------|----------------|---------|----------------|---------|----------------|---------|----------------|---------|----------------|---------|
|                         | Avocado peel   |         |                |         |                |         |                |         | Avocado seed   |         |                |         |                |         |                |         |
|                         | Procyanidins   |         | DPPH           |         | ABTS           |         | FRAP           |         | Procyanidins   |         | DPPH           |         | ABTS           |         | FRAP           |         |
|                         | (mg CE/g d.w.) |         | (mg TE/g d.w.) |         | (mg TE/g d.w.) |         | (mg TE/g d.w.) |         | (mg CE/g d.w.) |         | (mg TE/g d.w.) |         | (mg TE/g d.w.) |         | (mg TE/g d.w.) |         |
|                         | Effect         | p value | Effect         | p value | Effect         | p value | Effect         | p value | Effect         | p value | Effect         | p value | Effect         | p value | Effect         | p value |
| $\beta_0$               | 8.1298         | 0.0000* | 5.9622         | 0.0000* | 8.0353         | 0.0006* | 7.9278         | 0.0000* | 10.9987        | 0.0000* | 11.6117        | 0.0000* | 11.4461        | 0.0003* | 12.955         | 0.0001* |
| Lineal                  |                |         |                |         |                |         |                |         |                |         |                |         |                |         |                |         |
| $\beta_1$               | -5.9731        | 0.0001* | -7.5989        | 0.0000* | -2.4340        | 0.0385* | -9.3072        | 0.0002* | -6.1622        | 0.0004* | -10.7447       | 0.0002* | -7.9975        | 0.0038* | -9.988         | 0.0007* |
| $\beta_2$               | 2.2608         | 0.0008* | 1.8313         | 0.0007* | 4.2973         | 0.0128* | 0.7649         | 0.0319* | 2.4261         | 0.0023* | 2.1343         | 0.0059* | -2.4943        | 0.0369* | 1.676          | 0.0224* |
| $\beta_3$               | 2.8280         | 0.0005* | 1.4863         | 0.0010* | -1.9543        | 0.0579  | 1.3028         | 0.0114* | 2.0851         | 0.0031* | 1.5498         | 0.0123* | 1.0683         | 0.1625  | 3.402          | 0.0056* |
| Crossed                 |                |         |                |         |                |         |                |         |                |         |                |         |                |         |                |         |
| $\beta_{12}$            | 0.1898         | 0.15512 | 0.1202         | 0.1956  | 2.8571         | 0.0494* | -0.0866        | 0.6902  | 1.8613         | 0.0071* | 4.4914         | 0.0027* | 7.4787         | 0.0077* | 6.205          | 0.0030* |
| $\beta_{13}$            | -0.3980        | 0.0427* | 0.4422         | 0.0196* | 2.3784         | 0.0691  | 0.1210         | 0.5857  | 2.2557         | 0.0048* | 3.5437         | 0.0043* | 3.8723         | 0.0279* | 3.846          | 0.0078* |
| $\beta_{23}$            | -0.7402        | 0.0129* | -3.3287        | 0.0004* | -10.9273       | 0.0036* | -2.8379        | 0.0044* | -0.8383        | 0.0334* | -2.7257        | 0.0072* | -6.0253        | 0.0118* | 0.933          | 0.1123  |
| Quadratic               |                |         |                |         |                |         |                |         |                |         |                |         |                |         |                |         |
| $\beta_{11}$            | 11.0757        | 0.0000* | 6.1931         | 0.0000* | 11.7574        | 0.0009* | 6.8304         | 0.0002* | 4.7850         | 0.0003* | 6.0232         | 0.0004* | 12.0744        | 0.0008* | 9.550          | 0.0003* |
| $\beta_{22}$            | 4.2587         | 0.0001* | 1.9114         | 0.0003* | 2.2010         | 0.0235* | 1.6789         | 0.0034* | 1.3009         | 0.0039* | 0.6176         | 0.0365* | 3.6357         | 0.0088* | 2.903          | 0.0037* |
| $\beta_{33}$            | 2.4284         | 0.0003* | 1.2775         | 0.0007* | 2.9960         | 0.0129* | 1.4813         | 0.0043* | 0.9486         | 0.0074* | 1.3943         | 0.0075* | 2.3983         | 0.0200* | 2.016          | 0.0077* |
| $R^2$                   | 0.9996         |         | 0.9997         |         | 0.9635         |         | 0.9971         |         | 0.9907         |         | 0.9885         |         | 0.9878         |         | 0.9933         |         |
| p model                 | 0.0000*        |         | 0.0014*        |         | 0.0031*        |         | 0.0000*        |         | 0.0004*        |         | 0.0018*        |         | 0.0212*        |         | 0.0058*        |         |
| p lack of fit           | 0.1551         |         | 0.1956         |         | 0.1279         |         | 0.2794         |         | 0.0899         |         | 0.0762         |         | 0.1625         |         | 0.1123         |         |

\* Significant at  $\alpha \leq 0.05$ ; 1 Ethanol (%), 2 time (min), 3 amplitude (%).

In the avocado peel model, the lineal terms  $\beta_1$  and  $\beta_2$ , the crossed interaction  $\beta_{23}$  and all quadratic terms ( $\beta_{11}$ ,  $\beta_{22}$ , and  $\beta_{33}$ ) showed significant effects for the four response variables. In addition, the linear term  $\beta_3$  and the interaction between ethanol/water and amplitude ( $\beta_{13}$ ) for procyanidins and DPPH, as well as the interaction between ethanol/water and time  $\beta_{12}$  for ABTS, were significant. According to the effects reported in **Table 7.2**, the factor ethanol/water ( $\beta_1$ ) had the highest negative effect in the model, followed by the interaction between time and amplitude ( $\beta_{23}$ ). In contrast, all quadratic interactions showed positive effects. Moreover, we revealed a high correlation between the response variables and the factors ( $R^2 = 0.9996, 0.9997, 0.9635$  and  $0.9971$  for procyanidins, DPPH, ABTS and FRAP, respectively). The ANOVA test revealed the validity of the model, as all  $p$ -model values were significant, and the lack of fit was non-significant ( $p > 0.05$ ).

Concerning the avocado seed model, almost all linear, interacting, and quadratic terms were found to have significant effect. Only the amplitude ( $\beta_3$ ) for ABTS and the crossed interaction between time and amplitude ( $\beta_{23}$ ) for FRAP did not present significance. The correlation between factors and all responses was demonstrated because their determination coefficients were higher than 0.9 in all cases ( $0.9907, 0.9885, 0.9878$  and  $0.9933$  for procyanidins, DPPH, ABTS and FRAP, respectively). The validity of the model was confirmed based on ANOVA finding a lack of fit for  $p$  values  $> 0.05$  and significative  $p$ -model values.

### 3.3. Optimization of Sonotrode Extraction Conditions

The response surfaces graphs obtained for each variable were studied to select the optimal conditions for each by-product by establishing a compromise between them, aiming extracts with the highest total procyanidin contents and high antioxidant activity. Moreover, one of the objectives of this study was to develop an optimal sonotrode extraction process that could be industrially scaled up; thus, other reasons, such as economic and energy costs, were considered.

In **Figure 7.1** (from 1p to 12p), it is shown that the highest responses for the avocado peel model could be obtained at an ethanol/water ratio lower than 70:30, v/v but higher than 40%, with the highest response being near to the intermedium value evaluated (65%). Regarding amplitude, the highest responses were reached between 70 and 100%; thus, amplitude was selected to be as small as possible to reduce energy consumption. The time was also selected to be as short as possible, taking into account the other parameters, to achieve the fastest procedure.

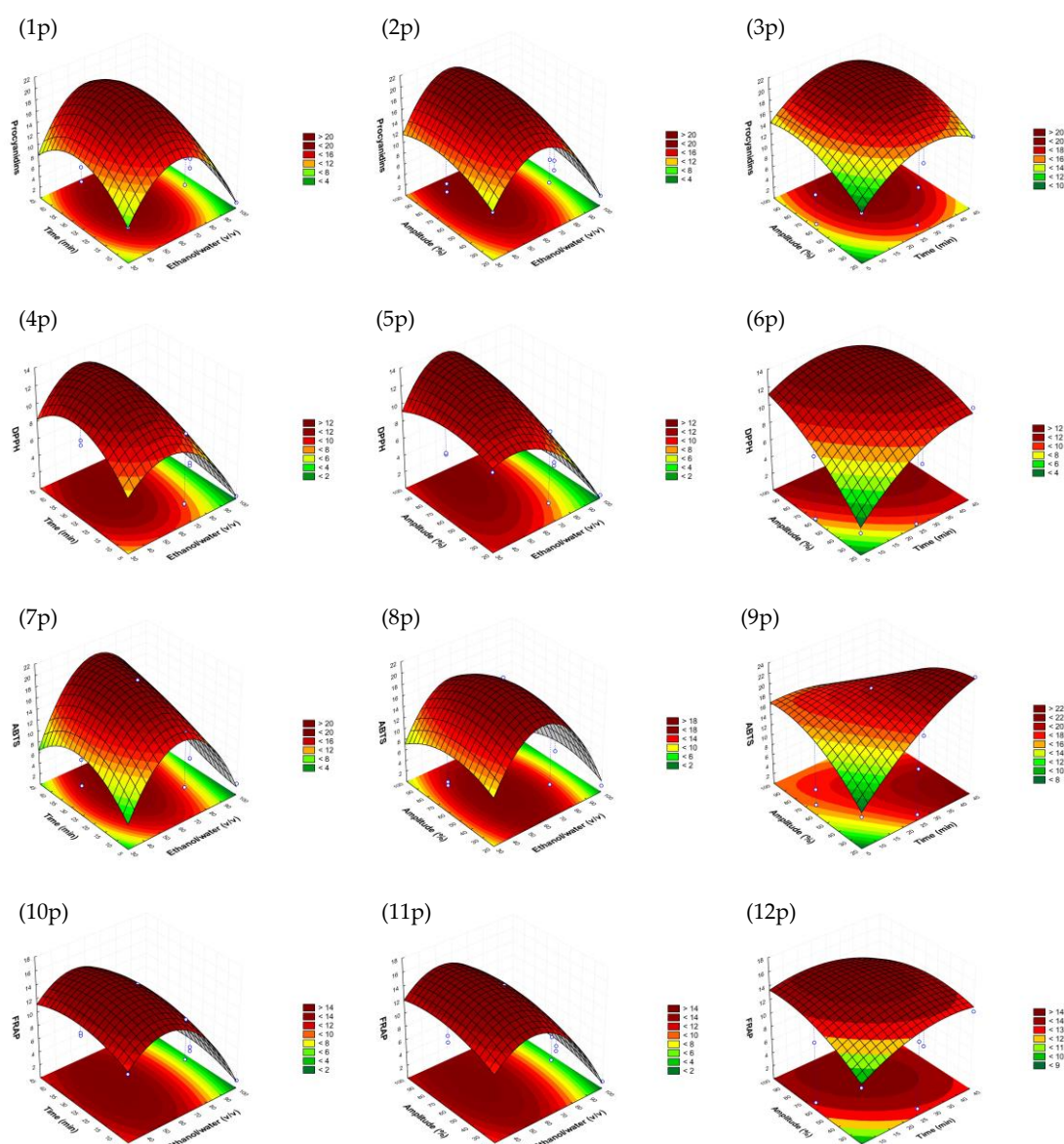


**Figure 7.1.** Response surface graphs (1p–12p) of the avocado peel sonotrode model that show the combined effects of the process variables: ethanol/water (v/v), time (min) and amplitude (%) for procyanidins (mg CE/g d.w.) and DPPH, as well as ABTS and FRAP antioxidant assays (mg TE/g d.w.).

Similar response surfaces were obtained for the avocado seed model, as can be seen in **Figure 7.2**. In this case, the ethanol/water ratio selected could be slightly lower than that of the peel. In fact, in terms of economic costs, it is advisable to choose lower percentages of ethanol and higher percentages of water. However, higher amplitudes of 90% seemed to be necessary to obtain better performances. This fact could essentially be attributed to the differences between matrices' structures. Once again, the time was selected to be as low as possible.

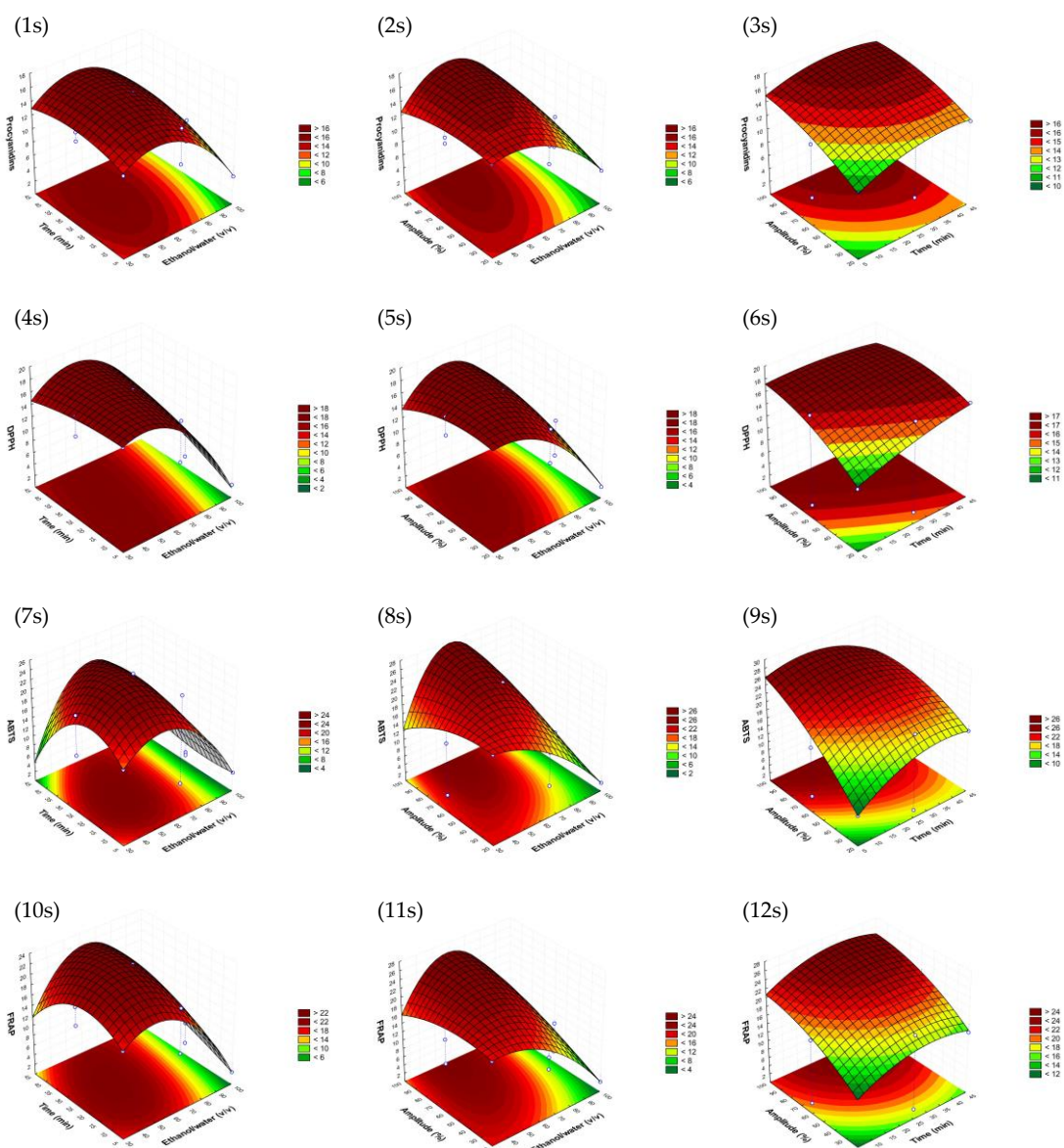


**Figure 7.2.** Response surface graphs (1s–12s) of the avocado seed sonotrode model that show the combined effects of the process variables: ethanol/water (v/v), time (min) and amplitude (%) for procyanidins (mg CE/g d.w.) and DPPH, as well as ABTS and FRAP antioxidant assays (mg TE/g d.w.).

In brief, the optimal conditions selected for the avocado peel model were 60:30, (v/v) ethanol/water, 30 min and amplitude 70%, while the optimal conditions for the avocado seed model 55:45 (v/v) ethanol/water, 30 min and amplitude 90% (**Table 7.3**).

**Table 7.3.** Optimal conditions selected and the predicted and obtained values expressed as the mean and standard deviation of the avocado peel and seed sonotrode models.

| Parameters                            | Optimal Conditions |              |                |              |              |              |              |              |
|---------------------------------------|--------------------|--------------|----------------|--------------|--------------|--------------|--------------|--------------|
|                                       | Avocado Peel       |              |                |              | Avocado Seed |              |              |              |
| Ethanol (%)                           | 60                 |              |                |              | 55           |              |              |              |
| Time (min)                            | 30                 |              |                |              | 30           |              |              |              |
| Amplitude (%)                         | 70                 |              |                |              | 90           |              |              |              |
|                                       | Procyanidins       | DPPH         | ABTS           | FRAP         | Procyanidins | DPPH         | ABTS         | FRAP         |
| <b>Predicted Value</b><br>(mg/g d.w.) | 20.73 ± 0.59       | 13.45 ± 0.44 | 19.20 ± 2.23.5 | 15.14 ± 0.63 | 16.99 ± 0.57 | 17.99 ± 0.83 | 25.41 ± 2.39 | 25.47 ± 1.24 |
| <b>Obtained value</b><br>(mg/g d.w.)  | 20.80 ± 0.10       | 13.80 ± 0.22 | 20.23 ± 0.12   | 14.97 ± 0.25 | 16.70 ± 0.20 | 17.76 ± 0.15 | 25.87 ± 0.33 | 26.62 ± 0.92 |
| <b>CV (%)</b>                         | 0.23               | 1.81         | 3.69           | 0.78         | 1.22         | 0.91         | 1.26         | 3.12         |
| <b>Control (mg/g d.w.)</b>            | 9.66 ± 0.24        | 5.26 ± 0.11  | 6.99 ± 0.34    | 5.00 ± 0.22  | 7.64 ± 0.34  | 5.36 ± 0.26  | 6.12 ± 0.19  | 8.00 ± 0.19  |



The accuracy of the two mathematical models was verified (**Table 7.3**). In all cases, there were no significant differences between the model's predicted values and the experimental results obtained based on the optimal conditions. Moreover, the coefficients of variation were lower than five for the total procyanidins and antioxidant assays in peel and seed models.

### 3.4. Comparison between Sonotrode and Ultrasonic Bath-Assisted Extractions

**Figure 7.3** shows a fluorescence chromatogram of the identified flavan-3-ols in avocado peel and seed extracts obtained via sonotrode at optimal conditions.

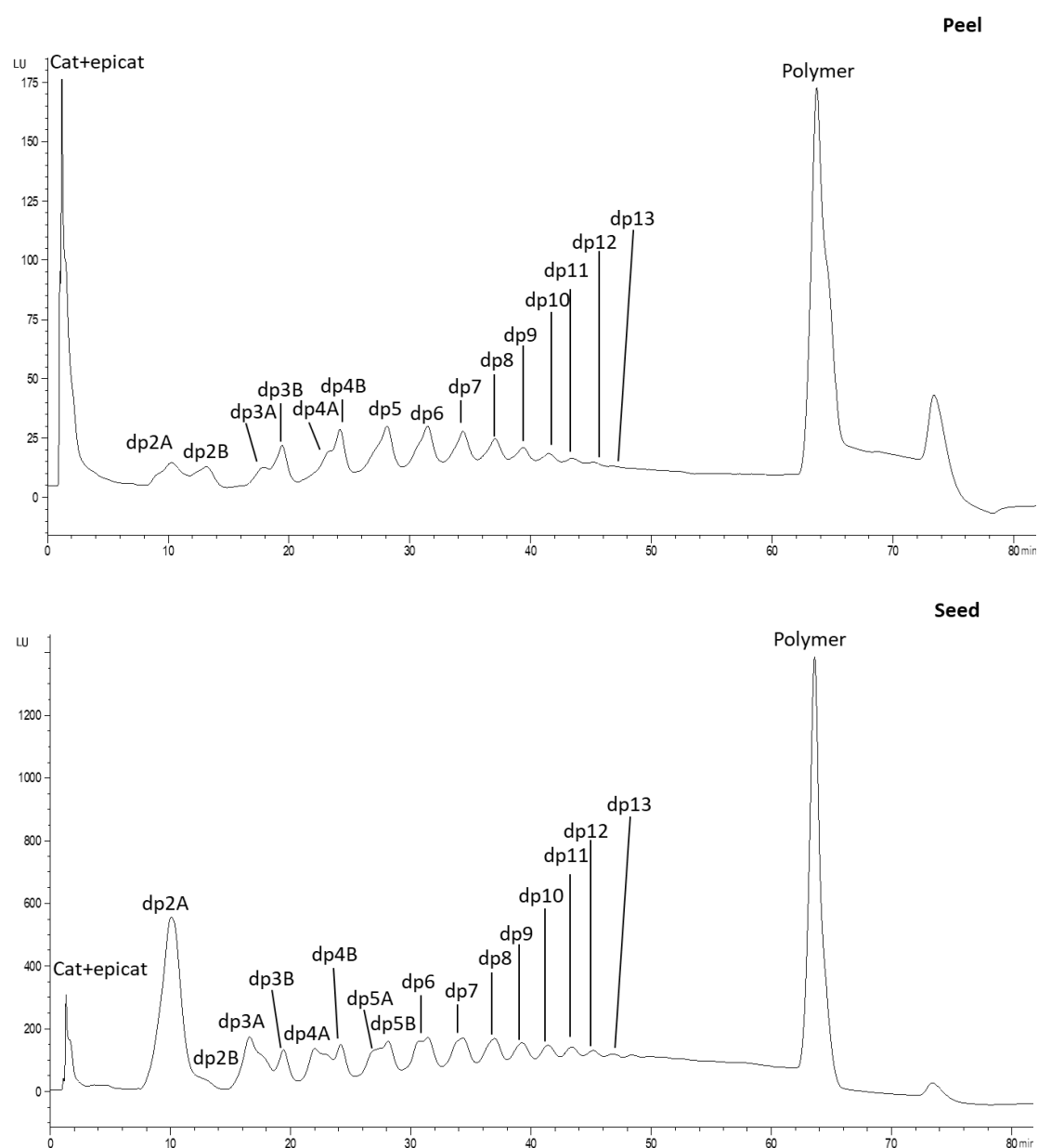


**Figure 7.3.** Fluorescence chromatograms of the identified flavan-3-ols in avocado peel and seed extracts obtained by sonotrode optimal conditions. Cat+epicat: catechin+epicatechin; dp: degree of polymerization.

The quantification via HPLC-FLD of procyanidins in both seed and peel extracts obtained via sonotrode and ultrasound bath technology are presented in **Table 7.4**.

**Table 7.4.** Distribution according to the polymerization degree of flavan-3-ols in avocado peel and seed optimal extracts obtained via sonotrode and ultrasound bath controls expressed as the mean and standard deviation.

|                           | Avocado Peel (mg/g d.w.) |                 | Avocado Seed (mg/g d.w.) |                 |
|---------------------------|--------------------------|-----------------|--------------------------|-----------------|
|                           | Sonotrode                | Ultrasound Bath | Sonotrode                | Ultrasound Bath |
| <b>Monomer</b>            | 8.17 ± 0.04              | 4.53 ± 0.06     | 0.72 ± 0.05              | 0.48 ± 0.05     |
| <b>dp2</b>                | 1.40 ± 0.01              | 0.76 ± 0.02     | 4.58 ± 0.02              | 2.68 ± 0.08     |
| <b>dp3</b>                | 1.15 ± 0.00              | 0.91 ± 0.04     | 1.65 ± 0.03              | 1.09 ± 0.06     |
| <b>dp4</b>                | 1.09 ± 0.02              | 0.91 ± 0.03     | 1.09 ± 0.02              | 0.68 ± 0.04     |
| <b>dp5</b>                | 0.90 ± 0.01              | 0.68 ± 0.01     | 0.75 ± 0.01              | 0.46 ± 0.03     |
| <b>dp6</b>                | 0.54 ± 0.00              | 0.49 ± 0.01     | 0.64 ± 0.01              | 0.38 ± 0.01     |
| <b>dp7</b>                | 0.48 ± 0.00              | 0.29 ± 0.01     | 0.48 ± 0.01              | 0.29 ± 0.02     |
| <b>dp8</b>                | 0.24 ± 0.00              | 0.16 ± 0.00     | 0.35 ± 0.00              | 0.21 ± 0.00     |
| <b>dp9</b>                | 0.06 ± 0.00              | 0.07 ± 0.00     | 0.23 ± 0.00              | 0.15 ± 0.00     |
| <b>dp10</b>               | 0.03 ± 0.00              | 0.02 ± 0.00     | 0.15 ± 0.00              | 0.09 ± 0.00     |
| <b>Polymer</b>            | 6.72 ± 0.03              | 0.83 ± 0.06     | 6.03 ± 0.05              | 1.15 ± 0.10     |
| <b>Total procyanidins</b> | 20.80 ± 0.10             | 9.66 ± 0.24     | 16.69 ± 0.20             | 7.64 ± 0.34     |

The total procyanidin content in the avocado peel and seed optimal extracts were 20.8 and 16.69 mg/g d.w., respectively. Total phenolic compounds from avocado peels and seeds measured by spectrophotometry (Folin Ciocalteu) or HPLC-MS have been reported by several authors using hydroethanolic liquid-liquid extraction [116], ultrasound bath [117,474,475], microwave [476–478], vacuum microwave [479] and pressurized fluid [452] assisted extractions. However, no previous references have been found about the use of sonotrode as ultrasound-assisted extraction technology, neither the optimization of extraction of procyanidins, in avocado peels and seeds. Checking other works of flava-3-ols analyzed by HPLC-FLD in other matrices, in cocoa powders the total procyanidin content ranged 3.31-28.58 mg/g d.w. [322], in *Psidium guayaba* leaves 10.5-15.8 mg/g d.w.[480], in cranberry extracts 0.73-22.29 mg/g d.w.[481], in barley flour 0.29-0.65 mg/g d.w.[482], and in grape seeds 3.91-14.18 mg/g d.w.[483]. So, the obtained results are in concordance. Besides, the optimal extracts were compared to controls by ultrasonic bath extraction. According to the results showed in **Table 7.4**, the sonotrode let to obtain a significative ( $p < 0.05$ ) increment, 54% higher, in the content of total procyanidins than ultrasound bath in avocado by-products. Besides, the avocado peel sonotrode extract had 62, 66 and 67% higher

antioxidant activity by DPPH, ABTS and FRAP, respectively, than the control. Similarly, regarding the avocado seed sonotrode extract, the results were 70, 76 and 70% higher than the control for DPPH, ABTS and FRAP, respectively. All the increments in the antioxidant activity in the extracts obtained with sonotrode were significative statistically ( $p < 0.05$ ) (**Figure 7.4**) compared to the obtained with ultrasound bath.

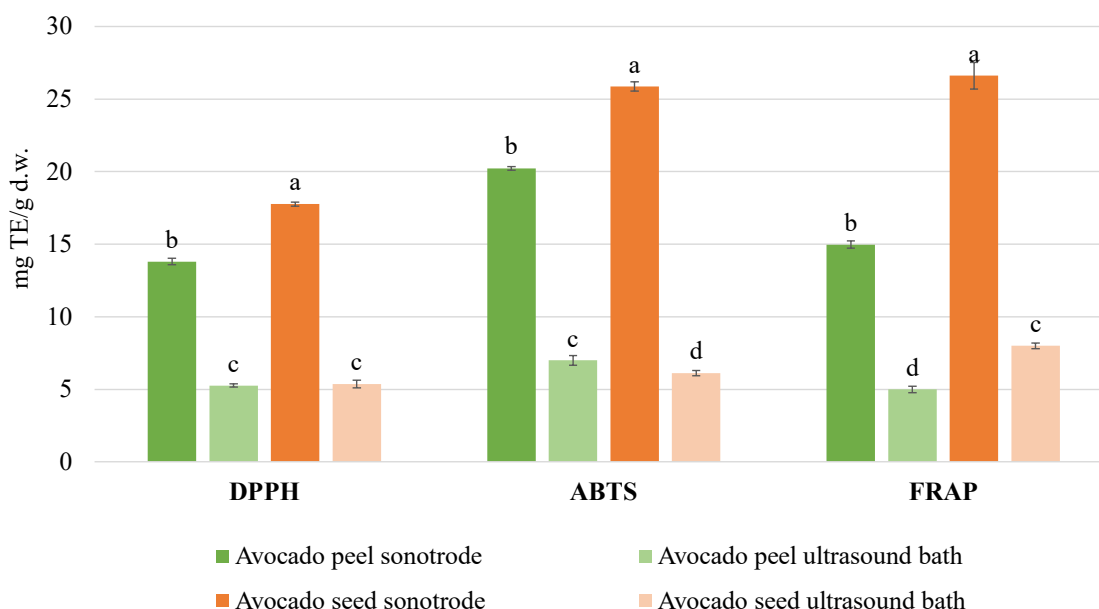


**Figure 7.4.** Representation of the antioxidant activity (DPPH, ABTS and FRAP) of the optimal extracts obtained by sonotrode in comparison with ultrasound bath. Different letters (a-d) in the same method indicate significative differences.

Comparing the antioxidant activities of the optimal extracts with the results obtained by other authors, few references were found. Overall, the results are in the same range of magnitude that the antioxidant activity reported by Figueroa et al. [476], Del Castillo-Llamosas et al. [484], Rodríguez-Martínez et al. [121] and Tremocoldi et al. [117] in avocado by-products extracted by other methods. Trujillo-Mayol et al. compared the antioxidant activity of avocado peel extracts obtained by using maceration (12 hours at room temperature), ultrasound bath (60 °C, 15 min), microwave (500 W, 120 min) and ultrasound-microwave combined. DPPH results were around 0.77 mg TE/g d.w. and FRAP values were about 0.16 mg TE/g d.w. with no significant differences between all the methods used [485], being results much lower than the reported in this work which could indicate that sonotrode let to obtain higher antioxidant results from avocado peel and seeds. The better performance of the sonotrode in comparison to the ultrasound bath has been previously reported in other works [330,486].

Moreover, regarding the conditions, using the ultrasound bath they were needed 45 min of treatment to reach the amount reported in **Table 7.4**, meanwhile with sonotrode the times were

reduced to 30 min for both the avocado peel and seed reaching higher extracting yields. Going further in the sonotrode extraction the sonde is introduced directly in contact with the extracting solvent and the sample, but when extracting with bath it does not happen. In the ultrasound bath the cavitation is transmitted from water through plastic to the solvent of extraction, attenuating the intensity of the treatment with energy losses [487]. So, all of this makes the sonotrode a better election than the ultrasound bath in time, energetic and economic terms. Besides sonotrode technology is scalable at pilot and industrial level.

As can be seen in **Table 7.4**, avocado peel was found to have a total procyanidin content 20% higher than avocado seed with the optimal conditions established. In contrast, avocado seed demonstrated to have significantly ( $p < 0.05$ ) higher antioxidant activity by the three methods measured (**Figure 7.4**). It can be explained by the presence of other phenolic compounds in the extracts as reported by López-Cobo et al. [461] that also have a role in the antioxidant activity. Besides, as reported by Sálazar-López et al. the polymerization degree of the procyanidins also affects directly the antioxidant activity being dimers and trimers more effective superoxide anion stabilizers compared to monomers [488]. So, as showed in **Table 7.4**, avocado peel extract was richer in monomer, meanwhile avocado seed extract was richer in dimer and trimer, which could affect to the values of the antioxidant activity reported here. Besides, it was confirmed statistically (**Figure 7.5**), as lower correlation was found between the monomer and the antioxidant assays ( $r = 0.0786-0.1193$ ,  $p > 0.05$ ) than for dimer and trimers ( $r = 0.5881-0.9735$ ,  $p < 0.05$ ).

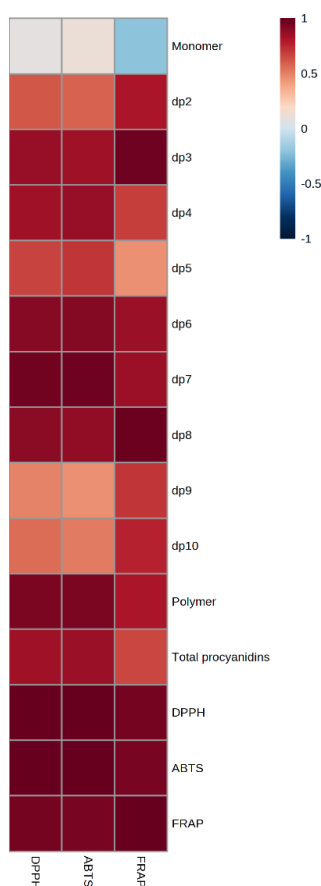


**Figure 7.5.** Correlation heatmap of the procyanidin profile and the antioxidant assays in the avocado by-products.

#### 4. Conclusions

Two Box–Behnken designs were performed to optimize the extraction of flavan-3-ols with high antioxidant activity from avocado peel and seed via sonotrode ultrasound-assisted extraction. The results of this study show that by applying sonotrode extraction, it is possible to increase the flavan-3-ols recovery by about 54% and the antioxidant activity by around 62–76% compared using ultrasound bath technology. In addition, the distributions of flavan-3-ols according to the degree of polymerization between the two avocado by-products were compared. As far as we are concerned, ultrasound-assisted extraction via sonotrode was used for the first time for the obtention of enriched avocado by-products extracts in procyanidins, and it demonstrated itself to be a useful method to take advantage of the flavan-3-ols present in avocado by-products. Furthermore, sonotrode ultrasound technology could be scaled-up to pilot and industrial scales, which would allow us to obtain avocado by-product extracts that could be used as functional ingredients for nutraceutical and food scopes.

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**CHAPTER 8. Enhanced extraction of procyanidins from avocado processing residues by pulsed electric fields pre-treatment.**





## Enhanced extraction of procyanidins from avocado processing residues by pulsed electric fields pre-treatment

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### Abstract

The waste generated by the global production of avocado (*Persea americana* Mill), which includes peels, seeds, and pulp, poses environmental challenges. The principles of circular economy offer a sustainable solution by valorizing this waste and transforming it into valuable products. This study investigates the potential of avocado by-products, particularly peels and seeds, as a source of procyanidins. Pulsed electric field (PEF) technology has been shown to increase the extraction yield of valuable compounds from food waste/losses. In our study, we optimized the PEF treatment of avocado peels and seeds for the extraction of procyanidins by using Box-Behnken designs with response surface methodology and desirability functions. Our study showed that the procyanidin content measured by HPLC-FLD significantly increased in PEF-pretreated avocado peel and seeds. The optimized treatment conditions include a frequency of 50 Hz, an electric field strength of 1.31 kV/cm, and 175 electric pulses for the avocado peels, resulting in a procyanidin content of  $9798.32 \pm 103.83$  µg CE/g d.w. For avocado seeds, the optimal conditions include a frequency of 100 Hz, an electric field strength of 0.75 kV/cm, and total operating time of 5.5 s, resulting in a procyanidin content of  $21103.12 \pm 234.76$  µg/g d.w. The precision and validity of the mathematical models confirmed the reliability of these optimal conditions. These results emphasize the potential of PEF technology for the extraction of procyanidin from avocado waste and contribute to environmentally friendly extraction techniques in the food industry.

**Keywords:** flavanols, avocado peel; avocado seed; HPLC-FLD; avocado wastes.

## 1. Introduction

Avocado (*Persea americana* Mill) is a fruit native to the tropical regions of America. It is classified under the *Persea* (Clus.) Miller genus within the *Lauraceae* family [57]. The *Lauraceae* family stands as one of the earliest among flowering plants. Avocado holds significant importance within this family as the primary edible fruit, boasting considerable commercial worth. Initially concentrated in Central and South America, its cultivation has now expanded globally [111]. Avocado consists mainly of the flesh (65–73%), the seed (16–20%) and the skin (11–15%). Avocado is predominantly eaten fresh, but the increasing global demand for its production and processing has led to a surge in avocado waste. When avocado by-products— such as peel and seeds — are discarded during processing, they cause environmental issues such as greenhouse gas emissions, soil and water pollution, and the attraction of pests. This waste poses urgent environmental challenges, with an estimated 40% of total avocado production being wasted or discarded as by-products [489]. To tackle these challenges, the valorization of avocado waste is crucial for sustainable development. It offers the opportunity to transform waste into valuable products while limiting the environmental impact [490]. Taking into account the principles of circular economy, this approach ensures efficient resource use and waste reduction, thereby lessening the detrimental environmental impacts associated with avocado waste disposal [110]. At the same time, it opens doors for economic growth by providing alternative raw materials for value-added products [491]. In recent years, avocado by-products have received increased attention due to their potential as sources for bioactive compounds like phenolics, carotenoids, and sterols. So far, avocado by-products have been used in producing biofuels, biodegradable plastics, animal feed, natural fertilizers, as a substrate for the growth of mushrooms and microorganisms, and as food ingredients and supplements [489]. In particular, the phenolic compounds known as procyanidins or flavan-3-ols from avocado peels and seeds have been attributed several bioactivities, such as in vitro antimicrobial activity against *Helicobacter pylori*, *Staphylococcus spp.* and *Candida spp.*, as well as cytotoxicity and anti-inflammatory and antidiabetic activities. In vivo studies have also shown the hypoglycemic and tissue-protective effects of hydroalcoholic procyanidins extracts from peel and seed on pancreas, kidneys, and liver in alloxan-induced albino rats. In food sector, ethanolic extracts from avocado seeds rich in procyanidin have shown a protective effect against the fatty acid degradation in sunflower oil [57].

Transparency Market Research registered a 6.1% annual growth rate in the market for natural-source polyphenols by 2020 [492]. Therefore, it is proposed to efficiently recover phytoconstituents from waste peels to benefit various industries such as biotechnology, pharmaceuticals, food, and cosmetics. The critical steps involve optimizing extraction procedures, quantifying the isolated extracts, ensuring their stability, and preserving their bioactivity during

storage. In extraction, the use of novel emerging extraction technologies can offer advantages such as mitigating environmental impact, reducing energy consumption, minimizing the use of organic solvents, and shortening extraction times compared to conventional methods. The application of these efficient extraction methods will be beneficial for the recovery of bioactives from fruit and vegetable waste [493]. Pulsed electric fields (PEF) is one of the most widely recognized emerging technology that has been shown to improve drying, diffusion, and extraction processes. In PEF extraction, short pulses of moderate to high electric field strengths, and relatively low energy (1–10 kJ/kg) are applied to products located between two electrodes in a batch or continuous treatment chamber, usually at or slightly above room temperature. The treatment chamber, which contain the products and two electrodes separated by an insulating material, is connected to a pulse generator consisting of a charger that converts alternating current into direct current, and charging an energy storage device such as a capacitor or an inductor [494].

PEF pre-treatment have previously been used to extract phenolic compounds from several matrices such as grape by-products [173,175,495,496], blueberry by-products [497], papaya peels and seeds [171,498], plum peels [496], mango peels [167], orange peels [169], and olive kernels [165], among others.

The aim of our work was to optimize the conditions for PEF as a pre-treatment to ultrasonic extraction of valuable procyanidins from avocado peels and seeds. The procyanidin content and profile were analyzed by liquid chromatography HPLC-FLD-DAD. The optimization was performed by using Box Behnken designs and quadratic desirability functions. The Box-Behnken design is a response surface methodology used for experiments with multiple variables. A quadratic desirability function complements this by allowing simultaneous optimization across these variables, enabling to find optimal conditions for procyanidin extraction.

## **2. Materials and Methods**

### **2.1. Samples and reagents**

Catechin standard was purchased from Sigma-Aldrich (St. Louis, MO, USA). Double-deionized water was obtained from Millipore (Bedford, MA, USA). HPLC-grade water, acetic acid, acetonitrile, and methanol were purchased from Merck KGaA (Darmstadt, Germany).

Avocado by-products of the 'Hass' variety were obtained from a local company located in the subtropical coast of the province of Málaga (Vélez-Málaga, Spain) in April 2021. The by-products from the production of guacamole, peels (moisture ~75%) and seeds (moisture ~45%), were provided separately. The peels were dried (as previously optimized) at 40 °C in a hot air dryer with an airflow of 1.6 m/s for 105 min (final moisture < 10%) [499]. The seeds were dried for

longer time (98 hours) at the same temperature until they reached a final moisture < 10%. The samples were stored at -18 °C until analysis.

## 2.2. Experimental design

The process to obtain avocado peels and seeds extracts enriched in procyanidins has been optimized using Box-Behnken designs. Each design consisted in 15 experiments with three levels (-1, 0, 1) carried out in duplicate. The independent factors in the case of avocado peels were the frequency (1, 50, 100 Hz), the electric field strength (0.8, 1.1, 1.5 kV/cm) and the number of pulses (100, 150, 200), while for the avocado seeds were the frequency (50, 100, 150 Hz), the total operating time (1, 5, 10 s) and the electric field (0.6, 0.7, 0.8 kV/cm). The dependent variable was total procyanidins expressed in µg CE (Catechin equivalents)/g d.w. (dry weight). The variables were fitted to a second order polynomial equation (Equation (16)) where  $Y$  represents the response variable,  $X_i$  and  $X_j$  are the independent factors that affect the response and  $\beta_0$ ,  $\beta_i$ ,  $\beta_{ii}$  and  $\beta_{ij}$  are the regression coefficients of the model (interception, linear, quadratic and interaction terms, respectively).

Equation (16). Second order polynomial equation.

$$Y = \beta_0 + \sum_{i=1}^4 \beta_i X_i + \sum_{i=1}^4 \beta_{ii} X_i^2 + \sum_{i=1}^4 \sum_{j=1}^4 \beta_{ij} X_i X_j \quad (16)$$

In order to evaluate the adjustment of the models, ANOVAs analyses were performed. Response surface methodology (RSM) was used for establishing the optimal conditions linked to desirability quadratic functions. The software used for the simulations and statistical analysis was Statistica 8.0 package (StatSoft, Tulsa, OK, USA).

## 2.3. Pulsed electric field treatment

PEF pre-treatments were performed using a lab-scale PEF unit delivering a maximum output voltage and current of 8 kV and 60 A, respectively (mod. SP7500, Alintel srl., Bologna, Italy). Peels were treated in pieces of 2-3 cm (3 g for each treatment) in tap water. The PEF treatments were performed after rehydrating the product for 20 min. The product had an initial conductivity of 0.21 mS/cm (measured by EC-Meter basic 30+, Crison). The PEF generator used provided monopolar rectangular-shape pulses and adjustable pulse duration (5-20 µs), and pulse frequency (50-500 Hz). The chamber size consisted into parallel stainless-steel electrodes with a 2 cm gap.

Before PEF treatment, avocado seeds were grounded, and a paste was prepared at a ratio of 1:2 avocado seeds:tap water. For each treatment 25 mL of this paste was used. The initial conductivity of the paste was 4.26 mS/cm. The PEF generator used was the same one of peels in a discontinuous mode with monopolar pulses. In this case the gap between electrodes was set to 1.8 cm.

The specific treatment conditions applied for each sample were given by the Box Behnken experimental designs. In both cases the pulse width was fixed to 10  $\mu$ s. After the PEF treatment the samples were subjected to freeze-drying (Thermo HETO, powerdry LYOLAB 3000; Waltham, USA) and kept at -18 °C till the extractions.

#### **2.4. Ultrasound assisted extraction**

For each experiment 0.5 g of sample was weighted and 100 mL of ethanol/water (v/v) was added in 250 mL beakers. They were submitted to extraction for 30 min by using a lab sonicator (UP400St ultrasonic processor, Hielscher, Germany) equipped with an ultrasonic probe of 22 mm of diameter with conditions previously optimized [500]. For the peel and seeds the ethanol at the concentration of 60% and 55%, respectively. Moreover, the amplitude of 70% and 90% was used for peel and seeds, respectively. The pulse was locked at 100% for all the analysis. After the extraction, the content of the beaker was transferred to a Falcon tube, and centrifugated at 9000 rpm for 10 minutes. The supernatants were collected, evaporated, and reconstituted in 2-4 mL of methanol/water (1:1, v/v). The final extracts were filtered using cellulose acetate filters of 0.2  $\mu$ m (Millipore, Bedford, MA, USA) and stored at -18 °C until analyses.

#### **2.5. Determination of procyanidins by HPLC-FLD**

The determination of procyanidins in avocado samples was carried out by a methods previously described with slight modifications [501]. The extracts were analyzed by high liquid performance chromatography (HPLC) in an Agilent 1200 Series (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump delivery system, a degasser, an autosampler and a fluorescence detector (FLD). The final selected column was Luna Hilic column (150  $\times$  2.0 mm; 3  $\mu$ m) (Phenomenex). The mobile phase consisted of 98% acetonitrile and 2% acetic acid (A) and 95% methanol, 3% water and 2% acetic acid (B). Samples were eluted with an increasing gradient of (B): 0 min, 7%; 3 min, 7%; 15 min, 30%; 40 min, 49%; 40.1 min, 7%; and 55 min, 7% at a flow rate of 0.350 mL/min. The total run time was 57 min, the mid-peak RT of dp 10 being 28 min. The injection volume was 2  $\mu$ L. The column temperature was held at 35 °C. The fluorescence detector wavelengths were 290 nm for excitation and 325 nm for emission. To ensure that all analytes were within the linear range of the detector, the photomultiplier tube gain was set to 9. Monomeric content of the extracts was calculated in relation to a response factor derived from a catechin standard curve over the range 0–100  $\mu$ g/mL. Oligomeric procyanidins(dp2-10) were calculated using the relative fluorescence response factors previously established by Hollands et al. [501].

### 3. Results and Discussion

#### 3.1. Fitting the model

When biological cells are exposed to an external electric field, the accumulation of charge on their membrane surfaces triggers an increase in transmembrane potential, leading to the formation of pores, either reversible or irreversible, in weaker areas of the membrane. This electroporation significantly increases membrane permeability, potentially resulting in cell disintegration. Electroporation enhances mass transfer rates, facilitating the release of intra- and inter-cellular compounds [502]. However, it is very important to find the best operating conditions when PEF treatment is applied before or during food processing.

To optimize the PEF pre-treatment conditions for the extraction of procyanidins from avocado by-products two Box-Behnken designs were performed and are presented in **Table 8.1** and **Table 8.2**, for the avocado peels and seeds, respectively.

**Table 8.1.** Box Behnken experimental design for the extraction of procyanidins from avocado peel by PEF.

|    | Frequency | Electric field | Number of pulses | Sum of procyanidins |
|----|-----------|----------------|------------------|---------------------|
| N  | X1        | X2             | X3               |                     |
| 1  | 1         | 0.8            | 150              | 5355.96 ± 59.23     |
| 2  | 1         | 1.5            | 150              | 4730.65 ± 338.34    |
| 3  | 100       | 0.8            | 150              | 8611.25 ± 282.99    |
| 4  | 100       | 1.5            | 150              | 8108.91 ± 337.97    |
| 5  | 50        | 0.8            | 100              | 4830.52 ± 373.40    |
| 6  | 50        | 1.5            | 100              | 6776.80 ± 126.38    |
| 7  | 50        | 0.8            | 200              | 7185.27 ± 65.78     |
| 8  | 50        | 1.5            | 200              | 9682.71 ± 525.30    |
| 9  | 1         | 1.1            | 100              | 6055.49 ± 738.28    |
| 10 | 100       | 1.1            | 100              | 6474.25 ± 311.03    |
| 11 | 1         | 1.1            | 200              | 6326.48 ± 182.87    |
| 12 | 100       | 1.1            | 200              | 7984.18 ± 363.01    |
| 13 | 50        | 1.1            | 150              | 9202.28 ± 186.81    |
| 14 | 50        | 1.1            | 150              | 8938.09 ± 161.52    |
| 15 | 50        | 1.1            | 150              | 8709.67 ± 127.12    |

**Table 8.2.** Box Behnken experimental design for the extraction of procyanidins from avocado seed by PEF.

| N  | Frequency<br>X1 | Electric field<br>X2 | Total operating<br>X3 | Sum of procyanidins |
|----|-----------------|----------------------|-----------------------|---------------------|
| 1  | 50              | 0.7                  | 1                     | 15681.11 ± 706.83   |
| 2  | 150             | 0.7                  | 1                     | 15956.48 ± 524.21   |
| 3  | 50              | 0.7                  | 10                    | 15864.38 ± 294.85   |
| 4  | 150             | 0.7                  | 10                    | 17160.18 ± 648.78   |
| 5  | 50              | 0.6                  | 5                     | 15693.00 ± 180.79   |
| 6  | 150             | 0.6                  | 5                     | 15368.60 ± 380.48   |
| 7  | 50              | 0.8                  | 5                     | 15313.34 ± 74.68    |
| 8  | 150             | 0.8                  | 5                     | 15624.00 ± 571.97   |
| 9  | 100             | 0.6                  | 1                     | 15549.79 ± 81.50    |
| 10 | 100             | 0.6                  | 10                    | 14094.46 ± 518.85   |
| 11 | 100             | 0.8                  | 1                     | 18994.77 ± 1020.65  |
| 12 | 100             | 0.8                  | 10                    | 20166.46 ± 261.37   |
| 13 | 100             | 0.7                  | 5                     | 20236.91 ± 205.84   |
| 14 | 100             | 0.7                  | 5                     | 20528.02 ± 332.81   |
| 15 | 100             | 0.7                  | 5                     | 20057.35 ± 237.53   |

The response evaluated was the sum of procyanidins in both cases. The procyanidins content ranged from 4730.65 to 9202.28 µg CE/g d.w. and from 14094.46 to 20528.02 µg CE/g d.w. in peel (**Table 8.1**) and seed (**Table 8.2**) models, respectively. The experimental data were fitted to second-order polynomial equations (Equation (16)). **Table 8.3** exhibits the regression coefficients along with their effects and corresponding *p* values. A significance level of *p* < 0.05 was applied across all cases. Non-significant terms were omitted, leading to the refinement of the models by considering only the significant ones.

**Table 8.3.** Regression coefficients effect of the avocado peel and seed optimization models.

| Regression coefficients | Avocado peel |                | Avocado seed |                |
|-------------------------|--------------|----------------|--------------|----------------|
|                         | Effect       | <i>p</i> value | Effect       | <i>p</i> value |
| $\beta_0$               | 6931.00*     | 0.0000         | 16301.72*    | 0.0000         |
| Linear                  |              |                |              |                |
| $\beta_1$               | 2425.17*     | 0.0000         | 295.08       | 0.1576         |
| $\beta_2$               | 431.31*      | 0.0001         | 1642.04*     | 0.0000         |
| $\beta_3$               | 1341.19*     | 0.0000         | 415.05*      | 0.0491         |
| Crossed                 |              |                |              |                |
| $\beta_{12}$            | -36.74       | 0.7832         | 317.53       | 0.2521         |

| Regression coefficients | Avocado peel |                | Avocado seed |                |
|-------------------------|--------------|----------------|--------------|----------------|
|                         | Effect       | <i>p</i> value | Effect       | <i>p</i> value |
| $\beta_{13}$            | 819.28*      | 0.0000         | 510.21       | 0.0701         |
| $\beta_{23}$            | 469.78*      | 0.0012         | 1313.51*     | 0.0000         |
| Quadratic               |              |                |              |                |
| $\beta_{11}$            | 1312.81*     | 0.0000         | 2881.89*     | 0.0000         |
| $\beta_{22}$            | 890.63*      | 0.0000         | 1846.06*     | 0.0000         |
| $\beta_{33}$            | 831.44*      | 0.0000         | 1218.78*     | 0.0000         |
| $R^2$                   | 0.9789       |                | 0.9510       |                |
| <i>p</i> model          | 0.0000*      |                | 0.0001*      |                |
| <i>p</i> lack of fit    | 0.7832       |                | 0.1035       |                |

\*significant  $p < 0.05$

In the avocado peel model, all linear terms ( $\beta_1$ ,  $\beta_2$ , and  $\beta_3$ ), crossed interactions ( $\beta_{13}$  and  $\beta_{23}$ ), and quadratic terms ( $\beta_{11}$ ,  $\beta_{22}$ , and  $\beta_{33}$ ) displayed significant effects on the response variable. As indicated in **Table 8.3**, both linear and quadratic terms exhibited positive effects. However, only the crossed term,  $\beta_{12}$  (related to frequency and voltage), displayed a non-significant negative impact on the model. Notably, the frequency factor ( $\beta_1$ ) demonstrated the most substantial positive influence, followed by the linear factor representing the number of pulses ( $\beta_3$ ). Moreover, a strong correlation between the response variable and the factors was observed ( $R^2 = 0.9789$ ). The ANOVA test confirmed the model's validity, showing a significant  $p$ -value for the model and a non-significant lack of fit ( $p > 0.05$ ).

Regarding the avocado seed model, all quadratic terms were found to have a significant influence. Besides, the linear terms  $\beta_2$  (corresponding to electric field strength),  $\beta_3$  (corresponding to total operating time) and the crossed term between them  $\beta_{23}$  were significant ( $p < 0.05$ ). In contrast the linear term corresponding to frequency ( $\beta_1$ ) and the rest of interactions ( $\beta_{12}$  and  $\beta_{13}$ ) did not have significance in the model. All the variables exhibited positive effect with the quadratic term of frequency ( $\beta_{11}$ ) being higher, followed by the quadratic term of electric field ( $\beta_{22}$ ). Additionally, a robust relationship was found between the dependent variable and the factors was noted, displaying a high  $R^2$  value of 0.9510. The model's validity was supported by ANOVA test, indicating a significant  $p$  value for the model and a non-significant lack of fit ( $p > 0.05$ ).

### 3.2. Optimization of PEF Extraction Conditions

The response surface graphs were analyzed to determine the best conditions for each by-product (i.e., peels or seeds), with the aim of achieving a balance between the factors evaluated and obtaining extracts with the maximum total procyanidin content.



**Figure 8.1** shows that the highest responses for the avocado peel model were obtained at electric fields strength higher than 1.1 kV/cm and a pulse number higher than 150 pulses. In terms of frequency, the highest responses were achieved between 40 and 80 Hz, therefore the lowest frequency possible was selected in order to reduce the energy consumption. The number of pulses also were selected as small as possible, taking into account the other parameters, to achieve the fastest possible procedure.

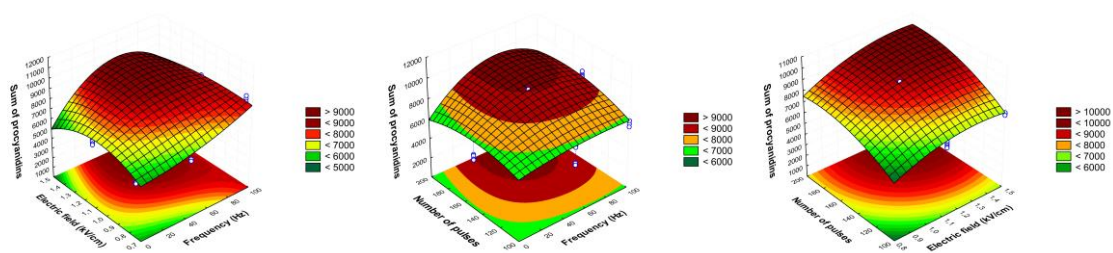


**Figure 8.1.** Response surface plots showing combined effects of process variables for the sum of procyanidins in avocado peel model expressed as  $\mu\text{g/g}$  d.w.

Similar responses surfaces were obtained for the avocado seed model as shown in **Figure 8.2**. In this case, the higher responses were achieved at a frequency between 80 and 120 Hz, higher than in the case of the peel model. The best electric field strength appeared to be in the range of 0.7-0.8 kV/cm. Regarding the total treatment time, the intermedium value tested (5 s) showed the best results.

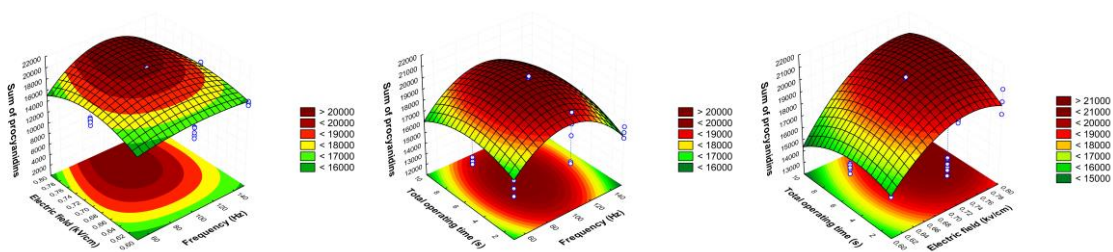


**Figure 8.2.** Response surface plots showing combined effects of process variables for the sum of procyanidins in avocado seed model expressed as  $\mu\text{g/g}$  d.w.

Thus, both models were processed and individually fitted to a quadratic function for predicting the optimum parameters to obtain the higher amount of procyanidins. For this purpose, the responses for each factor were normalized into a dimensionless scale between 0 and 1. This normalization allows for the combination of different factors, despite their different units or scales. It was considered also in combination with response surface methodology.

Briefly, the optimal conditions selected for the avocado peel model were a frequency of 50 Hz, an electric field strength of 1.31 kV/cm and pulse number of 175 with a desirability value of

0.9468 and a predicted procyanidin content of  $9845.41 \pm 222.60$   $\mu\text{g CE/g d.w.}$  Concerning the avocado seed model, a frequency of 100 Hz, a field strength of 0.75 kV/cm and a total operating time (i.e. calculated as number of pulses/frequency) of 5.5 s were selected as optimal, having a desirability value of 1 and a predicted procyanidin content of  $20955.06 \pm 452.35$   $\mu\text{g/g d.w.}$  of procyanidins. The accuracy of both mathematical models underwent confirmation (**Table 8.4**). In all cases, there were no significant deviations between the values predicted by the model and the actual results obtained under the optimal conditions. Furthermore, the coefficients of variation remained under 1 for both the peel and seed models respect to total procyanidins. Additional supplementary parameters derived from the optimized one are also presented in **Table 8.4**.

**Table 8.4.** Optimal conditions of the experimental models for extracting procyanidins from avocado peel and seed by PEF.

| Avocado peel                                   |                      | Avocado seed                    |                       |
|------------------------------------------------|----------------------|---------------------------------|-----------------------|
| Optimized parameters                           |                      | Optimized parameters            |                       |
| Frequency (Hz), $X_1$                          | 50                   | Frequency (Hz), $X_1$           | 100                   |
| Field strength (kV/cm), $X_2$                  | 1.31                 | Field strength (kV/cm), $X_2$   | 0.75                  |
| Number of pulses, $X_3$                        | 175                  | Total operating time (s), $X_3$ | 5.5                   |
| Other parameters                               |                      | Other parameters                |                       |
| Specific energy (kJ/kg)                        | 0.63                 | Specific energy (kJ/kg)         | 12.7                  |
| Pulse width ( $\mu\text{s}$ )                  | 10                   | Pulse width ( $\mu\text{s}$ )   | 10                    |
| Total treatment time (ms)                      | 1.75                 | Total treatment time (ms)       | 5.5                   |
| Total operating time (s)                       | 3.5                  | Number of pulses                | 550                   |
| Voltage (V)                                    | 2625                 | Voltage (V)                     | 1350                  |
| Sum of procyanidins                            |                      | Sum of procyanidins             |                       |
| Desirability value                             | 0.9468               |                                 | 1.0000                |
| Predicted value<br>( $\mu\text{g CE/g d.w.}$ ) | $9845.41 \pm 222.60$ |                                 | $20955.06 \pm 452.35$ |
| Obtained value<br>( $\mu\text{g CE/g d.w.}$ )  | $9798.32 \pm 103.83$ |                                 | $21103.12 \pm 234.76$ |
| CV (%)                                         | 0.34                 |                                 | 0.50                  |
| Predicted vs. Obtained                         |                      |                                 |                       |
| Control ( $\mu\text{g CE/g d.w.}$ )            | $6710.59 \pm 219.28$ |                                 | $19103.39 \pm 172.76$ |

CE: Catechin equivalents; CV: Coefficient of variation. Predicted, obtained and control sum of procyanidin values are expressed as average  $\pm$  standard deviation.

Several studies have reported the measurement of total phenolic compounds in avocado peels and seeds using various extraction methods such as hydroethanolic liquid-liquid extraction [116], ultrasound bath [117,474], sonotrode [500], microwave [503] and vacuum microwave [479] assisted extractions. However, the quantification was performed by Folin Ciocalteu (spectrophotometric method) or HPLC-MS, not comparable with the results obtained here.

Therefore, the optimal extracts were compared to non-PEF treated controls and the results are shown in **Figure 8.3**.

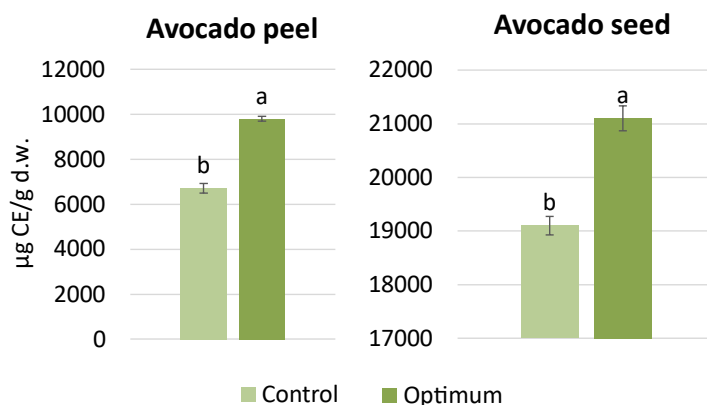


**Figure 8.3.** Sum of procyanidins of optimum PEF pre-treated extracts and the controls (not treated) for avocado peels and seeds, expressed as µg catechin equivalents/g d.w. Different letters (a,b) for each sample indicate significant differences ( $p < 0.05$ ).

According to the results, a significant ( $p < 0.05$ ) increase of 9.5 % in total procyanidins was achieved in the avocado seeds compared to the control. Regarding the avocado peel it a significant ( $p < 0.05$ ) increment of 31.5% in total procyanidins was achieved compared to control. Previous research have reported results in the range of 4.29-18.74 and 1.05- 23.68 mg CE/g d.w. for avocado seed and peel, respectively [461,499,500]. The differences in terms of numerical values compared to our previous studies could be due to differences in the ripening stage of avocados, the month of harvesting or minor differences in the processing and the avocado origin. We examined additional studies concerning flava-3-ols analyzed through HPLC-FLD in various matrices. In cocoa powders, the total procyanidin content varied between 3.31 and 28.58 mg/g d.w. [322]. *Psidium guajava* leaves exhibited a range of 10.5 to 15.8 mg/g d.w. [480], cranberry extracts showed values from 0.73 to 22.29 mg/g d.w. [481], barley flour demonstrated a range of 0.29 to 0.65 mg/g d.w. [482], and grape seeds displayed content ranging from 3.91 to 14.18 mg/g d.w.[483]. Thus, the obtained values are in the same range of magnitude, but none of them applied PEF.

Additionally, the complete procyanidin profile is shown in **Table 8.5**.

**Table 8.5.** Procyanidin distribution content of procyanidin in avocado peel and seed extract obtained using the optimal PEF conditions and the controls measured by HPLD-FLD-DAD and expressed in µg CE/g d.w. with average  $\pm$  standard deviation.

| Avocado peel |         | Avocado seed |         |
|--------------|---------|--------------|---------|
| Control      | Optimum | Control      | Optimum |

|                                  |                  |                 |                  |                   |
|----------------------------------|------------------|-----------------|------------------|-------------------|
| <b>Monomers<br/>(cat+epicat)</b> | 4721.58 ± 101.69 | 6673.37 ± 70.72 | 15475.72 ± 86.12 | 16975.65 ± 188.84 |
| <b>Dimers</b>                    | 1849.67 ± 75.03  | 2735.55 ± 28.99 | 980.04 ± 32.56   | 1245.75 ± 13.86   |
| <b>DP3</b>                       | 58.55 ± 19.94    | 172.52 ± 1.83   | 1559.39 ± 46.67  | 1683.27 ± 18.73   |
| <b>DP4</b>                       | 35.13 ± 10.09    | 96.77 ± 1.03    | 407.70 ± 7.46    | 453.12 ± 5.04     |
| <b>DP5</b>                       | 21.86 ± 6.13     | 58.32 ± 0.62    | 242.85 ± 3.91    | 267.08 ± 2.97     |
| <b>DP6</b>                       | 11.05 ± 3.10     | 28.58 ± 0.30    | 135.35 ± 1.04    | 145.78 ± 1.62     |
| <b>DP7</b>                       | 5.49 ± 1.44      | 15.48 ± 0.16    | 92.71 ± 1.69     | 101.26 ± 1.13     |
| <b>DP8</b>                       | 3.05 ± 0.55      | 6.30 ± 0.07     | 59.45 ± 1.01     | 64.49 ± 0.72      |
| <b>DP9</b>                       | 1.82 ± 0.01      | 3.62 ± 0.04     | 38.20 ± 0.04     | 39.77 ± 0.44      |
| <b>DP10</b>                      | 1.64 ± 0.24      | 2.66 ± 0.03     | 27.66 ± 0.28     | 27.85 ± 0.31      |
| <b>DP11</b>                      | < LOD            | 3.49 ± 0.04     | 26.34 ± 0.30     | 26.52 ± 0.30      |
| <b>DP12</b>                      | < LOD            | < LOD           | 25.86 ± 0.05     | 25.63 ± 0.29      |
| <b>Polymers</b>                  | 0.74 ± 1.05      | 1.65 ± 0.02     | 32.12 ± 7.02     | 46.94 ± 0.52      |

Cat+epicat: catechin + epicatechin; DP: degree of polymerization.

No significant changes in the procyanidin profile were observed between the optimum and control extracts. Regarding the avocado peel, monomers accounted for 68 and 70% of the total procyanidin content in control and optimum, respectively, and dimers accounted for 27% in both cases. In avocado seed, monomers, dimers, and trimers accounted for 80, 5 and 8%, respectively, in both extracts. All this is in agreement with previous research [500].

To the best of our knowledge, there are no previous references regarding the use of PEF in avocado by-products for any purposes. The only research regarding PEF and avocado was conducted by Castonera-García et al. [504] and reported the inactivation up to 85% of polyphenol oxidase enzyme from avocado by using an electric field strength of 18 kV/cm at frequencies above 1000 Hz.

However, there are few references on the use of PEF for improving polyphenol extraction from seeds. Rahmah et al. [505] reported an increase of 3.16-14.14% in the tannin content in *Areca catechu* L. seed powder treated with 1.92-12.44 kJ/cm<sup>3</sup> and 6000 Hz. Teh et al. [506] used the ratio 1/10 sample/solvent for treating with PEF canola seed cake powder and applied a frequency of 30 Hz and 1.1 kV/cm with a total operating time of 10 s as optimal parameter for extracting polyphenols. Parniakov et. al [498] PEF treated a suspension of powdered papaya seeds at a ratio solid/liquid of 1/10 using an electric field of 13.3 kV/cm and a total operating time of 2000 s for the extraction of phenolic compounds. Boussetta et. al [507] treated grape seeds, with a particle size of 4 mm, with a PEF at 20 kV/cm with a frequency of 0.33 Hz for a total treatment time of 20 ms increasing the polyphenol extraction. Boussetta et. al [508] applied PEF to flaxseed hulls at 20 kV/cm for 10 ms also with improvements in the polyphenol extraction. Thus, the obtained optimal parameters are in the range of values reported in other samples.

PEF has been reported previously by other authors to improve the extraction of polyphenols in other peels such as in papaya peels [171], grape peels [496] and pomegranate peels [153]. Shortskii et. al [509] reported an increase in the phenolic content of kiwifruit peel by 5.1% by using a PEF treatment at 100 V/cm and 0.96 kJ/kg. Frontuto et. al [510] used PEF as a pre-treatment for extracting phenolic compounds from potato peels with an increase of 10% in the yield using 1 kV/cm and 5 kJ/kg. Pataro et. al [511] reported an increase of 188% in the recovery of carotenoids from tomato peels by using PEF at a specific energy of 1 kJ/kg, electric field strength of 0.75 kV/cm, frequency of 10 Hz and pulse width of 20  $\mu$ s. Pataro et. al [512] reported an increase of 12-18% in the lycopene content in tomato peel by using PEF treatment at 5 kV/cm and 5 kJ/kg. Peiró et. al [170] improved the extraction of polyphenols from lemon peels using a PEF pre-treatment at 7 kV/cm and 1 Hz. Redondo et al. [513] identified the optimal condition of PEF treatment (5 kV/cm, 5.98 kJ/kg and 1 Hz) to improve the phenolic compounds recovery from the peach fruit peel. In orange peels, Luengo et. al [169] reported that a PEF treatment (7 kV/cm, 3.77 kJ/kg, 1 Hz) increased the antioxidant activity of the extracts by 192%. In our study, the optimal parameters determined are within the range of the values reported in literature.

In this context, it should be mentioned that the avocado seed required a higher specific energy (12.7 kJ/kg versus 0.63 kJ/kg in the avocado peel), a higher number of pulses (550 versus 175 in the avocado peel) and, consequently a longer treatment (5.5 ms versus 1.75 ms) and operating time (5.5 s versus of 3.5 s). In contrast, the applied electric field strength was higher for the peel (1.31 kV/cm) than for the seed (0.75 kV/cm). This could be attributed to the differences between the samples. In the case of avocado peel, pieces of 2-3 cm were used, while in the case of avocado seed it was created a paste in ratio 1:2 seed/water with milled seed of a size of 0.2-5  $\mu$ m. Besides, the initial electrical conductivity of the two types of samples was very different. It is a fact that the extent of electroporation relies on various factors including plant material compounds, cell physiology, electric field intensity, pulse waveform, treatment duration, number of pulses. This selective extraction process yields extracts of higher purity and increased extraction efficiency. Although the exact mechanisms of electroporation are not yet fully understood, it is evident that the membrane plays a pivotal role in amplifying the applied electric field. This is particularly significant due to the considerable difference in conductivity between intact membranes and the extracellular medium or cell cytoplasm [502].

#### **4. Conclusions**

The study's outcomes underline the effectiveness of pulsed electric fields (PEF) as a promising technology for procyanidin extraction from avocado peels and seeds. Optimized conditions, including a frequency of 50 Hz, electric field strength of 1.31 kV/cm, and 175 pulses for the avocado peel, and a frequency of 100 Hz, electric field strength of 0.75 kV/cm, and total operating

time of 5.5 s for the avocado seeds, resulted in substantial increase in procyanidin content. The procyanidin enhancement achieved— + 31.5% in avocado peels and + 9.5% in avocado seeds— compared to non-PEF treated control samples, demonstrates the effectiveness of the selective extraction process. The findings align with market trends for natural-source polyphenols, presenting a practical and scalable solution for industries seeking sustainable practices. Furthermore, the study's rigorous validation of mathematical models ensures the reliability of the proposed optimized conditions, paving the way for immediate applications in the fruit and vegetable processing industry. This research contributes to a paradigm shift in avocado waste management, emphasizing resource efficiency, environmental responsibility, and economic viability through innovative extraction technologies.

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## **CHAPTER 9. Effect of lactic acid bacteria fermentation on the polar compounds content with antioxidant and antidiabetic activity of avocado seed extracts**

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## Article

# Effect of Lactic Acid Bacteria Fermentation on the Polar Compounds Content with Antioxidant and Antidiabetic Activity of Avocado Seed Extracts

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## Abstract

Avocado seeds, a common waste in the avocado processing industry, have been found to have several bioactivities, such as anticancer, antimicrobial, hypocholesterolemic, antioxidant, and antidiabetic activities, among others. Nowadays, this wastage is causing an environmental problem, so the use of new technologies to take advantage of it is a novel field of research. In this study, the submerged fermentation by lactic acid bacteria was used as a novel tool for improving the bioactive compound extraction from avocado seeds. Avocado seeds were fermented by different strains, their polar compounds were identified and quantified by HPLC-ESI-TOF-MS, the antioxidant activity was measured by DPPH and FRAP assays, and the antidiabetic activity was analyzed by the alpha-amylase assay. A total of 32 polar compounds were identified, with 13 of them being described in avocado seeds for the first time. Avocado seeds fermented by *Pentosaceus acidilactici* showed the highest sum of polar compounds ( $6279.63 \pm 67.74 \mu\text{g/g}$  d.w.), and by extension, of hydroxytyrosol glucoside ( $2989.76 \pm 3.64 \mu\text{g/g}$  d.w.). *Lactiplantibacillus plantarum* CECT 9567 showed the highest antioxidant activity measured by both DPPH and FRAP assays ( $6294.67 \pm 19.44$  and  $6846.91 \pm 2.13 \mu\text{g TE/g}$  d.w., respectively). Furthermore, *Lactiplantibacillus plantarum* CECT 748T had the highest antidiabetic activity ( $52.15\% \pm 0.67\%$  inhibition of alpha-amylase activity), attributable to the polyphenols. According to the results, submerged fermentation by lactic acid bacteria led to an interesting increase of the polar compounds' extractability of avocado seeds, consequently improving the bioactivities of the extracts, which could then be used for food nutraceutical or cosmetic purposes.

**Keywords:** avocado seed waste; HPLC-ESI-TOF-MS; alpha-amylase; submerged fermentation; phenolic compounds

## 1. Introduction

Avocado (*Persea americana* Mill., *Lauraceae*) is a fruit dispersed worldwide in tropical and subtropical regions. There are numerous varieties of avocado according to the growth climate, with Hass being the most well-known and marketed [109]. The avocado processing industry, in the production of guacamole or essential oils, generates a solid waste residue that represents 21–30% of the fruit, consisting of seeds, peels, and exhausted pulp [110]. Usually, those residues are discarded as waste, representing an environmental problem, but they are also a prospective source of bioactive compounds [109]. The avocado seed is composed of the endocarp and the kernel. It has been previously reported to have anticancer activity in oral, breast, and liver cells, attributed to its triterpenoid compounds [514,515]. Additionally, antimicrobial activity of the avocado seed extract against Gram-positive and Gram-negative bacteria has been demonstrated for *Salmonella enteritidis*, *Citrobacter freundii*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Staphylococcus aureus*, methicillin-resistant *S. aureus*, *Escherichia coli*, *P. aeruginosa*, *Mycobacterium intracellulare*, and *Listeria monocytogenes* [516–518]. Furthermore, antifungal activity against *Candida* spp., *Cryptococcus neoformans*, and *Malassezia pachydermatis*, and larvicidal activity against *Artemia salina* and *Aedes aegypti*, were reported [519]. Its hypocholesterolemic activity was also demonstrated in mice, reducing cholesterol and low-density lipoprotein. In addition, the antioxidant [520] and antidiabetic activity [521] of avocado seed has been previously confirmed.

Lactic acid bacteria (LAB) are a heterogeneous group of Gram-positive food-grade microorganisms, historically used in food preservation. Usually, they are mostly reported to ferment carbohydrates as their main source of carbon to produce lactic acid at the industrial level. They can produce a variety of products, including bacteriocins, vitamins, amines, short-chain fatty acids, and exopolysaccharides, during metabolism [349]. Moreover, there are previous data that report increases in antioxidant and phenolic compounds in food submitted to fermentation by LAB, such as apple juice, mulberry juice, soy milk, and wheat dough. LAB enzymes have the capability to break the cell walls, improving the realization of phenolic acids and flavonoids or converting them into simple forms, in both cases improving their bioavailability [522]. Fermentation with acid lactic bacteria is a low-cost and sustainable technology for maintaining or improving the nutritional quality of food and its sensory properties. In fruits and their byproducts, fermentation can occur spontaneously via the lactic acid microbiota naturally present under adequate conditions. Otherwise, it can also be controlled using lactic acid bacteria starter cultures, aiming at different specific objectives, such as improving the digestibility. The fermentation of avocado seeds by LAB could lead to several benefits, such as improving their bioactivities, a field of research that has not been deeply studied. In avocado leaves, the fermentation by LAB has led to satisfactory results [188]. Recently, Rozan et al. reported a reduction in the anti-nutrients

tannins, oxalates, and alkaloids contents of 80.76%, 89.95%, and 70%, respectively, by fermenting avocado seed with LAB [388].

Based on the above and the results obtained in previous research [188], this study aimed to evaluate the effect of submerged fermentation with different LAB on the polar profile of avocado seeds, evaluating the antioxidant and the antidiabetic activities, in comparison to an unfermented avocado seed extract.

## **2. Materials and Methods**

### **2.1. Chemicals and Samples**

Most of the reagents, analytical standards, and the pancreatic alpha-amylase were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were previously reported [188]. Avocado seed byproduct samples of the variety Hass were provided by a local company after the guacamole production, in April 2022. The seeds were freeze-dried, sieved to a 100 µm particle size, and frozen at −18 °C until the analyses.

### **2.2. Fermentation of Avocado Seeds by Lactic Acid Bacteria**

The lactic acid bacteria (LAB) strains used were: *Pediococcus acidilactici* CECT 5765T, *Pediococcus pentosaceus* CECT 4695T, *Leuconostoc mesenteroides* subsp. *mesenteroides* CECT 219T, *Levilactobacillus brevis* CECT 5354, *Lactiplantibacillus plantarum* subsp. *plantarum* CECT 748T, and *Lactiplantibacillus plantarum* CECT 9567 (formerly strain C4), and they were obtained and grown as previously reported [188]. Fermentations were carried out according to the methodology described by De Montijo-Prieto et al. [188]. Avocado seeds were submerged in sterile water, previously sterilized at 90 °C, to eliminate the microorganisms previously present. After heat treatment, aliquots were taken to determine viable microorganisms on MacConkey agar (selective medium for enterobacteria, 37 °C), Tryptic soy agar (TSA, enriched medium for bacteria, 37 °C), and Sabouraud agar (medium for fungi, 25 °C). After incubations of the media, the number of viable microorganisms was confirmed to be below the detection limit of the tests. The quantification of the phenolic compounds was carried out at selected incubation times. Thus, *P. acidilactici* CECT 5765T, *P. pentosaceus* CECT 923, *Le. brevis* CECT 5354, and *La. plantarum* CECT 9567 were analyzed at 24 h of incubation, and *Leu. mesenteroides* CECT 219T and *La. plantarum* CECT 748T at 48 h of incubation.

### **2.3. Determination of Polar Compounds by HPLC-ESI-TOF-MS**

The extraction and analysis of polar compounds in the fermented and non-fermented avocado seeds were carried out following the procedure previously reported by De Montijo-Prieto et al. [188]. Analyses were performed in triplicate. The identification of the phenolic compounds was

performed according to the literature. For ensuring the mass accuracy, the tolerances chosen had a score higher than 90% and an error lower than 5 ppm. To quantify the phenolic compounds identified in the avocado seed extracts, calibration curves were used for vanillic acid ( $y = 4.1347x + 393.22$ ;  $R^2 = 0.982$ ), chlorogenic acid ( $y = 101.17x - 683.25$ ;  $R^2 = 0.9933$ ), ferulic acid ( $y = 10.502x + 353.77$ ;  $R^2 = 0.9964$ ), quercetin ( $y = 288.76x + 2462.1$ ;  $R^2 = 0.9673$ ), catechin ( $y = 315.87x - 415.59$ ;  $R^2 = 0.9950$ ), and rutin ( $y = 860.4x - 256.21$ ;  $R^2 = 0.9986$ ). The results are expressed as  $\mu\text{g/g d.w.}$

#### 2.4. Determination of Antioxidant Activity: DPPH and FRAP Assays

Avocado seeds fermented by different LAB were analyzed with DPPH and FRAP assays to determine the antioxidant activity via the procedures described in previous research [523]. The analyses were performed in triplicate and the results are expressed in mg of Trolox equivalents (TE)/g of dry weight (d.w.).

#### 2.5. Determination of Antidiabetic Activity: Alpha-Amylase Assay

The antidiabetic activity of the extracts was performed in vitro by the alpha-amylase assay according to previous research [524,525]. Briefly, 100  $\mu\text{L}$  of fermented or non-fermented methanolic extract of avocado seed (20 mg/mL) was added to 100  $\mu\text{L}$  of porcine pancreatic alpha-amylase ( $\geq 2$  ud/mL) and pre-incubated during 5 min at 37 °C. Then, 200  $\mu\text{L}$  of 0.2% starch solution in 20 mM of sodium phosphate buffer (pH 6.7) was added and incubated again for 5 min at 37 °C. The reaction was stopped with 1 mL of dinitrosalicylic acid (DNS) color reagent and kept in a boiling bath for 5 min. Once the tubes had cooled on ice until reaching room temperature, they were added to 6 mL of distilled water. The absorbance was measured at 540 nm in a UV-visible spectrophotometer (Spectrophotometer 300 Array, UV-Vis, single-beam, Shimadzu, Duisburg, Germany). The pharmacological inhibitor, acarbose, was included as a positive control at a concentration of 20 mg/mL. Analyses were performed in triplicate. The percentage of inhibition of the alpha-amylase activity was calculated as follows (Equation (26)):

$$\% \text{ Inhibition} = \left( 100 - \frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}} \right) \times 100 \quad (26)$$

#### 2.6. Data Processing

MassLynx 4.1 software (Waters Corporation, Milford, MA, USA) was used for elaborating the HPLC-ESI-TOF-MS results. The Statistica 7.0 package (StatSoft, Tulsa, OK, USA) was used for obtaining statistical differences (Tukey's test) by one-way ANOVA, and Pearson' correlations were performed. The rest of the statistical analyses were performed using MetaboAnalyst 5.0 (Xia Lab, McGill, Canada).

### 3. Results and Discussion

#### 3.1. Identification of Polar Compounds in Fermented Avocado Seeds by HPLC-ESI-TOF-MS

The avocado seeds fermented by the selected strains were analyzed by HPLC-ESI-TOF-MS, and a total of 32 polar compounds were identified. Among them were six phenolic acids, eighteen flavonoids, five terpenoid derivatives, one tyrosol derivative, and other organic compounds. They are presented in **Table 9.1**, along with their experimental and calculated  $m/z$ , time (min), error (ppm), score (%), molecular formula,  $m/z$  main in source fragments, and the tentative name for each compound. The peaks presented in **Table 9.1** correspond to the numbers shown in **Figure 9.1**, which is a representative chromatogram of a fermented avocado seed. To the best of our knowledge, 13 polar compounds were identified here for the first time in avocado seeds.

**Table 9.1.** Polar compounds identified by HPLC-ESI-TOF-MS in fermented and non-fermented avocado seeds.

| No. | Time  | $m/z$     | $m/z$     | Error | Score | Molecular                                       | $m/z$ in Source     | Compound                                |
|-----|-------|-----------|-----------|-------|-------|-------------------------------------------------|---------------------|-----------------------------------------|
| 1   | 0.26  | 343.0304  | 343.0301  | 0.9   | 94.75 | C <sub>13</sub> H <sub>12</sub> O <sub>11</sub> | 201.0226            | 5-O-Galloyl-1,4-galactarolactone        |
| 2   | 0.33  | 211.0813  | 211.0823  | -4.7  | 91.29 | C <sub>7</sub> H <sub>16</sub> O <sub>7</sub>   | 101.0223            | Perseitol                               |
| 3   | 0.37  | 191.0552  | 191.0556  | -2.1  | 91.72 | C <sub>7</sub> H <sub>12</sub> O <sub>6</sub>   | -                   | Quinic acid isomer a                    |
| 4   | 0.44  | 191.0551  | 191.0556  | -2.6  | 98.94 | C <sub>7</sub> H <sub>12</sub> O <sub>6</sub>   | -                   | Quinic acid isomer b                    |
| 5   | 1.48  | 315.1071  | 315.108   | -2.9  | 99.93 | C <sub>14</sub> H <sub>20</sub> O <sub>8</sub>  | 153.0197; 135.0442  | Hydroxytyrosol glucoside                |
| 6   | 2.57  | 353.0858  | 353.0873  | -4.2  | 99.99 | C <sub>16</sub> H <sub>18</sub> O <sub>9</sub>  | 179.0302; 191.0532  | Caffeoylquinic acid isomer a            |
| 7   | 3.49  | 337.0914  | 337.0923  | -2.7  | 99.67 | C <sub>16</sub> H <sub>18</sub> O <sub>8</sub>  | 163.0368; 191.0536  | Coumaroylquinic acid isomer a           |
| 8   | 3.89  | 337.0909  | 337.0923  | -4.2  | 99.99 | C <sub>16</sub> H <sub>18</sub> O <sub>8</sub>  | 163.0363; 191.0515  | Coumaroylquinic acid isomer b           |
| 9   | 4.12  | 443.1906  | 443.1917  | -2.5  | 99.76 | C <sub>21</sub> H <sub>32</sub> O <sub>10</sub> | 113.0245; 101.0224  | Pensternide                             |
| 10  | 4.58  | 353.0868  | 353.0873  | -1.4  | 99.99 | C <sub>16</sub> H <sub>18</sub> O <sub>9</sub>  | 179.0334; 191.0531  | Caffeoylquinic acid isomer b            |
| 11  | 4.90  | 337.0914  | 337.0923  | -2.7  | 91.94 | C <sub>16</sub> H <sub>17</sub> O <sub>8</sub>  | 163.0382; 191.0539  | Coumaroylquinic acid isomer c           |
| 12  | 5.02  | 387.1641  | 387.1655  | -3.6  | 99.95 | C <sub>18</sub> H <sub>28</sub> O <sub>9</sub>  | 279.0305; 207.0988; | Tuberonic acid glucoside                |
| 13  | 5.22  | 289.0698  | 289.0712  | -4.8  | 99.63 | C <sub>15</sub> H <sub>14</sub> O <sub>6</sub>  | 137.0219            | Catechin                                |
| 14  | 5.34  | 449.1082  | 449.1084  | -0.4  | 100   | C <sub>21</sub> H <sub>22</sub> O <sub>11</sub> | 287.0541            | Eriodictol 7-O-glucoside                |
| 15  | 5.57  | 337.0915  | 337.0923  | -2.4  | 99.85 | C <sub>16</sub> H <sub>18</sub> O <sub>8</sub>  | 163.0378; 191.0517  | Coumaroylquinic acid isomer d           |
| 16  | 5.62  | 577.1329  | 577.1252  | 13.3  | 90.86 | C <sub>19</sub> H <sub>30</sub> O <sub>20</sub> | 289.0682            | Procyanidin dimer                       |
| 17  | 5.77  | 415.1589  | 415.1604  | -3.6  | 99.83 | C <sub>19</sub> H <sub>28</sub> O <sub>10</sub> | 339 121             | 2-Phenylethyl beta-primeveroside        |
| 18  | 5.93  | 463.2179  | 463.2179  | 0.0   | 100   | C <sub>21</sub> H <sub>36</sub> O <sub>11</sub> | 285.0396; 125.0228  | Luteolin/kaempferol derivative isomer a |
| 19  | 6.85  | 463.2164  | 463.2179  | -3.2  | 99.71 | C <sub>21</sub> H <sub>36</sub> O <sub>11</sub> | 285.0390; 125.0202  | Luteolin/kaempferol derivative isomer b |
| 20  | 6.98  | 1151.2507 | 1151.2575 | -5.9  | 94.31 | C <sub>46</sub> H <sub>56</sub> O <sub>34</sub> | 863.1739; 289.0672  | Procyanidin tetramer                    |
| 21  | 7.10  | 441.1741  | 441.1761  | -4.5  | 99.97 | C <sub>21</sub> H <sub>30</sub> O <sub>10</sub> | 330.1303            | Hydroxyabscisic acid beta-D-glucoside   |
| 22  | 7.31  | 863.183   | 863.1823  | 0.8   | 90.3  | C <sub>45</sub> H <sub>36</sub> O <sub>18</sub> | 711.1354; 573.1021; | Procyanidin trimer                      |
| 23  | 7.79  | 625.1409  | 625.1405  | 0.6   | 97.84 | C <sub>27</sub> H <sub>30</sub> O <sub>17</sub> | 300.0247            | Quercetin-diglucoside isomer a          |
| 24  | 7.92  | 625.1389  | 625.1405  | -2.6  | 96.64 | C <sub>27</sub> H <sub>30</sub> O <sub>17</sub> | 300.0242            | Quercetin-diglucoside isomer b          |
| 25  | 8.30  | 463.2182  | 463.2179  | 0.6   | 100   | C <sub>21</sub> H <sub>36</sub> O <sub>11</sub> | 285.0363; 125.0241  | Luteolin/kaempferol derivative isomer c |
| 26  | 9.13  | 439.1009  | 439.1029  | -4.6  | 98.94 | C <sub>23</sub> H <sub>20</sub> O <sub>9</sub>  | 287.0497            | Triacetoxymethoxymethylflavone          |
| 27  | 9.25  | 439.1011  | 439.1029  | -4.1  | 98.07 | C <sub>23</sub> H <sub>20</sub> O <sub>9</sub>  | 287.0468            | Triacetoxymethoxymethylflavone          |
| 28  | 10.07 | 439.1037  | 439.1029  | 1.8   | 100   | C <sub>23</sub> H <sub>20</sub> O <sub>9</sub>  | 287.0533            | Triacetoxymethoxymethylflavone          |

|    |       |          |          |      |     |                                                 |          |                                            |
|----|-------|----------|----------|------|-----|-------------------------------------------------|----------|--------------------------------------------|
| 29 | 10.66 | 461.2382 | 461.2387 | -1.1 | 100 | C <sub>22</sub> H <sub>38</sub> O <sub>10</sub> | 285.0348 | Luteolin/kaempferol derivative II isomer a |
| 30 | 10.70 | 461.2405 | 461.2387 | 3.9  | 100 | C <sub>22</sub> H <sub>38</sub> O <sub>10</sub> | 285.0439 | Luteolin/kaempferol derivative II isomer   |
| 31 | 11.19 | 461.2397 | 461.2387 | 2.2  | 100 | C <sub>22</sub> H <sub>38</sub> O <sub>10</sub> | 285.0234 | Luteolin/kaempferol derivative II isomer c |
| 32 | 11.32 | 461.2387 | 461.2387 | 0.0  | 100 | C <sub>22</sub> H <sub>38</sub> O <sub>10</sub> | 285.0355 | Luteolin/kaempferol derivative II isomer   |

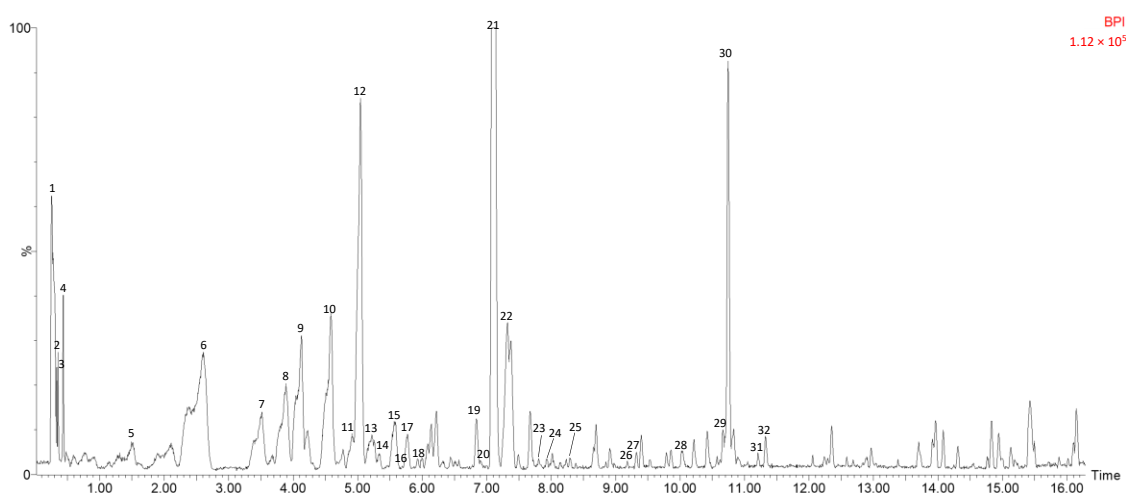


**Figure 9.1.** Base peak chromatogram of the polar compounds identified in the fermented avocado seed. Numbers 1–36 correspond to the peaks in **Table 9.1**.

Corresponding to peaks 3 and 4, two isomers of quinic acid were detected, named as isomers a and b, respectively. Some quinic acid derivatives were detected conjugated with other phenolic acids. Caffeoylquinic acid isomers a and b were detected at 2.57 and 4.58 min (peaks 6 and 10) with the  $m/z$  in source fragments 179 (C<sub>9</sub>H<sub>7</sub>O<sub>4</sub>)<sup>-</sup> of caffeic acid and 191 (C<sub>7</sub>H<sub>11</sub>O<sub>6</sub>)<sup>-</sup> of quinic acid, respectively. Besides, four isomers of coumaroylquinic acid, named as a, b, c, and d (peaks 7, 8, 11, and 15, respectively), were identified according to its  $m/z$  in source fragments 163 (C<sub>9</sub>H<sub>7</sub>O<sub>3</sub>)<sup>-</sup> and 191 (C<sub>7</sub>H<sub>11</sub>O<sub>6</sub>)<sup>-</sup>, belonging to coumaric and quinic acids, respectively [461].

With the  $m/z$  625, two quercetin-diglucoside isomers named a and b were detected according to its  $m/z$  in source fragment 300 (C<sub>15</sub>H<sub>8</sub>O<sub>7</sub>)<sup>2-</sup> (peaks 23 and 24), that corresponds to quercetin-7-olate, a conjugate base of quercetin arising from selective deprotonation of the 7-hydroxy group [461].

The organic compound perseitol, also known as D-glycero-D-galacto-heptitol, a sugar alcohol, was identified in avocado seeds at 0.33 min (peak 2) according to its  $m/z$  in source fragments 59 (C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>)<sup>-</sup>, 89 (C<sub>3</sub>H<sub>5</sub>O<sub>3</sub>)<sup>-</sup>, and 101 (C<sub>4</sub>H<sub>5</sub>O<sub>3</sub>)<sup>-</sup> [461].

The iridoid penstemide was identified corresponding to peak 9 with the  $m/z$  of 443 and the fragmentation according to López-Cobo et al. [461].

A phenethyl alcohol derivative, hydroxytyrosol glucoside (peak 5), was also found in fermented avocado seeds with the molecular formula  $C_{14}H_{20}O_8$ , the  $m/z$  315, and the  $m/z$  in source fragment 153 that corresponds to hydroxytyrosol [461].

At 7.20 min, the compound found with the  $m/z$  441 was named as hydroxyabscisic acid beta-D-glucoside, a derivative of abscisic acid, an isoprenoid plant hormone [461].

Catechin, procyanidin dimer, trimer, and tetramer were detected at 5.22, 5.62, 7.31, and 6.98, respectively, in accordance with other authors [461].

In addition, other compounds were identified here for the first time in avocado seed. With the  $m/z$  343, peak 1 was named as the galloyl ester 5-O-galloyl-1,4-galactarolactone according to the PUBCHEM database (PubChem CID: 59235421). The same molecule was also identified, but it was not quantified in *Phyllanthus emblica* L. fruit [526].

Tuberonic acid beta-D-glucoside (peak 12 at 5.02 min) was previously described by some authors in rice [527] and leaves of tobacco [528].

Corresponding to peak 14, a new flavonoid was detected with the  $m/z$  449 and the predicted formula  $C_{21}H_{22}O_{11}$ . It was named as eriodictyol 7-O-glucoside, in concordance with its main  $m/z$  in source fragment 287 ( $C_{15}H_{11}O_6$ )<sup>-</sup>, that is eriodictyol and the phenol explorer database [15]. This compound has been previously identified and quantified in other food, such as almonds [529].

With the  $m/z$  439, three flavonoid isomers were identified and called triacetoxymethoxymethylflavone isomer a, b, and c, according to the PUBCHEM database (PubChem CID: 129815511). Its main  $m/z$  in source fragment 287 ( $C_{15}H_{11}O_6$ )<sup>-</sup> suggests that it could also be an eriodictyol derivative, but it has not been previously described.

In addition, other flavonoid derivatives were identified. Three isomers (a, b, and c) of a luteolin/kaempferol derivative were identified according to  $m/z$  463 (peaks 18, 19, and 25) and the  $m/z$  in source fragments 285 ( $C_{15}H_9O_6$ )<sup>-</sup> and 125 ( $C_6H_9O_5$ )<sup>-</sup>, that correspond to luteolin and galloyl glucose with the loss of the carboxyl group, compounds that were previously identified and quantified in avocado seed [114]. Another luteolin derivative (II) was detected at 10.66, 10.70, 11.19, and 11.32 min, named as isomers a, b, c, and d, respectively. Their  $m/z$  in source fragment 285 ( $C_{15}H_9O_6$ )<sup>-</sup> is in agreement as they correspond to luteolin or kaempferol [114].

### 3.2. Lactic Acid Bacteria Growth in Avocado Seed

Avocado seed mixtures were supplemented with glucose and yeast extract to encourage the growth of LAB strains. As shown in **Table 9.2**, the number of viable bacteria in the avocado seed gradually dropped during incubations, until concentrations between  $3.88 \pm 0.09$  and  $4.17 \pm 0.03$  log CFU/mL at 48 h of incubation. Nevertheless, *La. plantarum* CECT 9567 slightly decreased

from  $7.31 \pm 0.02$  to  $6.71 \pm 0.03$  log CFU/mL at 24 and 48 h of incubation, respectively. As can be seen in **Table 9.2**, all LAB had a negative significant ( $p < 0.05$ ) correlation between the CFU/mL and the time ( $r = -0.9964$ ). Therefore, when increasing the time, a lower CFU/mL was found in all cases. Despite being part of the LAB metabolism, phenolic compounds are sometimes toxic for them, causing decreases in the growth. The specific pathway of polyphenols that produces bacterial inhibition is still unclear, but changes in the membrane fatty acid composition in *La. plantarum* were previously reported [370]. Therefore, plant-associated LAB have developed metabolic routes for degradation of those polyphenols by enzymes into less toxic compounds. Among the enzymes described in LAB strains capable of degrading or bio-transforming phenolic compounds are tannase, amylase, esterase,  $\beta$ -glucosidase, phenolic acid decarboxylase (PAD), reductase, and benzyl alcohol dehydrogenase enzymes [530]. Tannase enzymes can hydrolyze tannins and gallic acid esters. Hydroxycinnamic acids such as caffeic, p-coumaric, and ferulic acids can be bio-transformed into dihydrocaffeic, phloretic, or dihydroferulic acids, respectively, by reductase enzymes, or they can be decarboxylated into vinyl derivatives, such as vinyl phenol and vinyl guaiacol, by PAD enzymes. Subsequently, those vinyl derivatives can be reduced into ethyl derivatives [531]. Glucosidase enzymes release phenolic compounds by hydrolysis of glycosylated precursors, including flavonoid glucosides [532]. The tolerance to phenolic compounds depends on the LAB specie. A positive significant correlation was found between the content of quinic acid isomers a and b and perseitol, with the  $\text{Log}_{10}$  CFU/mL of all lactic acid bacteria ( $r = 0.6313$ ,  $0.6895$ , and  $0.9481$ , respectively). In these cases, those compounds could have inhibited the growth of LAB during the incubation time or were highly available during fermentation of the avocado seed and were accessible for the bacteria to be used or bio-transformed into other compounds as part of their metabolism. In contrast, quercetin-diglucoside isomer a and luteolin derivative II isomer b showed a negative significant correlation with  $\text{Log}_{10}$  CFU/mL ( $r = -0.4490$  and  $-0.5098$ , respectively). This means that those compounds were formed because of bio-transformations from precursor compounds and that were not toxic for LAB. Regarding *P. acidilactici* CECT 5765 T, it showed a significant ( $p < 0.05$ ) positive correlation of caffeoylquinic acid isomers a and b and the sum of procyanidins, with  $\text{Log}_{10}$  CFU/mL ( $r = 0.9999$ ,  $0.9999$ , and  $0.9624$ , respectively). Additionally, hydroxytyrosol glucoside and perseitol had the same tendency ( $p < 0.05$ ,  $r = 0.9999$ , in both cases). *P. pentosaceus* CECT 4695 T, *Le. brevis* CECT 5354, and *La. plantarum* CECT 9567 had similar tendencies. In the case of *Leu. mesenteroides* CECT 219 T, the phenolic acid precursor quinic acid isomers a and b showed a significant positive correlation with  $\text{Log}_{10}$  CFU/mL ( $r = 0.9278$  and  $0.8662$ , respectively). Furthermore, perseitol, catechin, and hydroxytyrosol glucoside showed a significant positive correlation with the  $\text{Log}_{10}$  CFU/mL ( $r = 0.9902$ ,  $0.8845$ , and  $0.9954$ , respectively). In contrast, caffeoylquinic acid isomers and coumaroylquinic acid isomers had significant negative correlations with  $\text{Log}_{10}$  CFU/mL ( $r$  from  $-0.7225$  to  $-0.9995$ ). For procyanidin dimer, trimer, and



tetramer, the opposite correlation was found with Log<sub>10</sub> CFU/mL compared to catechin. In the case of *La. plantarum* CECT 748 T, only quinic acid isomers a and b, perseitol, and hydroxytyrosol glucoside showed significant positive correlations with Log<sub>10</sub> CFU/mL ( $r > 0.9622$ ), and negative correlations were found for the rest of the compounds. Previously, the inhibition of the growth of *La. plantarum* with the presence of caffeic acid and its derivatives has been reported by other authors [370]. Others reported inhibition of *La. plantarum* by *p*-coumaric and ferulic acids present in wines [533]. Additionally, the inhibition of other LAB by hydroxycinnamic acids has been reported [534], or inhibition of other Gram-positive bacteria such as *Bacillus cereus* and *Enterococcus faecalis* by caffeoylquinic acid [535]. Furthermore, phenolic compounds found in olive products, such as oleuropein, tyrosol, sinapic acid, and syringic acid, showed inhibitory activity against *La. plantarum* strains [536]. Otherwise, there are no previous references of catechin inhibiting the growth of LAB [537]. The use of an avocado seed hydrolysate-based medium to support biomass growth and lactic acid production by *Lactobacillus* sp. has been previously reported [538]. As previously reported [118], avocado seed is a well-known source of carbohydrates (65%), with perseitol and D-mannoheptulose being the sugar compounds that stand out the most. Therefore, according to the results, the content of perseitol was reduced with the increase of Log<sub>10</sub> CFU/mL, which indicates that its content directly affects the growth of the LAB tested.

Taking everything into account, in order to compare between the LAB strains, the extracts fermented by *Leu. mesenteroides* CECT 219 T and *La. plantarum* CECT 748 T at 48 h and the rest at 24 h were selected. This is also supported by previous research [188].

The pH throughout the fermentations decreased from  $5.96 \pm 0.02$  (initial) to values between 4.5 and 5.2 at 48 h of incubation.

**Table 9.2.** Log CFU/mL and pH of lactic acid bacteria in avocado seed expressed as average  $\pm$  standard deviation.

| LAB                                  | 0 h                      | 24 h                     |     | 48 h                     |     |
|--------------------------------------|--------------------------|--------------------------|-----|--------------------------|-----|
|                                      | Log <sub>10</sub> CFU/mL | Log <sub>10</sub> CFU/mL | pH  | Log <sub>10</sub> CFU/mL | pH  |
| <i>P. acidilactici</i> CECT 5765 T   | $7.96 \pm 0.02$          | $7.14 \pm 0.02$          | 4.9 | $4.17 \pm 0.03$          | 4.9 |
| <i>P. pentosaceus</i> CECT 4695 T    | $7.57 \pm 0.01$          | $7.37 \pm 0.06$          | 4.9 | $4.10 \pm 0.09$          | 4.8 |
| <i>Leu. mesenteroides</i> CECT 219 T | $7.14 \pm 0.01$          | $7.04 \pm 0.04$          | 5.2 | $4.03 \pm 0.05$          | 5.2 |
| <i>Le. brevis</i> CECT 5354          | $7.05 \pm 0.03$          | $7.01 \pm 0.06$          | 5.0 | $4.04 \pm 0.04$          | 4.9 |
| <i>La. plantarum</i> CECT 748 T      | $7.95 \pm 0.02$          | $6.72 \pm 0.02$          | 4.9 | $3.88 \pm 0.09$          | 4.9 |
| <i>La. plantarum</i> CECT 9567       | $7.93 \pm 0.06$          | $7.31 \pm 0.02$          | 4.6 | $6.71 \pm 0.03$          | 4.5 |

### **3.3. Quantification of Phenolic Compounds by HPLC-ESI-TOF-MS and Its Bio-Transformations during Fermentation in Avocado Seeds**

The phenolic and other polar compounds identified in fermented and non-fermented avocado seeds are summarized in **Table 9.3**.

**Table 9.3.** Phenolic and other polar compounds quantified by HPLC-ESI-TOF-MS in the fermented avocado seeds and a control. Results are expressed as average  $\pm$  standard deviation.

| Compound                         | $\mu\text{g/g d.w.}$    |                        |                           |                         |                        |                        |                         |
|----------------------------------|-------------------------|------------------------|---------------------------|-------------------------|------------------------|------------------------|-------------------------|
|                                  | <i>P. acidilactici</i>  | <i>P. pentosaceus</i>  | <i>Leu. mesenteroides</i> | <i>Le. brevis</i>       | <i>La. plantarum</i>   | <i>La. plantarum</i>   | Control                 |
| 5-O-Galloyl-1,4-galactarolactone | 92.23 $\pm$ 1.26 a–c    | 86.57 $\pm$ 1.55 b–d   | 93.26 $\pm$ 1.46 a, b     | 83.46 $\pm$ 5.96 d      | 85.35 $\pm$ 0.93 c–d   | 88.19 $\pm$ 0.38 a–d   | 94.43 $\pm$ 1.02 a      |
| Quinic acid isomer a             | 107.68 $\pm$ 3.90 b, c  | 108.03 $\pm$ 3.99 b, c | 98.56 $\pm$ 1.79 b, c     | 112.11 $\pm$ 12.08 a, b | 102.87 $\pm$ 0.17 b, c | 123.77 $\pm$ 3.04 a    | 96.89 $\pm$ 0.99 c      |
| Quinic acid isomer b             | 146.31 $\pm$ 1.93 a–c   | 145.11 $\pm$ 1.31 a–c  | 139.39 $\pm$ 3.36 c, d    | 151.87 $\pm$ 3.85 a     | 137.38 $\pm$ 0.52 d    | 148.12 $\pm$ 4.09 a, b | 140.71 $\pm$ 1.35 b–d   |
| Perseitol                        | 12.42 $\pm$ 0.02 b      | 12.18 $\pm$ 0.14 b     | 10.36 $\pm$ 0.20 c        | 12.00 $\pm$ 0.35 b      | 10.90 $\pm$ 0.22 c     | 12.20 $\pm$ 0.22 b     | 15.46 $\pm$ 0.05 a      |
| Hydroxytyrosol glucoside         | 2989.76 $\pm$ 3.64 a    | 1547.58 $\pm$ 11.64 c  | 1740.31 $\pm$ 13.67 b     | 1574.06 $\pm$ 10.30 c   | 886.69 $\pm$ 8.32 e    | 128.44 $\pm$ 9.63 f    | 1255.66 $\pm$ 18.34 d   |
| Caffeoylquinic acid isomer a     | 888.91 $\pm$ 1.04 e     | 787.01 $\pm$ 6.32 f    | 922.80 $\pm$ 5.00 d       | 802.99 $\pm$ 2.25 f     | 1109.82 $\pm$ 4.88 c   | 1168.20 $\pm$ 12.57 a  | 1133.69 $\pm$ 4.70 b    |
| Caffeoylquinic acid isomer b     | 507.57 $\pm$ 2.48 e     | 449.37 $\pm$ 3.71 f    | 520.10 $\pm$ 0.57 d       | 421.71 $\pm$ 1.11 g     | 569.72 $\pm$ 1.57 c    | 652.06 $\pm$ 6.83 a    | 605.25 $\pm$ 7.33 b     |
| Coumaroylquinic acid isomer a    | 302.09 $\pm$ 4.99 a     | 318.50 $\pm$ 1.73 a    | 273.33 $\pm$ 11.65 b      | 238.75 $\pm$ 10.77 c    | 195.23 $\pm$ 9.63 d    | 194.92 $\pm$ 0.16 d    | 210.88 $\pm$ 4.20 d     |
| Coumaroylquinic acid isomer b    | 374.82 $\pm$ 13.54 c    | 341.59 $\pm$ 7.27 d    | 399.01 $\pm$ 4.21 c       | 301.14 $\pm$ 16.01 e    | 468.46 $\pm$ 7.37 b    | 514.91 $\pm$ 13.53 a   | 490.79 $\pm$ 3.34 a, b  |
| Coumaroylquinic acid isomer c    | 148.94 $\pm$ 3.80 a     | 147.85 $\pm$ 7.42 a    | 141.21 $\pm$ 1.22 a       | 119.56 $\pm$ 4.14 b     | 109.00 $\pm$ 5.16 b    | 111.02 $\pm$ 2.63 b    | 116.19 $\pm$ 2.57 b     |
| Coumaroylquinic acid isomer d    | 170.50 $\pm$ 6.77 d     | 153.54 $\pm$ 5.15 e    | 188.37 $\pm$ 0.35 c       | 141.33 $\pm$ 5.54 e     | 207.35 $\pm$ 0.94 b    | 227.54 $\pm$ 7.46 a    | 213.18 $\pm$ 5.62 a, b  |
| Pensternide                      | 50.45 $\pm$ 1.54 a      | 52.79 $\pm$ 3.15 a     | 55.24 $\pm$ 2.28 a        | 46.60 $\pm$ 11.55 a     | 56.91 $\pm$ 0.77 a     | 58.34 $\pm$ 1.06 a     | 58.77 $\pm$ 0.10 a      |
| Catechin                         | 48.00 $\pm$ 0.17 a      | 39.23 $\pm$ 2.03 b     | 38.46 $\pm$ 2.47 b        | 33.04 $\pm$ 4.68 b      | 33.37 $\pm$ 0.79 b     | 37.48 $\pm$ 1.78 b     | 45.75 $\pm$ 1.22 a      |
| Eriodictyol 7-O-glucoside        | 7.85 $\pm$ 0.08 b, c    | 7.99 $\pm$ 0.21 a–c    | 8.47 $\pm$ 0.28 a–c       | 7.33 $\pm$ 1.24 c       | 9.31 $\pm$ 0.07 a      | 8.81 $\pm$ 0.01 a, b   | 8.63 $\pm$ 0.04 a–c     |
| 2-Phenylethyl beta-primeveroside | 10.86 $\pm$ 0.26 a      | 11.24 $\pm$ 0.00 a     | 11.25 $\pm$ 0.08 a        | 10.68 $\pm$ 1.37 a      | 11.22 $\pm$ 0.01 a     | 12.00 $\pm$ 0.47 a     | 11.55 $\pm$ 0.13 a      |
| Procyanidin dimer                | 20.94 $\pm$ 0.31 b      | 18.58 $\pm$ 0.32 c, d  | 21.83 $\pm$ 0.24 a, b     | 18.00 $\pm$ 1.21 d      | 19.96 $\pm$ 0.12 b, c  | 23.04 $\pm$ 0.93 a     | 23.39 $\pm$ 0.91 a      |
| Procyanidin tetramer             | 18.79 $\pm$ 1.34 c      | 16.31 $\pm$ 0.24 c     | 21.97 $\pm$ 0.99 b        | 17.68 $\pm$ 0.19 c      | 23.74 $\pm$ 0.15 a, b  | 25.70 $\pm$ 1.53 a     | 22.82 $\pm$ 1.33 b      |
| Procyanidin trimer               | 166.92 $\pm$ 13.88 b, c | 133.81 $\pm$ 14.92 c   | 204.51 $\pm$ 19.56 a, b   | 128.79 $\pm$ 8.68 c     | 216.82 $\pm$ 1.29 a    | 240.41 $\pm$ 17.46 a   | 203.83 $\pm$ 11.81 a, b |

| Compound                                    | $\mu\text{g/g d.w.}$    |                         |                           |                          |                        |                       |                         |
|---------------------------------------------|-------------------------|-------------------------|---------------------------|--------------------------|------------------------|-----------------------|-------------------------|
|                                             | <i>P. acidilactici</i>  | <i>P. pentosaceus</i>   | <i>Leu. mesenteroides</i> | <i>Le. brevis</i>        | <i>La. plantarum</i>   | <i>La. plantarum</i>  | Control                 |
| Luteolin/kaempferol derivative isomer a     | 6.55 $\pm$ 0.12 a       | 6.64 $\pm$ 0.09 a       | 6.77 $\pm$ 0.12 a         | 6.30 $\pm$ 0.73 a        | 6.52 $\pm$ 0.06 a      | 6.90 $\pm$ 0.12 a     | 6.93 $\pm$ 0.09 a       |
| Luteolin/kaempferol derivative isomer b     | 12.28 $\pm$ 0.73 a      | 12.73 $\pm$ 0.34 a      | 12.84 $\pm$ 0.33 a        | 11.74 $\pm$ 1.56 a       | 12.21 $\pm$ 0.26 a     | 13.10 $\pm$ 0.42 a    | 13.01 $\pm$ 0.02 a      |
| Luteolin/kaempferol derivative isomer c     | 5.53 $\pm$ 0.08 a       | 5.72 $\pm$ 0.37 a       | 5.79 $\pm$ 0.09 a         | 5.55 $\pm$ 0.64 a        | 5.63 $\pm$ 0.41 a      | 5.82 $\pm$ 0.13 a     | 6.05 $\pm$ 0.08 a       |
| Quercetin-diglucoside isomer a              | 4.95 $\pm$ 0.08 b, c    | 5.00 $\pm$ 0.06 b, c    | 5.38 $\pm$ 0.14 a–c       | 4.72 $\pm$ 0.48 c        | 6.05 $\pm$ 0.48 a      | 5.52 $\pm$ 0.16 a, b  | 5.42 $\pm$ 0.04 a–c     |
| Quercetin-diglucoside isomer b              | 5.00 $\pm$ 0.14 b, c    | 5.13 $\pm$ 0.11 a–c     | 5.41 $\pm$ 0.01 a–c       | 4.87 $\pm$ 0.47 c        | 5.54 $\pm$ 0.14 a, b   | 5.58 $\pm$ 0.01 a     | 5.51 $\pm$ 0.02 a, b    |
| Triacetoxymethoxymethylflavone isomer a     | 3.81 $\pm$ 0.07 c, d    | 4.17 $\pm$ 0.02 b       | 4.84 $\pm$ 0.24 a         | 3.72 $\pm$ 0.19 d        | 4.14 $\pm$ 0.00 b, c   | 5.05 $\pm$ 0.04 a     | 4.84 $\pm$ 0.03 a       |
| Triacetoxymethoxymethylflavone isomer b     | 3.88 $\pm$ 0.04 d       | 3.91 $\pm$ 0.03 c, d    | 4.24 $\pm$ 0.09 b         | 3.84 $\pm$ 0.23 d        | 4.19 $\pm$ 0.03 b, c   | 4.54 $\pm$ 0.02 a     | 4.39 $\pm$ 0.11 a, b    |
| Triacetoxymethoxymethylflavone isomer c     | 4.66 $\pm$ 0.13 d       | 4.77 $\pm$ 0.14 c, d    | 5.42 $\pm$ 0.29 a, b      | 4.67 $\pm$ 0.36 c, d     | 5.23 $\pm$ 0.13 b, c   | 5.80 $\pm$ 0.06 a     | 5.49 $\pm$ 0.12 a, b    |
| Luteolin/kaempferol derivative II isomer a  | 8.57 $\pm$ 0.43 b       | 9.00 $\pm$ 0.67 a, b    | 9.51 $\pm$ 0.18 a, b      | 8.62 $\pm$ 1.20 b        | 10.39 $\pm$ 0.07 a     | 9.06 $\pm$ 0.13 a, b  | 9.34 $\pm$ 0.20 a, b    |
| Luteolin/kaempferol derivative II isomer b  | 12.43 $\pm$ 0.67 b, c   | 12.29 $\pm$ 0.14 b, c   | 12.79 $\pm$ 0.05 b        | 11.22 $\pm$ 1.02 c       | 25.67 $\pm$ 0.10 a     | 13.31 $\pm$ 0.03 b    | 13.18 $\pm$ 0.15 b      |
| Luteolin/kaempferol derivative II isomer c  | 5.42 $\pm$ 0.03 b, c    | 5.65 $\pm$ 0.02 b, c    | 5.79 $\pm$ 0.02 a–c       | 5.21 $\pm$ 0.58 c        | 6.41 $\pm$ 0.04 a      | 5.82 $\pm$ 0.14 a–c   | 5.90 $\pm$ 0.01 a, b    |
| Luteolin/kaempferol derivative II isomer d  | 8.31 $\pm$ 0.54 a, b    | 8.88 $\pm$ 0.55 a, b    | 8.78 $\pm$ 0.02 a, b      | 7.91 $\pm$ 0.89 b        | 9.54 $\pm$ 0.20 a      | 8.91 $\pm$ 0.55 a, b  | 8.85 $\pm$ 0.00 a, b    |
| <b>Sum of phenolic acids and precursors</b> | 5636.58 $\pm$ 42.08 a   | 3998.58 $\pm$ 48.55 d   | 4423.06 $\pm$ 41.82 b     | 3863.52 $\pm$ 66.05 d, e | 3786.53 $\pm$ 38.55 e  | 3268.97 $\pm$ 59.95 f | 4263.23 $\pm$ 48.44 c   |
| <b>Sum of procyanidins</b>                  | 254.65 $\pm$ 15.70 b, c | 207.93 $\pm$ 17.52 c, d | 286.77 $\pm$ 23.26 a, b   | 197.51 $\pm$ 14.75 d     | 293.89 $\pm$ 2.34 a, b | 326.63 $\pm$ 21.70 a  | 295.80 $\pm$ 15.27 a, b |
| <b>Sum of others</b>                        | 388.41 $\pm$ 9.96 a     | 391.68 $\pm$ 14.97 a    | 409.69 $\pm$ 8.12 a       | 373.12 $\pm$ 47.29 a     | 423.50 $\pm$ 8.16 a    | 417.40 $\pm$ 6.17 a   | 428.47 $\pm$ 6.19 a     |
| <b>Sum of polar compounds</b>               | 6279.63 $\pm$ 67.74 a   | 4598.19 $\pm$ 81.04 c   | 5119.52 $\pm$ 73.20 b     | 4434.15 $\pm$ 128.09 c   | 4503.92 $\pm$ 49.04 c  | 4013.01 $\pm$ 87.81 d | 4987.50 $\pm$ 69.90 b   |

Different letters (a–g) in the same line indicate significant differences.

As can be seen in **Table 9.3**, the sum of polar compounds ranged between 4013 and 6280 µg/g d.w. in the avocado seed fermented by LAB. Increments of 26% in avocado seed fermented by *P. acidilactici* and 4.3% by *Leu. mesenteroides* were observed compared to the control, but decreases were found in avocado seeds fermented by other LAB.

The sum of procyanidins decreased in the case of fermentation with *P. acidilactici*, *P. pentosaceus*, and *Le. brevis*, and did not show significant differences by *Leu. mesenteroides* or both strains of *La. plantarum* compared to the control. However, no significant increases were seen. There were no significant differences detected for the flavonoids eriodictyol 7-O-glucoside, 2-phenylethyl beta-primeveroside, and quercetin-diglucoside isomer a. Regarding luteolin derivatives, avocado seed fermented by *La. plantarum* CECT 748T stood out, with the highest content. Otherwise, some decreases were detected for quercetin diglucoside isomer b and the three isomers of triacetoxymethoxymethylflavone in the avocado seeds fermented by *Le. brevis*, *P. pentosaceus*, and *P. acidilactici*. Furthermore, perseitol significantly decreased in all cases; meanwhile, penstemide did not change compared to the control avocado seed. Most likely, perseitol was converted into D-mannoheptulose [539] by some of the LAB that have aldolase enzymes and consumed as a nutrient for their metabolism [540].

As shown in **Table 9.3**, the sum of phenolic acids and precursors increased by 32.21% and 3.74% after fermentations with *P. acidilactici* CECT 5765T and *Leu. mesenteroides* CECT 219T, respectively, but decreased with the other bacteria. Nevertheless, lactic acid bacteria metabolism led to interesting bio-transformations in the avocado seed compounds. Hydroxytyrosol glucoside, an important derivative of hydroxytyrosol in olive fruits [541,542], was significantly increased in fermentations with *P. acidilactici* CECT 5765T, *P. pentosaceus* CECT 923, *Leu. mesenteroides* CECT 219T, and *Le. brevis* CECT 5354. These increases can be explained by the fact that lactic acid bacteria can release phenolic compounds from plant matrices through their hydrolytic enzymes, such as amylase, xylanase, glucosidase, cellulase, tannase, esterase, or chitinase. These enzymes can disrupt the cell wall, where phenolic acids are found linked to lignin, cellulose, hemicellulose, or structural proteins through ether bonds via the hydroxyl group in aromatic rings, or they form esters via the carboxylic group with proteins and carbohydrates [357]. Thus, these enzymes play a key role in disintegrating plant cell wall matrices and increasing the bioavailability of phenolic compounds, which can be used as a new substrate for other bacterial enzymes or can be free for their extraction and recovery as added-value bioactive compounds. On the other hand, the fermentation of avocado seed with the two *La. plantarum* strains significantly decreased the hydroxytyrosol glucoside content. This decrease could be a result of the hydrolysis via the glucosidase activity of both strains to release hydroxytyrosol and glucose. Glycosidase activities are widespread in lactic acid bacteria; among them, *La. plantarum* glycosidases have been especially studied [543]. These enzymes have been reported to release a large number of

secondary metabolites from their glycosylated precursors, increasing their antioxidant activity and improving the flavor and aroma of fermented foods [532]. In addition to olive fruit, hydroxytyrosol and hydroxytyrosol glucoside have previously been found in avocado seed [461]. They have shown antioxidant, antibacterial, antifungal, anti-inflammatory, and antitumoral activities in both in vitro and in vivo assays [544–546]. In our previous research with avocado leaves [188], fermentation with both *La. plantarum* strains decreased the concentration of flavonoid glucosides and other glycosylated phenolic compounds, and in the case of *La. plantarum* CECT 748T, it led to a high antioxidant activity.

Regarding chlorogenic acids found in avocado seeds, a significant increase was found in caffeoylquinic acid in fermentation with *La. plantarum* CECT 9567 and in coumaroylquinic acid isomers a and c with *P. acidilactici* CECT 5765T, *P. pentosaceus* CECT 923, *Leu. mesenteroides* CECT 219T, and *Le. brevis* CECT 5354 fermentations. This group of phenolic compounds are produced in plants by esterification of the quinic acid and the hydroxycinnamic acids, including caffeic acid, *p*-coumaric acid, and ferulic acid [547]. They are widely distributed in plant-based foods, such as coffee, tea, and wine, as well as in avocado seed [117]. The most abundant compound is 5-caffeoylquinic acid. Their occurrence in plants has been associated with lignin biosynthesis, as an indirect defense agent, making tissues more resistant to pathogen attacks and inhibiting fungal toxins, and as a protective agent against oxidative damage caused by exposition to salinity and heat [547]. They have shown antioxidant, antimicrobial, antiviral, and anti-inflammatory activity, as well as risk reduction of developing type 2 diabetes and cardiovascular diseases [548]. Despite the antimicrobial activity, some microorganisms are resistant to chlorogenic acids because of their ability to degrade such compounds [548]. In this study, caffeoylquinic acid isomers a and b and coumaroylquinic acid isomers b and d decreased in fermentations with *P. acidilactici* CECT 5765T, *P. pentosaceus* CECT 923, *Leu. mesenteroides* CECT 219T, and *Le. brevis* CECT 5354, which could be due to the esterase activity of the strains. In human digestion, when chlorogenic acids reach the colon, the microbiota hydrolyses them, releasing hydroxycinnamic acid and quinic acid [549]. A significant increase in the concentration of quinic acid isomers a and b was found when avocado seeds were fermented with *Le. brevis* CECT 5354 and *La. plantarum* CECT 9567, indicating possible hydrolysis of hydroxycinnamic acids. The hydrolysis of these acyl-quinic acids is carried out by cinnamoyl esterases, which have been studied in *Lactobacillus* species, including *La. plantarum* [372] and *L. johnsonii* [550], as well as in *Escherichia coli*, *Bifidobacterium lactis*, and *Lactobacillus gasseri* [551]. Although hydroxycinnamic acids are efficiently absorbed after hydrolysis, intact chlorogenic acids are less well-absorbed only in the small intestine [552]. The hydrolysis of dietary phenolics by fermentation before intake can increase the bioavailability of bioactive compounds in case of dysbiosis of the fermentative microbiota [371].

### 3.4. Antioxidant Activity in Fermented Avocado Seeds

The antioxidant activity of the LAB-fermented avocado seed extracts was analyzed by DPPH and FRAP assays compared to non-fermented extract of avocado seeds (control), and the results are shown in **Figure 9.2**. A significant ( $p < 0.05$ ) positive correlation ( $r = 0.8884$ ) was found between the antioxidant activity measured by both methods, DPPH and FRAP.

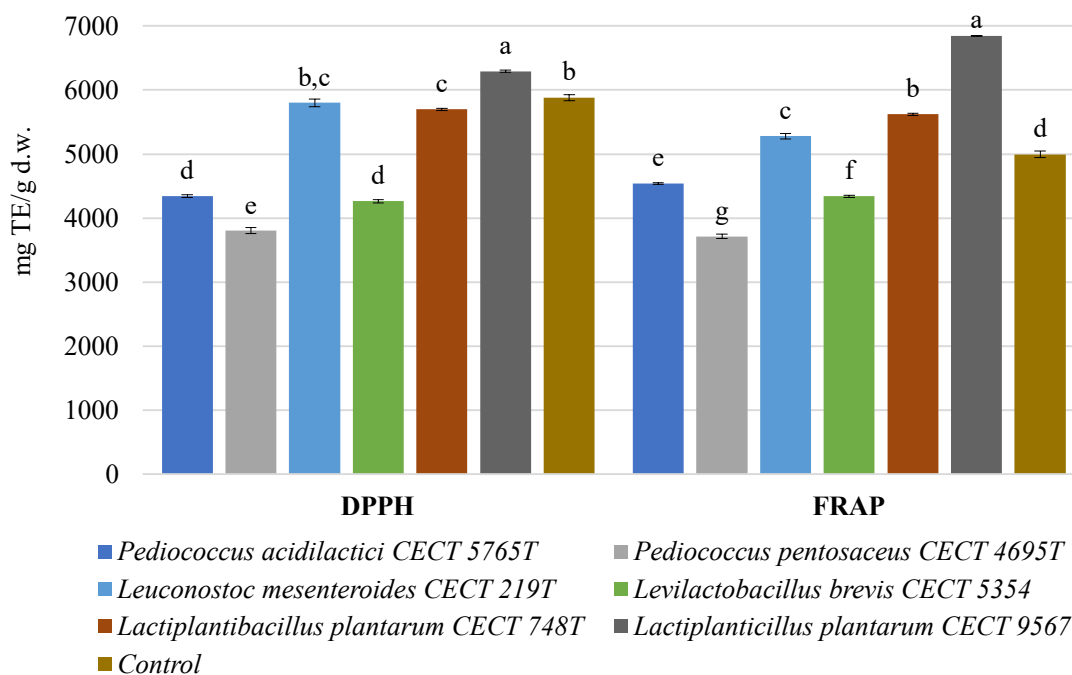


**Figure 9.2.** Antioxidant activity of avocado seed fermented by LAB strains and a control measured by DPPH and FRAP assays. Different letters (a–g) for each method indicate significant differences.

As can be seen in **Figure 9.2**, the antioxidant activity obtained for the non-fermented and fermented avocado seed extracts ranged 3803–6295 and 3714–6847  $\mu\text{g TE/g d.w.}$  for DPPH and FRAP assays, respectively. In both cases, the lowest result corresponded to *P. pentosaceus* CECT 4695T, and the highest to *La. plantarum* CECT 9567, with significant differences with respect to the control. For the FRAP assay, *La. plantarum* CECT 748T and *Leu. mesenteroides* CECT 219T also showed significant increases compared to the control. In the DPPH results, no significant reduction in the antioxidant activity was found for *Leu. mesenteroides* CECT 219T. Although avocado seed fermented by *P. acidilactici* CECT 5765T showed the highest content in the sum of polar compounds and, by extension, in the sum of phenolic acids and precursors, it showed a significant decrease in its antioxidant activity compared to the control. In contrast, those that had increases in their antioxidant activity did not show significant increases in the sum of polar compounds. This phenomenon was previously noticed by Li et al. [389] in apple juice fermented

by *La. plantarum* ATCC14917. They reported a significant decrease in total phenolic and total flavonoid compounds during fermentation, while the antioxidant activity increased over time. They attributed this to the increase of 5-O-caffeoylquinic acid, quercetin, and phloretin [389]. Results found here agree that *La. plantarum* CECT 9567, compared to the control, had significant increments of quinic acid isomer a, and caffeoylquinic acid isomers a and b. This was also statistically confirmed. According to the Pearson's correlation test, the antioxidant activity in the fermented avocado seed can be attributed to the polyphenol content. Furthermore, the sum of procyanidins showed a significant ( $p < 0.05$ ) strong correlation for both methods, DPPH ( $r = 0.9406$ ) and FRAP ( $r = 0.8865$ ). Besides, caffeoylquinic acid isomers a and b and coumaroylquinic acid isomers b and d, pensternide, eriodictyol 7-O-glucoside, quercetin-diglucoside isomers a and b, and triacetoxymethoxymethylflavone isomers a, b, and c also had significant ( $p < 0.05$ ) strong correlations with the antioxidant activity, as measured by both methods. Previously, Rozan et al. also reported an increment in the DPPH antioxidant activity in Hass avocado kernel flour fermented by *La. plantarum* [388].

### 3.5. Antidiabetic Activity in Fermented Avocado Seeds

The capacity of inhibiting alpha-amylase was analyzed in the LAB-fermented avocado seed compared to the non-fermented extract of the avocado seed (control). All were tested at 20 mg of avocado/mL, and the results are shown in **Figure 9.3**.

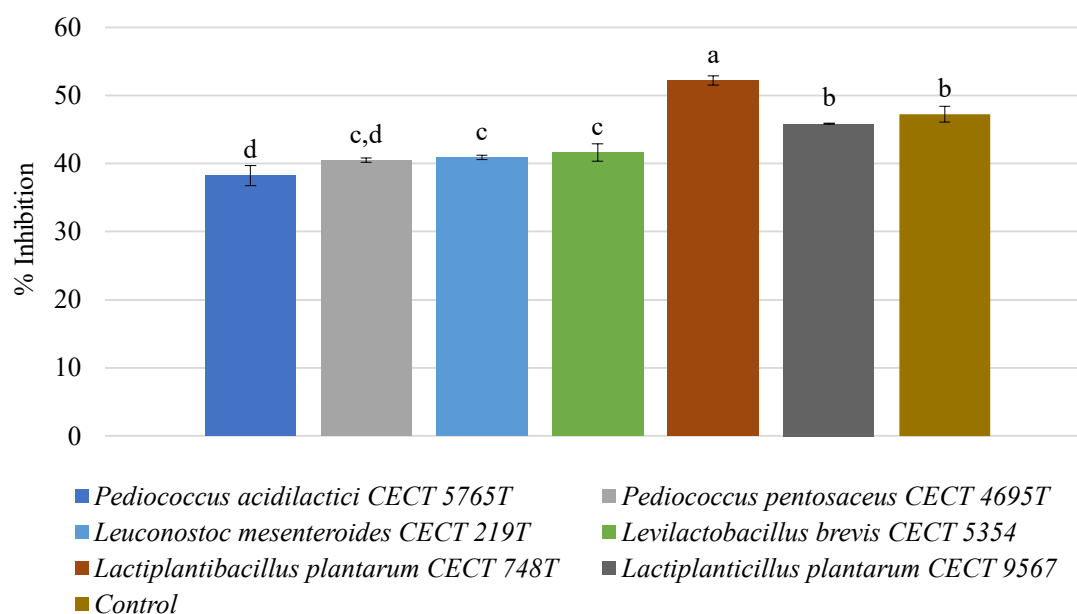


**Figure 9.3.** Inhibition of alpha-amylase activity of fermented avocado seed by different strains and non-fermented avocado seed. Different letters (a–d) indicate significant differences.



As can be seen in **Figure 9.3**, all the LAB tested presented a % of inhibition higher than 25%. *La. plantarum* CECT 748T had the highest % of inhibition; meanwhile, *P. acidilactici* CECT 5765T had the lowest. *La. plantarum* CECT 9567 did not show significant differences with the unfermented avocado seed, but the rest had significant decreases in the capacity of inhibiting alpha-amylase activity. Ojo et al. reported values similar to those obtained here, with a % of inhibition  $\approx 20\%$  of alpha-amylase at  $\approx 20$  mg/mL of avocado seed extract [521]. Moreover, Abd Elkader et al. reported values of the same range of magnitude with avocado leaves and fruits [553]. The Pearson correlation test revealed a significant positive correlation between the antidiabetic activity and the compounds caffeoylquinic acid (isomers a and b,  $r = 0.7786$  and  $0.6163$ , respectively), penstemide ( $r = 0.5154$ ), procyanidin trimer ( $r = 0.6112$ ) and tetramer ( $r = 0.6881$ ), quercetin-diglucoside isomers a ( $r = 0.7749$ ) and b ( $r = 0.6540$ ), eriodictyol 7-O-glucoside ( $r = 0.7022$ ), triacetoxymethoxymethylflavone isomers b ( $r = 0.5741$ ) and c ( $r = 0.5421$ ), and luteolin/kaempferol derivative isomers a–d ( $r = 0.5929$ – $0.7970$ ). Moreover, a positive correlation ( $r = 0.7568$ ) with total flavan-3-ols content was also found. Younis et al. [554] studied the antidiabetic activity of seven varieties of avocado seed by the alpha-amylase method and reported that it could be attributed to its content of polyunsaturated fatty acids, as well as several polyphenolic compounds, such as eriodictyol-7-oneohesperidoside, 4-caffeoylquinic acid, and linoleic acid ethyl ester [554]. Furthermore, the inhibition of alpha-amylase activity showed a significant positive correlation with the antioxidant activity measured by DPPH ( $r = 0.6376$ ) and FRAP assays ( $r = 0.5503$ ). This makes sense as most of the compounds correlated with the antioxidant activity also showed correlation with the antidiabetic activity, and by extension, flavonoids. The differentiation compounds between both activities were luteolin derivative isomers. In fact, flavonoids are reported by other authors to have antidiabetic activity [555].

### 3.6. Clustering Heatmap

A hierarchical clustering heatmap (**Figure 9.4**) was performed to provide an intuitive visualization of all the polar compounds quantified by HPLC-ESI-TOF-MS and the antioxidant (DPPH and FRAP) and antidiabetic (alpha-amylase) assays carried out in the fermented avocado seeds by the selected strains, and the non-fermented control.

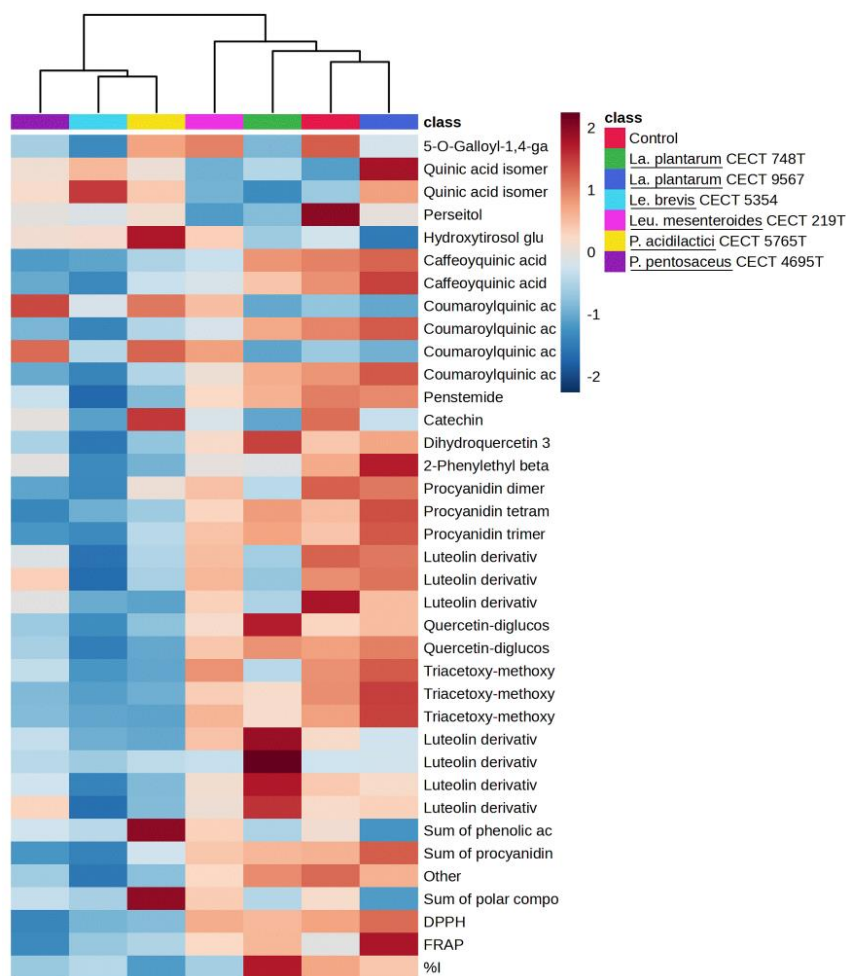


**Figure 9.4.** Clustering heatmap of the avocado seeds fermented by LAB and the control according to the analyses performed.

As can be seen from **Figure 9.4**, avocado seed fermented by *La. plantarum* CECT 9567 was clustered with the control samples, showing minor differences. Close to them were the avocado seeds fermented by *La. plantarum* CECT 748T and *Leu. mesenteroides* CECT 219T. Although fermentation of the avocado seed with *La. plantarum* CECT 9567 showed a phenolic profile similar to that of the unfermented seed, key bio-transformations carried out by this strain can be visualized in the figure by color changes. These include increased concentrations of quinic acid and chlorogenic acids, such as some isomers of caffeoylquinic acid, as well as decreased concentrations of hydroxytyrosol-glucoside, by enzymatic hydrolysis for the release of free hydroxytyrosol and glucose. Furthermore, **Figure 9.4** highlights the antioxidant activity by the FRAP assay found in the fermentation with this strain. Another clear group was formed by the rest of the LAB-fermented avocado seeds. In a nutshell, this clustering confirms all the results discussed previously, and shows the differences in the metabolism of the tested LAB on the polar compounds present in avocado seeds.

#### 4. Conclusions

In this research, the chemical bio-transformations and changes in antioxidant and antidiabetic activity induced by LAB strains were studied in avocado seeds using submerged fermentations. A total of 32 polar compounds were identified by HPLC-ESI-TOF-MS and, to our knowledge, 13 of them were tentatively identified for the first time. It was found that among all the compounds, caffeoylquinic and coumaroylquinic acids' isomers could have inhibited the growth of *L. mesenteroides*. No effect was found for procyanidins in LAB growth. Quinic acid and perseitol seemed to be the most available compounds to be bio-transformed by LAB or consumed as part of their metabolism. Avocado seeds fermented by *P. acidilactici* showed the highest sum of polar compounds, and by extension, of hydroxytyrosol glucoside, quantified by HPCL-ESI-TOF-MS. Among all the tested LAB, *La. plantarum* CECT 9567 showed the highest significant increase in the antioxidant activity, measured by both DPPH and FRAP, mainly attributable to its content in hydroxycinnamic acids, procyanidins, and flavonoids. Furthermore, *La. plantarum* CECT 748T had the highest alpha-amylase inhibitory activity, attributable to the polyphenols. In conclusion, submerged fermentation by lactic acid bacteria can be used in the exploitation and valorization of avocado seed waste for its use in food formulations or the production of enriched extracts.

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## SECTION 3. FINAL PRODUCT





## **CHAPTER 10. Fortification of orange juice with hesperidin, hydroxytyrosol and catechin: sensorial and chemical analysis**





## **Fortification of orange juice with hesperidin, hydroxytyrosol and catechin: sensorial and chemical analysis**

### **Abstract**

Given the widespread consumption of orange juice, fortifying it with bioactive compounds from by-products offers a dual benefit: enhancing the juice's health benefits and addressing food waste through a circular economy. This study investigates the fortification of orange juice with bioactive compounds, specifically hesperidin, catechin and hydroxytyrosol, derived from orange peels, avocado by-products, and olive leaves, respectively. 2 mg/mL was selected as the fortification degree based on sensory acceptability ( $n = 15$ ). Then, the stability of the fortified juice was evaluated over a 5-day period, considering the effects of pasteurization on pH, TSS, color, phenolic compounds by HPLC-ESI-TOF-MS, vitamin C by HPLC-UV/VIS and antioxidant activity by DPPH and FRAP. Pasteurization significantly impacted the color parameters of orange juice, initially resulting in increased luminosity, but darkening effect and the reversion of that condition over the days. While pasteurization initially lowers naturally present flavonoids ( $\approx 49\%$ ) and vitamin C content ( $\geq 10\%$ ), the stability over a 5-day storage period remains in a fortified juices with 30% higher phenolic content than the control with consistent vitamin C content, suggesting not only an increase in the total phenolic content but also a protective effect of the added extracts in phenolic acids and vitamin C content over the days. Fortified orange juice exhibited higher antioxidant activity compared to non-fortified juice, and while pasteurization initially reduced antioxidant levels, it also helped stabilize antioxidant activity over time compared to non-pasteurized juice. Taking everything into account this study demonstrated that the fortification of orange juice with mixed extract from by-products makes it a superior choice for maintaining health benefits during storage with acceptable appealing.

**Keywords:** food enrichment; acceptability evaluation; by-product utilization, bioactive extract.

## 1. Introduction

The growing consumer demand for functional beverages has spurred the exploration and development of fortified drinks with enhanced nutritional and health benefits [556]. Recent advancements in food science have focused on incorporating bioactive compounds, such as polyphenols, to improve the antioxidant properties and overall health benefits of these beverages [557].

Orange juice, being the most consumed juice globally [450], serves as an ideal candidate for fortification due to its high consumer acceptance and established nutritional profile. Traditionally, orange juice has been fortified with a variety of compounds, such as plant probiotics [558], tuna oil powder [559], fructooligosaccharides [560], minerals such as iron [561] and calcium [562], and vitamin D [563,564], or vitamin C [565]. The only references regarding the fortification of orange juice with polyphenols was in tetrapack orange juice UHT with hydroxytyrosol [566].

Thus, in the context of fortifying orange juice, the incorporation of polyphenolic extracts from byproducts like orange peel, olive leaves, and avocado peel not only enhances the antioxidant capacity of the beverage but also aligns with sustainable development practices. Utilizing byproducts for the extraction of these valuable compounds reduces waste and supports environmental sustainability [567]. Hesperidin is the major flavonoid found in the orange peels with amounts up to 1.1 mg/g dry peel [74,387]. It has been shown to exhibit significant antioxidant, anti-inflammatory, and cardioprotective effects, among others [436]. Hydroxytyrosol, one of the major phenolic compounds found in olive leaves in amounts of 1.0 mg/g dry leaf [568], is recognized for its exceptionally high antioxidant capacity [545]. Procyanidins is a class of polyphenols present in avocado peel and seeds in amounts up to 20.8 and 16.7 mg/g dry sample [500]. They have demonstrated a wide range of biological activities, including antioxidant, anti-inflammatory, and anticancer properties, mainly attributed to the monomer catechin [569], which is found in avocado peel in amounts of 8 mg/g dry weight.[500] Altogether, these compounds not only could maintain or enhance the bioactive value of fortified foods but also offer potential therapeutic benefits.

Despite their benefits, the stability of polyphenols in fortified beverages during storage remains a critical challenge, as these compounds are sensitive to environmental factors such as light, temperature, and oxygen exposure [570].

Another critical aspect of producing fortified beverages is the choice between pasteurized and non-pasteurized processes. Pasteurization is commonly used to extend the shelf-life of juices by inactivating microorganisms and enzymes that could spoil the product. However, this heat treatment can also lead to the degradation of sensitive nutrients such as vitamin C [571,572]. Non-pasteurized juices, while preserving more of the natural nutrients, pose a higher risk of microbial

contamination and a shorter shelf-life. Balancing the benefits and drawbacks of these processing methods is essential for optimizing the nutritional and sensory qualities of fortified juices.

Understanding the impact of fortification on nutrient stability and antioxidant activity under different processing conditions will help in formulating beverages that retain their health benefits over time. This is particularly important in the context of increasing consumer awareness and demand for products that not only offer convenience but also contribute positively to health and wellness.

This study aims to evaluate the impact of fortifying orange juice with a mixture of polyphenolic extracts (hesperidin, catechin, and hydroxytyrosol). We first determined the optimal concentration of the extracts based on sensory acceptability tests, ranging from 0.5 to 8 mg of extract per mL of juice. We also measured pH, total soluble solids, and color. After selecting the highest acceptable concentration, we assessed the fortified juice's total polyphenol profile using HPLC-ESI-MS, vitamin C content using HPLC-UV/VIS, and antioxidant activity through DPPH and FRAP assays over a 5-day storage period. Additionally, we examined the effect of pasteurization on these parameters.

This research addresses the challenges and opportunities in developing fortified functional beverages. The findings will contribute to the creation of more stable and nutritionally superior functional beverages, offering enhanced health benefits.

## **2. Materials and methods**

### **2.1. Chemicals and reagents**

Double-deionized water was obtained from Millipore (Bedford, MA, USA). The reagents used, Trolox (6-hydroxy-2,5,7,8-tetramethylchromen-2-carboxylic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), and FRAP (ferric reducing antioxidant power) reagents including TPTZ (2,4,6-Tri(2-pyridyl)-1,3,5-triazine), acetonitrile, methanol and acetic acid along with standards such as ascorbic acid, hesperetin, catechin, hydroxytyrosol, apigenin, rutin, ferulic acid and vanillic acid were sourced from Sigma-Aldrich (St. Louis, MO, USA).

Hesperidin extract was purchased from Monteloeder sl. (Alicante, Spain), procyanidin extract was provided by Silvateam (Mondovì, Italy) and hydroxytyrosol extract was from Deretil (Almeria, Spain). All the extract were in conformity for food grade in agreement with all European regulations.

## **2.2. Juice production**

Fresh orange juice was produced from oranges (var. Navelina) in a juice extractor (Zümex, Valencia, Spain) in a local company the same day of the experiments and kept in refrigeration in darkness at 4 °C until the addition of the extracts or/and pasteurization.

## **2.3. Preparation of mixed extract**

It was created a mixed extract combining the three extracts: orange peel hesperidin, avocado by-product procyanidins and olive leaves hydroxytyrosol extracts.

According to the European Union law for commercialized products [573], it was established the healthy statement “Polyphenols in olive oil contribute to the protection of blood lipids against oxidative damage” but can only be used for olive oil containing a minimum of 5 mg of hydroxytyrosol and its derivatives (e.g. oleuropein complex or tyrosol) per 20 g of olive oil. Although this healthy statement is not extensible to other food products, we decide to add hydroxytyrosol extract to the orange juice. The minimum amount that should be added to the juice to theoretically have the claim is 0.02 mg of hydroxytyrosol/mL juice. Thus, also based in preliminary sensory analysis, the addition range established was between 0.0312 and 0.5 mg of olive leaf hydroxytyrosol extract/mL of juice.

Orange peel hesperidin extract adding concentrations were established according to most reported bioactivities for an adult of 80 kg and a consumption of a glass of 250 mL of juice a day, it should be between 6.4-32 mg/mL [436,574,575]. Thus, combined with preliminary sensory test, it was established in the range 0.4375-7 mg/mL.

Avocado by-products procyanidin extract was which caused the most changes in the organoleptic attributes of the juice, especially in the color, so it was kept in the same range of hydroxytyrosol between 0.0312 and 0.5 mg/mL.

By this way, they were established 4 different levels of fortified orange juice with mix extract (0.5, 2, 5 and 8 mg extract/mL juice) composed by 87.5% of hesperidin extract, 6.25% of procyanidin extract and 6.25% of hydroxytyrosol extract.

## **2.4. Pasteurization**

The pasteurization of the juices was carried out in water bath (Buchi B-100) controlling the temperature outside and inside the product, at 80 °C for 10 minutes with a lag of 5 minutes, in agreement with other authors [576–579]. After it, the samples were immediately chilled in a mixture of water/ice and kept in refrigeration in darkness at 4 °C.

## **2.5. Color measurement**

The color of all the juices was measured by a PCE-CSM 4 colorimeter (PCE Instruments, Albacete, Spain) and the results are expressed by the CIE  $L^*a^*b^*$ , after initial calibration against standard white. The samples were filled in a 1 cm cell with the same white background, and three replicate measurements were performed.

## **2.6. pH and total soluble solids**

pH of the orange juices was measured using a pHmetre LAQUA PH1100 (Horiba, Spain) equipped with an electrode LAQUA 9615S (Horiba, Spain). Total soluble solids (TSS) were evaluated using a portable hand refractometer with ATC 0-32 Brix (Zuzi HPC014, Auxilab S.L., Zaragoza, Spain). Three replicate measurements were performed for both analyses.

## **2.7. Sensory evaluation of orange juice samples**

The sensory analysis was carried out to determine the consumer acceptability of fortified orange juices with various extract amounts. The sensory evaluation was conducted in blind through hedonic test for an untrained panel of 15 participants from the department of Nutrition and Bromatology of the University of Granada. Each juice was presented in a randomized order as a 30 mL sample in transparent plastic cups with a code number, with a total of 5 samples. Using a 1 to 9-point hedonic scale, participants rated their own appealing in appearance, color, odor, taste, sweetness, bitterness, bouquet, mouthfeel, and overall acceptability. The significance of the points was: 1, “I dislike it extremely”; 2 “I dislike it very much”; 3, “I dislike it moderately”; 4, “I dislike it slightly”; 5, “I neither like it nor dislike it”; 6, “I like it slightly”; 7, “I like it moderately”; 8, “I like it very much”; and 9, “I like it extremely” [580].

## **2.8. Phenolic compounds determination by HPLC-ESI-TOF-MS**

Extraction of phenolic compounds from the orange juices was carried out as previously reported [581]. Briefly, 8 mL of the homogenized juice underwent lyophilization using a Zirbus lyophilizer (Bad Grund, Germany) at  $-50\text{ }^{\circ}\text{C}$  for 120 hours under a pressure of 0.4 mbar. The resulting lyophilized product was reconstituted in 1 mL of methanol:water (1:1, v/v). Subsequently, the extracts were filtered through 0.2  $\mu\text{m}$  regenerated cellulose filters (Millipore, Bedford, MA, USA) and stored at  $-18\text{ }^{\circ}\text{C}$  until analysis.

The analysis was performed using a BRUKER COMPACT system (Waters Corporation, Billerica, MA, USA) equipped with an electrospray ionization (ESI) source in negative mode and a time-of-flight (TOF) mass spectrometer (Bruker). The compounds were separated on an ACQUITY UPLC BEH Shield RP18 column (1.7  $\mu\text{m}$ ,  $2.1 \times 100\text{ mm}$ ; Waters Corporation, Milford, MA, USA) at  $40^{\circ}\text{C}$ , following a gradient method previously used [387]. The mobile phases were  $\text{H}_2\text{O}$  with 1% acetic acid (phase A) and acetonitrile (phase B). The analysis of the data was conducted using the software DataAnalysis 6.1 for identifying the compounds and QuantAnalysis 5.2 for

quantification (Bruker, MA, USA). A representative base peak total ion chromatogram of the fortified orange juice is included as **Figure 10.1**. Identification of phenolic compounds was carried out in agreement with previous published research [74]. They are listed in **Table 10.1** with its time (min), experimental and calculated  $m/z$ , error (ppm), score (%), molecular formula and compound name. Quantification of phenolic compounds was achieved using calibration curves for hesperetin ( $y = 18900x + 92214$ ;  $R^2 = 0.9858$ ), hydroxytyrosol ( $y = 8971.1x - 35808$ ;  $R^2 = 0.9989$ ), catechin ( $y = 2341.1x - 7282.5$ ;  $R^2 = 0.9980$ ), rutin ( $y = 1835.7x - 4974.7$ ;  $R^2 = 0.9922$ ), ferulic acid ( $y = 1278.6x - 788.82$ ;  $R^2 = 0.9903$ ) and vanillic acid ( $y = 3987.6x - 4060.3$ ;  $R^2 = 0.9986$ ).

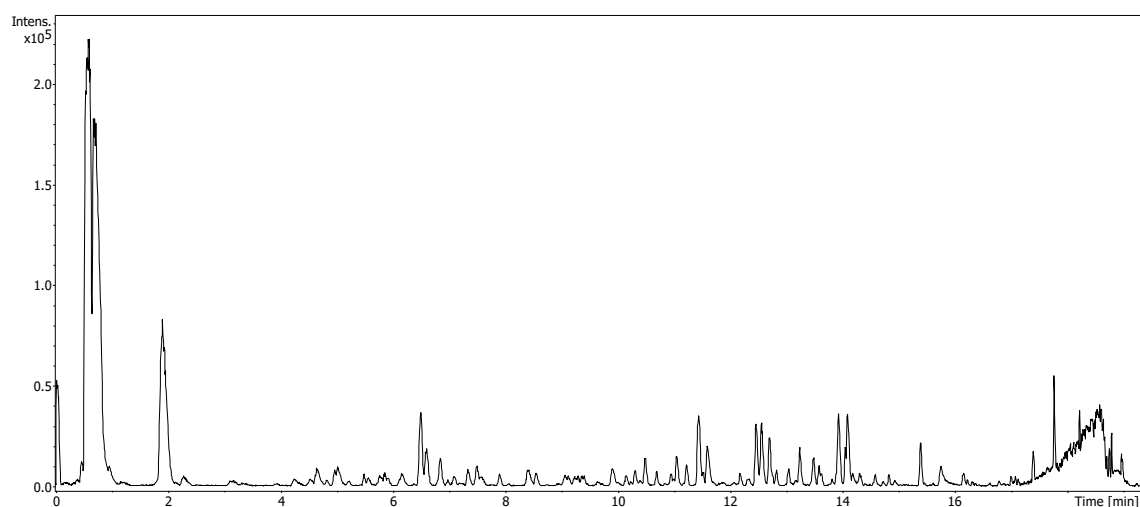


**Figure 10.1.** Base peak chromatogram of fortified orange juice.

**Table 10.1.** Identified compounds in the fortified orange juice by HPLC-ESI-TOF-MS.

| N  | Time (min) | $m/z$ experimental | $m/z$ calculated | Error (ppm) | Score (%) | Molecular formula                               | Compound                                    |
|----|------------|--------------------|------------------|-------------|-----------|-------------------------------------------------|---------------------------------------------|
| 1  | 0.6        | 133.0144           | 133.0142         | -0.9        | 100       | C <sub>4</sub> H <sub>6</sub> O <sub>5</sub>    | Tartaric acid                               |
| 2  | 0.7        | 191.0185           | 191.0197         | 1.4         | 100       | C <sub>6</sub> H <sub>8</sub> O <sub>7</sub>    | Citric acid                                 |
| 3  | 1.9        | 153.0555           | 153.0557         | 1.3         | 100       | C <sub>8</sub> H <sub>10</sub> O <sub>3</sub>   | Hydroxytyrosol                              |
| 4  | 2.3        | 313.0563           | 313.0565         | 0.7         | 100       | C <sub>13</sub> H <sub>14</sub> O <sub>9</sub>  | Norbergenin isomer I                        |
| 5  | 3.2        | 355.0673           | 355.0671         | -0.6        | 100       | C <sub>15</sub> H <sub>16</sub> O <sub>10</sub> | Caffeic acid 3-O-glucuronide isomer I       |
| 6  | 4          | 355.0677           | 355.0671         | -1.8        | 100       | C <sub>15</sub> H <sub>16</sub> O <sub>10</sub> | Caffeic acid 3-O-glucuronide isomer II      |
| 7  | 4.3        | 385.0785           | 385.0776         | -2.2        | 100       | C <sub>16</sub> H <sub>18</sub> O <sub>11</sub> | Feruloyl-galactaric acid isomer I           |
| 8  | 4.4        | 355.0676           | 355.0671         | -1.6        | 100       | C <sub>15</sub> H <sub>16</sub> O <sub>10</sub> | Caffeic acid 3-O-glucuronide isomer III     |
| 9  | 4.5        | 313.0566           | 313.0565         | -0.3        | 100       | C <sub>13</sub> H <sub>14</sub> O <sub>9</sub>  | Norbergenin isomer II                       |
| 10 | 4.7        | 355.0676           | 355.0671         | -1.6        | 100       | C <sub>15</sub> H <sub>16</sub> O <sub>10</sub> | Caffeic acid 3-O-glucuronide isomer IV      |
| 11 | 5.0        | 385.0786           | 385.0776         | -2.4        | 100       | C <sub>16</sub> H <sub>18</sub> O <sub>11</sub> | Feruloyl-galactaric acid isomer II          |
| 12 | 5.1        | 355.0677           | 355.0671         | -1.6        | 100       | C <sub>15</sub> H <sub>16</sub> O <sub>10</sub> | Caffeic acid 3-O-glucuronide isomer V       |
| 13 | 5.2        | 385.0784           | 385.0776         | -1.9        | 100       | C <sub>16</sub> H <sub>18</sub> O <sub>11</sub> | Feruloyl-galactaric acid isomer III         |
| 14 | 5.6        | 385.0778           | 385.0776         | -0.3        | 100       | C <sub>16</sub> H <sub>18</sub> O <sub>11</sub> | Feruloyl-galactaric acid isomer IV          |
| 15 | 5.7        | 399.0953           | 399.0933         | -5.0        | 100       | C <sub>17</sub> H <sub>20</sub> O <sub>11</sub> | Sinapic acid-O-glucuronide isomer I         |
| 16 | 5.8        | 289.0717           | 289.0718         | 0.2         | 100       | C <sub>15</sub> H <sub>14</sub> O <sub>6</sub>  | Catechin                                    |
| 17 | 5.8        | 337.0574           | 337.0565         | -2.7        | 100       | C <sub>15</sub> H <sub>14</sub> O <sub>9</sub>  | <i>p</i> -coumaroyl-isocitric acid isomer I |

| N  | Time (min) | m/z experimental | m/z calculated | Error (ppm) | Score (%) | Molecular formula                               | Compound                                               |
|----|------------|------------------|----------------|-------------|-----------|-------------------------------------------------|--------------------------------------------------------|
| 18 | 5.9        | 325.0937         | 325.0929       | -2.4        | 100       | C <sub>15</sub> H <sub>18</sub> O <sub>8</sub>  | <i>p</i> -coumaroyl glucose                            |
| 19 | 5.9        | 385.0784         | 385.0776       | -1.9        | 100       | C <sub>16</sub> H <sub>18</sub> O <sub>11</sub> | Feruloyl-galactaric acid isomer V                      |
| 20 | 6.2        | 337.0573         | 337.0565       | -2.3        | 100       | C <sub>15</sub> H <sub>14</sub> O <sub>9</sub>  | <i>p</i> -coumaroyl-isocitric acid isomer II           |
| 21 | 6.2        | 355.0671         | 355.0671       | -0.1        | 100       | C <sub>15</sub> H <sub>16</sub> O <sub>10</sub> | Caffeic acid 3- <i>O</i> -glucuronide isomer VI        |
| 22 | 6.5        | 355.1043         | 355.1035       | -2.5        | 100       | C <sub>16</sub> H <sub>20</sub> O <sub>9</sub>  | Ferulic acid- <i>O</i> -glucoside                      |
| 23 | 6.6        | 367.0679         | 367.0671       | -2.3        | 100       | C <sub>16</sub> H <sub>16</sub> O <sub>10</sub> | Feruloyl-isocitric acid isomer I                       |
| 24 | 6.6        | 225.077          | 225.0768       | -0.6        | 100       | C <sub>11</sub> H <sub>14</sub> O <sub>5</sub>  | Dihydrosinapic acid                                    |
| 25 | 6.6        | 369.0835         | 369.0827       | -2.2        | 100       | C <sub>16</sub> H <sub>18</sub> O <sub>10</sub> | Ferulic acid 4- <i>O</i> -glucuronide isomer I         |
| 26 | 6.8        | 369.0835         | 369.0827       | -2.1        | 100       | C <sub>16</sub> H <sub>18</sub> O <sub>10</sub> | Ferulic acid 4- <i>O</i> -glucuronide isomer II        |
| 27 | 6.9        | 385.1145         | 385.114        | -1.2        | 100       | C <sub>17</sub> H <sub>22</sub> O <sub>10</sub> | Sinapic acid- <i>O</i> -glucoside                      |
| 28 | 7.0        | 385.0777         | 385.0776       | -0.1        | 100       | C <sub>16</sub> H <sub>18</sub> O <sub>11</sub> | Feruloyl-galactaric acid isomer VI                     |
| 29 | 7.1        | 367.0679         | 367.0671       | -2.3        | 100       | C <sub>16</sub> H <sub>16</sub> O <sub>10</sub> | Feruloyl-isocitric acid isomer II                      |
| 30 | 7.4        | 399.0939         | 399.0933       | -1.4        | 100       | C <sub>17</sub> H <sub>20</sub> O <sub>11</sub> | Sinapic acid- <i>O</i> -glucuronide isomer II          |
| 31 | 7.5        | 399.0943         | 399.0933       | -2.5        | 100       | C <sub>17</sub> H <sub>20</sub> O <sub>11</sub> | Sinapic acid- <i>O</i> -glucuronide isomer III         |
| 32 | 7.6        | 367.0683         | 367.0671       | -3.2        | 100       | C <sub>16</sub> H <sub>16</sub> O <sub>10</sub> | Feruloyl-isocitric acid isomer III                     |
| 33 | 8.4        | 337.0572         | 337.0565       | -2.1        | 100       | C <sub>15</sub> H <sub>14</sub> O <sub>9</sub>  | <i>p</i> -coumaroyl-isocitric acid isomer III          |
| 34 | 8.6        | 381.0834         | 381.0827       | -1.7        | 100       | C <sub>17</sub> H <sub>18</sub> O <sub>10</sub> | Caffeic acid derivative                                |
| 35 | 8.9        | 337.057          | 337.0565       | -1.6        | 100       | C <sub>15</sub> H <sub>14</sub> O <sub>9</sub>  | <i>p</i> -coumaroyl-isocitric acid isomer IV           |
| 36 | 9.1        | 193.059          | 193.0506       | -1.4        | 100       | C <sub>10</sub> H <sub>10</sub> O <sub>4</sub>  | Ferulic acid                                           |
| 37 | 9.2        | 367.0684         | 367.0671       | -3.7        | 100       | C <sub>16</sub> H <sub>16</sub> O <sub>10</sub> | Feruloyl-isocitric acid isomer IV                      |
| 38 | 9.3        | 337.0586         | 337.0565       | -6.2        | 100       | C <sub>15</sub> H <sub>14</sub> O <sub>9</sub>  | <i>p</i> -coumaroyl-isocitric acid isomer V            |
| 39 | 9.6        | 367.0691         | 367.0671       | -5.4        | 100       | C <sub>16</sub> H <sub>16</sub> O <sub>10</sub> | Feruloyl-isocitric acid isomer V                       |
| 40 | 9.9        | 337.0576         | 337.0565       | -3.1        | 100       | C <sub>15</sub> H <sub>14</sub> O <sub>9</sub>  | <i>p</i> -coumaroyl-isocitric acid isomer VI           |
| 41 | 10.4       | 367.0681         | 367.0671       | -2.9        | 100       | C <sub>16</sub> H <sub>16</sub> O <sub>10</sub> | Feruloyl-isocitric acid isomer VI                      |
| 42 | 10.6       | 367.0686         | 367.0671       | -4.1        | 100       | C <sub>16</sub> H <sub>16</sub> O <sub>10</sub> | Feruloyl-isocitric acid isomer VII                     |
| 43 | 10.7       | 429.1787         | 429.1766       | -4.8        | 100       | C <sub>20</sub> H <sub>30</sub> O <sub>10</sub> | Phenethyl rutinoside                                   |
| 44 | 10.8       | 367.0689         | 367.0671       | -4.9        | 100       | C <sub>16</sub> H <sub>16</sub> O <sub>10</sub> | Feruloyl-isocitric acid isomer VIII                    |
| 45 | 11.1       | 579.1724         | 579.1719       | -0.9        | 100       | C <sub>27</sub> H <sub>32</sub> O <sub>14</sub> | Narirutin                                              |
| 46 | 11.4       | 609.1827         | 609.1825       | -0.3        | 100       | C <sub>28</sub> H <sub>34</sub> O <sub>15</sub> | Hesperidin                                             |
| 47 | 12.2       | 623.1637         | 623.1618       | -3.1        | 100       | C <sub>28</sub> H <sub>32</sub> O <sub>16</sub> | Isorhamnetin-3- <i>O</i> -rutinoside isomer I          |
| 48 | 12.2       | 477.0686         | 477.0675       | 2.3         | 100       | C <sub>21</sub> H <sub>18</sub> O <sub>13</sub> | Quercetin-3- <i>O</i> -glucuronide                     |
| 49 | 12.2       | 443.1933         | 443.1923       | -2.2        | 100       | C <sub>21</sub> H <sub>32</sub> O <sub>10</sub> | Cynaroside A                                           |
| 50 | 12.4       | 623.1615         | 623.1618       | 0.6         | 100       | C <sub>28</sub> H <sub>32</sub> O <sub>16</sub> | Isorhamnetin-3- <i>O</i> -rutinoside isomer II         |
| 51 | 13.5       | 593.1875         | 593.1876       | 0.1         | 100       | C <sub>28</sub> H <sub>34</sub> O <sub>14</sub> | Didymin                                                |
| 52 | 13.6       | 575.1413         | 575.1406       | -1.1        | 100       | C <sub>27</sub> H <sub>28</sub> O <sub>14</sub> | Apigenin 7-[6''-(3-Hydroxy-3-methylglutaryl)glucoside] |
| 53 | 13.9       | 517.2294         | 517.2291       | -0.7        | 100       | C <sub>24</sub> H <sub>38</sub> O <sub>12</sub> | Vomifoliol 9-[xylosyl-(1->6)-glucoside] isomer I       |
| 54 | 14.1       | 517.229          | 517.2291       | 0.0         | 100       | C <sub>24</sub> H <sub>38</sub> O <sub>12</sub> | Vomifoliol 9-[xylosyl-(1->6)-glucoside] isomer II      |

## 2.9. Vitamin C determination by HPLC-UV/VIS

The determination and analysis of AA, DHA and total Vitamin C was carried out by the method described in previous published research [331,581]. The standard used was ascorbic acid in the range between 1-250 ppm ( $y = 85.9807x - 146.5146$ ,  $R^2 = 0.9997$ ). Analysis were performed in duplicate and the results are expressed in  $\mu\text{g}$  ascorbic acid equivalents/mL of juice.

## 2.10. Antioxidant assays

The DPPH radical scavenging activity was assessed using a method suggested by various authors [247,248]. Specifically, 100  $\mu\text{L}$  of each extract was mixed with 2.9 mL of DPPH solution. After rapid stirring, the extract's bleaching effect was monitored over a period from 0 to 30 minutes at a wavelength of 517 nm.

The FRAP assay was conducted following the technique described by Pulido et al., (2000). This assay relies on the reduction of  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  by antioxidant compounds. A volume of 30  $\mu\text{L}$  of each extract was combined with 90  $\mu\text{L}$  of distilled water and 900  $\mu\text{L}$  of the FRAP reagent. The mixture was incubated for 30 minutes at 37 °C and then measured with a spectrophotometer at 595 nm.

Standard curves of Trolox equivalents (TE) from 1-200 ppm were created for each assay. The resulting equations were  $y = 0.0029x + 0.0423$  ( $R^2 = 0.9965$ ), and  $y = 0.0037x + 0.1174$  ( $R^2 = 0.9914$ ) for DPPH, and FRAP assays, respectively. Analyses were performed in duplicate and the results are reported as  $\mu\text{g TE/g d.w.}$

## 2.11. Statistical analysis

Statistical analysis was performed using Tukey's test with STATISTICA software version 7.0 (StatSoft, Tulsa, OK, USA).

## 3. Results and discussion

### 3.1. Evaluation of the orange juice fortification level

Fortification of orange juice with hesperidin, catechin and hydroxytyrosol mixed extract was carried out at 0.5, 2, 5 and 8 mg/mL and they were evaluated the pH, total soluble solids (TSS), color and acceptability sensory analysis.

#### 3.1.1. pH and total soluble solids (TSS)

**Table 10.2** details the pH and TSS measurements for the orange juices used in the sensory evaluation. No significant differences were observed in pH values, which ranged from 3.62 to 3.68, or in TSS values, which ranged from 10.85 to 11.05 °Brix. These results indicate that the fortification with extracts did not significantly alter the acidity or the soluble solid content of the juices. The reported values are in the range usually reported [566,582,583].

**Table 10.2.** pH and TSS of the different orange juices used in the acceptability analysis.

| Sample  | pH              |   | ° Brix           |   |
|---------|-----------------|---|------------------|---|
| Control | 3.62 $\pm$ 0.53 | a | 11.05 $\pm$ 0.78 | a |



|           |             |   |              |   |
|-----------|-------------|---|--------------|---|
| 0.5 mg/mL | 3.68 ± 0.51 | a | 10.85 ± 0.77 | a |
| 2 mg/mL   | 3.68 ± 0.51 | a | 10.90 ± 0.77 | a |
| 5 mg/mL   | 3.65 ± 0.51 | a | 10.85 ± 0.77 | a |
| 8 mg/mL   | 3.65 ± 0.52 | a | 11.05 ± 0.78 | a |

### 3.1.2. Colorimetric analysis

The colorimetric analysis of the orange juice samples used in the acceptability sensory test, as depicted in **Figure 10.2**, illustrates the impact of different concentrations of the mixed extract on the color properties of the juice. The analysis shows a distinct variation in the  $L^*$ ,  $a^*$ , and  $b^*$  values among the samples.

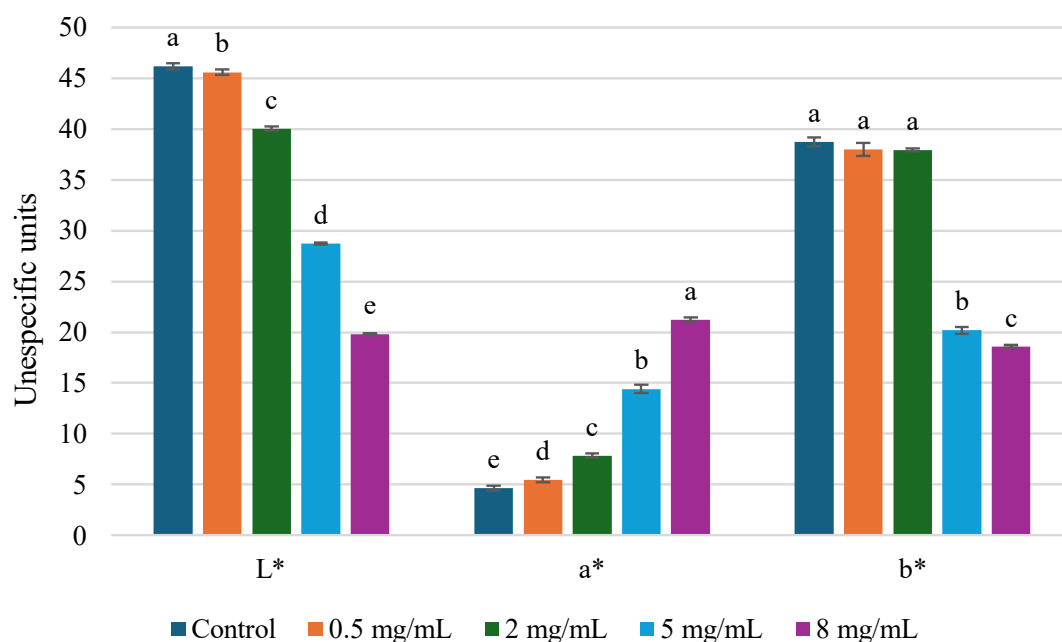


**Figure 10.2.** Colorimetric analysis results of orange juice samples fortified with different concentrations of the mixed extract (0.5, 2, 5, and 8 mg/mL). The control sample (non-fortified juice) is also included for comparison.

The results obtained for the control orange juice were in the range of previous articles [584]. The control sample exhibited the highest  $L^*$  value, indicating the lightest color, significantly ( $p < 0.05$ ) different from the rest of samples. As the concentration of the mixed extract increased, the  $L^*$  value decreased, signifying a darker juice. The sample with 8 mg/mL extract showed the lowest  $L^*$  value, highlighting the most pronounced darkening effect. In addition, the  $a^*$  value increased with higher extract concentrations, indicating a shift towards a more reddish hue. This is likely due to the color properties of the procyanidin extract, which has a more brownish tone.

Moreover, the  $b^*$  value demonstrated a decrease with increasing extract concentration, showing a reduction in the yellow component of the juice color. The hydroxytyrosol and hesperidin extracts, being pale yellow, contributed less to the yellow color as its concentration increased, combined with the darker tones from the procyanidin extract.

When other authors added hydroxytyrosol extract to orange juice did not report significant changes in  $L^*a^*b^*$  color values [566]. In pomegranate juices fortified with pomegranate peel rich in flavan-3-ols extract it was reported a decrease in  $L^*$  and  $b^*$  values and an increase in  $a^*$  value compared to non-fortified juice [585], the same tendency reported here. Other authors enriched strawberry and raspberry juices with grape skin extract rich in procyanidins also reported decreased in  $L^*$  values and significant increases in  $a^*$  value compared to not enriched [586].

Therefore, the color changes observed in the fortified orange juice samples can be attributed to the intrinsic colors of the added extracts. The hydroxytyrosol extract, being pale yellow, hesperidin with a medium orangish color, and the procyanidin extract, with its brownish hue, combined to alter the original yellowish-orange color of the juice. The overall effect was a darker, more reddish-orange hue as the concentration of the mixed extract increased. The results suggest that while the addition of these polyphenolic extracts could enhance other attributed of the juice, they also impart significant changes to its appearance, which may affect consumer acceptance.

### **3.1.3. Acceptability sensory evaluation**

The average results of the acceptability sensory test are shown in Figure 10.3. As can be seen they were evaluated several attributed such as appearance, color, odor, taste, sweetness, bitterness, bouquet, mouthfeel, and overall acceptability. It can be clearly appreciated that those juices fortified with 5 and 8 mg/mL were evaluated with a mark lower than 5 categorized as “I slightly dislike it” in most of the attributes, with significant ( $p < 0.05$ ) differences with the control. Otherwise, those orange juices fortified with 0.5 and 2 mg/mL did not show significant differences with the control in overall acceptability and taste relative attributes. The appearance, color and odor of the juices were the attributes that differentiated the juice fortified with 2 mg/mL from the fortified with 0.5 mg/mL and the control, but those differences were no significant ( $p < 0.05$ ). It can be due that the used extracts to fortify, despite being food grade, have their own specific sensory profile not usually linked to food. Panelists noted that the higher concentration of extracts introduced unfamiliar aromatic notes that were less appealing. Thus, it can be affirmed that the orange juices fortified with 0.5 or 2 mg/mL of extract were generally accepted with marks higher than 6 (categorized as “I like it slightly”) in all the attributes, as well as the non-fortified juice.

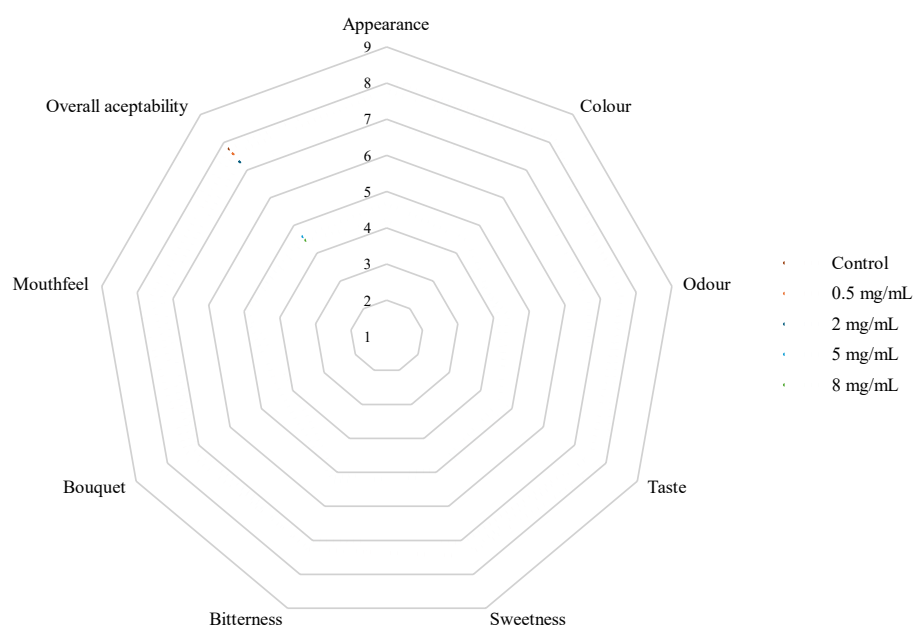


**Figure 10.3.** Average sensory analysis results of the evaluated orange juices.

### 3.2. Evaluation of stability shelf-life

After the previous analysis it was selected an addition of 2 mg/mL of extract that had minor color changes, stable pH and ° Brix, and overall good acceptability through the sensory analysis. There are few previous references regarding fortification of juices that reported different addition amounts. Altunkaya et al., (2013) fortified apple juice with pomegranate peel extracts reported that a concentration between 5 and 10 mg/mL was acceptable after a 10-panelist sensory evaluation. Hashemi et al. (2018) for fortifying guava whey juice with moringa leaf extract reported that 15-20 mg/mL prologued shelf life of the juice, but they did not do a sensory analysis [588]. Toaldo et al. (2013) evaluated the effect of fortifying grape juices with grape seeds with an addition of 200 mg/mL, but with no sensory evaluation [576]. Tamer et al. (2017) enriched lemonade with different herbal extract at concentrations between 0.05-0.1 mg/mL finding that the ones enriched with 0.1 mg/mL of heather and ginger were the most preferred after a sensory analysis with a 15 judges panel [577]. Gil et al. (2023) evaluated the effect of enrichment of apple juice with different plant materials between 0.5-50 mg/mL. They found that the juice enriched with 1 mg/mL of *C. sativus* flower juice, berry extract and persimmon fruit were which had better acceptance in a sensory evaluation (n = 9) [578]. Trigo et al. (2020) fortified carrot juice with pomegranate peel extract at 2.5 mg/mL, selected by sensory analysis (n = 10) [579]. Gunathilake et al. (2013) designed a beverage made of apple, blueberry, and cranberry juice with addition of ginger. They found that an addition of 20 mg/mL of ginger extract was acceptable after a sensory analysis (n = 20) [589]. Singla et al. (2024) evaluated watermelon juice incorporated of 15 mg/mL

of papaya peel extract determined by sensory evaluation [590]. De Beer et al. (2014) formulated a red-fleshed plum nectars enriched with 24% plum skin extract decided with a sensory analysis ( $n = 8$ ) [591]. The differences between similar published research and ours in the amounts of extracts used for juice fortification can be influenced by the type of extract, the properties of the base juice, variability in the sensory analysis, the purpose of fortification, and differences in processing methodologies.

The stability and shelf-life of the fortified orange juice with extract of 2 mg/mL were assessed over a period of 5 days, with measurements taken for pH, TSS, color, phenolic compounds, vitamin C content, and antioxidant activity.

### **3.2.1. pH and TSS**

The pH and TSS values of the orange juice samples, including both control and fortified samples, were monitored daily for 5 days.

Table 10.3 shows that the pH values remained relatively stable throughout the 5-day period for all samples, with no significant differences observed between the fortified and control juices, neither for the pasteurized and fresh juices. The pH values ranged from 3.62 to 3.84, indicating that the addition of extracts did not significantly alter the acidity of the juice.

TSS values, expressed in degrees Brix, were consistent across all samples, fluctuating slightly but showing no significant differences. The TSS values ranged from 12.10 to 12.90 °Brix. It demonstrates that the soluble solid content of the juice was maintained throughout the storage period regarding the addition of the extract or pasteurization.

Some authors reported the similar results. Cortés et al. (2008) did not report changes in the pH and TSS between untreated and pasteurized orange juices over 7 days of storage [584]. Souza et al. (2004) did not find significant changes in pH and TSS during 72 hours of unpasteurized orange juices [592]. Figueroa et al. (2016) evaluated the stability of pH and TSS in orange juice fortified with orange pulp extracts reporting no significant changes over a 8-week period [593].

The primary components contributing to the pH and Brix of orange juice are organic acids and sugars, that are relatively resistant to high temperature [594–596]. Thus, the stability of organic acids and sugars in the juice were the responsible for finding no changes in pH and TSS in the evaluated orange juices over the 5-days time period regarding the pasteurization or the addition of the extract.

**Table 10.3.** pH and TSS (° Brix) of orange juice samples over 5 days.

|                       | DAY 1       |              | DAY 2       |              | DAY 3       |              | DAY 4       |              | DAY 5       |              |
|-----------------------|-------------|--------------|-------------|--------------|-------------|--------------|-------------|--------------|-------------|--------------|
|                       | pH          | ° Brix       | pH          | ° Brix       | pH          | ° Brix       | pH          | ° Brix       | pH          | ° Brix       |
| Extract               | 3.71 ± 0.07 | 12.90 ± 0.20 | 3.75 ± 0.16 | 12.60 ± 0.20 | 3.76 ± 0.08 | 12.90 ± 0.20 | 3.79 ± 0.15 | 12.90 ± 0.20 | 3.71 ± 0.11 | 12.60 ± 0.20 |
| Extract + pasteurized | 3.73 ± 0.01 | 12.90 ± 0.20 | 3.75 ± 0.06 | 12.10 ± 0.20 | 3.84 ± 0.07 | 12.20 ± 0.20 | 3.81 ± 0.10 | 12.20 ± 0.20 | 3.74 ± 0.10 | 12.20 ± 0.20 |
| Control + pasteurized | 3.62 ± 0.01 | 12.30 ± 0.20 | 3.72 ± 0.14 | 12.60 ± 0.20 | 3.67 ± 0.06 | 12.60 ± 0.20 | 3.64 ± 0.10 | 12.90 ± 0.20 | 3.70 ± 0.06 | 12.60 ± 0.20 |
| Control               | 3.69 ± 0.06 | 12.80 ± 0.20 | 3.71 ± 0.12 | 12.60 ± 0.20 | 3.75 ± 0.13 | 12.60 ± 0.20 | 3.70 ± 0.06 | 12.80 ± 0.20 | 3.69 ± 0.03 | 12.60 ± 0.20 |

### 3.2.2. Colorimetric analysis

It was performed a colorimetric analysis of the fortified orange juice and control samples over a period of 5 days, specifically examining the L\*, a\*, and b\* values. It was also evaluated the effect of pasteurization and the results are showed in Figure 10.4.

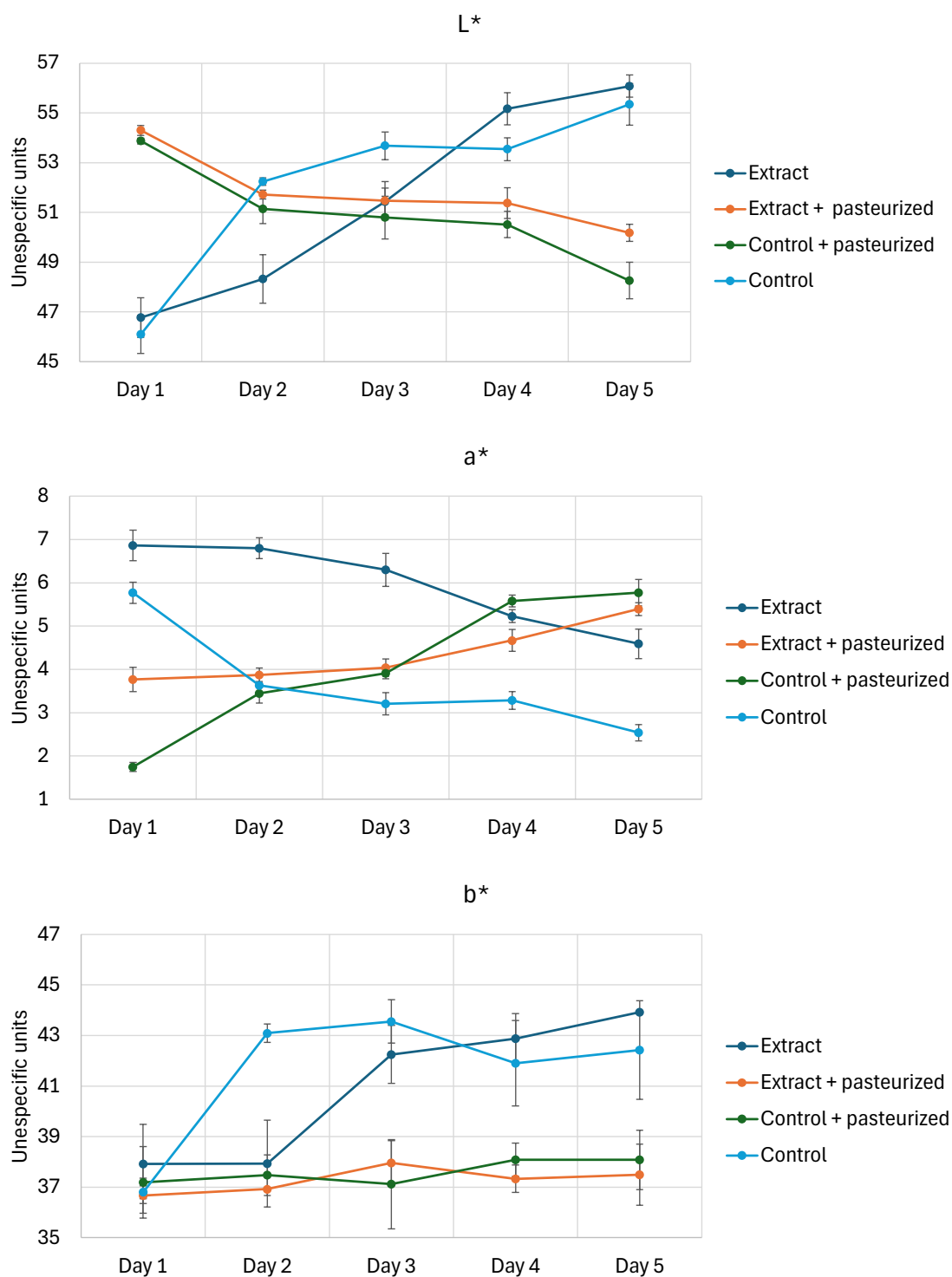


**Figure 10.4.** Color L\*, a\* and b\* values for the orange juicer for 5 days.

The L\* values for both fortified and control juices showed a significant increase over the 5-day period, indicating improved luminosity. This increase can be attributed to several factors. Over time, the pigments in the juice undergo oxidation, which degrades certain color compounds, resulting in a lighter appearance. Specifically, the oxidation of carotenoids and natural pigments in orange juice can cause it to become lighter as these compounds break down [595]. Additionally,

during storage, solid particles such as pulp may settle at the bottom of the container, leading to a clearer, lighter liquid at the top, reflected in an increased  $L^*$  value [597].

Pasteurized fortified and non-fortified orange juices demonstrated a noticeable increase in  $L^*$  value on day 1, suggesting that pasteurization contributes to initial juice clarification by precipitating solids or destruction of compounds, resulting in a clearer and lighter appearance immediately after processing. This phenomenon was previously reported by in pasteurized apple [598] and orange juices [599]. However, over the 5-day period, pasteurized juices showed a decrease in  $L^*$  values compared to their non-pasteurized counterparts. This could be due to the combined effect of the destruction of pigments that lead the visualization of the no enzymatic browning processes occurring during storage, which are more likely in pasteurized juices and result in gradual darkening and reduced  $L^*$  values over time. Previously, Chaikham et al. (2014) reported that pasteurized longan juice showed significant browning due to no enzymatic reactions, resulting in decreased  $L^*$  values during storage compared to non-pasteurized samples in a 4 weeks period at 4 °C [600]. Similar to our results, Cortés et al., (2008) reported an increase in the  $L^*$  value in pasteurized orange juice ( $52.41 \pm 0.12$ ) compared to untreated ( $51.36 \pm 0.54$ ). Besides, over time they reported a significant decrease in  $L^*$  value after 1 week of refrigerated storage [584]. Similar tendency was reported by Rivas et al. (2006) in the  $L^*$  value during storage in orange-carrot juice refrigerated during storage at 2 °C for 4 weeks [601].

The  $a^*$  values for fortified and control samples exhibited significant decreases over the 5 days. This decrease is mainly attributed to the oxidation of carotenoids, other pigments, or even the added extract in the case of the fortified juice, responsible for the red color in orange juice. As these pigments degrade over time, the red component ( $a^*$  value) decreases, potentially increasing the green component [584,599].

Pasteurized juices, both fortified and non-fortified, showed lower initial  $a^*$  values compared to non-pasteurized samples, indicating a reduction in red color intensity immediately after processing. Cortés et al., (2008) also reported higher  $a^*$  values in fresh orange juices ( $4.56 \pm 0.40$ ) compared to pasteurized ( $1.57 \pm 0.03$ ).

The  $b^*$  values for the fortified and control orange juices indicated an increase in the yellow color component over the 5 days. It goes linked to the  $a^*$  values reported. As orange juice is stored the pigments responsible for red hues, such as carotenoids, can degrade. This degradation leads to a reduction in the red color (decreased  $a^*$  values) and can simultaneously enhance the perception of yellow tones (increased  $b^*$  values). Besides, over time, especially in fortified juices where additional particles or components may be present, clarification processes and settling of suspended solids (like pulp) can occur. This can lead to changes in light reflection and transmission characteristics, affecting the perceived color balance and potentially increasing the

b\* value. In agreement to our results, Tiwari et al. (2009) reported that as orange juice is stored, the degradation of red pigments and clarification processes can lead to an increase in yellow tones, resulting in higher b\* values [602].

Otherwise, pasteurized juices had lower b\* values, indicating a decrease in the yellow color component due to pasteurization with resulting stable b\* values over time. The lower b\* values in pasteurized juices immediately after treatment indicate a decrease in the yellow color component due to the heat sensitivity of pigments responsible for yellow hues especially on lutein [599]. Pasteurization involves heating the juice to eliminate microorganisms, which can degrade or alter these pigments. However, over the subsequent storage period, the b\* values stabilize. This stabilization could be attributed to protein-pigment interactions and controlled oxidative processes during storage that further contribute to maintaining the yellow color component in pasteurized juices [599,603]. It ensures that pasteurized juices exhibit stable b\* values over time, despite the initial impact of heat on their color profile.

Thus, the colorimetric analysis revealed that pasteurization has a significant impact on the color parameters of orange juice. Pasteurized samples generally show an increase in luminosity (L\* value) while exhibiting a decrease in the red color component (a\* value) and yellow color component (b\* value) compared to non-pasteurized samples. These changes can be attributed to the thermal processes involved in pasteurization, which likely affect the pigments responsible for the color of the juice.

### **3.2.3. Phenolic compounds**

Phenolic compounds present in the orange juices comparing those with and without a mixed extract, and subjected or not to pasteurization over a 5-day storage period were analyzed by HPLC-ESI-TOF-MS and the results are collected in Table 10.4.



**Table 10.4.** Phenolic compounds quantified by HPLC-ESI-TOF-MS in orange juice samples over a 5-day storage period expressed in µg/mL with average ± standard deviation.

|                                     | 1                |                   | 2 | Extract<br>3     | 4                | 5                | 1                | 2                | Extract + pasteurization<br>3 | 4                | 5                | 1               | 2                 | Control<br>3      | 4               | 5               | 1                 | 2                 | Control + pasteurization<br>3 | 4               | 5            |
|-------------------------------------|------------------|-------------------|---|------------------|------------------|------------------|------------------|------------------|-------------------------------|------------------|------------------|-----------------|-------------------|-------------------|-----------------|-----------------|-------------------|-------------------|-------------------------------|-----------------|--------------|
| Caffeic acid derivative             | 27.03 ± 0.53a,b  | 25.94 ± 0.16a-c   |   | 24.90 ± 0.14a-d  | 24.47 ± 0.59a-d  | 22.94 ± 0.49d-f  | 26.61 ± 1.46a,b  | 24.14 ± 1.10b-d  | 20.06 ± 1.35f,g               | 18.24 ± 0.54g    | 12.71 ± 0.98h    | 27.05 ± 0.07a   | 23.27 ± 1.62c-e   | 18.42 ± 1.06g     | 12.48 ± 0.11h   | 12.35 ± 0.02h   | 24.14 ± 1.47b-d   | 20.80 ± 1.69c-e   | 19.04 ± 0.51g                 | 11.06 ± 0.90i   | 8.62 ± 0.59i |
| Sum of caffeic acid-3-O-glucuronide | 148.41 ± 6.79b   | 99.54 ± 4.52d     |   | 59.07 ± 2.17f,g  | 52.74 ± 2.29g,h  | 38.13 ± 1.46i,j  | 165.49 ± 6.27a   | 77.03 ± 4.73e    | 46.03 ± 3.48h,i               | 32.99 ± 1.58j    | 18.39 ± 1.59k    | 151.97 ± 3.70b  | 67.09 ± 3.53e,f   | 37.61 ± 2.90i,j   | 17.54 ± 0.99k   | 11.22 ± 0.49k   | 111.26 ± 4.45c    | 53.58 ± 2.92g,h   | 36.81 ± 1.22i,j               | 14.76 ± 0.73k   | 8.72 ± 0.39k |
| Sum of caffeic acid derivatives     | 175.44 ± 7.32b   | 125.47 ± 4.69c    |   | 83.98 ± 2.30c,f  | 77.21 ± 2.88f,g  | 61.07 ± 1.95h,i  | 192.11 ± 7.73a   | 101.17 ± 5.83d   | 66.10 ± 4.83g,h               | 51.23 ± 2.12i    | 31.09 ± 2.58j    | 179.03 ± 3.77b  | 90.36 ± 5.15d,e   | 56.02 ± 3.96h,i   | 30.02 ± 1.10j,k | 23.58 ± 0.52j,k | 135.40 ± 5.91c    | 74.39 ± 4.61f,g   | 55.85 ± 1.73h,i               | 17.35 ± 0.97j,k | 25.8 ± 1.63k |
| Ferulic acid                        | 19.92 ± 0.46e    | 26.36 ± 1.09c     |   | 26.93 ± 1.35c    | 39.62 ± 1.26b    | 42.25 ± 1.05a    | 12.88 ± 0.37g    | 9.17 ± 0.87h     | 6.79 ± 0.13j,i                | 5.40 ± 0.16k     | 3.75 ± 0.37k     | 19.91 ± 0.22e   | 7.42 ± 0.44h-j    | 7.90 ± 0.45h,i    | 3.50 ± 0.06k    | 3.40 ± 0.27k    | 13.89 ± 0.41g     | 21.74 ± 0.86e     | 23.98 ± 0.83d                 | 16.48 ± 0.77f   | 12.5 ± 0.09g |
| Sum of ferulic acid 4-O-glucuronide | 32.79 ± 1.20a    | 27.98 ± 0.29b     |   | 24.64 ± 1.40c    | 23.12 ± 0.76c,d  | 21.46 ± 0.67d,e  | 32.09 ± 1.25a    | 24.26 ± 0.39c    | 18.79 ± 1.39e,f               | 15.06 ± 0.64g    | 9.39 ± 0.87h     | 31.20 ± 1.65a   | 20.66 ± 0.34d,e   | 16.30 ± 0.93f,g   | 9.70 ± 0.52h    | 8.07 ± 0.41h    | 24.84 ± 1.40c     | 17.88 ± 0.35f     | 16.20 ± 0.19f,g               | 7.69 ± 0.45h,i  | 5.23 ± 0.14i |
| Ferulic acid-O-glucoside            | 211.68 ± 5.54c,d | 204.38 ± 6.54c-e  |   | 201.00 ± 0.98c-e | 200.84 ± 3.11c-e | 198.61 ± 1.85d,e | 255.35 ± 7.38a   | 235.42 ± 2.50b   | 215.50 ± 1.50c                | 172.75 ± 5.31j   | 111.99 ± 8.68k,l | 239.02 ± 5.79b  | 206.31 ± 6.18c-e  | 178.82 ± 6.70f,j  | 119.30 ± 2.10k  | 101.28 ± 4.63l  | 240.69 ± 6.15a,b  | 196.14 ± 8.11d,e  | 192.52 ± 0.36e,f              | 98.61 ± 6.27l   | 49.1 ± 3.39m |
| Sum of feruloyl isocitric acid      | 95.08 ± 2.88b    | 83.52 ± 2.87c-e   |   | 76.11 ± 2.10d-f  | 72.46 ± 2.31f,h  | 66.20 ± 1.72g-i  | 118.19 ± 5.64a   | 92.01 ± 3.55b,c  | 74.95 ± 2.78e-g               | 58.57 ± 2.39i,j  | 37.63 ± 2.75k    | 109.36 ± 4.27a  | 84.32 ± 4.41c,d   | 61.44 ± 3.82i,j   | 38.31 ± 1.92k   | 31.59 ± 1.55k,l | 79.97 ± 3.22d-f   | 64.09 ± 2.00b-j   | 55.19 ± 1.79j                 | 26.88 ± 1.97l,m | 19.1 ± 1.16m |
| Sum of feruloyl galactaric acid     | 94.11 ± 2.90a    | 59.73 ± 2.14c     |   | 41.95 ± 2.04e    | 36.47 ± 1.41e,f  | 28.64 ± 0.99g,h  | 93.65 ± 5.41a    | 50.19 ± 1.53d    | 33.74 ± 2.31f,g               | 23.83 ± 1.10h    | 12.36 ± 1.03i    | 89.67 ± 2.59a   | 42.26 ± 1.51e     | 27.56 ± 2.00g,h   | 12.87 ± 0.67i   | 8.63 ± 0.46i    | 72.61 ± 1.98b     | 38.32 ± 1.78e,f   | 27.52 ± 1.70g,h               | 11.23 ± 0.51i   | 6.82 ± 0.32i |
| Sum of ferulic acid derivatives     | 453.58 ± 13.00b  | 401.96 ± 12.92c,d |   | 370.63 ± 7.87d,e | 372.50 ± 8.85d,e | 357.16 ± 6.27e   | 512.17 ± 20.05a  | 411.05 ± 8.84c   | 349.78 ± 8.11e,f              | 275.61 ± 9.61h   | 175.11 ± 13.71i  | 489.16 ± 14.53a | 360.97 ± 12.88e   | 292.03 ± 13.89g,h | 183.67 ± 5.27i  | 152.95 ± 7.31i  | 432.00 ± 13.16b,c | 338.15 ± 13.12e,f | 315.42 ± 4.88f,g              | 160.88 ± 9.97j  | 92.9 ± 5.09j |
| Sum of p-coumaroyl isocitric acid   | 200.08 ± 6.60a   | 188.54 ± 4.60a,b  |   | 178.89 ± 6.79b,c | 169.96 ± 7.00c   | 163.54 ± 5.80c,d | 164.59 ± 6.54c,d | 146.84 ± 6.01d,e | 122.82 ± 3.40g                | 118.77 ± 5.08g,h | 83.59 ± 7.71i    | 199.17 ± 5.89a  | 143.08 ± 11.24e,f | 102.04 ± 7.41h    | 81.24 ± 2.43i   | 73.05 ± 2.10i,j | 124.46 ± 6.51g    | 126.17 ± 2.43f,g  | 103.34 ± 3.94h                | 59.51 ± 3.17j,k | 43.8 ± 2.27k |
| p-coumaroyl glucose                 | 30.05 ± 0.55a    | 29.66 ± 0.40a,b   |   | 28.94 ± 0.53a-c  | 27.24 ± 0.49b-d  | 26.33 ± 0.33c-e  | 21.95 ± 0.08f    | 22.88 ± 0.32f    | 25.83 ± 2.53d,e               | 22.78 ± 0.63f    | 12.93 ± 0.99h,i  | 30.08 ± 0.35a   | 22.34 ± 0.37f     | 22.52 ± 1.38f     | 16.38 ± 0.55g   | 17.03 ± 0.87g   | 21.99 ± 0.18f     | 22.91 ± 1.08f     | 23.70 ± 0.49f                 | 14.60 ± 0.71g,h | 10.8 ± 0.85i |
| Sum of coumaric acid derivatives    | 230.13 ± 7.15a   | 218.20 ± 5.00a,b  |   | 207.83 ± 7.32b,c | 197.21 ± 7.49c,d | 189.87 ± 6.13c,d | 186.54 ± 6.62d,e | 169.71 ± 6.33e,f | 148.65 ± 5.93g,h              | 141.55 ± 5.72h-j | 96.52 ± 8.70k    | 229.26 ± 6.24a  | 165.42 ± 11.61f,g | 124.56 ± 8.79j    | 97.62 ± 2.98k   | 90.08 ± 2.97k,l | 146.45 ± 6.69g-i  | 149.08 ± 3.51g,h  | 127.04 ± 4.43i,j              | 74.12 ± 3.88l,m | 54.7 ± 3.11m |
| Sinapic acid O-glucoside            | 63.67 ± 2.36a,b  | 57.42 ± 3.03b-d   |   | 53.02 ± 4.79d,e  | 48.43 ± 1.32e,f  | 47.67 ± 0.99e,f  | 67.90 ± 2.41a    | 64.84 ± 3.05a    | 56.71 ± 2.85c,d               | 40.03 ± 1.60g    | 32.99 ± 2.32h,i  | 67.16 ± 1.30a   | 61.00 ± 1.02a-c   | 39.63 ± 3.45      | 27.16 ± 0.67g,h | 27.19 ± 0.90i,j | 54.60 ± 1.34c-e   | 49.16 ± 2.60e,f   | 44.69 ± 1.42f,g               | 22.31 ± 1.38j,k | 15.7 ± 0.91k |
| Dihydrosinapic acid                 | 71.00 ± 2.98a    | 69.16 ± 6.55a     |   | 59.24 ± 5.54b    | 57.99 ± 3.54b    | 55.77 ± 2.80b    | 71.48 ± 3.19a    | 68.90 ± 1.30a    | 56.17 ± 4.60b                 | 41.90 ± 3.72c    | 40.55 ± 3.96c    | 71.04 ± 0.22a   | 2.74 ± 0.24d      | 3.21 ± 0.18d      | 2.44 ± 0.05d    | 2.61 ± 0.04d    | 2.44 ± 0.05d      | 2.68 ± 0.21d      | 2.96 ± 0.28d                  | 2.35 ± 0.02d    | 1.82 ± 0.13d |
| Sum of sinapic acid O-glucuronide   | 85.54 ± 4.52a    | 52.81 ± 2.81c     |   | 39.73 ± 0.87d,e  | 37.95 ± 1.43d-f  | 32.20 ± 0.89e-g  | 73.75 ± 4.45b    | 44.32 ± 1.54d    | 31.75 ± 2.15f,g               | 23.87 ± 1.09h    | 13.73 ± 1.20i    | 85.80 ± 5.77a   | 37.45 ± 1.90d-f   | 27.63 ± 2.24g,h   | 13.63 ± 0.65i,j | 6.05 ± 0.52j,k  | 57.82 ± 3.19c     | 35.56 ± 2.68e,f   | 25.85 ± 0.87g,h               | 10.16 ± 0.75i-k | 2.77 ± 0.05k |

| Day                                                    | 1                 | 2                 | Extract<br>3       | 4                  | 5                  | 1                | 2                   | 3                   | 4                 | 5                 | 1                 | 2                   | 3                 | 4                 | 5                 | 1                  | 2                  | 3                 | 4                | 5                    |
|--------------------------------------------------------|-------------------|-------------------|--------------------|--------------------|--------------------|------------------|---------------------|---------------------|-------------------|-------------------|-------------------|---------------------|-------------------|-------------------|-------------------|--------------------|--------------------|-------------------|------------------|----------------------|
| Sum of sinapic acid derivatives                        | 220.21 ± 9.85a    | 179.38 ± 12.38b   | 151.99 ± 11.19c    | 144.36 ± 6.30c     | 135.63 ± 4.69c,d   | 213.13 ± 10.05a  | 178.05 ± 5.89b      | 144.62 ± 9.61c      | 105.80 ± 6.41c,f  | 87.27 ± 7.48f,g   | 224.00 ± 7.29a    | 101.19 ± 3.16c,f    | 70.47 ± 5.86g     | 43.22 ± 1.36h     | 35.85 ± 1.45h,i   | 114.86 ± 4.58d,e   | 87.40 ± 5.49f,g    | 73.50 ± 2.57g     | 34.82 ± 2.14h,i  | 20.3 ± 1.09 i        |
| SUM OF PHENOLIC ACIDS                                  | 1079.35 ± 37.32a  | 925.02 ± 34.99b   | 814.43 ± 28.69c,d  | 791.28 ± 25.51c-e  | 743.72 ± 19.04d,e  | 1103.95 ± 44.44a | 859.98 ± 26.89b,c   | 709.14 ± 28.48e,f   | 574.19 ± 23.86g,h | 390.00 ± 32.46i   | 1121.44 ± 31.83a  | 717.94 ± 32.80e,f   | 543.09 ± 32.49h   | 354.54 ± 10.71i,j | 302.46 ± 12.25j   | 828.71 ± 30.34c,d  | 649.02 ± 26.73f,g  | 571.81 ± 13.61g,h | 295.64 ± 17.62j  | 185. ± 7k            |
| Apigenin 7-[6''-(3-Hydroxy-3-methylglutaryl)glucoside] | 18.08 ± 0.48a     | 16.75 ± 1.21a,b   | 14.27 ± 0.18c,d    | 13.32 ± 0.48d,e    | 12.99 ± 0.18d-f    | 17.04 ± 0.67a,b  | 13.97 ± 0.09c,d     | 12.88 ± 0.34d-f     | 11.42 ± 0.32f     | 6.35 ± 0.51g,h    | 17.89 ± 0.71a     | 13.09 ± 1.15d-f     | 11.67 ± 0.61e,f   | 8.10 ± 0.58g      | 7.24 ± 0.36g,h    | 15.67 ± 0.58b,c    | 13.94 ± 0.15c,d    | 11.74 ± 0.66e,f   | 7.27 ± 0.01g,h   | 5.77 ± 0.16 h        |
| Cynaroside A                                           | 9.04 ± 0.12g      | 10.89 ± 0.03f     | 11.03 ± 0.42e,f    | 13.88 ± 0.20b,a    | 14.13 ± 0.05a      | 11.56 ± 0.44d-f  | 12.07 ± 0.11c-e     | 12.29 ± 0.48c,d     | 11.88 ± 0.28c-f   | 6.44 ± 0.58j      | 9.16 ± 0.14g      | 11.48 ± 0.66d-f     | 11.86 ± 0.44c-f   | 8.87 ± 0.34g,h    | 7.75 ± 0.29h,i    | 10.99 ± 0.34e,f    | 12.90 ± 0.24b,c    | 12.27 ± 0.62c,d   | 8.98 ± 0.42g     | 7.18 ± 0.20 i,j      |
| Didymin                                                | 92.31 ± 0.65b     | 69.90 ± 1.10 c    | 51.19 ± 0.08 d     | 43.35 ± 0.28 e     | 29.96 ± 0.04 j     | 29.33 ± 1.56 j   | 32.96 ± 1.28 h      | 34.61 ± 0.18 g,h    | 32.49 ± 0.63 h,i  | 17.73 ± 0.48 m    | 97.05 ± 0.37a     | 33.36 ± 1.12 h      | 36.07 ± 0.88 f,g  | 29.82 ± 0.30 j    | 30.34 ± 0.68 i,j  | 34.67 ± 0.38 g,h   | 35.62 ± 0.07 f,g   | 37.06 ± 0.06 f    | 25.31 ± 0.90 l   | 22.0 ± 0.63 k        |
| Sum of isorhamnetin-3-O-rutinoside                     | 13.50 ± 0.12a     | 13.31 ± 0.22a-b   | 12.10 ± 0.42c,d    | 11.28 ± 0.10d-f    | 11.01 ± 0.12c-g    | 11.55 ± 0.33c-e  | 11.68 ± 0.29c-e     | 12.06 ± 0.31c,d     | 10.99 ± 0.14c-g   | 6.23 ± 0.46j      | 13.59 ± 0.21a     | 11.94 ± 0.44c,d     | 12.05 ± 0.51c,d   | 10.30 ± 0.05g,h   | 9.76 ± 0.27h,i    | 12.22 ± 0.34c      | 12.41 ± 0.10b,c    | 12.14 ± 0.28c,d   | 10.63 ± 0.30 f,h | 9.11 ± 0.26 i        |
| Narirutin                                              | 39.06 ± 0.66a,b   | 37.32 ± 0.54a-c   | 36.66 ± 1.46c      | 34.23 ± 0.20d      | 32.31 ± 0.39d,e    | 36.65 ± 0.16c    | 36.39 ± 0.05c       | 36.93 ± 0.57b,c     | 30.32 ± 0.54e-g   | 23.16 ± 1.08i     | 38.97 ± 0.48a-c   | 29.92 ± 0.24f,g     | 27.45 ± 1.04h     | 19.77 ± 0.34j     | 26.34 ± 0.50h     | 32.08 ± 0.32d-f    | 33.00 ± 1.19d      | 28.15 ± 1.20g,h   | 22.64 ± 1.18i    | 23.0 ± 0.98 i        |
| Sum of norbergenin                                     | 22.73 ± 0.54a     | 17.86 ± 0.34c     | 8.91 ± 0.50f,g     | 7.91 ± 0.24g,h     | 5.46 ± 0.16i       | 22.71 ± 0.89a    | 15.82 ± 0.57d       | 9.70 ± 0.59e,f      | 6.62 ± 0.22h,i    | 3.22 ± 0.13j      | 22.75 ± 0.68a     | 10.57 ± 0.33c       | 6.68 ± 0.24h,i    | 3.89 ± 0.05j      | 2.98 ± 0.05j      | 19.65 ± 0.81b      | 9.82 ± 0.62c,f     | 6.41 ± 0.27i      | 3.71 ± 0.08j     | 2.78 ± 0.03 j        |
| Phenethyl rutinoside                                   | 18.55 ± 0.38a,b   | 16.09 ± 1.19c-f   | 16.80 ± 0.99b-e    | 16.69 ± 0.21b-e    | 16.03 ± 0.05c-f    | 17.26 ± 0.41a-d  | 18.48 ± 0.49a,b     | 18.90 ± 0.70a       | 16.84 ± 0.33b-e   | 11.45 ± 0.78h     | 18.49 ± 0.29a,b   | 17.85 ± 0.29a-c     | 14.57 ± 1.33f,g   | 13.63 ± 0.12g     | 14.84 ± 0.00e-g   | 16.99 ± 0.03a-d    | 15.63 ± 0.31d-g    | 14.37 ± 0.97f,g   | 10.77 ± 0.95h,i  | 9.28 ± 0.69 i        |
| Quercetin 3-O-glucuronide                              | 17.77 ± 0.34b,c   | 16.28 ± 1.17c,d   | 16.76 ± 1.58c,d    | 19.44 ± 0.36a,b    | 15.80 ± 0.24d      | 19.87 ± 0.99a    | 15.72 ± 0.95d       | 15.10 ± 0.88d       | 15.03 ± 0.47d     | 8.02 ± 0.52e      | 17.73 ± 0.03b,d   | n.d.                | n.d.              | n.d.              | n.d.              | n.d.               | n.d.               | n.d.              | n.d.             | n.d.                 |
| Sum of vomifolliol 9-xylosyl-(1->6)glucoside]          | 1192.03 ± 9.94a   | 1011.50 ± 13.30b  | 643.12 ± 5.42d,e   | 689.25 ± 8.00c,d   | 672.67 ± 6.41c,d   | 547.34 ± 15.08f  | 604.82 ± 8.62e      | 673.81 ± 22.47c,d   | 697.89 ± 14.78c   | 459.17 ± 9.58h,j  | 1190.14 ± 5.45a   | 450.40 ± 25.43i,j   | 499.91 ± 21.04f,h | 427.44 ± 22.40c   | 483.28 ± 23.34h-j | 451.68 ± 26.88b-j  | 517.13 ± 7.28f,g   | 530.18 ± 15.03f,g | 430.34 ± 4.58 j  | 319. ± 19.8 7k       |
| SUM OF FLAVONOIDS                                      | 1423.06 ± 13.24a  | 1209.90 ± 19.11 b | 810.85 ± 11.02c,d  | 849.36 ± 10.08 c   | 810.36 ± 7.66 c,d  | 713.30 ± 20.52c  | 764.90 ± 12.44d,e   | 826.28 ± 26.53c     | 833.48 ± 17.71c   | 541.77 ± 14.11h-j | 1425.76 ± 8.37a   | 578.61 ± 29.70g-i   | 620.27 ± 26.09f,g | 521.83 ± 24.19i,j | 582.53 ± 25.54g,h | 593.95 ± 29.68f-h  | 650.45 ± 10.01f    | 652.32 ± 19.13f   | 516.38 ± 8.16j   | 401. ± 23. 17k       |
| Hesperidin                                             | 1309.68 ± 0.10a   | 802.01 ± 0.20c    | 650.84 ± 0.13e     | 540.33 ± 0.09h     | 469.28 ± 0.05j     | 850.73 ± 0.36b   | 654.13 ± 0.25d      | 584.68 ± 0.09f      | 543.34 ± 0.14g    | 504.97 ± 0.06i    | 3.86 ± 0.22m,n    | 4.22 ± 0.34l,m      | 5.04 ± 0.17k      | 3.54 ± 0.05n      | 3.56 ± 0.02n      | 4.38 ± 0.04l,m     | 4.61 ± 0.09k,l     | 5.10 ± 0.07k      | 4.08 ± 0.24m,n   | 3.90 ± 0.31 l-n      |
| Hydroxytyrosol                                         | 108.49 ± 0.92a    | 105.11 ± 0.88a    | 97.64 ± 0.87c      | 96.44 ± 0.77c      | 82.90 ± 0.64e      | 87.69 ± 0.84d    | 87.59 ± 0.83d       | 82.70 ± 0.82e       | 67.96 ± 0.53f     | 43.58 ± 0.43g     | n.d.              | n.d.                | n.d.              | n.d.              | n.d.              | n.d.               | n.d.               | n.d.              | n.d.             | n.d.                 |
| Catechin                                               | 19.48 ± 0.32a     | 7.77 ± 0.55c      | 6.91 ± 0.32d       | 6.38 ± 0.12d,e     | 5.75 ± 0.11e       | 14.86 ± 0.69b    | 6.87 ± 0.17d        | 6.00 ± 0.51e        | 5.71 ± 0.21e      | 4.84 ± 0.44f      | n.d.              | n.d.                | n.d.              | n.d.              | n.d.              | n.d.               | n.d.               | n.d.              | n.d.             | n.d.                 |
| SUM OF EXTRACT PHENOLIC COMPOUNDS                      | 1437.66 ± 1.34a   | 914.90 ± 1.63c    | 755.39 ± 1.31d     | 643.14 ± 0.98g     | 557.93 ± 0.80i     | 953.28 ± 1.88b   | 748.58 ± 1.25e      | 673.38 ± 1.43f      | 617.01 ± 0.88h    | 553.39 ± 0.93j    | -                 | -                   | -                 | -                 | -                 | -                  | -                  | -                 | -                | -                    |
| SUM OF PHENOLIC COMPOUNDS                              | 3940.08 ± 51.90 a | 3049.82 ± 55.72 b | 2380.67 ± 41.02d,e | 2283.78 ± 36.57e,f | 2112.01 ± 27.50g,h | 2770.52 ± 66.85c | 2373.46 ± 40.58 d,e | 2208.80 ± 56.44 f,g | 2024.68 ± 42.45 h | 1485.16 ± 47.51 i | 2551.07 ± 40.44 d | 1300.77 ± 62.85 j,k | 1168.40 ± 58.79 k | 879.90 ± 34.97 l  | 880.55 ± 37.82 l  | 1427.04 ± 60.06 ij | 1304.08 ± 36.83 ,k | 1229.23 ± 32.84 k | 815.93 ± 26.12 l | 591. ± 32 ± 33.6 8 m |

n.d.: non-detected; different letters (a-o) in the same line indicate significant ( $p < 0.05$ ) differences.

The sum of phenolic compounds quantified in the juices was in the range 591-3940  $\mu\text{g/mL}$ , corresponding the minor value to day 5 non-fortified pasteurized juices and the major to day 1 fortified non-pasteurized juice. Those values are in the range of previous reported values in orange juices [604–606].

As can be seen in **Table 10.4** the added compounds could be clearly identified in the fortified juices, as they had hydroxytyrosol and catechin, compounds usually not found in orange juices, and significantly higher amounts of hesperidin than the naturally present in the juice. There were added 1.75 mg/mL of hesperidin extract with purity 80% (1.4 mg/mL), 0.125 mg/mL of hydroxytyrosol extract with purity 80% (0.1 mg/mL) and 0.125 mg/mL of catechin extract with purity 20% (0.025 mg/mL), and were found 1.3 mg/mL of hesperidin, 0.1 mg/mL of hydroxytyrosol and 0.019 mg/mL of catechin. Thus, the solubility of the extract was adequate, and the juice was correctly homogenized as no significant differences were found between the added and quantified amounts. In these compounds it could be appreciated the effect of storage time with significant decreases of 70.5, 64.2 and 23.6% in fortified juices, and 67.4, 40.6 and 50.3% in pasteurized fortified juices, of catechin, hesperidin and hydroxytyrosol, respectively, over the 5 days. Storage time leads to oxidative degradation of phenolic compounds, including catechin, hesperidin, and hydroxytyrosol. This aligns with studies showing significant decreases in these compounds over time [607–609]. Catechin seemed the most sensitive over time with the major reductions. Hesperidin, being a glycosylated flavonoid, and hydroxytyrosol, being a simple phenolic compound, both have structural features that contribute to their stability in acidic environments like orange juice. The glycosidic linkage in hesperidin and the simpler phenolic structure of hydroxytyrosol render them less susceptible to degradation compared to catechin with exposition to light and oxygen [574]. Catechin has a more complex structure compared to hesperidin and hydroxytyrosol containing multiple hydroxyl groups and a catechol structure, which are prone to oxidation and other chemical reactions under various environmental conditions [610]. Thus, oxygen can react with catechin molecules, leading to the formation of quinones and other oxidized products that degrade its structure and reduce its bioactivity [611,612].

Pasteurization also affected them with significant reductions of 23.7, 35.0 and 19.2% in catechin, hesperidin and hydroxytyrosol, respectively. In this case hesperidin was the most sensitive to temperature among the three compounds. Hesperidin is a flavonoid glycoside, meaning it consists of a flavonoid (hesperetin) attached to a sugar molecule (rutinose). Glycosidic bonds are generally more susceptible to hydrolysis (breaking apart) under certain conditions, including elevated temperatures [574]. In contrast, hydroxytyrosol seemed to be the most resistant to temperature. Hydroxytyrosol has a relatively simple structure compared to hesperidin and catechin. It is a phenolic compound with a single phenol group (-OH) attached to a benzene ring. This structure

contributes to its stability under heat because it lacks labile groups that are prone to degradation under elevated temperatures [613–615].

Looking to the phenolic compounds profile at day 1, the control juice was composed 44% of phenolic acids and 56% flavonoids. By adding the extract, the profile was modified to 27% of phenolic acids and 70% flavonoids and 3% hydroxytyrosol. Inside the phenolic acids, it was composed by  $\approx 43\%$  ferulic acid derivatives,  $\approx 20\%$  coumaric acid derivatives,  $\approx 20\%$  sinapic acid derivatives and  $\approx 16\%$  caffeic acid derivatives. The flavonoid group, letting out the extract compounds, was composed of  $\approx 84\%$  sum of vomifoliol 9-[xylosyl-(1 $\rightarrow$ 6)-glucoside] isomers a and b,  $\approx 6.5\%$  of didymin,  $\approx 2.8\%$  narirutin, and other compounds in minor amounts ( $\leq 1.5\%$ ). The profile is in agreement with previous research [581]. They were not found significant differences in the amount of any compound between fortified and non-fortified juices at day 1.

At day 1, looking to the fortified juice, no significant reduction was observed in the sum of phenolic acids, but there were in the sum of flavonoids ( $\approx 50\%$ ). In the non-fortified juice significant reduction were achieved both for the sum of phenolic acids ( $\approx 26\%$ ) and sum of flavonoids ( $\approx 58\%$ ). Thus, it could be affirmed that pasteurization affected the most flavonoids in agreement with other authors [594]. In the case of pasteurization, the extract seemed also to have a protective effect against phenolic compounds degradation, but higher in the case of phenolic acids. de Beer et al., (2014) found the same effect in red-fleshed plum nectars by addition of plum skin extract. Phenolic acid derivatives, such as those found in orange juice that are linked to isocitric acid or glycosides, may be more prone to oxidation under heat stress [616,617]. However, the presence of hesperidin, catechin, and hydroxytyrosol in the juice could have mitigated this oxidation, thereby reducing their degradation compared to if they were unprotected.

Evaluating the shelf life of the phenolic compounds in the 5-days period in both fortified and non-fortified juices, letting apart the extract compounds, they were seen significant differences in all the phenolic acids and flavonoids quantified. The control juice had a reduction 33.6% higher in the sum of phenolic acids than the fortified juice. In contrast, the fortified juices showed a reduction 19% higher in the sum of flavonoids than the control. The same tendencies were maintained in fortified and non-fortified pasteurized juices. This could mean that the extract had an antioxidant effect among the intrinsic juice phenolic compounds avoiding their degradation over time, especially phenolic acids. However, further research is needed to find the possible mechanisms.

Looking to the sum of phenolic compounds, including the added extracts, both controls and fortified samples had the same reduction over the 5-days period, resulting in a content 33% higher in the fortified one, both in the case of pasteurized and non-pasteurized juices.

#### **3.2.4. Vitamin C**

The vitamin C content, including ascorbic acid (AA) and dehydroascorbic acid (DHA), was measured using HPLC-UV/VIS.

The stability of vitamin C in the fortified and non-fortified orange juice samples was assessed over a period of 5 days, considering both ascorbic acid (AA) and dehydroascorbic acid (DHA) levels. As illustrated in **Table 10.5**, the total vitamin C content, including the AA/DHA ratio, remained relatively stable throughout the storage period. However, notable differences were observed between the pasteurized and non-pasteurized samples.

**Table 10.5.** Vitamin C content of orange juices analyzed by HPLC-UV/VIS, expressed in  $\mu\text{g/mL}$  as average  $\pm$  standard deviation.

| Sample                   | Day | AA ( $\mu\text{g/mL}$ ) |     | DHA ( $\mu\text{g/mL}$ ) |     | Ratio AA/DHA | Total Vitamin C ( $\mu\text{g/mL}$ ) |     |
|--------------------------|-----|-------------------------|-----|--------------------------|-----|--------------|--------------------------------------|-----|
| Extract                  | 1   | 529.66 $\pm$ 38.06      | a   | 375.53 $\pm$ 26.64       | a-h | 1.410        | 905.19 $\pm$ 64.69                   | a,b |
|                          | 2   | 517.44 $\pm$ 0.20       | a,b | 390.88 $\pm$ 14.80       | a-g | 1.324        | 908.32 $\pm$ 14.99                   | a   |
|                          | 3   | 499.88 $\pm$ 29.37      | a-c | 396.15 $\pm$ 0.13        | a-f | 1.262        | 896.02 $\pm$ 29.50                   | a-e |
|                          | 4   | 490.69 $\pm$ 1.09       | a-e | 410.71 $\pm$ 2.74        | a-d | 1.195        | 901.40 $\pm$ 3.83                    | a-c |
|                          | 5   | 481.22 $\pm$ 0.91       | b-e | 418.56 $\pm$ 32.51       | a,b | 1.150        | 899.78 $\pm$ 33.42                   | a-d |
| Control                  | 1   | 500.34 $\pm$ 25.75      | a-c | 392.36 $\pm$ 28.62       | a-g | 1.275        | 892.70 $\pm$ 54.37                   | a-e |
|                          | 2   | 494.91 $\pm$ 0.19       | a-d | 398.83 $\pm$ 7.26        | a-e | 1.241        | 893.74 $\pm$ 7.45                    | a-e |
|                          | 3   | 491.62 $\pm$ 13.43      | a-e | 410.25 $\pm$ 4.10        | a-d | 1.198        | 901.87 $\pm$ 17.52                   | a-c |
|                          | 4   | 489.18 $\pm$ 5.56       | a-e | 412.45 $\pm$ 11.85       | a-c | 1.186        | 901.62 $\pm$ 17.41                   | a-c |
|                          | 5   | 488.95 $\pm$ 0.05       | a-e | 420.52 $\pm$ 25.43       | a   | 1.163        | 909.47 $\pm$ 25.48                   | a   |
| Control + pasteurization | 1   | 430.03 $\pm$ 1.17       | f-j | 335.32 $\pm$ 22.76       | h   | 1.282        | 765.35 $\pm$ 23.94                   | f   |
|                          | 2   | 421.31 $\pm$ 14.87      | g-j | 350.52 $\pm$ 3.86        | f-h | 1.202        | 771.84 $\pm$ 18.73                   | f   |
|                          | 3   | 417.00 $\pm$ 9.44       | h-j | 358.89 $\pm$ 5.00        | e-h | 1.162        | 775.88 $\pm$ 14.44                   | f   |
|                          | 4   | 415.45 $\pm$ 7.26       | i,j | 364.97 $\pm$ 0.78        | d-h | 1.138        | 780.43 $\pm$ 8.03                    | f   |
|                          | 5   | 406.09 $\pm$ 0.12       | j   | 364.48 $\pm$ 9.31        | d-h | 1.114        | 770.57 $\pm$ 9.43                    | f   |
| Extract + pasteurization | 1   | 462.98 $\pm$ 3.84       | c-f | 349.16 $\pm$ 7.49        | g,h | 1.326        | 812.14 $\pm$ 11.33                   | f   |
|                          | 2   | 462.17 $\pm$ 4.09       | c-g | 360.77 $\pm$ 4.44        | e-h | 1.281        | 822.94 $\pm$ 8.53                    | d-f |
|                          | 3   | 458.11 $\pm$ 0.66       | d-h | 367.78 $\pm$ 10.12       | c-h | 1.246        | 825.88 $\pm$ 10.78                   | c-f |
|                          | 4   | 456.28 $\pm$ 1.76       | d-i | 373.51 $\pm$ 0.74        | b-h | 1.222        | 829.78 $\pm$ 2.51                    | b-f |
|                          | 5   | 453.00 $\pm$ 2.58       | e-i | 367.54 $\pm$ 9.77        | c-h | 1.233        | 820.54 $\pm$ 12.35                   | e,f |

Different letters (a-j) in the same column indicate significant differences.

The values obtained for AA, DHA and total vitamin C content are in the range of previous published research [331,581].

The fortified and control juices maintained high levels of vitamin C, with minor fluctuations, and without significant differences between them, indicating that the addition of the extract did not affect the vitamin C content. Besides, total vitamin C levels (sum of AA and DHA) generally were consistent over time with no significant increases or decreases between days. This could be attributed to proper handling and storage practices, such as refrigeration at 4 °C and light

protection, that helped to preserve vitamin C levels and prevent degradation [618]. No significant changes in the vitamin C content in orange juice over days were previously reported [619].

Going further, in general the AA concentrations generally decrease ( $\geq 2\%$ ) over the 5 days in all samples, indicating potential degradation of ascorbic acid over time. In contrast, DHA concentrations tend to increase ( $\geq 5\%$ ), suggesting conversion or accumulation of dehydroascorbic acid during the storage period. However, none of these changes over the time were significant for any sample ( $p < 0.05$ ). The same tendency was reported by Plaza et al. (2006) in orange juices during 40 days of storage [619].

The ratio of AA to DHA fluctuates across samples and days, reflecting the balance between reduced (AA) and oxidized (DHA) forms of vitamin C. Higher ratios indicate a higher concentration of ascorbic acid relative to dehydroascorbic acid. As can be seen, for all samples the ratio AA/DHA is reduced over the 5 days of storage time in agreement with the decreases in the AA content and increases in the DHA content. DHA is the oxidized form of AA, so DHA can be formed through the oxidation of AA, which may occur as a natural part of vitamin C metabolism or due to external factors such as oxygen exposure [620].

Pasteurization appears to have an effect on total vitamin C levels, with significant ( $p < 0.05$ ) decreases of 14.3 and 10.3% in control and fortified juices, respectively, compared to non-pasteurized samples at day 1. However, over the 5-day period, pasteurized samples show relatively stable trends in vitamin C levels, similar to the non-pasteurized juices, suggesting that the process may stabilize vitamin C content despite initial reductions. Similarly, while pasteurization also lowers initial AA and DHA levels significantly in fortified and non-fortified juices, it does not significantly alter the rate of AA degradation and DHA generation during subsequent storage, similarly to non-pasteurized juices. The decrease in vitamin C content in the pasteurized samples aligns with the known impact of heat treatment on vitamin C stability, as pasteurization can cause degradation of vitamin C due to its sensitivity to heat. The same effect of reducing the vitamin C content in orange juice because of the pasteurization process followed by stability during refrigerated storage has been previously reported by several authors [596,621]. Besides, they proposed alternative methods for ensuring microbial stability with lower reduction in the vitamin C content such as pulse electric fields and high pressure processing [565,622,623].

This stability is critical as vitamin C is a significant nutrient in orange juice, contributing to its health benefits. The retention of vitamin C ensures that the fortified juice remains a valuable source of this essential vitamin, which is crucial for various bodily functions, including immune support and antioxidant defense [619]. The findings suggest that the fortified orange juice provides a stable source of vitamin C over a typical consumption period.

### 3.2.5. Antioxidant activity

The antioxidant activity of the fortified orange juice samples was measured using DPPH and FRAP assays over a 5-day period. The results, as presented in **Figure 10.5** and **Figure 10.6**, demonstrate that the antioxidant capacity of the juice samples remained throughout the storage period, with significant activity observed in both fortified and control samples.

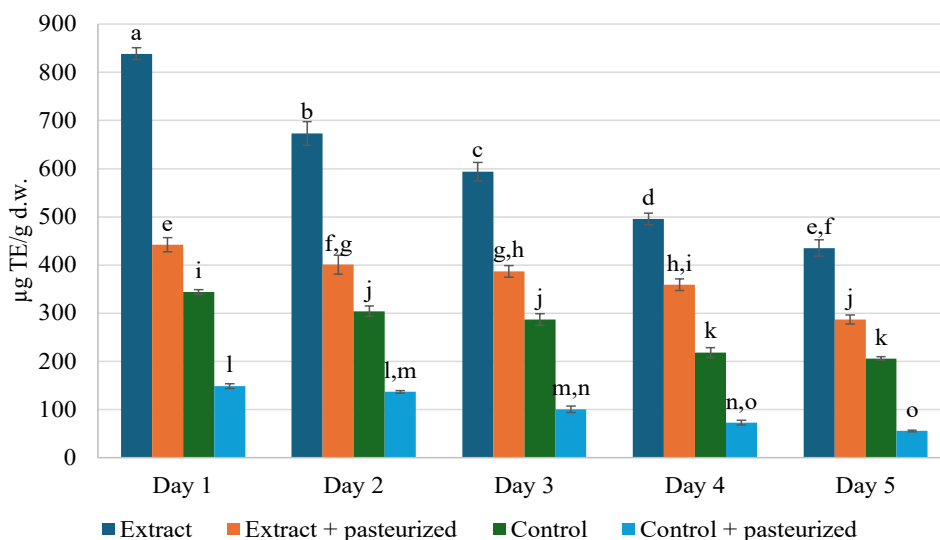


**Figure 10.5.** DPPH antioxidant activity results over 5 days expressed with average and standard deviation. Different letters (a-o) indicate significant differences. Extract: Orange juice added of 2 mg/mL of extract.

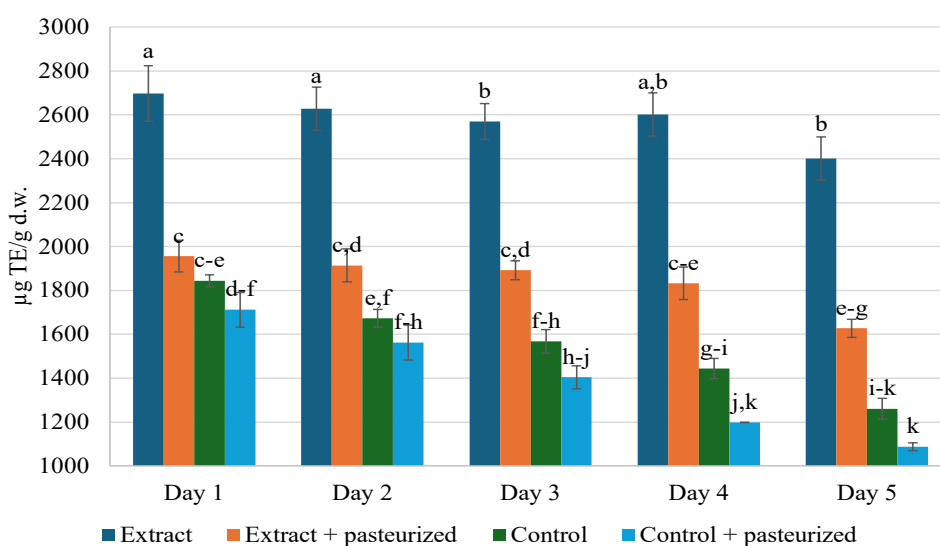


**Figure 10.6.** FRAP antioxidant assay results over 5 days expressed as average and standard deviation. Different letters (a-k) indicate significant differences. Extract: Orange juice added of 2 mg/mL of extract.

DPPH results ranged 55-838  $\mu\text{g TE/g d.w.}$  and FRAP between 1087 and 2698  $\mu\text{g/g d.w.}$  in the evaluated juices. The results are in the same range of magnitude than previous reported research in orange juices [280,622]. Both antioxidant assays showed the same tendency among the days and between the samples, also corroborated by the very strong significant ( $p < 0.01$ ) correlation showed between them ( $r = 0.9219$ ). It was revealed a significant decrease ( $p < 0.05$ ) in the antioxidant activity over time in all the evaluated juices individually as reported by other authors [622]. The fortified samples consistently exhibited higher antioxidant activity compared to the controls. Looking to day 1 it can be clearly appreciated that the fortified juice exhibited 59% and 36% higher antioxidant activity than the control by DPPH and FRAP assays, respectively. In day 5, fortified juice showed  $\geq 48\%$  higher antioxidant activity than the control. This enhancement in antioxidant activity can be attributed to the added polyphenolic extracts, which are known for their potent antioxidant properties. The stability of these antioxidants in the juice matrix suggests that the formulation effectively protects these compounds from degradation. Synowiec-Wojtarowicz et al. (2018) reported that supplementation of lime and mandarin juices with polyphenols like resveratrol, hesperidin, and chlorogenic acid significantly increased their antioxidant potential, showcasing the benefits of fortification [624]. Savatovic et al. (2009) demonstrated that the addition of polyphenols from apple pomace significantly improved the antioxidant activity of apple juice [625].

The consistent antioxidant activity over the storage period is crucial for ensuring that the health benefits of the food product. Factors such as oxidative degradation, enzymatic activity, storage conditions, and time-dependent changes can contribute to the observed decline in antioxidant activity over time. In fortified juice it was appreciated a significant decrease of 11 and 48% in the antioxidant activity measured by FRAP and DPPH, respectively, between day 1 and day 5. However, in this time higher decreases of 31% for FRAP and 62% for DPPH were showed in the non-fortified juices. The fact that the fortified juice shows a smaller decline in antioxidant activity compared to non-fortified juice suggests that the fortification process effectively stabilizes the antioxidant content of the juice over time. This stabilization is important because it helps maintain the potential health benefits associated with antioxidants.

Evaluating the effect of pasteurization, it was revealed that affected in a significantly negative way the antioxidant activity of the fortified and non-fortified juices, reaching reductions up to 58%. Antioxidants are sensitive to heat and can undergo chemical changes or breakdown, thereby reducing their antioxidant activity. This effect was previously reported in orange juices by other authors [626,627].

According with the DPPH results, in pasteurized samples it was decreased significantly the slope of decrease both in fortified and non-fortified samples. This means that the rate at which



antioxidant activity decreases over time was slower in pasteurized samples compared to non-pasteurized samples. Pasteurization appears to have a stabilizing effect on antioxidant activity as measured by the DPPH assay. Pasteurization can exert a stabilizing effect on antioxidant activity in juices by several mechanisms. Pasteurization reduces microbial activity, thereby minimizing potential microbial degradation of antioxidants [627,628]. These combined effects contribute to the observed phenomenon where pasteurized juices, both fortified and non-fortified, exhibit a slower rate of decline in antioxidant activity as measured by the DPPH assay compared to non-pasteurized counterparts. Therefore, while pasteurization is primarily aimed at ensuring food safety by eliminating harmful microorganisms, it also plays a critical role in maintaining the functional quality of juices, particularly in preserving their antioxidant benefits over extended storage periods.

However, the tendency in FRAP assay was different as no significant differences were appreciated in the decrease slope in the fortified samples submitted or not to pasteurization, and in the non-fortified samples the decrease slope was significantly higher in the pasteurized sample. In this case pasteurization did not have a consistent effect on the antioxidant activity as measured by the FRAP assay. In fortified samples, antioxidant activity remained relatively stable regardless of pasteurization. However, in non-fortified samples, pasteurization led to a faster decline in antioxidant activity over time.

The differing effects observed between DPPH and FRAP assays suggest that different bioactive compounds or mechanisms may be affected by pasteurization differently. Besides, they exhibit distinct mechanisms and specific bioactive compounds targeted by each assay.

It was found a significant ( $p < 0.01$ ) correlation between the antioxidant activity measured by the two methods and the ascorbic acid ( $r = 0.7092$  and  $0.6646$ ). In the same way, significant positive correlation was found between the sum of phenolic compounds and the antioxidant activity measured by both methods ( $r = 0.8439$  for FRAP and  $r = 0.9173$  for DPPH). DPPH showed slightly higher positive correlation ( $r = 0.7611$ ) with sum of flavonoids (not including extract) and FRAP with sum of phenolic acids ( $r = 0.7054$ ), both significant. The sum of compounds present in the extract also had significant positive correlation with the antioxidant results by DPPH ( $r = 0.8845$ ) and FRAP ( $r = 0.7968$ ), and so each compound individually. Thus, all the determined compounds seemed to contribute to the antioxidant activity.

#### **4. Conclusions**

This study presents a novel approach to enhancing the bioactive value of orange juice through the fortification with bioactive compounds derived from food industry by-products. By utilizing hesperidin from orange peels, catechin from avocado by-products, and hydroxytyrosol from olive leaves, we have demonstrated that it is possible to not only improve the phenolic content of orange

juice but also contribute to a circular economy by repurposing waste materials. Our findings reveal that fortification at a concentration of 2 mg/mL effectively balances the enhancement of the juice's phenolic content and antioxidant properties while maintaining consumer acceptance. Furthermore, this study provides new insights into the effects of pasteurization on the quality of fortified orange juice. While pasteurization initially decreases the levels of vitamin C and flavonoids, it also stabilizes the antioxidant activity and phenolic content over a 5-day storage period, demonstrating that fortification can sustain the health benefits of orange juice even under processing conditions. This approach not only could increase the functional value of orange juice but also extends its shelf life and stability, making it a promising strategy for the development of functional foods with enhanced health benefits. Further studies could expand on different juice matrices and longer storage periods to fully understand the implications of fortification including microbial tests.

# **INTEGRATIVE DISCUSSION**

Orange and avocado by-products are often overlooked or discarded but converting them into valuable ingredients can reduce environmental impact and promote a circular economy by creating new revenue streams and decreasing landfill waste. Embracing these by-products reflects a commitment to sustainable practices and innovative resource management [3,6].

The drying is an essential step to ensure the storage of by-products at an industrial level, in order to use them for managing and re-use as functional ingredients or as nutraceuticals [629]. The mathematical modelling allows to reduce drying times while achieving the final moisture goal by predicting the samples behavior as similar as actually is [630]. Among all the tested mathematical models, Page regression model gave better adjustment, reporting higher coefficient of regressions and lower squared and absolute errors for the major temperatures and air flows tested, both in orange (CHAPTER 1) and avocado peels (CHAPTER 6). This model was also found to predict the best drying kinetics in other fruit by-products such as shea [631], lima [632], tomato [633] or dragon fruit [634], among others. The mathematical modelling of the drying kinetics of avocado seed was already studied by other authors and reported Avhad and Marchetti model to be the best [244]. To our knowledge, it was the first time that the **mathematical modelling of convective drying** of orange and avocado peels was studied.

Looking to the effect of the drying parameters, temperature and air-flow demonstrated to have a direct effect on the time needed to achieve the final moisture content. At 40 and 60 °C, when increasing the air flow, the time needed to reach the final moisture content was reduced, so the exposure to temperature was reduced and, in consequence, the polyphenol reduction was lower. However, at 80 °C, the case-hardening phenomenon was increased when increasing the air flow, reducing the bioactive content. These effects were previously reported [235,456]. Thus, based on that, for the orange peel the best drying conditions selected were 60 °C with an air flow of 1.6 m/s and taking 315 min that allowed a lower reduction of phenolic compounds, especially flavanones such as hesperidin and narirutin, antioxidant activity, and ascorbic acid content. Most authors that aimed to extract phenolic compounds dried the orange peel at 60 °C for an excess of time (12-48 hours) [260,291,635]. When focusing to dry till moisture content < 10 %, the best drying conditions for the orange peel allows reductions of  $\approx 43$  hours compared to sun drying, or of  $\approx 31$  hours compared to vacuum oven drying [212]. Otherwise, in the avocado peel, the drying conditions that showed the lowest decrease in procyanidin and antioxidants were 40 °C and an airflow of 1.6 m/s for 105 min. In both cases, orange and avocado peels, the airflow resulted beneficial reducing losses in bioactive compounds meanwhile there were differences in temperature and drying time. These differences can be attributed to different factors: First, they had different moisture diffusivity and activation energy: orange peels had moisture diffusivity values between  $10^{-5}$  and  $10^{-9}$  m<sup>2</sup>/s with and activation energy of 139.57 kJ/mol, meanwhile the avocado peel presented moisture diffusivity values between  $10^{-7}$  and  $10^{-9}$  m<sup>2</sup>/s and an activation

energy of 14.69 kJ/mol. As the avocado peel presented a higher average moisture diffusivity values and lower activation energy, the drying should be quicker than the orange peel explaining the minor time needed. And second, they had different thickness: The avocado peel by-product consisted of the avocado skin of  $\approx 5$  mm meanwhile the orange peel by product was composed by two layers consisting of flavedo and albedo of  $\approx 1$  cm. When the sample is thinner the time needed for drying is lower as the conduction of heat and diffusion of water inside the material are easier [636,637].

To take advantage of agro-industrial wastes one possible approach is to isolate or extract specific compounds. Into this scope, bioactive compounds such as phenolic compounds have been attributed several bioactivities [638]. The extraction of phenolic compounds has been extensively studied over years using conventional extraction methods that had drawbacks such as using hazardous solvents during long times at high temperatures. However, recently there are some innovative and non-thermal extracting methods such as ultrasound assisted extraction that overcome those drawbacks [639].

Thus, it was optimized the extraction of phenolic compounds from orange peel (CHAPTER 2) and avocado peel and seed (CHAPTER 7) by **ultrasound assisted extraction by sonotrode**. The use of sonotrode instead of ultrasound bath allows the future scalability of the extraction procedure to pilot or industrial levels. Besides, bath systems exhibit non-uniform energy distribution and a reduction of power over time, leading to lower efficiency compared to probe systems. In probe systems, energy delivery is concentrated in a specific sample zone, resulting in a more efficient cavitation effect [141,142,330,487].

For all the optimization processes they were used Box Behnken experimental designs. This kind of designs require fewer experimental runs compared to other response surface methodology designs, such as central composite designs, especially when the number of factors increases. Unlike factorial or central composite designs, Box-Behnken designs do not include combinations where all factors are at their extreme levels simultaneously. This feature helps avoid potentially unsafe or unfeasible conditions. Moreover, Box-Behnken designs are capable of fitting a full quadratic model (second-order polynomial) without the need for additional points. This capability allows for accurate modelling of curvature in the response surface [640].

All the models showed high determination coefficients ( $R^2 \geq 0.9$ ) with no significant lacks of fit. Besides, they were validated with no significant differences between model predicted and experimental obtained values.

The optimal ultrasound extraction conditions obtained for all samples were 45% ethanol, 90% amplitude, and 35 min for orange peel, 60% ethanol, 70% amplitude and 30 min for avocado peel, and 55% ethanol, 90% amplitude and 30 min for avocado seed. In all cases, it was the first time

that the ultrasound extraction by sonotrode was performed in these by-products. With these conditions in all cases, they were achieved significant increments in comparison to controls. Other authors extracting phenolic compounds from orange peel by maceration used 100% organic solvents such as acetone or methanol and required times of 3-72 hours [267,268,273], or temperatures of 60 °C [267,269,272]. Besides, using other quicker technologies than maceration such as high voltage electrical discharges [262], microwave-assisted extraction [269], and pressurized liquid extraction [60], they were noticed lower yields compared to our results. Previous references using ultrasound bath reported ethanol 40-60% with 15-30 min, but achieving also lower yield [60,274,275]. Total phenolic compounds from avocado peels and seeds measured by spectrophotometry (Folin Ciocalteu) or HPLC-MS have been reported by some authors using hydroethanolic liquid-liquid extraction with higher times (1 hour) [116] and ultrasound bath with similar ethanol:water ratio but with times between 65-120 min [474,475]. Besides, the same for other technologies such as microwave [476–478], vacuum microwave [479] and pressurized fluid [452] assisted extractions. However, no previous references have been found about the optimization of extraction of procyanidins measured by HPLC-FLD in avocado peels and seeds.

Overall, the optimal conditions are quite similar for orange and avocado by-products analysed with ethanol concentrations 45-60%. In all cases pure ethanol or water alone resulted not adequate for extracting phenolic compounds. It could be due to their individual solvent properties and the diverse nature of phenolic compounds. Water, being highly polar, is effective at dissolving highly polar phenolic compounds, while ethanol, with intermediate polarity, is better suited for less polar compounds. An ethanol-water mixture offers a balanced polarity, enhancing the solubility range and extraction efficiency [641]. The minor differences in the percentage of ethanol between samples could be attributable to the different aiming compounds and different matrix composition [642]. For the orange peel, it was the total phenolic compounds that consist of majorly phenolic acids and its derivatives, followed by flavonoids. In contrast, avocado peel and seed aimed compounds were procyanidins with a wide range of polymerization degree. Going further, avocado peel was richer in the monomers catechin + epicatechin, meanwhile avocado seed in procyanidins with a polymerization degree between 2 and 4. Catechin and epicatechin are less polar than procyanidins (DP 2-4) due to their simpler structures and fewer hydroxyl groups thus. It resulted in avocado peel needing slightly higher percentage of ethanol (60%) than avocado seed (55%). In addition, orange peel and avocado seed needed higher amplitude (90%) meanwhile avocado peel lower (70%), which could indicate that avocado peel has a less dense structure that is more easily penetrated and disrupted, facilitating efficient extraction at lower power energy.

Besides, optimal orange peel extracts were exhaustively characterized, and they were identified a total of 74 compounds being 32 of them tentatively identified in orange by-products for the first

time such as caffeic, ferulic and sinapic acids derivatives, and apigenin, naringenin and kaempferol derivatives.

The next step carried out was to try two different pre-treatments to enhance even more the extraction of phenolic compounds. Firstly, **pulse electric field pre-treatment** was optimized in orange peel (CHAPTER 3), and avocado peel and seed (CHAPTER 8). It was performed with Box Behnken designs that showed high correlation coefficient and models validity was confirmed in all cases. It was the first time that pulse electric field was optimized as a pre-treatment to ultrasound extraction for extracting phenolic compounds in orange and avocado by-products.

Previously, PEF has been applied to orange peel for increasing the efficiency of extracting soluble dietary fiber [323], extraction of limonene [324], or shorten the drying process [325]. However, few research have been done using PEF for extracting polyphenols from orange wastes [149,169] and they reported electric fields 1-7 kV/cm. None of them optimized the PEF conditions as in the present work. The only research regarding PEF and avocado was conducted by Castonera-García et al. [504] and the aim was to inactivate the polyphenol oxidase enzyme. To the best of our knowledge, there are no previous references regarding the use of PEF in avocado by-products for any purposes. Otherwise, there are some references on the use of PEF for improving polyphenol extraction from seeds such as canola seed cake powder [506], papaya seeds [498], grape seeds [507] and flaxseed hulls [508] with electric fields in the range 1.1-20 kV/cm, and from peels such as papaya peels [171], grape peels [496], potato peel [510], tomato peel [511], lemon peels [170], peach peels [513], kiwifruit peel [509] and pomegranate peels [153] with electric fields of 0.1-7 kV/cm and specific energies inputs ranging 0.96-5 kJ/kg.

When comparing the PEF treatment of orange peel, avocado peel, and avocado seed, several key differences in parameters arise due to their physical forms and inherent material properties. First there are differences in the electric field strength and the specific energy inputs applied to each sample. **Electric field strength** is a critical factor in PEF treatments as it determines the intensity of the electric field applied to the sample. This intensity affects how effectively the electric field can permeate the cell membranes and induce electroporation. The electric field strength varies significantly between the samples due to their resistance and structure. For orange peel, a higher field strength of 1.4 kV/cm is used because the thick, dense peel requires a stronger electric field to penetrate and achieve effective electroporation. Avocado peel has a slightly lower electric field strength of 1.31 kV/cm since it is less resistant than orange peel but still needs a substantial electric field to induce electroporation. In contrast, avocado seed required a much lower field strength of 0.75 kV/cm because it was treated in a paste form that facilitated a more uniform application of the electric field, reducing the need for a high field strength. **Specific energy input** represents the amount of energy delivered per unit mass of the sample. It reflects the efficiency and intensity of

the treatment. Orange peel required a moderate energy input of 1.9 kJ/kg due to the need to overcome the resistance of the thick peel. Avocado peel has a lower specific energy input of 0.63 kJ/kg because it is less resistant compared to orange peel. Avocado seed demanded a high specific energy input of 12.7 kJ/kg due to the dense nature of the seed and the high resistance of the paste, which requires substantial energy for effective treatment.

Secondly, another difference between the PEF treatments among the samples was the way in which this electric field and specific energy input was delivered. It is function of the frequency, number of pulses and pulse duration, that determines the total treatment and total operating time. In the case of orange peel, they were applied at a frequency of 1 Hz, 30 pulses of 100  $\mu$ s, which resulted in 3.3 ms of treatment time 30 s of total operating time. Otherwise, for the avocado peel, 50 Hz and 175 pulses of 10  $\mu$ s, with a total treatment time of 1.75 ms and total treatment time of 3.5 s, and avocado seed was treated with 100 Hz and 550 pulses of 10  $\mu$ s, resulting in a total treatment time of 5.5 ms and total operating time of 5.5s. These differences highlight how PEF treatment parameters are specifically adjusted for each material's unique characteristics. In the case of orange and avocado peels, pieces of 2-3 cm were used, while in the case of avocado seed it was created a paste in ratio 1:2 seed/water with milled seed of a size of 0.2-5  $\mu$ m. Besides, the initial electrical conductivity of the three types of samples was very different. Although the exact mechanisms of electroporation are not yet fully understood, it is evident that the membrane plays a pivotal role in amplifying the applied electric field. This is particularly significant due to the considerable difference in conductivity between intact membranes and the extracellular medium or cell cytoplasm [502].

Another pre-treatment that was applied was the **submerged fermentation with lactic acid bacteria**. They were achieved significant increments in the phenolic compounds in orange peels (CHAPTER 4) and avocado seeds (CHAPTER 9), but decreases in the case of avocado peels (Data not included in the thesis).

LAB have been previously studied in orange peel with different purposes such as creating a prebiotic [360] or for producing lactic acid [350,361]. Otherwise, the only previous reference regarding fermenting avocado seed with LAB was from Rozan et al. [388] who reported a reduction in the anti-nutrients. However, as far as we are concerned, it was the first time that this biotechnology was applied to orange and avocado by-products to enhance the phenolic content. In the case of avocado seed, the fermentation with *P. acidilactici* resulted in the higher increment in the total polar content, meanwhile fermenting with *La. plantarum* CECT 9567 resulted in the highest antioxidant activity and with *La. plantarum* CECT 748T in the highest antidiabetic activity measured by alpha-amylase inhibitory activity. Those differences could be explained by the different activity of the different identified compounds. The anti-diabetic activity was



correlated with the content in flavan-3-ols meanwhile the antioxidant with phenolic acid derivatives, the iridoid pensternide and quercetin derivatives. In contrast, in the orange peel, the fermentation with *La. plantarum* CECT 9567 showed the highest increase in phenolic acids and highest antioxidant activity measured by DPPH and fermenting with *Le. Brevis* CECT 5354 resulted in increased flavonoid content with the highest antioxidant activity measured by ABTS. The differences are due to that different LAB strains are more efficient at enhancing specific types of bioactive compounds in different substrates. This is due to their distinct enzymatic activities and metabolic pathways [532]. The results highlight the importance of selecting specific LAB strains for targeted bioactive enhancements, depending on the desired health benefit. For instance, *La. plantarum* CECT 9567 is suitable for increasing antioxidant activity in both substrates. Besides, the differences between the chemical compositions and physical structures of orange peel and avocado seed are key factors in determining which bacteria enhance their bioactive contents the most.

The previous chapters focused only in the flavan-3-ols content measured by HPLC-FLD. In this case it was exhaustively characterized the polar content of avocado seed by HPLC-ESI-TOF-MS and a total of 32 polar compounds were identified, with 13 of them being described in avocado seeds for the first time including luteolin/kaempferol derivatives.

These approaches of pre-treatments (PEF or LAB submerged fermentation) and extraction (ultrasound assisted-extraction with probe) can be integrated with other processes to enable component separation, fractionation, and concentration. **Membrane technologies** offer an appealing avenue for addressing these processes effectively. Thus, a first trial of sonotrode scalability followed by membrane separation steps in compliance with food grade regulations was carried out for orange peels (CHAPTER 5). Differently to CHAPTER 2, in this case the ultrasound extraction was performed for 12 hours at 40% of amplitude using just water as solvent. Besides, it was carried out a previous enzymatic hydrolysis of pectins. The performance of the extraction of phenolic compounds was undesirable as no increases and low yields were achieved in the phenolic content. This outcome arises from several factors. As was previously demonstrated in CHAPTER 2, the presence of ethanol notably impacts the effectiveness of ultrasound, amplifying its effects compared to a purely aqueous suspension. Moreover, higher intensity (amplitude 90%) during lower times (30 min) resulted in higher phenolic yields. In contrast, in CHAPTER 5 it was used a prolonged exposure (12 hours) with lower energy (amplitude 40%) operating through a distinct mechanism, resulting in the distinct outcomes observed. A previous research involving the use of pectinases enzymes combined to ultrasound bath extraction also reported low outcomes of phenolic content than without enzymes [270], Thus the enzymatic hydrolysis prior to extracting phenolic compounds should be extensively studied. Otherwise, the membranes used in the process did not allow the isolation of any specific phenolic compounds,

although a slight increase in the concentration was achieved. The use of ultrasound technology and the purification membrane steps, as it was observed in the food grade analysis, let that the final product resulted conform microbiologically, allergens free, pesticides free, mycotoxins free and DNA free, aligned with European food grade laws. Further research in the membrane processes, the scalability of the all the optimized technologies and the possible integration of an enzymatic hydrolysis step should be carried out.

Finally, it was assessed the viability of formulating a fortified food product with extracts from by-products. Thus, orange juice was fortified with hesperidin from orange peels, catechin from avocado by-products and hydroxytyrosol from olive leaves (CHAPTER 10). It was possible to enrich the orange juice with 2% of a mixed extract with high organoleptic acceptability demonstrated by sensorial analysis. Thus, the fortified juice showed higher total phenolic content and higher antioxidant activity than non-fortified, even after pasteurization.

Overall, this thesis is significant for advancements in extraction and processing technologies and the development of value-added products from agricultural waste. It bridges the gap between scientific research and practical applications in the food and nutraceutical industries, potentially benefiting both consumers and producers alike.





# CONCLUSIONS





## Main conclusions

1. The mathematical modelling of the drying kinetics of orange peel allows, at lower times, to minimize the reduction of phenolic compounds, ascorbic acid and antioxidant activity.
2. The optimized ultrasound assisted extraction with sonotrode allowed to increase 60% the phenolic recovery in orange peel compared to a conventional extraction. Besides, 32 new phenolic compounds were identified for the first time.
3. The combination of optimized pulse electric fields pre-treatment with ultrasound extraction has let to obtain orange peel enriched extracts with 27.5% higher content of polyphenols and 53% higher antioxidant activity.
4. Using lactic acid bacteria fermentation as a pre-treatment to ultrasound extraction by bacteria as *Lantiplantibacillus plantarum* and *Levilactobacillus brevis* enhanced the phenolic compounds recovery up to 20% with significant increments in the antioxidant activity.
5. The scalable and non-thermal ultrasound extraction process for phenolic compounds from orange peels was successfully demonstrated at a 1000 L scale, producing a food-grade product, though further optimization of enzymatic hydrolysis is needed for better performance of membrane filtration purification steps.
6. The mathematical modelling of the air-drying of avocado peels has led to minimize the flavan-3-ols and antioxidant activity losses while minimizing the drying time.
7. Applying ultrasound assisted extraction by sonotrode at optimized conditions for avocado peels and seeds, it is possible to increase the flavan-3-ols recovery by about 54% and the antioxidant activity by around 70% compared using ultrasound bath technology.
8. The use of pulsed electric fields prior to ultrasound extraction let to achieve higher extraction of procyanidins of 31.5% in avocado peels and of 9.5% in avocado seeds.
9. Fermentation by lactic acid bacteria as *Lactiplantibacillus plantarum* and *Pediococcus acidilactici* of avocado peels, as a pre-treatment to ultrasound extraction, allowed increases of 26% in the phenolic compounds and precursors, with significant increments in the antioxidant and anti-diabetic activities.
10. The fortification of orange juice with by-products extracts in a concentration of 2 mg/mL effectively enhance the phenolic content by 33% up to 5 days, while maintaining consumer acceptance. It also protected vitamin C and antioxidant activity of the juices among pasteurization and during shelf-life.





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