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VALORIZATION OF AGRI-FOOD BY-PRODUCTS FOR PHARMACEUTICAL, COSMETIC, AND NUTRACEUTICAL APPLICATIONS IN A CIRCULAR ECONOMY PERSPECTIVE

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Abstract

The global food system faces a critical sustainability challenge with significant food loss and waste, substantially contributing to greenhouse gas emissions. Global efforts aim to reduce this waste by adopting circular economy principles. Indeed, the large amounts of agricultural waste generated are rich in valuable bioactive compounds like polyphenols and proteins, with applications in nutraceutical, pharmaceutical, and cosmetic industries. This research focuses on valorizing underutilized by-products from *Fruttagel*®, an Italian agri-food company processing many fruits (apples, apricots, peaches and tomato) and legumes (beans, green beans, peas and soy). By-products from fruit juice (pomace, peels, etc.) and tomato sauce production (peels), as well as legume by-products (skins, leaves, okara), were analyzed and compared to final products. Analytical methodologies, including Ultrasound-Assisted Extraction (UAE) for polyphenol recovery and chromatographic methods (HPLC-DAD and UHPLC-DAD-ESI-MS^n), were developed and optimized for chemical characterization and stability evaluation. The Total Phenolic Content (TPC) and Total Antioxidant Status (TAS) assays were applied for functional screening. Protein content was determined using the Kjeldahl method. Pesticide analysis was also conducted on fruit by-products. Chemical analysis revealed significant amounts of different polyphenols in the by-products, such as chlorogenic acid in biological peach and apricot, feruloyl glucaric acid derivatives in beans, flavonols in green beans, and isoflavones in soy. Many by-products, especially soy, also showed high protein content suggesting potential in nutraceuticals and cosmetics. In collaboration with the pharmaceutical company *Valpharma S.p.A.*, an enteric-coated tablet incorporating UAE extracts as active ingredients was formulated, exploiting the prebiotic potential of polyphenols. Purification and concentration methods for the extracts were optimized, and a "marker Tablet" strategy, employing a substitute active ingredient, was used to evaluate tablet properties. The obtained results demonstrate the significant potential of agri-food by-products as valuable and sustainable ingredients within a circular bio-economy, highlighting their nutritional and functional benefits for new formulations.

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1. STATE OF THE ART

1.1 CIRCULAR ECONOMY

1.1.1 From Waste to Resource: The Circular Economy Approach to Food

The global food system faces a significant sustainability challenge, characterized by excessive food waste with serious economic and environmental consequences[7]. According to the latest report from the Food and Agriculture Organization (FAO) of the United Nations (2022), approximately 14% of global food is lost during harvest, production, and distribution, while an additional 17% is wasted by consumers[8]. This results in an annual global food waste of 1.3 billion tones, with 90 million tones wasted in the European Union alone, as noted in the FAO report[1].

Historically, food waste was often ignored or undervalued, with surplus food frequently redirected to animal feed or compost. However, growing alarms over resource reduction, climate change, and food security have encouraged a paradigm shift[9].

Moreover, concerning the climate change, the UNEP Food Waste Index 2021 Report[10] highlights the significant contribution of unconsumed food to global greenhouse gas emissions, estimating that it accounts for 8-10% of the total[11]. This finding underscores the urgent need to address food waste as a critical factor in climate change mitigation efforts[10].

As a result, the United Nations General Assembly approved the Sustainable Development Goals for 2030, which include a 50% reduction in food waste and at least a 25% reduction in food losses along supply and production chains[2]. To address this challenge, advanced supply chain management practices have emerged as a crucial strategy to optimize resource use and minimize waste[12].

Moreover, in 2020 The European Commission exposed the new Circular Economy Action Plan (CEAP)[13]. This initiative serves as a basis of the European Green Deal, a comprehensive strategy for sustainable growth in Europe[13]. By transitioning to a circular economy, the EU aims to improve pressure on natural resources, stimulate sustainable economic growth, create jobs, and contribute to achieving the 2050 climate neutrality goal[13]. The CEAP outlines the circular economy processes, sustainable consumption practices, and waste prevention, with the ultimate goal of maximizing resource use within the EU economy[13]. The circular economy model has been proposed as a framework for sustainable development by shifting from a linear economy (*Figure* 1 and *Figure* 2), where resources are extracted, used, and discarded, to a circular economy where waste is minimized, and materials are recovered and reused[3,4]. This circular approach aligns with the principles of sustainable development, promoting resource efficiency, reducing environmental impact, and enhancing economy[7,12].

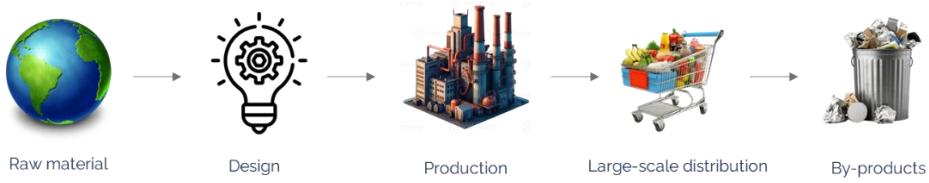


Figure 1. The linear economy model: extract, produce, consume, dispose

Figure 2. The Circular economy model: from Waste to New products

1.1.2 Agri-Food Waste: A Source of Bioactive Compounds

In the context of the circular economy, the food sector emerges as a prime candidate for adopting sustainable practices. Indeed, the food chain, about production, treatment, storage, processing, packaging, distribution, marketing, and consumption, generates billions of tons of agricultural food waste annually, including plant material such as fruits, vegetables, cereals, and their derivatives[5]. This waste stream represents a negative impact on the environment and human health. Therefore, the idea of finding innovative ideas for the reuse of food wastes arises. Knowing the unused potential of agri-food by-products, researchers have increasingly focused on valorizing these residues into high-value products. Agri-food by-products is a rich source of bioactive compounds, including phenols, peptides, polyphenols, tannins, alkaloids, carotenoids, terpenes, flavonoids, flavanols, anthocyanins, essential oils, carbohydrates, enzymes, fatty acids, dietary fibers, lipids, biopolymers, nitrogen compounds, minerals, vitamins, amino acids, and other phytoconstituents[5] (see *Figure* 3). Among these bioactive molecules, polyphenols, particularly flavonoids and phenolic acids

(hydroxybenzoic and hydroxycinnamic acids), are of particular interest due to their effective antioxidant and antimicrobial properties[14]. These compounds offer significant potential for the development of nutraceuticals, functional foods, and food additives. By employing advanced separation, identification, and characterization techniques, it is possible to extract and isolate these valuable bioactive molecules from agri-food by-products. These extracted compounds can then be used in many different industries, including food, pharmaceutical, and cosmetic sectors[5]. Current research activities are primarily directed towards the innovative application of agri-food by-products-derived bioactives to create novel, high-value products, thus contributing to a more sustainable and circular food system[5].

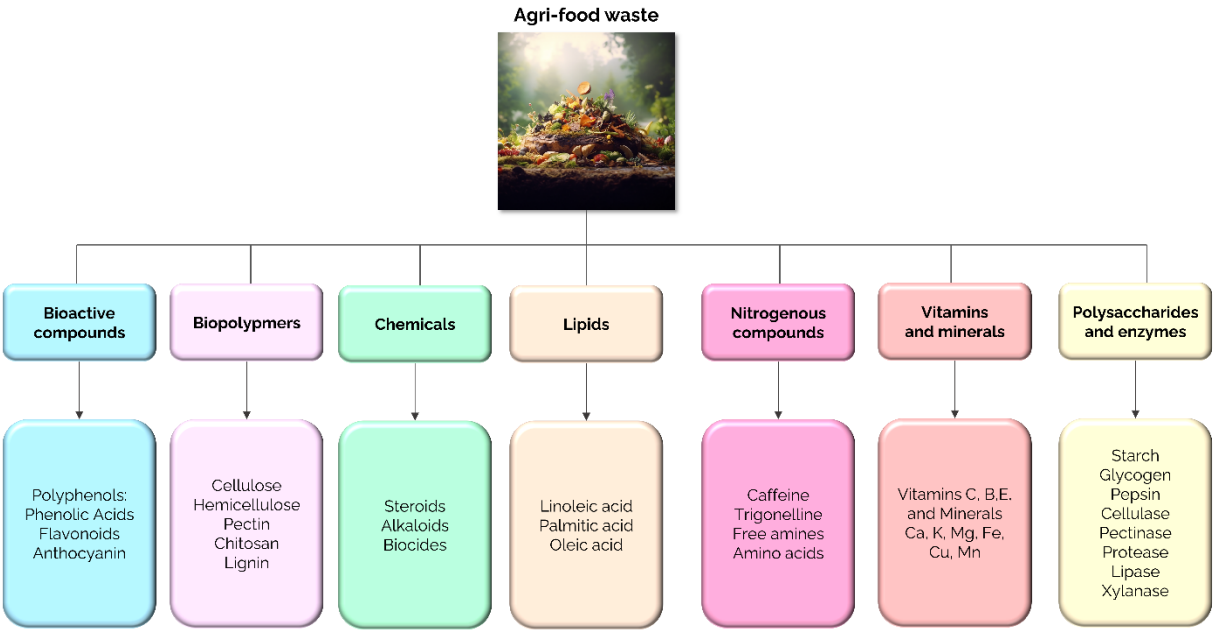


Figure 3 The Chemical Composition of Agri-Food Waste[5]

1.1.3 Pharmaceutical, nutraceutical, and cosmetic applications of agri-food by-products

The high amount of bioactive molecules within agri-food by-products has unlocked new paths for their use in the pharmaceutical, nutraceutical, and cosmetic industries. Indeed, agri-food waste serves as a rich source of phenolic compounds, including flavonoids and anthocyanins[15]. These compounds hold a wide range of therapeutic properties, such as antibacterial, antifungal, anti-inflammatory, and immunomodulatory activities, as well as significant antioxidant potential, which can mitigate oxidative stress and cellular damage[16]. Furthermore, they have exhibited antiviral properties, capable of inhibiting viral replication and cell infection. For instance, flavonoid compounds like tangeretin, nobiletin, and hesperidin have shown promise as potential antiviral agents[17]. In addition to their antiviral properties, bioactive compounds, particularly phenolic compounds, present in agri-food waste have potent antioxidant activity particularly interesting for the

cosmetics industry. These compounds effectively neutralize free radicals and protect cellular components from oxidative damage[15]. The cosmetic industry extensively exploits antioxidants in formulations such as creams, powders, and skin oils to prevent cellular aging and promote skin health[18]. Notably, antioxidants, when incorporated into sunscreen products, enhance UV protection, preventing collagen degradation and photoaging[19].

1.1.4 Valorization of Agri-Food By-products from Fruttagel®

This research aims to valorize underutilized by-products and waste generated from the industrial production of *Fruttagel*®, an agri-food company based in the Emilia-Romagna region. *Fruttagel*® specializes in the processing of fresh fruits, cereals, and legumes into a varied range of products, including fruit juices, tomato sauce, frozen legumes, and plant-based milk alternatives like soy milk. The fruit samples provided by the company included a variety of cultivars such as apples, apricots, peaches, and tomatoes. Similarly, the legume samples included peas, green beans, beans, and soy. All samples were grown using three different cultivation methods: biological, conventional, and "Lotta Integrata" (LI)[20]. These practices differ in the types and amount of pesticides used. Conventional agriculture is an intensive farming that maximizes land use and yields to meet global food demands. However, this approach is often based on heavy use of chemical pesticides and fertilizers, leading to significant environmental concerns and high energy consumption[21]. In contrast, biological farming, regulated by EU Regulation 2018/848, is emerging as a sustainable agricultural alternative. This method emphasizes the use of natural substances and processes, exploiting the soil fertility and respecting natural cycles. Although biological systems may produce lower yields than conventional methods, they are generally more environmentally friendly[21]. "Lotta Integrata" (LI) offers another approach to agricultural production, which represents a middle way between conventional and organic farming, aiming to minimize the use of chemicals and reduce environmental impact. This strategy balances economic and production needs with environmental and health considerations[22].

Concerning fruits, the agri-food consortium provided by-products generated during fruit juice production[23]. The various processing steps involved in juice production result in the separation and transformation of different fruit components, leading to the formation of by-products with distinct compositions and biomolecular profiles (see *Figure* 4)[24].

The specific fruit by-products examined in this study include apple pomace comprising pulp, peels, seeds, and stalks, constitutes approximately 25% of the total apple processed for juice production[24],[25].

Peach and apricot pomaces composed primarily of pulp and peel tissues, account for approximately 10% of the initial fruit weight used in juice production[26],[27]. Tomato by-products consisting mainly of seeds (60%) and skin (40%), represent nearly 5% of the total waste generated during tomato paste production[28] (see Figure 5). These by-products offer significant potential as sources of value-added compounds such as polyphenols[25]-[27],[29] which possess a wide range of bioactivities that contribute to human health. Their primary functions include

antioxidant and photoprotective activities, which help combat oxidative stress implicated in aging and degenerative diseases[30]-[31]. Plant-based proteins which provide nutritional value[28],[32]. Indeed, these peptides, derived from plant-based proteins, exhibit various bioactivities[33],[34].

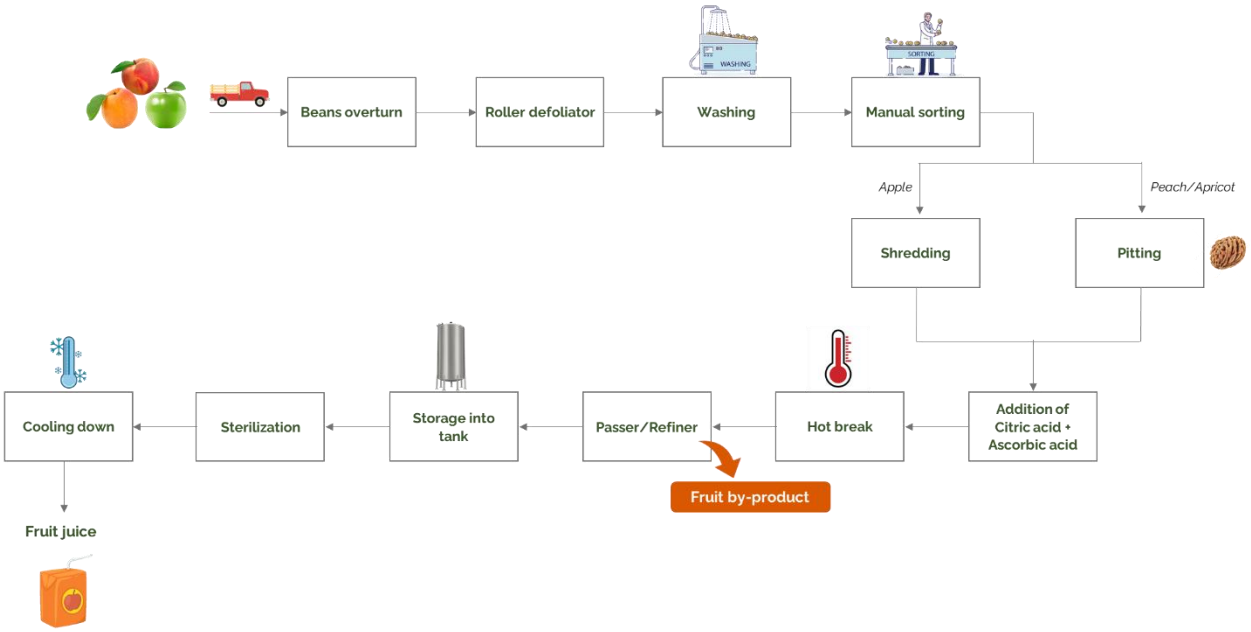


Figure 4 Fruttagel® Fruit juices production

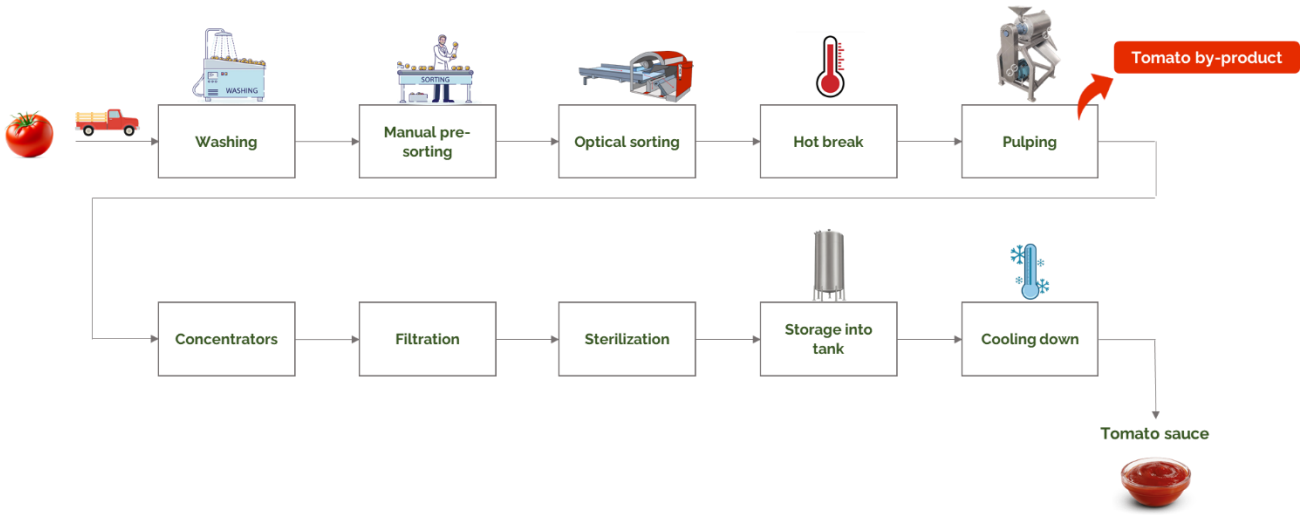


Figure 5 Fruttagel® Tomato sauce production

Regarding legumes, the agri-food consortium provided samples of both beans and green beans (*Phaseolus vulgaris L.*) from the final production of frozen legumes (see *Figure* 6). The bean samples included both by-products (peels, leaves, hulls) and the final product for sale, while the green bean samples consisted only of waste. Additionally, soy samples were provided, specifically the “soy raw material” refers to seeds used as the initial source in soy milk production while the "legume by-product", called okara, denotes the waste material

generated during the industrial processing stage (see *Figure* 7). The literature provides many examples showing that legume waste is still rich in active metabolites[5],[15]. Recent research by Sayegh et al. (2023)[35] investigated the impact of incorporating bean hull, a by-product of bean processing, into bread since it represents a dietary fiber and metabolites, including polyphenols, source. These results demonstrated the potential of bean peel-enriched bread in preventing chronic diseases revealing that regular consumption of such bread can reduced the risk of type 2 diabetes and the cancer development. In addition to bean hull, other agricultural by-products may still have potential for secondary applications in human health. For example, the nutritional value and potential health benefits of green beans has been already demonstrated[36]. Indeed, they are rich in proteins, vitamins, minerals, and dietary fiber, which have been linked to various health benefits, such as reduced risk of cardiovascular disease, improved digestive health, and potential cancer prevention[36]. While the majority of research focuses on the edible part, it is reasonable to hypothesize that green bean by-products, such as pods, may retain significant nutritional value. Given their similarity to the edible portion, it is likely that they share similar biochemical compositions, including the presence of essential nutrients and bioactive compounds. Moreover, the by-product of soy processing, okara, has been the subject of recent wide research regarding its potential for reuse. Recent literature studies have highlighted the potential of okara as a valuable source of protein, carbohydrates, and bioactive compounds, including isoflavones. This nutritional profile makes okara a promising substrate for microbial fermentation and a versatile ingredient in various food formulations. As reported by Swallah et al.[37], okara can be incorporated into a wide range of food products, such as beverages, bakery items, meat products, and confectionery or used to promote gut health and prevent chronic diseases like diabetes, hyperlipidemia, and obesity. Thus, okara represent a valuable food ingredient and a sustainable solution for food waste.

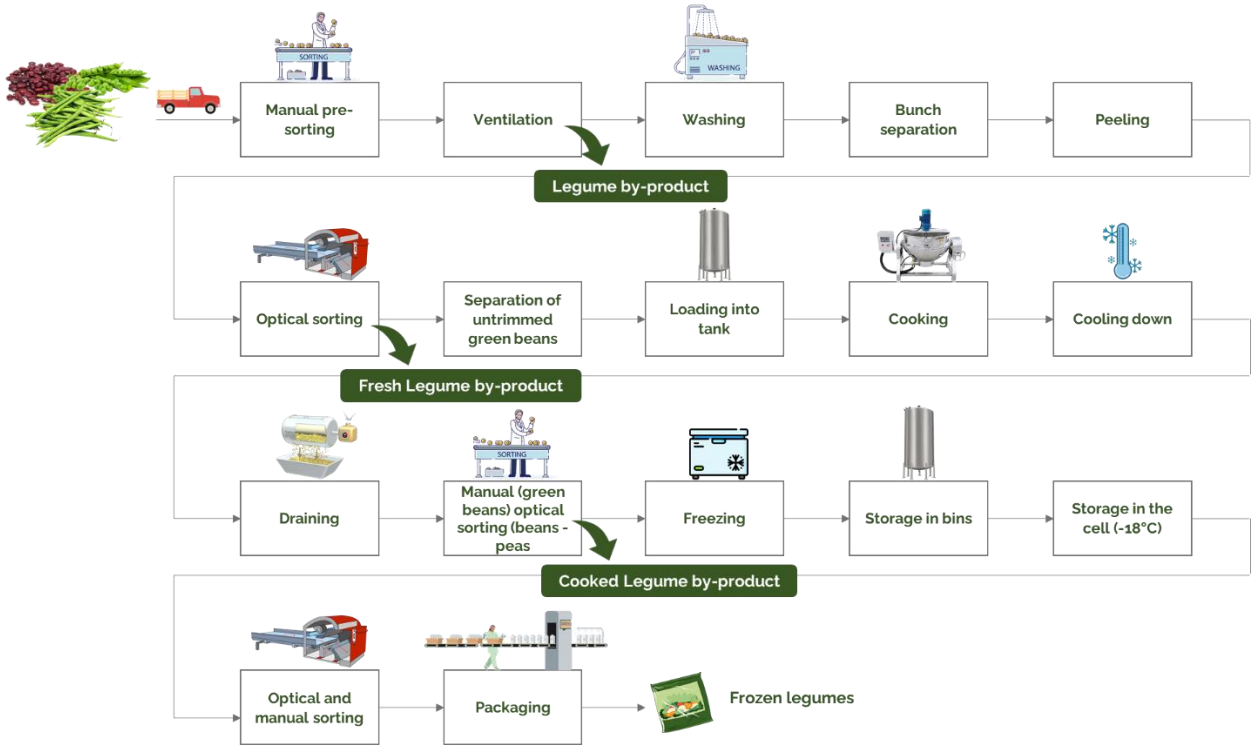


Figure 6 Fruttagel® Frozen Legumes production

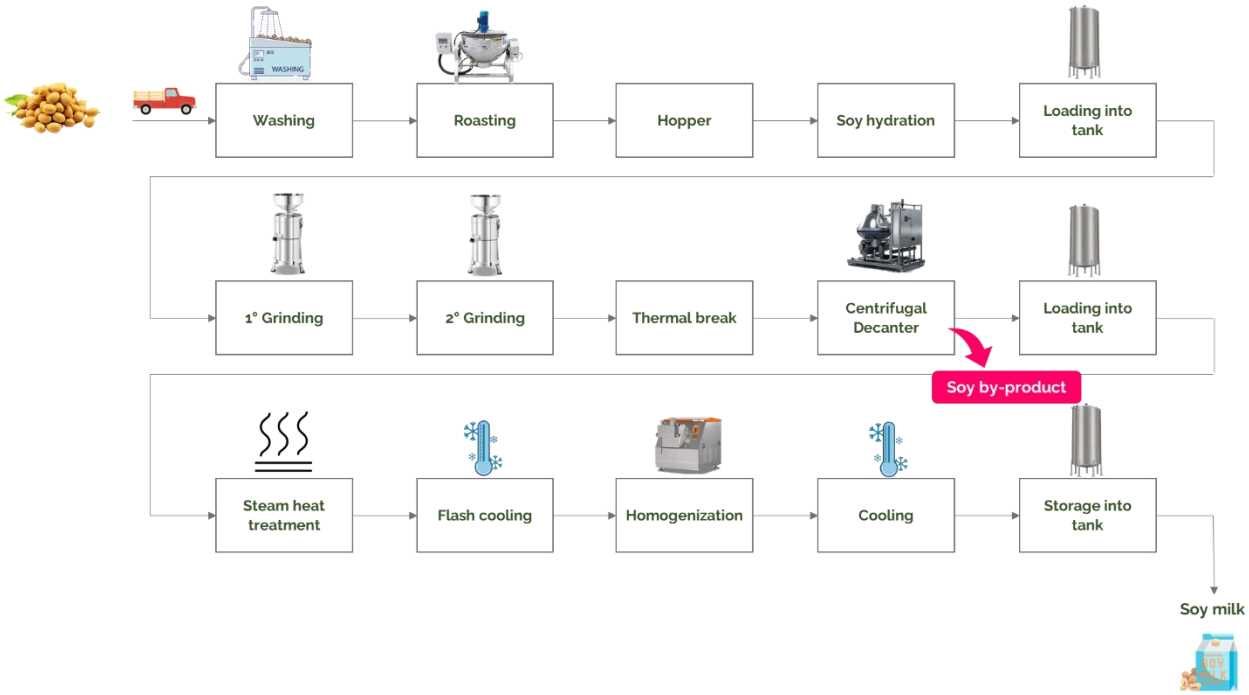


Figure 7 Fruttagel® Soy milk production

1.2 BIOACTIVE COMPOUNDS IN AGRI-FOOD WASTES

1.2.1 Polyphenols: chemical classification and beneficial effects on human health

In nature, plants produce a varied range of secondary metabolites known as polyphenols. These compounds are essential for plant survival and play critical roles in several biological functions. Indeed, polyphenols serve as a vital defense mechanism against biotic and abiotic stressors, including infections, ultraviolet radiation, and pollutants. They prevent herbivores and pathogens through the emission of unpleasant odors[38].

Furthermore, polyphenols significantly contribute to the sensory properties of plants and their derived foods. Anthocyanins, for instance, impart the red, blue, and purple colors observed in fruits like strawberries, plums, and grapes. Flavanones contribute to bitterness, as exemplified by olives, while proanthocyanidins give astringency, a characteristic flavor of wine[39].

From a chemical standpoint, polyphenols are organic compounds characterized by an aromatic ring with one or more hydroxyl groups. Despite this common structural feature, polyphenols exhibit remarkable variety and are classified into many subgroups with distinct properties.

Polyphenols are primarily categorized into two major groups: flavonoids and non-flavonoids[39],[40].

A) FLAVONOIDS

Flavonoids present a characteristic fifteen-carbon skeleton composed of two aromatic rings (A and B, *Figure* 8) linked by a heterocyclic pyran ring (C, *Figure* 8), resulting in a C6-C3-C6[39] structure. While the basic structure of flavonoids is aglycone, they are predominantly found in plants in a glycosylated form, with sugar moieties attached at various positions on the flavonoid skeleton. The most common glycosylation sites are positions 3 and 7. The types of sugars involved can include L-rhamnose, D-glucose, glucorhamnose, galactose, and arabinose.

The structural variety of flavonoids, particularly in terms of glycosylation patterns, significantly impacts their biological activities[41],[42].

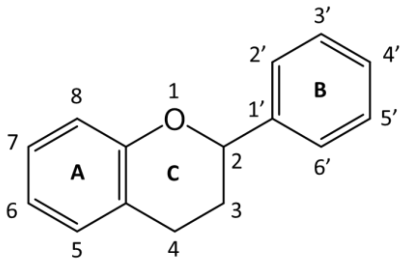


Figure 8 Flavonoids skeleton

The structural variety of flavonoids arises from variations in the linkage position between the heterocyclic ring

(C, *Figure* 9) and the second aromatic ring (B, *Figure* 9), as well as the degree of unsaturation and oxidation within the heterocycle. This structural variability gives rise to a wide range of subclasses, including flavones, flavonols, isoflavones, flavanones, flavan-3-ols, anthocyanidins, and anthocyanins. Additionally, chalcones, which serve as biosynthetic precursors to flavonoids, are also considered part of this family[39],[43].

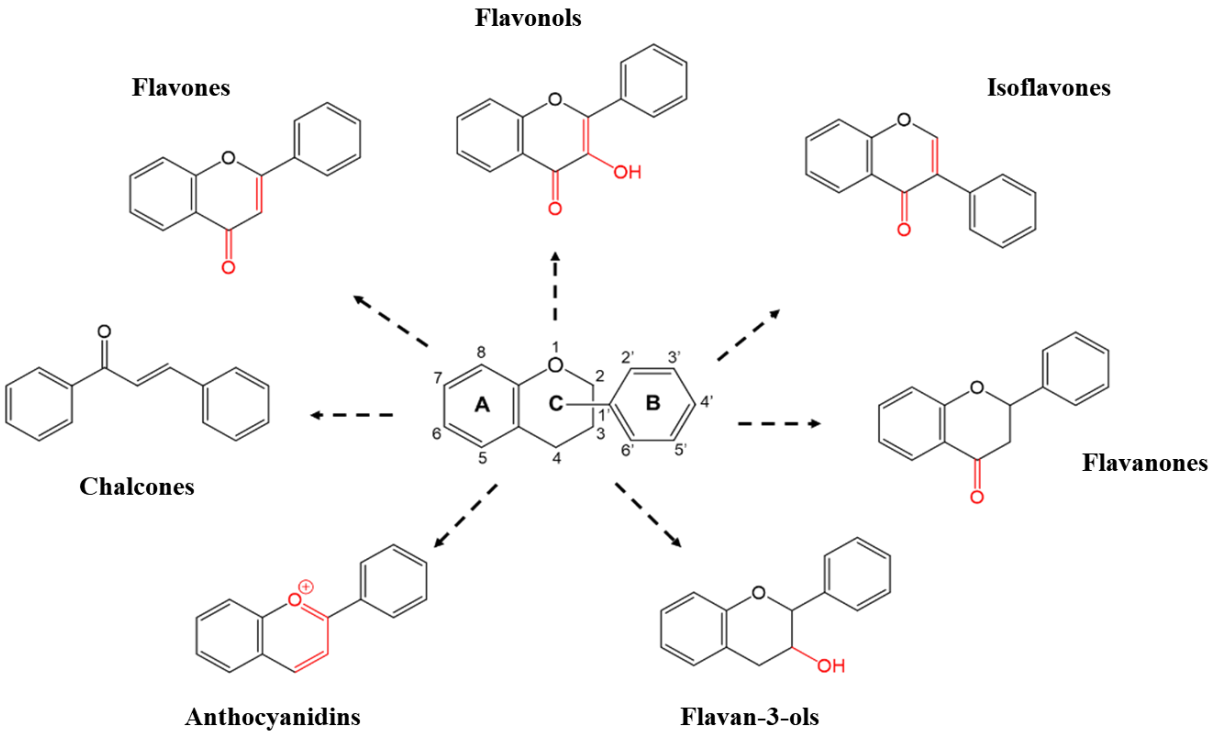
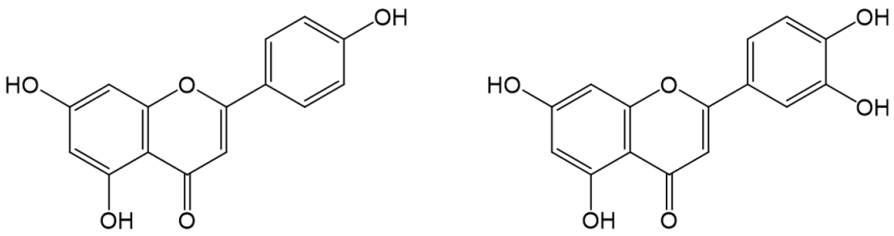


Figure 9 Subclasses of Favonoids

Flavones

Flavones are characterized by a carbonyl group at the C4 position, a B ring linked to the heterocyclic ring at C2, and a double bond between C2 and C3 (*Figure* 8)[42]. These compounds are primarily found in plants as glycosides, with apigenin and luteolin being notable examples (*Figure* 10)[42],[44].

Luteolin, in particular, stands out for its potent ability to modulate the immune response compared to other natural compounds. It exhibits a wide range of pharmacological properties, including antimicrobial, anti-inflammatory, anti-allergic, chemopreventive, chemotherapeutic, cardioprotective, anti-diabetic, and neuroprotective effects. Apigenin, another significant flavone, is recognized for its anti-inflammatory and antioxidant properties, which are mediated by inhibiting cytokine secretion and downregulating enzymes like COX-2[45]. Additionally, apigenin has been implicated in various therapeutic and health benefits, showing potential efficacy against conditions such as diabetes, amnesia, Alzheimer's disease, depression, insomnia, and cancer[40].



Apigenin **Luteolin**

Figure 10 Apigenin and Luteolin: major flavones

Flavonols

Flavonols are characterized by unsaturation between C2 and C3 in the heterocyclic ring and a ketone group at the C4 position (*Figure* 8). Distinguishing them from flavones is the presence of a hydroxyl group at the C3 position[42],[44]. The flavonol skeleton is essentially a 3-hydroxyflavone. The hydroxyl group at position 3 can undergo glycosylation or sugar binding.

Among the most documented and widely studied flavonols are quercetin, kaempferol, myricetin, and isorhamnetin (*Figure* 11). Quercetin and kaempferol, in particular, exhibit extensive structural diversity, with over 270 and 340 glycosidic forms, respectively, making them highly versatile compounds[46]. Quercetin, isoquercetin, kaempferol, and myricetin play crucial roles in modulating oxidative stress and treating inflammatory and infectious diseases. Kaempferol, specifically, is known for its anti-inflammatory, anti-tumor, and cardioprotective properties[40].

Another notable compound is quercetin-3-O-rutinoside, commonly known as rutin. Often referred to as "vitamin P," rutin is known for its ability to reduce blood vessel permeability. It has been extensively researched and marketed for its diverse pharmacological effects[45].

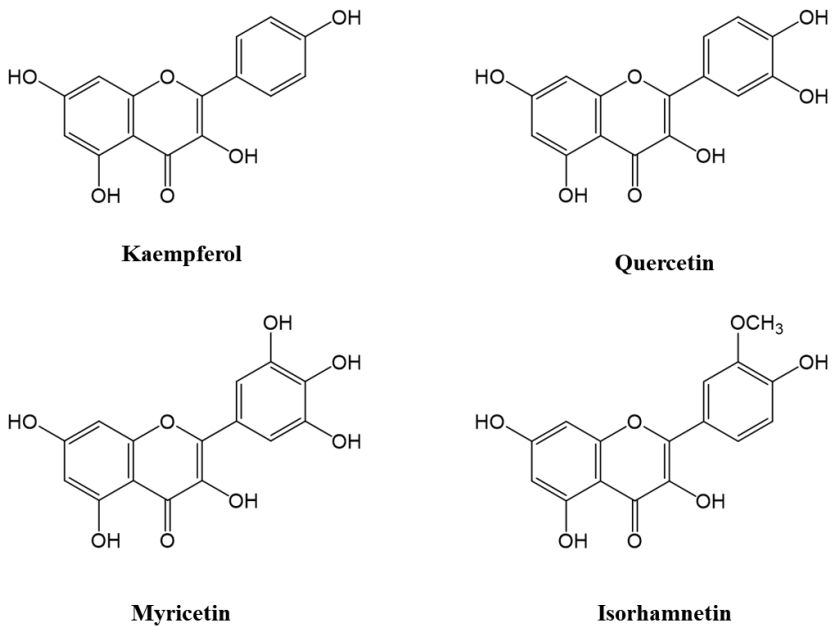


Figure 11 Flavonols

Isoflavones

Unlike most flavonoids, which have the B ring linked to the C ring at position 2, isoflavones are characterized by the B ring being connected to the heterocyclic ring (C) at position 3 (see *Figure* 8)[46],[47]. In plants, isoflavones exhibit antimicrobial activity and are produced as a defense mechanism against bacterial and fungal attacks. However, their occurrence is limited to specific taxa, primarily found in legumes. Soy and its derivatives, such as soy milk, tofu, tempeh, and miso, are the primary dietary sources of isoflavones for humans[47].

Despite not being steroids, isoflavones share structural similarities with estrogens, particularly estradiol. This similarity endows them with pseudohormonal properties, including the ability to bind to estrogen receptors. Consequently, they are classified as phytoestrogens, or plant estrogens. The most abundant isoflavones in legumes include genistein, daidzein, and glycitein (see *Figure* 12), which can be found in both aglycone and glycone forms[47].

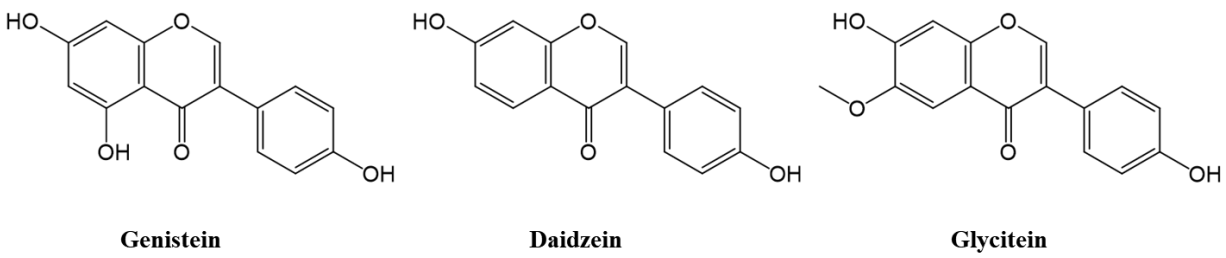


Figure 12 Isoflavones

Flavanones

Flavanones share structural similarities with flavones, featuring a carbonyl group at C4 but lacking a hydroxyl group at C3 (*Figure* 8). A key difference is the absence of a double bond between positions 2 and 3[47]. Glycosylation in flavanones typically occurs through disaccharide substitution at position C7. These compounds are primarily found in citrus fruits, such as oranges and lemons, contributing to the bitter taste of their juice and peel[46].

Most flavanones exist in aglycone form, with naringenin and hesperetin being notable examples (*Figure* 13)[47]. Research suggests that flavanone consumption may be linked to a reduced risk of diabetes and obesity[40]. Naringenin, in particular, has been extensively studied for its potential benefits against oxidative stress, inflammation, neurological disorders, and especially cardiovascular and metabolic diseases. It is documented for its ability to lower LDL cholesterol and triglycerides while raising HDL cholesterol and inhibiting glucose uptake[40].

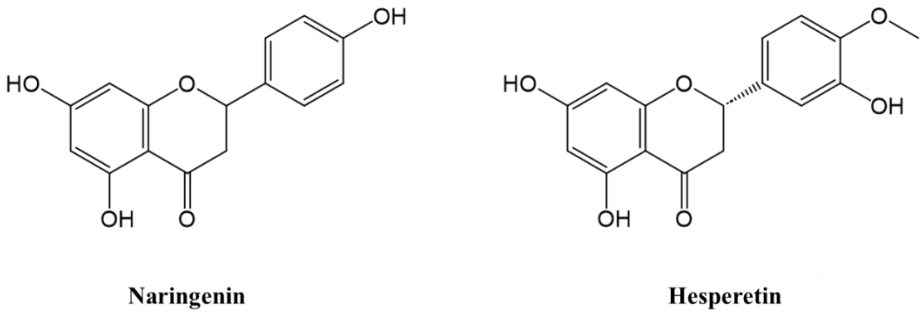


Figure 13 Flavanones

Flavan-3-ols

Flavan-3-ols, also known as flavanols, are a significant class of polyphenols characterized by a saturated heterocyclic ring (C, *Figure* 8) lacking a double bond between C2 and C3, and a carbonyl group at position 4 (*Figure* 8). They are often referred to as flavan-3-ols due to the hydroxyl group at the C3 position of the C ring. Primarily found as aglycones, flavanols are abundant in many fruits, including grapes, apples, blueberries, tea, and cocoa beans.

The hydroxyl group at C3 introduces two chiral centers, distinguishing catechins and epicatechins as diastereomers. Catechins have a trans configuration, represented by (+)-catechin and (-)-catechin, while epicatechins have a cis configuration, represented by (+)-epicatechin and (-)-epicatechin (*Figure* 14)[46]. Flavanols can also form conjugates with gallic acid, such as epicatechin gallate, epigallocatechin, and epigallocatechin gallate[48].

Catechins are the major polyphenols in tea, linked to its associated health benefits. Epigallocatechin gallate, the most abundant catechin in green tea, is thought to play a significant role in reducing vascular inflammation, lowering blood pressure, and decreasing oxidized LDL levels. Similarly, flavanols in cocoa and chocolate have garnered clinical attention for their potential in preventing cardiovascular and metabolic diseases[40].

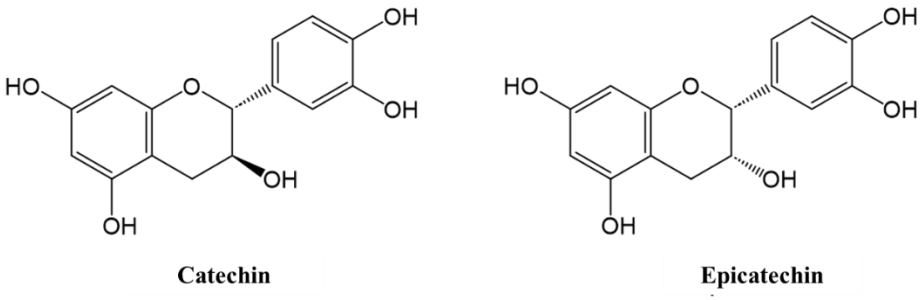


Figure 14 Flavan-3-ols

Anthocyanidins and anthocyanins

Anthocyanidins and anthocyanins are unique among flavonoids due to the presence of two double bonds in their heterocyclic rings. Anthocyanins, the glycosylated forms of anthocyanidins, are characterized by an oxonium ion in the C ring (*Figure* 8), contributing to their intense color[49]. They also exhibit specific patterns of hydroxylation and methoxylation on the B ring. Variations in the number of hydroxyl groups and the presence of sugar units, such as glucose, galactose, and arabinose, result in a diverse array of anthocyanins[46]. Anthocyanins are responsible for the red, blue, and purple colors observed in various fruits like strawberries, plums, grapes, and radishes, as well as certain vegetables and flower petals. Their color can change with pH levels, appearing red in acidic conditions and blue in basic conditions[39]. These compounds are of significant interest to humans due to their technological and health implications. From a technological perspective, they influence the sensory properties of products, while from a health standpoint, they are believed to protect against cardiovascular risks[50].

Chalcones

Chalcones represent a subclass of flavonoids notable for the absence of the C-ring, a characteristic feature of the basic flavonoid structure (*Figure* 15). This unique structure has led to their alternative name: open-chain flavonoids[47]. Key representatives of this class include phloridzin, arbutin, phloretin, and naringenin chalcone (*Figure* 15)[51]. These compounds are found in significant quantities in tomatoes, pears, strawberries, cranberries, and certain wheat products.

Chalcones and their derivatives have attracted significant interest due to their various nutritional and biological benefits. Dietary intake of flavonoids is considered a simple and safe method for potential disease prevention[51].

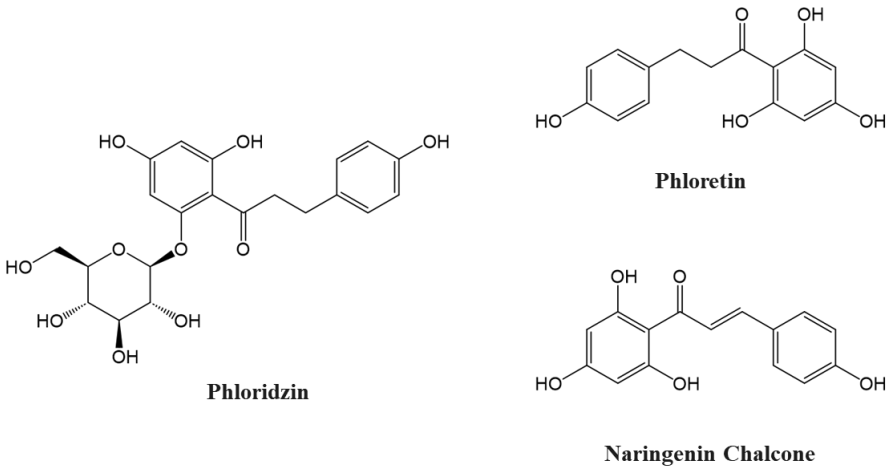


Figure 15 Chalcones

B) NOT-FLAVONOIDS

Polyphenols share a structural backbone characterized by multiple hydroxyl groups attached to aromatic rings. In contrast, non-flavonoids have a single aromatic ring, while tannins feature multiple aromatic rings. This family of polyphenols includes phenolic acids, stilbenes, lignans, and tannins[42],[39].

Phenolic Acids

Phenolic acids are non-flavonoid polyphenols characterized by a phenolic ring containing at least one carboxylic acid group. They are primarily categorized into two main types: benzoic acid derivatives and cinnamic acid derivatives, with C6-C1 and C6-C3 skeletons, respectively (*Figure* 16)[52],[53].

While fruits and vegetables primarily contain free phenolic acids, cereals and seeds predominantly contain bound forms. These bound forms can only be released through acid or alkaline hydrolysis or enzymatic action[52]. The main sources of phenolic acids include cranberries, pears, cherries, apples, oranges, grapefruits, cherry and apple juice, lemons, peaches, potatoes, lettuce, spinach, coffee, tea, and coffee beans[54].

The most common hydroxybenzoic acid derivatives include gallic acid, p-hydroxybenzoic acid, protocatechuic acid, vanillic acid, and syringic acid[40]. However, the levels of these hydroxybenzoic acids, whether free or esterified, are generally low in edible plants. As a result, they have not been extensively studied and are not currently considered to be of significant nutritional relevance[53].

In contrast, hydroxycinnamic acids are a predominant class of phenolic compounds, including caffeic acid, ferulic acid, p-coumaric acid, and sinapic acid among the most common[40],[54]. These hydroxycinnamic acids are widely distributed in foods, with chlorogenic acid being the most abundant. Chlorogenic acid, a combination of caffeic acid and quinic acid, is found in high concentrations in various fruits and coffee[53].

Interest in phenolic acids stems from their significant potential for food preservation and therapeutic applications. Biological studies on hydroxycinnamic acids have explored their possible beneficial effects on neurodegenerative diseases, highlighting their anti-inflammatory, antioxidant, and neuroprotective properties.

Hydroxybenzoic acids are primarily recognized for their antioxidant capabilities, which may offer benefits in managing chronic diseases. Additionally, epidemiological studies have revealed an inverse relationship between the consumption of hydroxybenzoic acids and the risk of cardiovascular diseases and obesity[40].

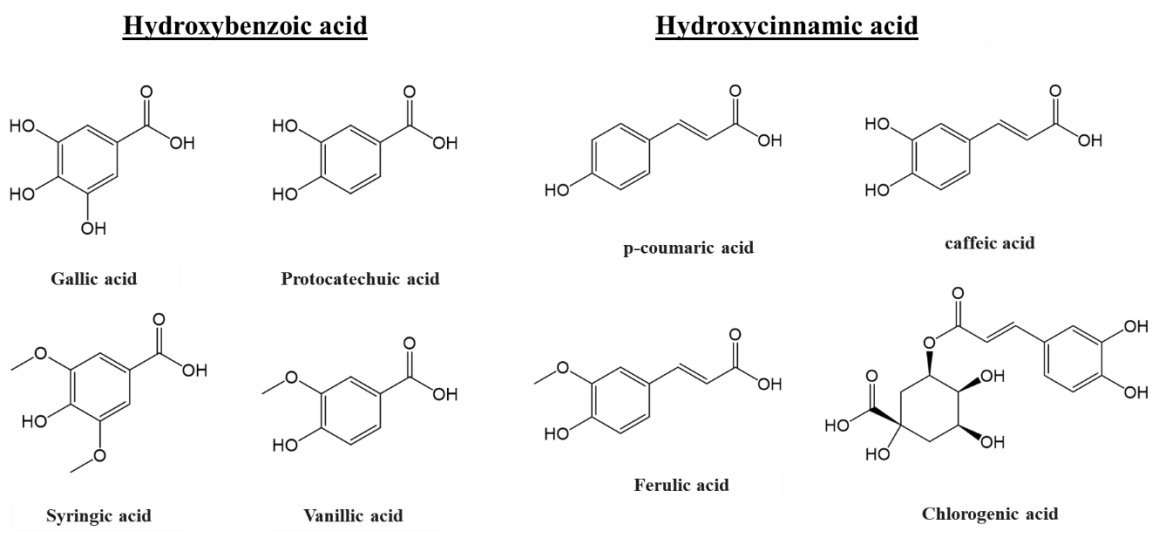


Figure 16 Phenolic acids

Stilbenes

Stilbenes possess a C6-C2-C6 structure, characterized by two benzene rings linked by a methylene bridge to two carbon atoms. These compounds can exist in two isomeric forms: cis and trans. They can be found in both free and glycosylated forms, with the latter being more abundant. Key dietary sources of stilbenes include grapes, wine, soybeans, peanuts, beans, blueberries, and cranberries[54].

These substances function as antifungal molecules synthesized in response to infections or injuries. Consumption of stilbenes has been associated with protection against oxidative stress related to age-related diseases, as well as a reduced risk of hypertension, diabetes, and obesity.

The most well-known and extensively studied stilbene is resveratrol, renowned for its high biological activity and primarily found in grapes and red wine[40],[54].

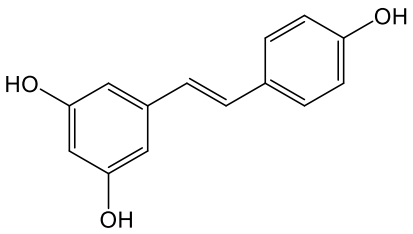


Figure 17 Stilbenes

Lignans

Lignans are a class of non-flavonoid compounds characterized by two phenylpropane units linked by a carbon-carbon bond between the β-positions at C8 of the propane side chains, referred to as the β-β' bond. Thus, their chemical structure can be represented as (C6-C3)2[46]. The C9 and C9' positions of lignans can undergo various substitutions, resulting in a diverse range of structural forms. Primarily, lignans are found in aglycone form, with a smaller presence of glycosides[46].

The primary dietary sources of lignans include oilseeds, particularly flaxseed, while secondary sources encompass whole grains, legumes, and vegetables such as garlic, asparagus, and carrots, as well as fruits like apricots, pears, peaches, and strawberries. Notable dietary lignans include secoisolariciresinol, matairesinol, lariciresinol, pinoresinol, medioresinol, and syringaresinol[53].

In the plant kingdom, lignans are produced as secondary metabolites, serving as defensive functions against pathogenic fungi and bacteria. They also possess antioxidant properties. Additionally, lignans are natural non-flavonoid phytoestrogens. Epidemiological and physiological studies in humans have demonstrated their beneficial effects in preventing lifestyle-related diseases, such as type II diabetes and certain estrogen-dependent tumors, including breast cancer in postmenopausal women[40].

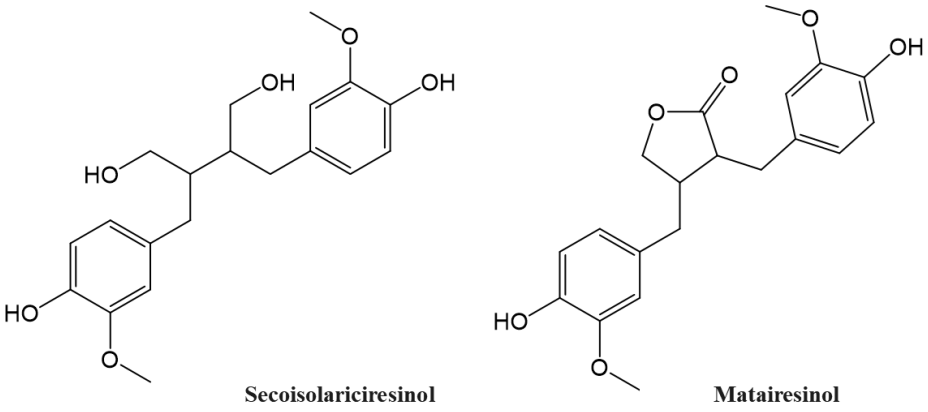


Figure 18 Lignans

Tannins

Tannins are water-soluble phenolic compounds with molecular weights ranging from 500 to 3000 Daltons. They can form strong complexes with carbohydrates and proteins and are classified into two main categories: hydrolyzable tannins and condensed tannins.

Hydrolyzable tannins are esters of gallic and ellagic acids, including gallotannins and ellagitannins. Condensed tannins, also known as proanthocyanidins, are oligomers or polymers composed of two to more than 200 flavan-3-ol monomers. Additionally, a third group known as phlorotannins, composed entirely of phloroglucinol units, has been isolated from various genera of brown algae[43],[54].

Proanthocyanidins significantly impact the sensory properties of foods by forming complexes with salivary proteins. They are responsible for the astringent taste found in fruits such as grapes, peaches, persimmons,

apples, pears, and berries, as well as in beverages like wine, cider, tea, and beer, and even contribute to the bitterness of chocolate[39,53].

Like many other polyphenols, tannins exhibit several biological effects, including antioxidant, antimicrobial, anticancer, and anti-inflammatory activities. However, it is important to be aware of the potential toxicity of tannins when consumed in excess. Their ability to form complexes with proteins and digestive enzymes may disrupt normal physiological activities in the body, increasing the risk of adverse health effects. Generally, it is not advisable to consume large quantities of tannins due to these potential health risks. Many questions about tannins remain, as they have not been studied as extensively as simpler polyphenols[55].

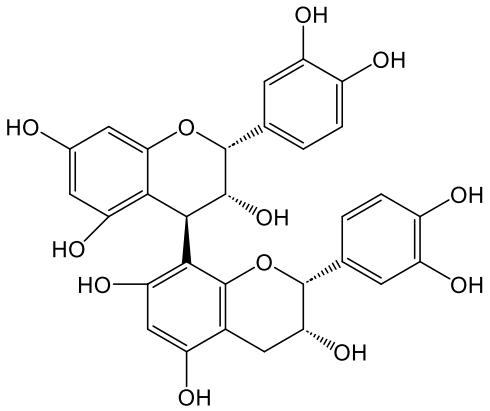


Figure 19 Tannins

1.2.2 Structure-Activity Relationship of Polyphenols: Impact on Antioxidant Activity

The antioxidant properties of polyphenols[42] are closely linked to their chemical structure, particularly the number and arrangement of hydroxyl groups. The primary mechanism of antioxidant action involves the scavenging of free radicals through hydrogen atom donation from the phenolic hydroxyl group[56]:

$$F\text{-}OH + R\bullet \rightarrow F\text{-}O\bullet + RH$$

where F-OH represents the phenolic compound, R• is the free radical, and F-O• is the phenoxyl radical.

In phenolic acids, antioxidant activity is primarily determined by the number and position of hydroxyl groups[57,58]. Gallic acid, with three hydroxyl groups, exhibits high antioxidant capacity. The electron-withdrawing nature of the carboxyl group in hydroxybenzoic acids generally decreases their H-donating ability. In contrast, hydroxycinnamic acids, with the presence of the electron-withdrawing but weaker -CH=CH-COOH side chain, tend to exhibit higher antioxidant activity than their benzoic acid counterparts[57,58].

The position of electron-donating substituents (such as hydroxyl or methoxy groups) on the aromatic ring significantly influences antioxidant activity. Ortho and para substitutions generally enhance antioxidant activity by increasing electron density on the aromatic ring and facilitating hydrogen atom donation[57].

In flavonoids, antioxidant activity is significantly influenced by the arrangement of functional groups on the

core structure[59]. Key structural features contributing to high radical scavenging capacity include:

- B ring 3',4'-catechol: The presence of two adjacent hydroxyl groups on the B ring enhances radical stability[59].
- 2,3-double bond conjugated with a 4-oxo function: This structural arrangement contributes to electron delocalization and increased radical stability[59].
- Free 3-hydroxyl group: This group is crucial for hydrogen atom donation and subsequent radical stabilization[59].

Structural modifications can significantly impact antioxidant activity. For example, O-methylation of hydroxyl groups can decrease antioxidant activity by perturbing the molecular planarity and reducing hydrogen-donating ability. Glycosylation typically reduces antioxidant activity due to steric hindrance and the removal of free hydroxyl groups[56]. Conversely, increasing the degree of polymerization in compounds like proanthocyanidins can enhance antioxidant activity against various radical species[56].

Quercetin serves as a prime example of a potent antioxidant flavonoid, fulfilling all the critical structural requirements. Rutin, the 3-O-rutinoside of quercetin, exhibits lower antioxidant activity due to glycosylation. Similarly, luteolin, lacking the 3-hydroxyl group, demonstrates reduced antioxidant capacity compared to quercetin. Flavan-3-ols, which lack the 2,3-double bond conjugated with the 4-oxo function, generally exhibit lower antioxidant activity than flavonols[57].

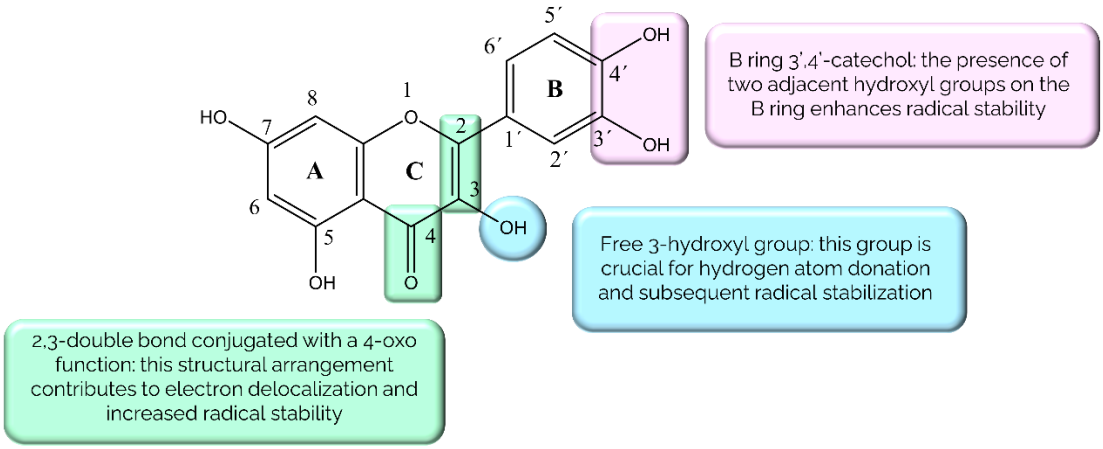


Figure 20 Flavonoids structure-antioxidant activity relationship[59]

1.2.3 Types of polyphenols in agri-food by-products under investigation

Knowing the potential of agri-food by-products to contribute to a more sustainable future following the circular economy perspective, numerous research studies have investigated their valorization. This chapter reviews the state-of-the-art research on by-products from apples, peach, apricots and tomatoes, highlighting their polyphenolic content, reported in *Figure* 21, and their subsequent potential applications on human health.

Concerning apple, as reported by Szabo et al.[60], its by-products has been demonstrated to be rich in phenolic compounds with significant concentrations found in the seeds, peel and stem. The major phenolic compounds in apple by-products include phlorizin from the dihydrochalcone family, chlorogenic acid from the hydroxycinnamic acid family, and epicatechin from the flavan-3-ol family[60]. Interestingly, phlorizin represents the prominent phenolic compound in apple by-products making them attractive for nutraceutical applications, given its potent antioxidant, anti-inflammatory, and antimicrobial properties[60]. Indeed, phlorizin has shown particular promise in managing diabetes, owing to its ability to influence glucose absorption and excretion. Literature studies on diabetic mice have demonstrated that a phlorizin-enriched diet can significantly mitigate blood glucose spikes[61].

Rodríguez-González et al.[62] led a research study on peach by-products (*Prunus persica L*), containing peel and remnant pulp, revealing their richness in polyphenols and dietary fiber. Specifically, these by-products exhibited high levels of 3-O-caffeoylquinic acid (chlorogenic acid), p-coumaroylquinic, and 4-feruloylquinic acids, as well as kaempferol derivatives. These findings underline the potential of peach by-products as valuable sources of bioactive compounds for functional foods and food supplements. Peach juice by-products, in particular, due to their high polyphenol content, have been shown to mitigate obesity-related complications[62]. A study reported in the literature[62] demonstrated that treatment of obese rats with hepatic steatosis using peach by-products resulted in significant hypoglycemic effects, improved insulin sensitivity, increased hepatic glycogen content, and decreased hepatic activity.

A research reported in the literature[63] has demonstrated the potential of apricot (*Prunus armeniaca L.*) by-products, particularly the leaves, as functional nutraceutical and bioactive ingredients. This study[63] identified twelve phenolic compounds in apricot leaves, with 3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, and 5-O-caffeoylquinic acid being the most predominant phenolic compounds. These compounds are analogous to those found in peach by-products[62] (mentioned above). Additionally, apricot leaves were rich in quercetin-3-O-glucosides and kaempferol. Given the similarity in polyphenol profiles of peach by-products, these results suggest that apricot by-products hold significant promise for preventing cardiovascular diseases, diabetes, and obesity[62].

Literature study[60] indicates that tomato by-products are rich in polyphenols, particularly tomato peels, which contain 83% of the total flavonoid content. While tomato processing by-products are primarily rich in carotenoids, such as lycopene, the synergistic effects with other bioactive compounds, including polyphenols, contribute to their overall antioxidant and antimicrobial activities[64]. The valorization of tomato by-products represents an opportunity to use these natural bioactive molecules for human health. Among polyphenols, quercetin, naringenin, and rutin are the predominant compounds in tomato peels and by-products. These compounds exhibit significant antioxidant and antimicrobial properties[60]. Additionally, due to their hydrophobic nature, flavonoid polyphenols can penetrate bacterial cell membranes, exerting their antibacterial activity within the cell[60].

Figure 21 Most common polyphenols found in fruit waste, based on previous studies[60-62]

Moving to legume samples (*Figure* 22), it has been already reported in literature by Colletti et al.[65] that okara, the by-product of soymilk production, is rich in polyphenols, particularly isoflavones. Among them, the specific isoflavones content in okara includes aglycone isoflavones such as daidzein, genistein, and glycitein[65]. The pharmaceutical interest in these compounds stems from their role as phytoestrogens, which has been extensively investigated in the literature. Isoflavones are believed to have potential benefits in preventing hormone-related cancers, osteoporosis, and menopausal symptoms[66].

Species of *Phaseolus vulgaris L.*, including both beans and green beans, have been documented for their rich polyphenol content. A study by Yang et al.[67] investigated the polyphenol composition of various bean species, identifying a significant presence of both phenolic acids and flavonoids. While this study examined the entire plant matrix, it is reasonable to assume that bean by-products also contain substantial amounts of these bioactive compounds.

Zilani et al.[68] investigated the polyphenol content of peas (*Pisum sativum L.*). Among the phenolic compounds found, ellagic acid has been shown to enhance pancreatic secretion, increase glucose absorption and glycogen storage in the liver and muscles, reduce glucose intolerance, and inhibit glucose absorption by inhibiting glucosidase[69]. The flavonoid kaempferol has been reported to improve insulin-stimulated glucose uptake in mature adipocytes[70], while naringenin suppresses carbohydrate absorption from the intestine, exerting an extra-pancreatic effect[71]. These findings suggest potential mechanisms underlying the antidiabetic activity of pea extracts.

Figure 22 Most common polyphenols found in legume and legume wasste, based on previous studies[65-71]

1.2.4 Proteins and peptides

Proteins are macromolecules composed of linear polymers of amino acid residues linked by peptide bonds[32]. They exhibit diverse structural levels, including primary, secondary, tertiary, and quaternary structures[72]. In plants, proteins fulfill crucial roles, serving as enzymes, structural components, and functional molecules. Moreover, they act as storage reserves to support the growth and development of seedlings[73]. Plants possess the biosynthetic capacity to synthesize all 20 proteinogenic amino acids, encompassing alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine[74]. The unique combination of amino acids, their sequence, and the intricate interactions between them determine the protein's physicochemical properties, including structure, surface hydrophobicity, net charge, and the presence of reactive groups[32]. These properties, in turn, significantly influence the protein's biological functions[75]. Based on their structural characteristics, proteins can be broadly classified as globular, fibrous, or flexible. While fibrous proteins are generally water-insoluble, globular proteins exhibit varying degrees of solubility in water, acids, and bases. Notably, plant proteins predominantly consist of globular proteins, often existing as multimeric complexes linked by covalent bonds. These proteins are further categorized into distinct groups: albumins (water-soluble), globulins (soluble in dilute salt solutions), prolamins (soluble in aqueous ethanol solutions), and glutelins (soluble in dilute acids or alkalis, or insoluble in water). Albumins and globulins constitute the major protein fractions in most pulses (>50%) and some pseudocereals (e.g., quinoa, amaranth). In contrast, prolamins (predominant in wheat, maize, barley, and rye) and glutelins (predominant in wheat) represent a significant portion of the total protein content in cereals and pseudocereals[32]. Legumes, such as beans, peas, and lentils, are recognized for their substantial protein content[72]. Bioactive peptides, derived from proteins, can be classified as endogenous or exogenous. Exogenous peptides are inactive within their parent protein source but are released through proteolytic processes, such as fermentation, hydrolysis, and food

processing. In contrast, endogenous peptides exert biological activities within the plant itself, playing roles in defense mechanisms, cell division control, and reproductive processes. A vast array of both endogenous and exogenous bioactive peptides has been identified in plant sources, including cereals and legumes[76].

1.3 ANALYTICAL CHARACTERIZATION OF POLYPHENOLS AND PROTEINS

1.3.1 Pre-treatment and extraction techniques

Extraction of bioactive compounds from plant matrices is a complex task due to the inherent complexity of these matrices. Indeed, plant matrices exhibit significant heterogeneity, including a different range of compounds. Moreover, bioactive compounds are often confined within the inner cellular areas of fruit, vegetable, and legume, requiring targeted extraction processes for their release[5].

The suitability of an extraction technology depends on several critical factors, including the desired purity of the extract, the physicochemical properties of the target compound, its localization within the vegetal matrices (both free and bound within cellular structures), and the economic feasibility and value of the resulting product[5]. Before the extraction process, many preliminary procedure or pre-treatments are typically applied to enhance yield of bioactives extracted[5]. Examples of these operations include washing, cutting, size reduction, and drying[77]. It has been demonstrated that an appropriate sample preparation, including drying (freeze-drying is generally preferred over air-drying to preserve phenolic content) and size reduction (milling, grinding) is essential for efficient extraction[78].

Extraction techniques are largely categorized into conventional and non-conventional methods. Conventional or traditional methods include Soxhlet extraction, maceration, infusion, percolation, decoction, steam distillation, and hydrodistillation[5]. The problems of conventional techniques include high organic solvent consumption, stringent purity requirements, elevated costs, reduced extraction efficiency, prolonged processing times, high temperatures, and the release of potentially damaging residues[5].

On the contrary, non-conventional extraction techniques such as supercritical fluid extraction (SFE), pulsed electric fields (PEF), ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), enzyme-assisted extraction (EAE), and pressurized liquid extraction (PLE) offer several advantages, including economic feasibility, enhanced safety, and innovative process design, making them increasingly preferred in contemporary applications[5]. Concerning extraction solvent, selection is crucial, with factors like polarity and compatibility with target compounds considered. Common solvents include methanol, ethanol, acetone, and water. Additionally, acidification of solvents (with weak acids like formic acid) is often necessary for stabilizing and extracting compounds like anthocyanins[78]. Many factors influence the extraction such as solvent-to-solid ratio that impacts extraction yield, with an optimal balance needed; particle size, smaller

particle size generally enhances extraction efficiency and temperature and time: higher temperatures can increase extraction rates but also increase the risk of compound degradation[78].

Furthermore, it is crucial to rigorously evaluate the economic feasibility and applicability of the extraction process, particularly within sectors such as agri-food and pharmaceuticals, where ensuring the production of specific compounds with low or non-toxicity is necessary[5].

Regarding safety, the potential toxicity associated with different solvents in the extracts, coupled with low yields, has encouraged a growing interest in green and modern extraction technologies to maximize product yield while minimizing the adverse environmental impact of solvents[5]. From an economic perspective, the desired phytochemicals should be extracted using appropriate methods that facilitate the recovery of all value-added components, allowing the complete use of agri-food wastes[5].

Emergent green extraction techniques are founded on non-thermal principles, aimed at facilitating extraction without the risk of overheating the waste matrix while simultaneously reducing energy consumption[5]. Among the targeted solvents, water is frequently employed, with ethanol emerging as a favorite alternative. In conclusion, non-conventional extraction methods offer several advantages over conventional approaches, notably enhanced selectivity in the isolation of bioactive compounds, allowing the production of safe extracts, reduced energy consumption, and minimized environmental impact[5].

1.3.2 Analytical characterization of polyphenols

Following extraction, the potential of bioactive compounds can be measured based on their characteristic properties. In the case of polyphenols, antioxidant (radical scavenging capacity) and antimicrobial (microbial inhibition) activities are of primary significance[5].

Antioxidant activity is extensively investigated through the radical scavenging mechanisms of the active compounds, employing methods such as the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) assay. In addition to these, the ferric reducing antioxidant power (FRAP) assay measures the capacity of bioactive compounds to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). Furthermore, the oxygen radical absorbance capacity (ORAC) assay evaluates the ability of bioactive compounds to scavenge peroxyl radicals, resulting in a diminished level of fluorescence inhibition within the reaction[5].

Traditional spectrophotometric assays offer convenient and rapid screening methods for quantifying classes of phenolic compounds within crude plant extracts. However, the complexity of plant phenolic compounds, coupled with their varying reactivity towards assay reagents, requires the use of a different range of analytical methods[78]. Several techniques are employed for the separation and identification of bioactive compounds, including gas chromatography-mass spectrometry (GC-MS), ultra-high-performance liquid chromatography (UHPLC), and attenuated total reflectance infrared spectroscopy (ATR-FTIR)[5].

Gas chromatography (GC) has been widely employed, particularly for the analysis of phenolic acids and flavonoids. However, the characteristic low volatility of these compounds requires derivatization before the GC analysis, such as methylation or trimethylsilylation[78].

High-performance liquid chromatography (HPLC) has emerged as the favorite technique for analyzing phenolic compounds due to its versatility and reliability. Reversed-phase chromatography using C18 columns is the most widely used approach. Mobile phases typically consist of acetonitrile or methanol, often acidified with acetic, formic, or phosphoric acid, to optimize separation. Both isocratic and gradient elution modes are employed, depending on the complexity of the sample matrix. HPLC allows simultaneous analysis of multiple phenolic compounds and their potential derivatives or degradation products[78].

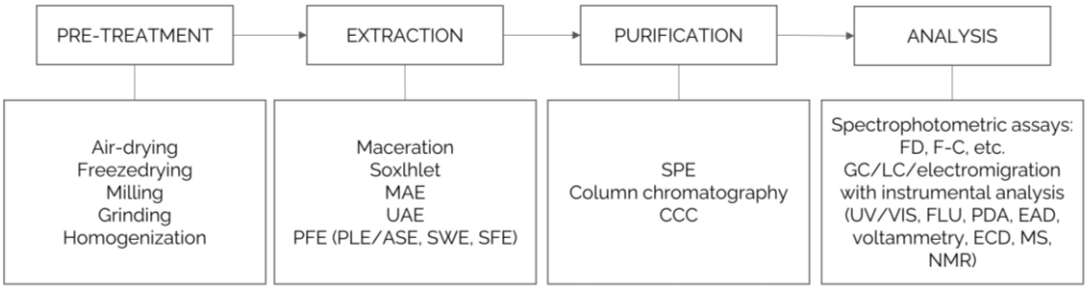


Figure 23 From Sample to Analysis[78]

1.3.3 Protein Extraction and Quantification from Plant Matrices

Several methodologies are employed for extracting plant-based proteins, including acid, alkaline, and ionic strength modulation. Alkaline extraction, utilizing a medium with a pH significantly above the protein's isoelectric point (pI), enhances protein solubility by disrupting electrostatic interactions. Conversely, acid extraction exploits protein solubility at acidic pH values below the pI. Salt extraction influences the "salting-in" and "salting-out" phenomenon, where increasing salt concentration initially increases protein solubility followed by precipitation at higher salt concentrations[34].

To improve extraction efficiency, various pre-treatments are used, including enzymatic and physical methods. Ultrasound-assisted extraction (UAE) has gained significant attention due to its ability to disrupt plant cell walls through cavitation, leading to enhanced protein release[34].

Protein quantification in food samples is commonly achieved through the determination of total nitrogen content. The Kjeldahl and Dumas methods are widely used for this purpose[79]. The Kjeldahl method involves the conversion of organic nitrogen to ammonium ions, followed by acid-base titration. The Dumas method combusts the sample, releasing nitrogen gas, which is then quantified using a thermal conductivity detector. However, these indirect methods can overestimate protein content due to the presence of non-protein nitrogenous compounds (like nitrates, nitrites, nucleic acids)[79].

Direct protein determination based on amino acid analysis offers a more accurate approach. This method involves hydrolyzing the protein into its constituent amino acids, followed by their separation and quantification using liquid chromatography (LC) with post-column ninhydrin derivatization[79]. The protein content is then calculated by summing the individual amino acid residues after subtracting the molecular mass of water. Amino acid analysis provides a more accurate assessment of protein content as it eliminates interference from non-protein nitrogenous compounds[79].

2 DEVELOPMENT AND APPLICATION OF ANALYTICAL METHODS FOR CHEMICAL AND FUNCTIONAL CHARACTERIZATION OF POLYPHENOLS IN AGRI-FOODS

2.1 MATERIAL AND METHODS

2.1.1 Chemicals and Reagents

Methanol for HPLC-MS, Water for HPLC-MS, Acetic acid for HPLC-MS, Methanol HPLC grade ≥ 99.9%, methanol ≥ 99.8%, acetone HPLC grade ≥ 99.8%, ethanol and acetic acid ≥ 99.8%, potassium persulfate ≥ 99.0%, ABTS (2,2-azinobis-(3ethylbenzothiazoline-6-sulfonate), Trolox® (6-hydroxy-2,5,7,8-tetramethyl-3,4-dihydrochro mene-2-carboxylic acid), sodium hydroxide ≥ 98% in pellets, sodium carbonate ≥ 99.5%, gallic acid ≥ 95%, protocatechuic acid ≥ 97%, trans-cinnamic acid ≥ 98.0%, caffeic acid ≥ 98.0%, p-coumaric acid ≥ 98.0%, ferulic acid ≥ 99.5%, (+)-catechin ≥ 99.0%, isoquercitrin ≥ 98.0%, daidzein ≥ 97.0%, genistein ≥ 97.0%, and phloridzin dihydrate ≥ 98.5% were purchased from Sigma-Aldrich (Taufkir-chen, Germany); chlorogenic acid ≥98.0% was purchased from Apollo Scientific (Bredbury, UK); and Quercitrin ≥ 98% was purchased from Cayman Chemical (Ann Arbor, MI, USA). Folin–Ciocalteu reagent was purchased from VWR Chemicals (Darmstadt, Germany); copper (II) sulfate pentahydrate and potassium sodium tartrate tetrahydrate EMSURE® were purchased from Merck (Darmstadt, Germany); (−)-epicatechin ≥ 97.0%, hesperetin ≥ 97.0%, and apigenin ≥ 97.0% were purchased from TCI (Zwijndrecht, Belgium); hyperoside ≥ 92.0% was purchased from HWI group (Rulzheim, Germany); (+)-rutin trihydrate ≥ 97% was purchased from Alfa Aesar (Haverhill, MA, USA); myricetin ≥ 98%, naringenin ≥ 97% and kaempferol ≥ 98.0% were purchased from ThermoFisher (Kandel, Germany); and naringenin chalcone ≥ 95% was purchased from PhytoLab (Vestenbergsgreuth, Germany). Ultrapure (type 1) water for HPLC was obtained using a Direct-Q® 5 UV system (Merck, Darmstadt, Germany). VWR Syringe Filter, nylon, 0.45 μm, was purchased from VWR International Srl (Milan, Italy). VWR Syringe Filter, PTFE, 0.22 μm, was purchased from VWR International Srl (Milan, Italy).

2.1.2 Equipment

The analytical HPLC system employed was Agilent 1260 Infinity, equipped with ChemStation software 3D system Rev. B 04.03, G1312C 1260 Bin Pump VL, and G1315D 1260 DAD VL detector (Agilent, Santa Clara, CA, USA), a Kinetex XB-C18 column (4.6 mm × 150 mm, 5 μm, 100 Å) (Phenomenex, Torrance, CA, USA). The Spectrophotometer: Jasco UV-VIS V-630 (Jasco Europe, Lecco, Italy). Ultrasound Bath: Elmasonic S 40 H (GEASS S.R.L., Turin, Italy). The centrifuge: Awel International MF 20-R multifunction centrifuge (MedWrench, East Point, GA, USA). Freeze-dryer: Alpha 1-4 LO plus (Martin Christ, Harz, Germany).

Rotatory evaporator: IKA Rotary Evaporators RV 10 basic (IKA-Werke GmbH & Co. KG, Staufen im Breisgau, Germany). Dionex UltiMate 3000 RS system including a Diode Array Detector (DAD) coupled to a LTQ XLTM linear ion-trap mass spectrometer-ESI ion source from Thermo Fisher Scientific (Waltham, MA, USA). The Thermo XCalibur software, version 3.1.66.10 (Waltham, MA, USA), was employed for the acquisition of mass chromatograms and spectral data. Ultrasound Bath from Bandelin Sonorex (Berlin, Germany); Analytical balance from Sartorius; Centrifuge from Heraeus Biofuge Pico (Barcellona, Spain).

2.1.3 Agri-food samples

Materials derived from fruits (such as apricot, peach, apple juice, and tomato sauce) and legumes (including beans, peas, green beans, and soy) were supplied by ***Fruttagel***® S.C.p.A (Alfonsine, Italy). The vegetal matrices were freeze-dried by the supplier and were then processed in two grinding steps. In the first step, a domestic mixer was used to break down the solid materials and reduce their particle size. The second step involved milling with the IKA Tube Mill 100 control to obtain fine powders. These powders were subsequently cryo-lyophilized at -60 °C for 24 hours and stored at -20 °C until further use. The fruit and legume by-products (ByP), the final products (FinalP) and the raw material (RM) being studied are listed in ***Table*** 1and ***Table*** 2.

Table 1 Fruit samples

Sample		Acronym
Apricot	Biological by-product	*Ac-Bio-ByP*
	Conventional by-product	*Ac-Conv-ByP*
	Biological final product	*Ac-Bio-FinalP*
	Conventional final product	*Ac-Conv-FinalP*
Peach	Biological by-product	*Pch-Bio-ByP*
	Conventional by-product	*Pch-Conv-ByP*
	Biological final product	*Pch-Bio-FinalP*
	Conventional final product	*Pch-Conv-FinalP*
Apple	Biological by-product	*Apl-Bio-ByP*
	Conventional by-product	*Apl-Conv-ByP*
	Apple final product	*Apl-FinalP*
Tomato	Biological peels	*T-Bio-ByP*
	LI* peels	*T-LI-ByP*
	Biological final product	*T-Bio-FinalP*
	Conventional final product	*T-Conv-FinalP*

Table 2 Legume samples

Sample		Acronym
Beans	Biological by-product	*B-Bio-ByP*
	Conventional by-product	*B-Conv-ByP*
	Final product	*B-FinalP*
	Biological fresh by-product	*B-Fr-Bio-ByP*
	Biological cooked by-product	*B-Co-Bio-ByP*
	LI* fresh by-product	*B-Fr-LI-ByP*

	LI* cooked by-product	*B-Co-LI-ByP*
Green Beans	LI* fresh by-product	*GB-Fr-LI-ByP*
	LI* cooked by-product	*GB-Co-LI-ByP*
	Biological fresh by-product	*GB-Fr-Bio-ByP*
	Biological cooked by-product	*GB-Co-Bio-ByP*
Soy	Biological raw material	*S-Bio-RM*
	Conventional raw material	*S-Conv-RM*
	Biological by-product	*S-Bio-ByP*
Peas	Biological fresh by-product	*P-Fr-Bio-ByP*
	Conventional fresh by-product	*P-Fr-Conv-ByP*
	Final product	*P-FinalP*

Lotta integrata is a crop defense practice that aims to reduce pesticide use through the implementation of multiple measures. It is the intermedium between conventional and biological methodologies*

2.1.4 Ultrasound-Assisted Extraction (UAE) procedure of Polyphenols from Agri-Food Samples

Approximately 0.5 grams of each lyophilized sample was weighed (n = 3), and 4 mL of a 50:50 methanol:water mixture containing 3.4% acetic acid (Solution A) was added. The resulting suspension was vortexed for 1 minute and then subjected to an ultrasonic bath (Elmasonic S 40 H) at 25 °C for 15 minutes. Afterward, the solution was centrifuged at 3680× g (4400 rpm) at 20 °C for 15 minutes, and the supernatant was collected in a 50 mL Falcon tube. This process was repeated two more times. The residual biomass underwent a second extraction step with 4 mL of a 70:30 acetone:water solution (Solution B). This mixture was also vortexed for 1 minute and treated with ultrasound at 25 °C for 15 minutes. The solution was then centrifuged at 3680× g (4400 rpm) at 20 °C for 15 minutes, and the supernatant was placed in a 50 mL Falcon tube. This extraction procedure was carried out twice more. Once all six supernatants were collected, a final centrifugation at 3680× g (4400 rpm) at 20 °C for 5 minutes was performed to remove any residual particles. The obtained extracts were filtered through a nylon 0.45 μm filter, transferred to a round-bottom flask, and dried using a rotary evaporator at 40 °C followed by cryo-lyophilization for 24 hours. The dried extracts were weighed for gravimetric determination and then stored in plastic Eppendorf tubes at −80 °C until use. The entire procedure is reported in *Figure* 24.

The UAE extraction method detailed above has been reported in the literature[23],[80].

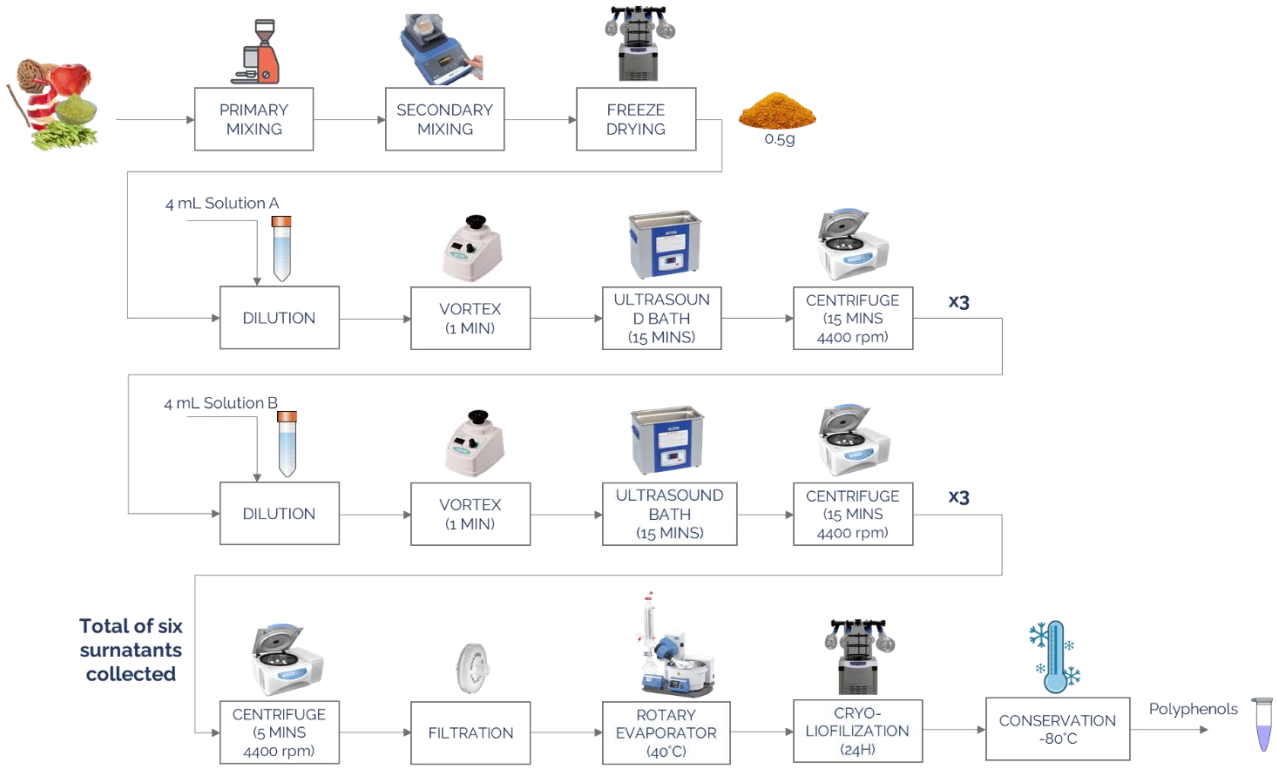


Figure 24 Ultrasound-Assisted Extraction (UAE) procedure

2.1.5 Gravimetric Determination (Yield of Extraction)

Gravimetric determination was performed on all dried extracts obtained through UAE to calculate the extraction yield (%):

$$yield\% = (grams\ of\ dried\ extract / grams\ of\ sample) \times 100$$

The Gravimetric Determination has been reported in the literature[23],[80].

To perform the TPC assay, TAS assay, and HPLC-DAD analysis, the dried extracts were reconstituted with 25 mL of a 50:50 mixture of Solution A and Solution B (v/v), as described in *Section* 2.1.4.

2.1.6 Total Phenolic Content (TPC) Assay

The phenolic content of the extracts was measured following the procedure described by Redmile-Gordon et al.[81]. The method involves preparing two reagents: Reagent A, which reacts with proteins to eliminate their interference, and Reagent B, which is non-reactive with proteins, allowing for the measurement of polyphenols. Both reagents consist of three different stock solutions: (1) 3.5 g of $CuSO_4 \cdot 5\ H_2O$ dissolved in 100 mL of H_2O, (2) 7 g of sodium potassium tartrate dissolved in 100 mL of H_2O, (3) 70 g of Na_2CO_3 dissolved in 1 L of 0.35 N NaOH solution. To prepare Reagent A, these three solutions were combined sequentially in a volume ratio of 1:1:100 (v/v/v). Reagent B was prepared similarly, except that the first solution was replaced

with deionized water. A standard calibration curve was created using gallic acid as the standard (MW: 170.12 g/mol), yielding solutions ranging from 15.71 to 31.43 µg/mL. Standard and sample solutions were prepared as described in *Section* 2.1.4 and subsequently diluted 1:2 with deionized water. Each solution was prepared in triplicate. Next, 400 µL of the standard or sample dilution was mixed with 400 µL of Reagent A. The same procedure was applied for Reagent B. The samples and standards were then incubated at room temperature in the dark for 10 minutes. After incubation, 400 µL of Folin–Ciocalteu reagent (diluted 1:10) was added to all standards and samples. This dilution was prepared just before the end of the incubation by mixing 2N Folin with deionized water in a 1:10 ratio and stored in the dark. The reaction proceeded in the dark for 30 minutes at room temperature, after which the absorbances were measured at a wavelength of 750 nm (λmax). A blank was prepared by adding 400 µL of water, 400 µL of either Reagent A or Reagent B (depending on which was used), and 400 µL of the Folin–Ciocalteu reagent diluted 1:10. The absorbance values measured at 750 nm (λmax) for Reagent A (AbsA) and Reagent B (AbsB) were used to calculate the absorbance of proteins and the absorbance of polyphenols following equations reported in the literature[81]:

$$Abs\ proteins = 1.25 \times (AbsA - AbsB)$$

$$Abs\ polyphenols = AbsB - 0.2 \times (Abs\ proteins)$$

The Abs polyphenols were used to determine the TPC values, expressed in mmol of gallic acid equivalents (GAE) 100 g−1 of DW of lyophilized fruit sample powder[23]. While in the case of legumes, the TPC values were expressed as mg of gallic acid equivalents (GAE) g-1 of dry extract (Ext)[80]. For legumes, expressing TPC results over dry extract (Ext) facilitated comparison with existing literature.
The TPC method detailed above has been reported in the literature[23],[80].

2.1.7 TAS—ABTS•+ Radical Cation Scavenging Activity

TAS was determined through the Trolox Equivalent Antioxidant Capacity (TEAC) assay by spectrophotometric measurement of the ABTS•+ radical cation[82]. A 7 mM solution of ABTS salt was prepared in water, along with a 2.45 mM solution of potassium persulfate, using sonication for 5 minutes. The ABTS stock solution (referred to as solution A) and the potassium persulfate solution (solution B) were then combined in a 2:1 (v/v) ratio to produce the ABTS•+ radical cation (solution C). The oxidation of ABTS was completed by incubating the mixture in the dark at room temperature for 8 hours. Before measurement, solution C was diluted 1:3 (v/v) in ethanol to create a working solution (solution D) with an absorbance of approximately 0.7 at 734 nm (λmax). A calibration curve was established using Trolox as a reference antioxidant, which was dissolved and diluted in ethanol to achieve final concentrations ranging from 0.74 to 23.73 µM. The D solution, along with Trolox solutions and samples (prepared in triplicate as outlined in *Section* 2.1.4), was equilibrated at 30°C using a thermomixer. The reaction was conducted directly in a cuvette, in the dark, by adding 10 µL of each Trolox solution and sample to 1.0 mL of solution D (with an absorbance at 734 nm of 0.712). The mixture was then gently mixed and allowed to stand for 1 minute before measuring the absorbances at 734 nm.

Ethanol was used as a blank for the measurements. After recording all absorbances at 734 nm, the change in absorbance (ΔAbs) and the percentage of inhibition (I%) of ABTS•+ relative to each Trolox solution and sample were calculated as follows:

$$\Delta Abs_{734nm} = A - A1 \text{ (sample or Trolox solution)}$$

and

$$I\% = [(\Delta Abs_{734nm})/A] \times 100$$

A: control absorbance (ABTS•+);

A1: sample absorbance.

The TAS was expressed as mmol equivalent of Trolox (TE) 100 g−1 of dry weight (DW) of lyophilized fruit powder[23]. While in the case of legumes, the TAS values were expressed as μmol equivalent of Trolox (TE) g−1 of dry extract (Ext)[80]. For legumes, expressing TAS results over dry extract (Ext) facilitated comparison with existing literature.

The TAS method detailed above has been reported in the literature[23],[80].

2.1.8 Liquid Chromatography–Diode Array Detection (HPLC-DAD) Analysis of Polyphenols

Table 10 reports the concentration of the 22 standard polyphenols whose separation was achieved with HPLC analysis in gradient mode. The column temperature was maintained at 20.0 ± 0.8 °C. The mobile phase comprised 100% methanol (solvent A) and a 2% acetic acid aqueous solution (solvent B). The optimized gradient was as follows: 2-30% solvent A over the first 10 minutes, transitioning to 30-46% solvent A from 10 to 65 minutes, with a flow rate of 1.0 mL/min. The injection volume was set at 20 μL. Detection was carried out at five different wavelengths (λ = 250 nm, 280 nm, 320 nm, 360 nm, and 370 nm) to cover the maximum absorptions of the various standards in the mixture. Chromatographic peaks were identified by comparing the elution order, retention times (RT), and UV-Vis absorption spectra with those of the known standards. For compounds without available standards, identification was based on phenolic class or family spectral parameters, and quantification was achieved using calibration curves from a member of the phenolic class that had similar spectra, as described by Monteiro[83]. For sample preparation, all extracts (25 mL each) were concentrated by a factor of 10 and then filtered through a PTFE 0.22 μm filter before injection. A volume of 20 μL from these solutions was injected into the HPLC-DAD system under the established chromatographic conditions. Samples were extracted in triplicate, and the resulting solutions were analyzed twice daily to determine polyphenol content, following the previously described chromatographic conditions. The concentration of each polyphenol in the samples was determined by interpolating the peak area against the respective calibration curve. The polyphenol content was expressed in mg 100 g-1 DW (DW) of the lyophilized sample powder.

The HPLC-DAD chromatographic method detailed above has been reported in the literature[23].

2.1.8.1 HPLC-DAD Method Validation

Sensitivity. The limit of detection (LoD) and limit of quantification (LoQ) were obtained by considering the standard deviation of signals (LoD = 3.3 × (SD of intercept/m); LoQ = 10 × (SD of intercept/m); SD of intercept = SE of intercept × √N[23].

Linearity. Stock solutions (SS) of each reference standard were prepared in 100% methanol to a concentration of 1.5 mg mL−1. Only apigenin was dissolved in 100% ethanol at the same concentration. Several dilutions of each standard were prepared using methanol: water 50:50 with 2% acetic acid (employed to avoid peak tailing). The standard calibration curves were obtained by analyzing each standard in the linearity ranges described in *Table* 6. Calibration curves were then obtained via linear least-squares regression analysis (R^2) by plotting the peak areas versus polyphenol concentration[23].

Precision. The intra-day precision was evaluated by analyzing the mean concentration of the linearity range of each compound and diluted in 50:50 MeOH:H_2O 2% acetic acid on the same day (n = 3) (*Table* 8). The inter-day precision was obtained by analyzing the same solutions two times a day on three different days for one week (n = 6) (*Table* 7)[23].

Accuracy (Recovery). The % recovery was carried out by adding a 22 polyphenol standard mixture at a fixed concentration (reported in *Table* 9) in a vegetal matrix before and after being subjected to ultrasound-assisted extraction (n = 4), as reported in *Section* 2.4. The obtained solutions were concentrated 10 times and injected twice in the HPLCDAD system under the chromatographic conditions described in *Section* 2.9. The % recovery (reported in *Table* 9) was obtained using the following formula[23]:

$$\% \text{ Recovery} = [(A \text{ post-UAE}/A \text{ pre-UAE})] \times 100$$

A pre-UAE: peak area of standard spiked in the sample, obtained before UAE. A post-UAE: peak area of standard spiked in the sample obtained after UAE[23].

Specificity. The method specificity was determined using three vegetal matrix samples and comparing the chromatograms obtained after injecting the non-spiked and spiked samples, respectively. Moreover, each sample analysis was followed by a double solvent injection. No signal at polyphenol retention times demonstrated the absence of any carryover effect.

The HPLC-DAD chromatographic method validation detailed above has been reported in the literature[23].

2.1.9 Ultra High-Performance Liquid Chromatography-Diode Array Detection-Electron Spray Ionization-Ion Trap Mass Spectrometry (UHPLC-DAD-ESI-MS^n)

Polyphenolic compounds in legume UAE extracts were analyzed using a UHPLC-DAD-ESI-MS^n system. Chromatographic separation was achieved on a Kinetex C18 column (50 × 2.1 mm, 1.7 µm) kept at 20 °C. A gradient elution was employed, using a mobile phase consisting of 0.1% formic acid in water [A] and 100% methanol [B] at a flow rate of 0.250 mL/min. The gradient profile started with 2% [B] for 5 min, increasing to 10% [B] at 7.78 min, 30% [B] at 9.72 min, 37% [B] at 14 min, 60% [B] at 23.0 min, then dropping back to 2% [B] at 23.23 min and keeping this composition until the end (27 min). UV-Vis spectra were recorded on a DAD detector in the range of 190–500 nm, with signal acquisition at 280 nm.

Mass spectrometric analysis was performed using a linear ion trap mass spectrometer equipped with an electrospray ionization (ESI) source. Both positive and negative ion modes were applied. The ESI source parameters were set as follows: source voltage of 4.20 kV (positive) or 4.00 kV (negative), capillary temperature of 330 °C, source heater temperature of 250 °C, sheath gas flow of 50 arbitrary units, and auxiliary gas flow of 8 arbitrary units. Mass spectra were acquired over an m/z range of 50–2000.

Regarding sample preparation, all UAE extracts of 25 mL were subjected to a 10-times concentration and subsequent centrifugation at 13,000 rpm for 10 min before injection.

A semi-quantitative analysis was performed using DAD total scan chromatograms. Peak areas of standard reference compounds with known concentrations were compared to those of identified polyphenols in the UAE extracts to estimate their relative amount employing the following equation:

$$Area\ Standard : Concentration\ Standard\ \left(\frac{\mu g}{mL}\right) = Area\ Peak\ Sample : Concentration\ Peak\ Sample\ \left(\frac{\mu g}{mL}\right)$$

In the absence of a standard reference compound, semi-quantitative calculations were performed using the areas of polyphenol standards from the same class or family, as previously reported by Mesquita and Monteiro[84]. The quantity of polyphenols was expressed in milligrams per 100 g of dry weight (DW) of the lyophilized sample powder.

The UHPLC-DAD-ESI-MS^n chromatographic method detailed above has been reported in the literature[80].

2.1.10 Analysis of Pesticide Residues

The procedure applied to determine pesticide content in UAE fruit extract was adopted from the agri-food company, *Fruttagel*®. This procedure, known as UNI EN 15662 of 2018, originates from UNI (Ente di Normazione Italiano), a private Italian association[85]. This is a multimethod for detecting pesticide residues using gas chromatography (GC) and liquid chromatography (LC) after extraction.

A total of 10 mL of extract was combined with 10 mL of acetonitrile containing an internal standard, Tetrakis(4-chlorophenyl)carbohydrate (TPCC). Salts were then added to the mixture for dehydration, and it

was vortexed thoroughly. The mixture was centrifuged at 4000 rpm for 5 minutes, and the supernatant was collected and dried under nitrogen. The resulting dry extract was solubilized in 1 mL of ethyl acetate, from which 0.5 mL was injected for gas chromatography-mass spectrometry (GC-MS). The remaining 0.5 mL was dried again under nitrogen and reconstituted with 0.1 mL of acetone and 0.9 mL of acetonitrile, followed by filtration through a 0.45 µm filter for HPLC-MS analysis. The analytical procedures for pesticide determination using GC-MS and HPLC-MS were carried out entirely at *Fruttagel*®, which provided the results. The legal pesticide limit in food is 0.01 ppm[86].

2.1.11 Evaluation of the Stability of Polyphenolic Compounds

Stability studies were performed on both the UAE extracts (refer to *Section* 2.1.4) and the sample powder used for the extractions. A single representative sample, the biological apple by-product (Apl-Bio-ByP), was used to optimize the study. The results obtained from this sample were considered valid for all samples analyzed in the project.

2.1.11.1 Long-Term Stability of Polyphenols in Stored UAE Extract

To evaluate the stability of the UAE extract, samples were stored in plastic falcon tubes at -20°C for 15 months (t = 15 mth). Polyphenol content was assessed at the beginning (t = 0) and at the end of the storage period (t = 15 months) using the HPLC-DAD chromatographic method described in *Section* 2.1.8. This analysis aimed to investigate potential changes in polyphenol composition and stability over the extended storage period (*Figure* 25).

2.1.11.2 Long-Term Stability of Polyphenols in Stored sample powder

To evaluate the stability of polyphenols in the powdered samples, two extractions were performed: an initial extraction at time zero (t=0 months) and a subsequent extraction after 15 months of storage at -20°C in its original plastic container. The amount of polyphenols in each extract was determined using the HPLC-DAD chromatographic method (described in *Section* 2.1.8). By comparing the polyphenol content between the two time points, potential changes in polyphenol stability over the 15-month storage period were assessed (*Figure* 25).

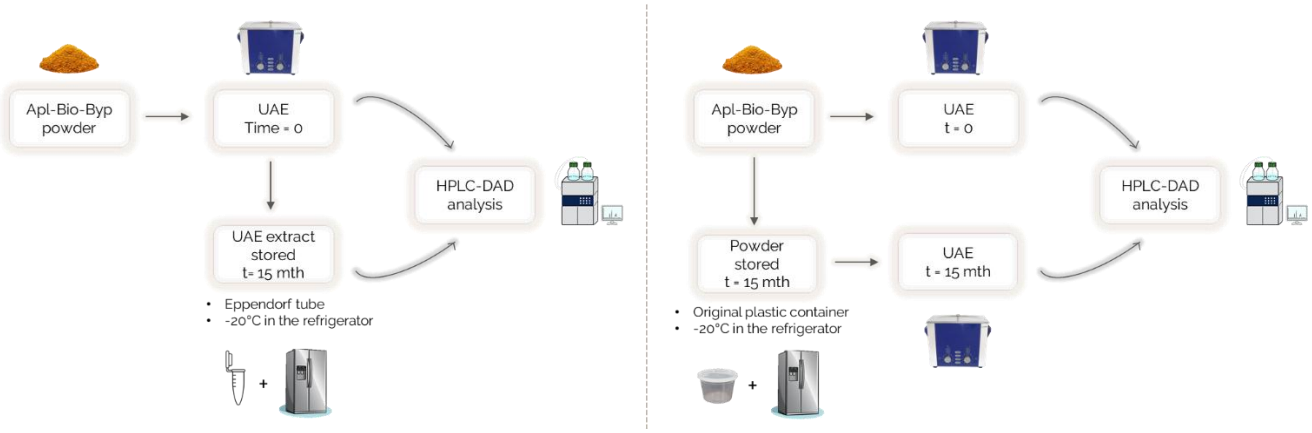


Figure 25 Left-hand side: HPLC-DAD analysis of fresh extraction of powdered apple bio by-product vs. stored extract. Right-hand side: HPLC-DAD analysis of the effect of UAE on freshly extracted powdered apple bio by-product and stored powdered apple bio by-product

2.1.12 Statistical Evaluation

All results were expressed as the mean ± SD of 3 independent experiments. Statistical analyses were performed using ordinary one-way ANOVA and Sidak's multiple comparison tests. The statistical software GraphPad 10.0 version (GraphPad Prism, San Diego, CA, USA) was used, and p-values < 0.05 were considered statistically significant[23],[80].

2.2 RESULTS AND DISCUSSION

2.2.1 Characterization of Fruit samples

2.2.1.1 Gravimetric Determination—Extract Yield (%) of UAE fruit extracts

The graph presented in *Figure* 26 compares the extract yield (%) from fruit by-products with that of the final products. The results indicate that the yields from the by-products are significantly lower than those of their respective final products, with the exception of peach by-products and their corresponding final products. However, these by-products still possess the potential for further valorization. On the other hand, it was expected that the final juice products would yield a higher amount of extractable material compared to their by-products. In the cases of apricots and peaches, the extraction yields from the by-products are slightly lower than those from the final products, while the yield from the final products of apple (Apl-FinalP) is nearly 90% (*Table* 3). Notably, the unexpectedly low yield of the peach conventional by-product (Pch-Conv-ByP) may be attributed to variations in the composition of the initial samples. Concerning tomatoes, the extractable portions from the by-products were expected to be significantly lower than those from the final products. This is because tomato by-products consist mainly of the peels and seeds, and no pulp remains in the waste, which differs from the other fruits[23].

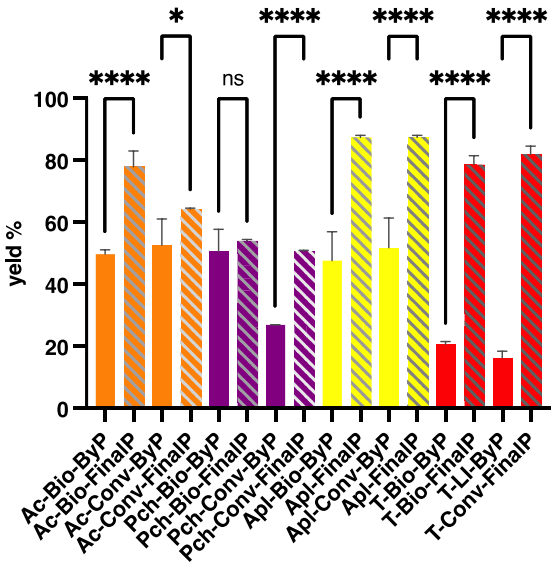


*Figure 26 The yield (%) values of the extracts obtained from fruit by-products (ByP) and final products (FinalP) after ultrasound-assisted extraction (UAE). The results are the mean value of 3 extractions ± SD of the same sample. **** p < 0.0001; *** p < 0.005 *p<0.05. ns=not significant*[23]

Table 3 Extract Yields (%) of UAE Fruit extracts[23]

Samples analyzed	Gravimetric Determination

	Yield (%) ±SD (n=3)
Ac-Bio-ByP	49.8±7.29E-01
Ac-Conv-ByP	52.6±4.23E+00
Ac-Bio-FinalP	78.0±2.53E+00
Ac-Conv-FinalP	64.3±1.42E-01
Pch-Bio-ByP	50.7±3.57E+00
Pch-Conv-ByP	27.0±4.58E-02
Pch-Bio-FinalP	54.1±2.31E-01
Pch-Conv-FinalP	50.7±1.59E-01
Apl-Bio-ByP	47.7±4.65E+00
Apl-Conv-ByP	51.8±4.81E+00
Apl-FinalP	87.5±3.24E-01
T-Bio-ByP	20.7±9.08E-01
T-LI-ByP	16.3±2.18E+00
T-Bio-FinalP	78.6±2.96E+00
T-Conv-FinalP	82.1±2.55E+00

2.2.1.2 Total Phenolic Content (TPC) of UAE fruit extracts

The calibration curve of standard gallic acid (15.71–31.43 μg mL−1), obtained by following the procedure reported in *Section* 2.7, showed a linear equation (y = 0.0512x − 0.1882; LoD = 5.07 μg mL−1; LoQ = 15.35 μg mL−1) with an R^2 = 0.997 and was used to determine the TPC of all the UAE extracts. *Figure* 27 (*Table* 4) shows the TPC values expressed as mmol GAE 100 g−1 DW obtained for all the samples.

The TPC for those by-products with a high pulp content, such as apricot and peach by-products, was very high and comparable to those obtained for their final products. In particular, in the case of apricots, the TPC values of all the UAE extracts are very close to each other, indicating that the amounts of polyphenols remaining in the by-products are comparable to that present in the final products. As expected for apple samples, the by-products show lower but more valuable TPC than the final products. For tomato samples, the TPC values of the by-products are significantly lower than those of their final products, which is explained, as said before, by the composition of by-products and by the low extraction yield observed in the gravimetric determination (*Section* 2.1.5)[23].

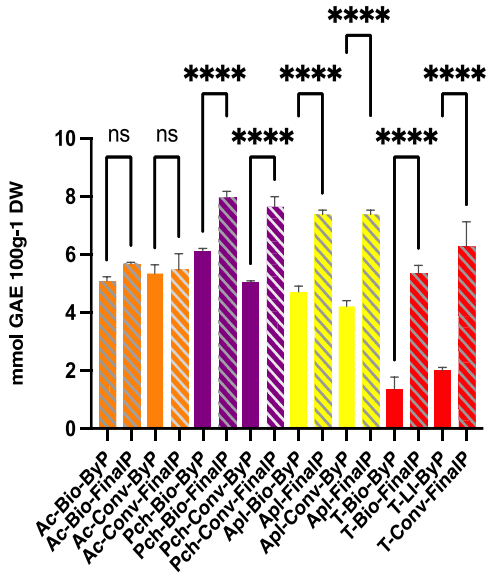


*Figure 27 Total phenolic content (TPC) values of extracts obtained from ByP and FinalP via ultrasound-assisted extraction (UAE). The results are the mean value of 3 extractions ± SD of the same sample. **** $p < 0.0001$. ns = not significant*[23]

Table 4 TPC of UAE Fruit extracts[23]

Samples analyzed	Total Phenolic Content (TPC) Average mmol GAE $100g^{-1}$ DW ±SD (n=3)
Ac-Bio-ByP	5.14±1.71E-01
Ac-Conv-ByP	5.42±3.22E-01
Ac-Bio-FinalP	5.73±1.18E+00
Ac-Conv-FinalP	5.52±1.34E+00
Pch-Bio-ByP	6.06±4.71E-01
Pch-Conv-ByP	4.99±1.58E-01
Pch-Bio-FinalP	7.87±1.05E+00
Pch-Conv-FinalP	7.56±5.09E-01
Apl-Bio-ByP	4.68±3.34E-01
Apl-Conv-ByP	4.25±4.97E-01
Apl-FinalP	7.38±1.04E+00
T-Bio-ByP	1.42±5.98E-01
T-LI-ByP	1.99±9.35E-02
T-Bio-FinalP	5.35±2.95E-01
T-Conv-FinalP	6.27±8.81E-01

2.2.1.3 Total Antioxidant Status Assay (TAS) of UAE fruit extracts

The calibration curve of Trolox (0.74–23.73 µM) was performed to obtain a linear equation (y = 3.8189x − 3.3127; LoD = 0.04 µM; LoQ = 0.11 µM; R2 = 0.9935) for evaluating the antioxidant activity of the UAE extracts, which was expressed as mmol Trolox 100 g−1 DW. *Figure* 28 (*Table* 5) shows the TAS values for all the UAE extracts obtained following the methodology described in *Section* 2.1.7. Overall, the results

showed that final products display a similar TAS value compared to that of the by-products, except for Pch-Bio-ByP. This is an interesting result since the TPC levels evaluated for both the ByPs and FinalPs were found to be significantly different for all the fruit samples except for apricot products[23].

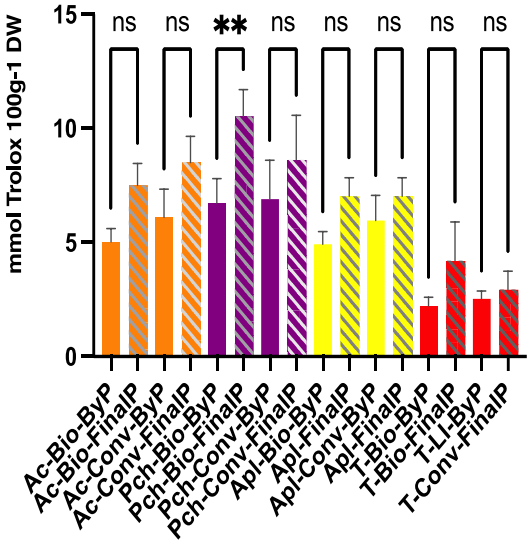


*Figure 28 Total antioxidant status (TAS) values of extracts obtained from fruit ByP and FinalP via UAE. The results are the mean value of 3 extractions ± SD of the same sample. ** $p < 0.005$. ns = not significant*[23]

Table 5 TPC of UAE Fruit extracts[23]

Samples analyzed	Total Antioxidant Status (TAS) Average mmol Trolox $100g^{-1}$ DW ±SD (n=3)
Ac-Bio-ByP	5.04±5.58E-01
Ac-Conv-ByP	6.12±1.26E+00
Ac-Bio-FinalP	7.51±9.60E-01
Ac-Conv-FinalP	8.51±1.14E+00
Pch-Bio-ByP	6.73±1.05E+00
Pch-Conv-ByP	6.88±1.69E+00
Pch-Bio-FinalP	10.57±1.17E+00
Pch-Conv-FinalP	8.66±1.93E+00
Apl-Bio-ByP	7.06±7.96E-01
Apl-Conv-ByP	4.95±5.69E-01
Apl-FinalP	5.98±1.10E+00
T-Bio-ByP	2.27±3.65E-01
T-LI-ByP	2.53±3.69E-01
T-Bio-FinalP	4.21±1.66E+00
T-Conv-FinalP	2.94±7.71E-01

2.2.1.4 Relationship Between Total Phenolic Content, Antioxidant Activity, and Individual Phenolic Compounds in Fruit By-products

In general, it can be noted that peach samples tend to have the highest antioxidant activity, followed by apricot ones. For almost all the UAE extracts, it is interesting to note that the by-products exhibit a TAS value comparable to that of the final products. This feature observed for apple by-products is in contrast to the previous results, where Apl-FinalP revealed the highest difference between the by-product and final product related to the extract yield (%) and to TPC values. The unexpectedly high TAS value obtained for these by-products may be explained by the composition of such extracts. Indeed, despite Pch-Bio, Pch-Conv, Apl-Bio, Apl-Conv, T-Bio, and T-LI by-products having lower TPC values than their final products, they may contain a remarkable percentage of phenolic compounds, such as flavonoids, whose chemical structure is linked to a higher capacity of scavenging free radicals[57],[31]. The analysis of the graph reported in *Figure* 29 in which TPC and TAS values are correlated, indicates that a higher content of phenolic compounds might not be necessarily related to a higher antioxidant activity. This aspect confirms the importance of characterizing the bioactive compounds since the type and the nature of the polyphenol contained in the sample might influence the TAS values greatly. Other important considerations that can help in considering by-products for their valorization have to be conducted on the results obtained by correlating the TPC values to the extract yield (%) (*Figure* 30). According to the results previously discussed, a higher yield of extraction does not necessarily relate to a proportional increase in the content of phenolic compounds. This observation is more evident for the peach and apricot samples studied, whereas for apple and tomato samples, a higher correlation is visible between by-products and final products.

The TPC value for each group of samples was also correlated to the sum of the individual phenolic compounds (SPC) determined by HPLC. The graphs reported in *Figure* 31 suggest a good correlation between the TPC and the Sum of the individual quantities of Phenolic Compounds (SPC) values except for apricot (Ac) samples. A TPC assay is indeed a quick method, which can be used for a preliminary indication of the polyphenol yield when optimizing the extraction, but many chemically similar compounds interfere with the test results[87]. Therefore, the HPLC method is strictly required for an accurate quantification of polyphenols. The difference in TPC and SPC can be ascribed to all the compounds that interfere in the TPC assay as well as to the types and levels of interferents, which depend on the natural source of the extract[88]. This evidence allows for the characterization of the samples from both a chemical and functional point of view, thus optimizing the reuse of the studied by-products in a circular economy prospect. This discussion has been reported in literature[23].

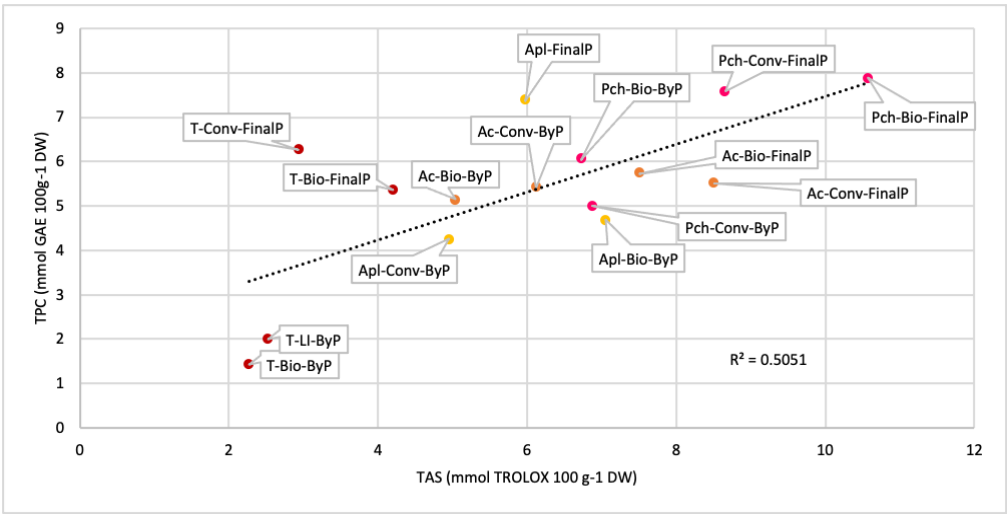


Figure 29 Correlation between TPC and the TAS values of fruit ByP and finalP[23]

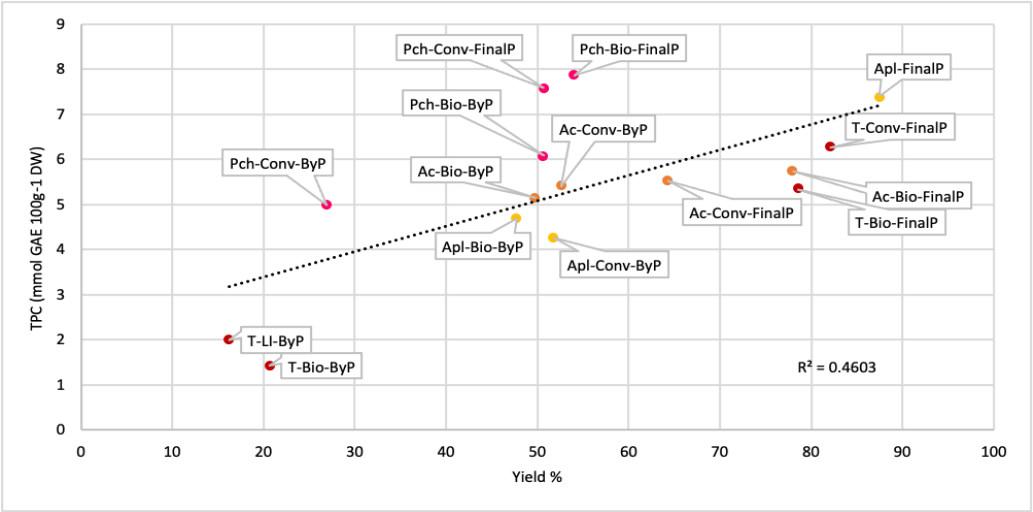


Figure 30 Correlation between the extract yield (%) and the TPC values of fruit by-products and final products[23]

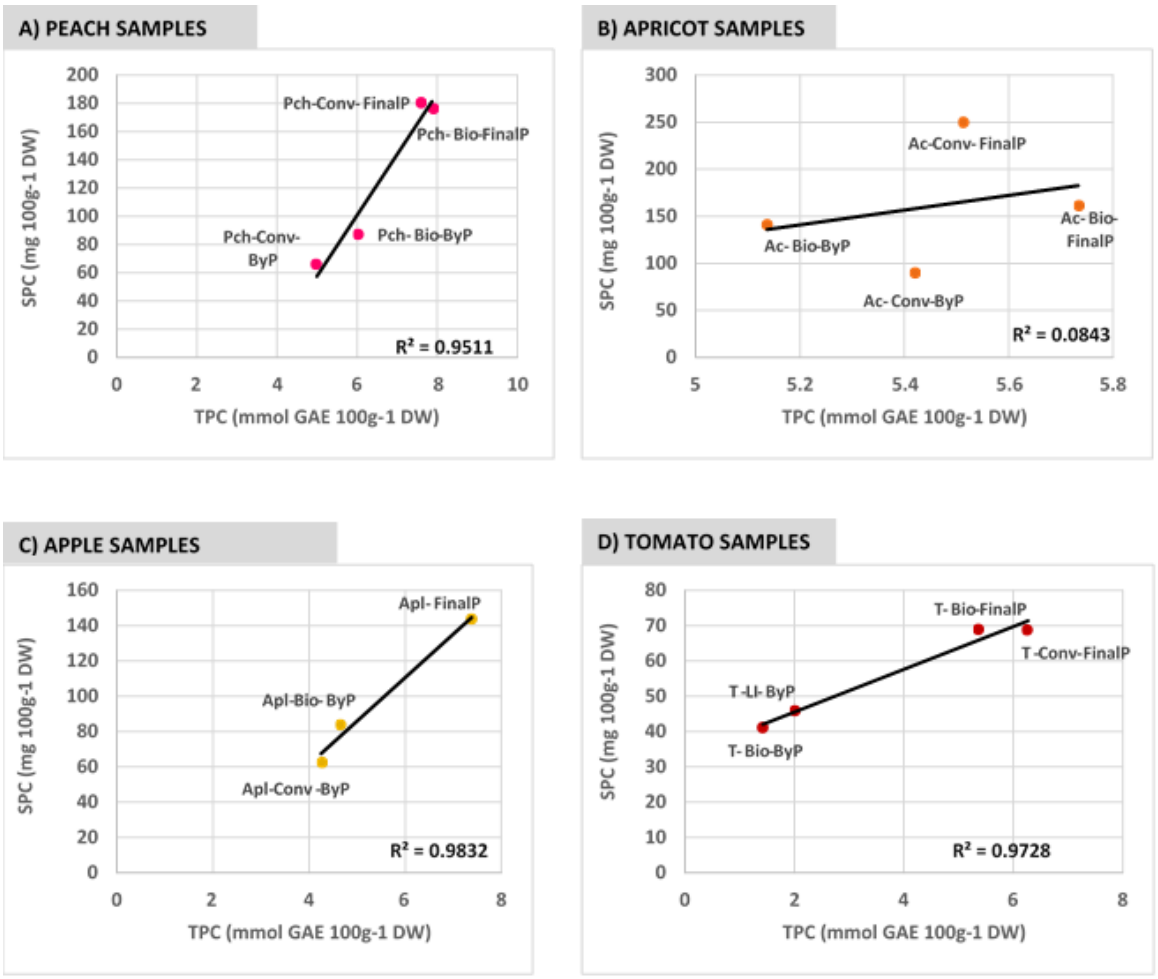


Figure 31 Correlations between the Sum of Individual Quantities of Phenolic Compounds (SPC), as determined by HPLC-DAD, and the Total Phenolic Content (TPC) values. Each graph corresponds to the results obtained for both fruit by-products and the final products of each sample type: A) peach samples; B) apricot samples; C) apple samples; D) tomato samples. The results are expressed as the mean value of three replicates (n=3)[23]

2.2.1.5 HPLC-DAD Method Validation

The results of the HPLC-DAD analytical method validation, as described in the *Section* 2.1.8.1, are presented below. All results have been reported in literature[23].

Sensitivity and Linearity.

Table 6 Data concerning calibration curves of polyphenols standards including LoD and LoQ values[23]

Std	Compound	λ (nm)	rt (min)	Linear Range µg mL^{-1}	Calibration Curves	R^2	LoD (µg mL^{-1})	LOQ
1	Gallic Acid	280	3.685	31.60-240.00	y = 71.466x + 198.82	0.9994	10.40	31.60
2	Protocatechuic acid	250	6.175	7.00-100.00	y = 44.709x + 49.354	0.9998	2.30	7.00

3	(+)-Catechin	280	9.324	19.40-240.00	y = 19.454x + 52.856	0.9998	6.40	19.40
4	Chlorogenic acid	320	10.597	49.90-300.00	y = 84.437x + 353.85	0.9999	16.50	49.90
5	Caffeic acid	320	11.522	3.60-60.00	y = 127.5x + 74.584	0.9999	1.20	3.60
6	(-)-Epicatechin	280	12.204	10.70-128.00	y = 14.039x + 21.127	0.9997	3.50	10.70
7	P-coumaric acid	320	14.982	0.60-10.00	y = 241.61x + 23.811	0.9999	0.20	0.60
8	Ferulic acid	320	16.484	2.60-32.00	y = 99.2x + 35.838	0.9998	0.90	2.60
9	Hyperoside	250	24.975	7.30-150.00	Y=49.26x + 44.529	0.9999	2.40	7.30
10	Isoquercitrin	250	25.828	2.80-40.00	y = 49.507x + 17.742	0.9998	0.90	2.80
11	(+)-Rutin Trihydrate	250	26.301	5.00-64.00	y = 37.121x + 13.267	0.9998	1.70	5.00
12	Phloridzin dihydrate	280	27.814	7.30-96.00	y = 41.09x + 44.011	0.9998	2.40	7.30
13	Trans-cinnamic acid	280	32.306	1.50-24.00	y = 156.96x + 30.134	0.9998	0.50	1.50
14	Quercitrin	250	32.934	13.30-256.00	y = 31.202x + 64.347	0.9999	4.40	13.30
15	Myricetin	370	33.915	7.50-128.00	y = 118.7x - 39.818	0.9999	2.50	7.50
16	Daidzein	250	37.583	8.50-160.00	y = 111.22x + 142.73	0.9999	2.80	8.50
17	Naringenin	280	44.200	5.20-64.00	y = 64.552x + 39.8	0.9998	1.70	5.20
18	Genistein	250	50.684	7.70-96.00	y = 160.73x + 172.32	0.9998	2.50	7.70
19	Hesperetin	280	50.702	12.60-160.00	y = 114.62x + 213.99	0.9998	4.20	12.60
20	Naringenin chalcone	370	57.585	8.10-96.00	y = 125.18x + 120.48	0.9997	2.70	8.10
21	Kaempferol	370	60.822	5.30-96.00	y = 193.95x + 70.837	0.9999	1.70	5.30
22	Apigenin	320	65.028	6.70-96.00	y = 112.74x + 66.409	0.9998	2.20	6.70

Precision.

Table 7 inter-day variation of polyphenols standards[23]

Std	Compound	Conc. µg mL^{-1}	Day 1 ±SD n=2	Day 2 ±SD n=2	Day 3 ±SD n=2	Average of Areas ±SD n=6	RSD%
1	Gallic acid	120	8749.600±2.022E+01	8770.900±8.771E+03	8877.750±1.768E+00	8799.417±6.8670E+01	0.78
2	Protocatechuic acid	50	2299.750±1.520E+01	2336.500±6.081E+00	2383.950±5.586E+00	2340.067±4.2213E+01	1.80
3	(+)-Catechin	120	2540.800±5.233E+00	2566.450±9.192E-01	2584.700±1.980E+00	2563.983±2.2054E+01	0.86
4	Chlorogenic acid	150	10681.000±1.273E+01	10687.000±1.414E+01	10800.500±1.202E+01	10722.833±6.7328E+01	0.63
5	Caffeic acid	30	3792.800±5.233E+00	3915.350±1.690E+01	4068.600±7.071E+00	3925.583±1.3818E+02	3.52
6	(-)-Epicatechin	64	986.350±1.216E+00	994.080±1.004E+00	1001.700±1.556E+00	994.043±7.6751E+00	0.77
7	p-coumaric acid	5	1091.650±1.485E+00	1125.650±4.738E+00	1172.500±5.233E+00	1129.933±4.0595E+01	3.59
8	Ferulic acid	16	1583.900±1.414E-01	1591.300±5.657E-01	1605.300±2.404E+00	1593.500±1.0868E+01	0.68
9	Hyperoside	32	3769.550±2.072E+01	3868.150±1.237E+01	4023.700±1.442E+01	3887.133±1.2813E+02	3.30
10	Isoquercitrin	20	970.415±1.973E+00	974.000±2.404E+00	983.190±3.437E+00	975.868±6.5892E+00	0.68
11	(+)-Rutin Trihydrate	75	1200.200±9.192E+00	1228.400±1.556E+00	1236.450±2.044E+01	1221.683±1.9036E+01	1.56
12	Phloridzin dihydrate	48	2127.250±1.025E+01	2154.050±3.465E+00	2192.750±4.313E+00	2158.017±3.2930E+01	1.53
13	Trans-cinnamic acid	12	1895.150±2.758E+00	1904.300±5.657E-01	1925.250±1.061E+00	1908.233±1.5431E+01	0.81
14	Quercitrin	128	3866.200±3.111E+00	3993.500±1.202E+01	4156.050±1.421E+01	4005.250±1.4528E+02	3.63

15	Myricetin	64	7852.050±3.316E+01	7901.650±4.525E+00	7995.650±9.405E+00	7916.450±7.2935E+01	0.92
16	Daidzein	80	32849.500±4.738E+01	32660.000±2.121E+01	32715.500±5.162E+01	32741.667±9.7422E+01	0.30
17	Naringenin	32	2118.750±4.830E+01	2080.700±2.828E-01	2103.000±1.414E+01	2100.817±1.9119E+01	0.91
18	Genistein	48	7557.650±7.071E-02	7787.400±1.966E+01	8103.000±3.154E+01	7816.017±2.7380E+02	3.50
19	Hesperetin	80	9721.650±1.153E+01	9865.300±4.525E+00	10028.500±1.626E+01	9871.817±1.5353E+02	1.56
20	Naringenin chalcone	48	14759.500±2.051E+01	14712.500±1.626E+01	14685.500±7.071E-01	14719.167±6.8670E+01	0.25
21	Kaempferol	48	18385.000±4.243E+01	18365.500±2.616E+01	18396.500±2.051E+01	18382.333±4.2213E+01	0.09
22	Apigenin	48	12293.000±3.536E+01	12324.000±7.071E+00	12331.500±2.616E+01	12316.167±2.2054E+01	0.17

Table 8 Intra-day variation of polyphenols standards[23]

Std	**Compound**	**Concentration µg mL^{-1}**	**Area 1 ±SD n=2**	**Area 2 ±SD n=2**	**Area 3 ±SD n=2**	**Average of Areas ±SD n=6**	**RSD%**
1	Gallic acid	120	8892.8±4.09E+01	8982.250±1.28E+02	9046.850±8.59E+01	8973.967±7.74E+01	0.86
2	Protocatechuic acid	50	2341.800±3.39E+00	2372.150±2.69E+01	2382.800±2.88E+01	2365.583±2.13E+01	0.90
3	(+)-Catechin	120	2660.450±7.97E+01	2773.100±1.03E+02	2820.950±1.10E+02	2751.500±8.24E+01	2.99
4	Chlorogenic acid	150	10846.000±1.20E+01	10947.500±7.00E+01	11004.500±1.03E+02	10932.667±8.03E+01	0.73
5	Caffeic acid	30	3889.550±5.16E+00	3947.250±2.30E+01	3956.350±2.45E+01	3931.050±3.62E+01	0.92
6	(-)-Epicatechin	64	1033.600±3.82E+00	1075.700±3.97E+01	1093.750±4.31E+01	1067.683±3.09E+01	2.89
7	p-coumaric acid	5	1119.300±1.70E+00	1137.150±6.15E+00	1138.850±7.42E+00	1131.767±1.08E+01	0.96
8	Ferulic acid	16	1657.900±3.11E+00	1726.650±6.46E+01	1754.800±6.99E+01	1713.117±4.98E+01	2.91
9	Hyperoside	32	3854.450±9.97E+00	3900.450±1.34E+01	3900.750±2.24E+01	3885.217±2.66E+01	0.69
10	Isoquercitrin	20	991.735±2.88E+00	998.445±5.87E-01	995.775±4.28E+00	995.318±3.38E+00	0.34
11	(+)-Rutin Trihydrate	75	1214.800±2.01E+01	1215.900±1.56E+01	1233.100±3.41E+01	1221.267±1.03E+01	0.84
12	Phloridzin dihydrate	48	2159.450±1.91E+00	2185.950±2.50E+01	2196.700±2.86E+01	2180.700±1.92E+01	0.88
13	Trans-cinnamic acid	12	1929.350±4.88E+00	1951.500±1.20E+01	1955.800±1.67E+01	1945.550±1.42E+01	0.73
14	Quercitrin	128	3977.650±7.14E+00	4032.650±1.85E+01	4033.200±2.57E+01	4014.500±3.19E+01	0.79
15	Myricetin	64	7676.300±4.67E+00	7651.850±5.95E+01	7709.900±4.98E+01	7679.350±2.91E+01	0.38
16	Daidzein	80	33093.500±2.98E+02	33054.500±5.37E+02	33116.000±6.18E+02	33088.000±3.11E+01	0.09
17	Naringenin	32	2287.850±7.07E-02	2301.250±1.90E+01	2283.350±7.45E+01	2290.817±9.31E+00	0.41
18	Genistein	48	9083.350±1.05E+03	8975.550±1.20E+03	8953.250±1.23E+03	9004.050±6.96E+01	0.77
19	Hesperetin	80	9801.900±2.15E+02	9627.500±1.22E+02	9673.050±2.76E+02	9700.817±9.05E+01	0.93
20	Naringenin chalcone	48	14718.000±3.82E+01	14724.500±7.07E-01	14729.500±6.15E+01	14724.000±5.77E+00	0.04

21	Kaempferol	48	18548.500±2.74E+02	18579.000±3.28E+02	18613.500±3.27E+02	18580.333±3.25E+01	0.18
22	Apigenin	48	12423.000±2.19E+02	12466.000±2.08E+02	12487.500±2.47E+02	12458.833±3.28E+01	0.26

Accuracy (Recovery).

Table 9 %Recovery of HPLC-DAD data[23]

Standard	Compound	Concentration µg mL-1	% Recovery±SD n= 2	RDS%
1	Gallic Acid	7.031	103.16±5.42E+00	2.20
2	Protocatechuic acid	2.813	91.71±7.73E+00	6.11
3	(+)-Catechin	15.625	92.81±1.20E+01	5.27
4	Chlorogenic acid	2.344	92.47±4.94E+00	5.53
5	Caffeic acid	0.938	91.72±1.21E+01	6.10
6	(-)-Epicatechin	7.031	101.67±1.73E+00	1.17
7	p-coumaric acid	0.469	104.44±1.23E+01	3.07
8	Ferulic acid	1.406	100.36±9.06E-01	0.25
9	Hyperoside	9.375	93.95±2.13E+00	4.41
10	Isoquercitrin	9.375	97.94±5.80E+00	1.47
11	(+)-Rutin Trihydrate	4.688	100.48±1.87E+00	0.34
12	Phloridzin dihydrate	7.031	104.35±7.66E+00	3.01
13	Trans-cinnamic acid	1.875	90.54±2.65E+01	7.02
14	Quercitrin	14.063	95.50±1.23E+01	3.25
15	Myricetin	4.950	101.29±5.95E+00	0.91
16	Daidzein	4.250	99.63±1.81E+01	0.26
17	Naringenin	1.875	99.72±6.42E-01	0.20
18	Genistein	3.645	99.20±2.72E+01	0.57
19	Hesperetin	7.050	99.75±5.46E+00	0.18
20	Naringenin chalcone	4.125	90.78±1.35E+01	6.84
21	Kaempferol	3.000	101.63±5.30E+00	1.15
22	Apigenin	3.300	102.06±6.96E+00	1.44

2.2.1.6 Characterization of fruit UAE extracts by HPLC-DAD

To determine the polyphenolic content profile of each UAE extracts, an optimized and validated HPLC-DAD method was employed. A mixture consisting of 22 standard compounds was used to optimized the chromatographic conditions. The chromatographic separation of this reference standard mixture is illustrated in *Figure* 32. The HPLC analysis, conducted according to the conditions outlined in *Section* 2.1.6, successfully separated and identified all 22 compounds in under 70 minutes. Although hesperetin (peak 18) and genistein (peak 19) were overlaid, quantification remained feasible due to their different wavelengths (λmax) of 280 nm and 370 nm, respectively. The chromatographic method was validated for sensitivity, specificity, linearity, precision, and recovery, with detailed data provided in *Table* 10 (*Section* 2.1.8.1). The results indicated that the method was sensitive, with limits of detection (LoD) ranging from 0.2 to 16.5 µg/mL and limits of

quantification (LoQ) between 0.6 and 49.9 µg/mL. The precision, assessed both intra- and inter-day, averaged 2.5% and 3.5%, respectively. The method demonstrated optimal linearity, confirmed by R^2 values exceeding 0.9994 for all standard compounds. Recovery rates ranged from 90.54% to 104.44%, supporting the method's suitability for the quantitative analysis of polyphenols in the UAE extracts. All the characterization of UAE fruit extracts has been reported in literature[23].

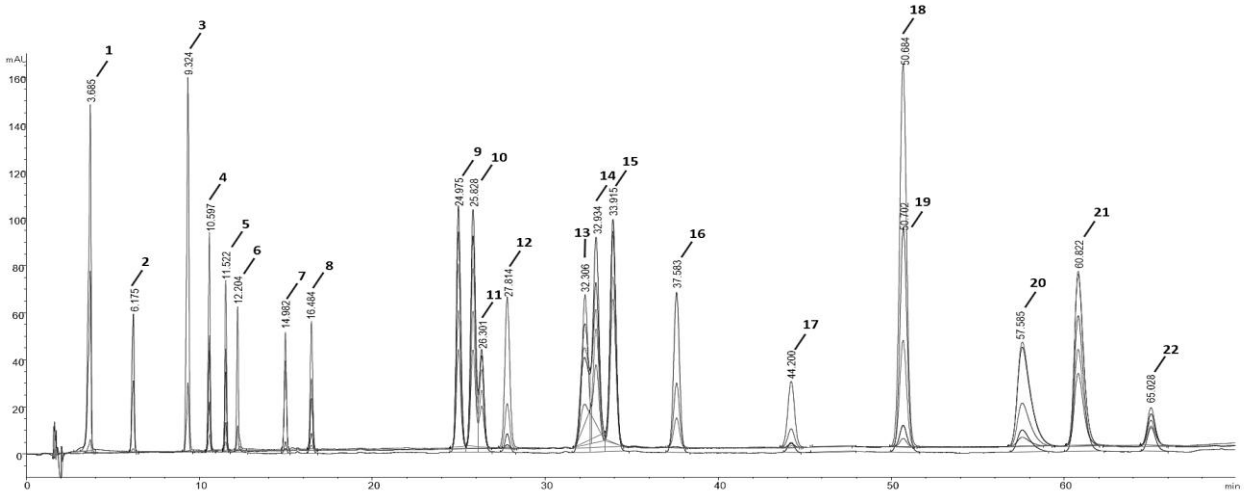


Figure 32 The chromatographic and spectrophotometric analysis of the standard polyphenolic mixture. The overlaid chromatograms were registered at λ = 280 nm; λ = 320 nm; λ = 370 nm; λ = 360 nm; λ = 250 nm; 1 = gallic acid; 2 = protocatechuic acid; 3 = (+)-catechin; 4 = chlorogenic acid; 5 = caffeic acid; 6 = (−)-epicatechin; 7 = p-coumaric acid; 8 = ferulic acid; 9 = hyperoside; 10 = isoquercitrin; 11 = (+)-rutin trihydrate; 12 = phloridzin; 13 = trans-cinnamic acid; 14 = quercitrin; 15 = myricetin; 16 = daidzein; 17 = naringenin; 18 = hesperetin; 19 = genistein; 20 = naringenin chalcone; 21 = kaempferol; 22 = apigenin. The retention time for each compound, the concentrations applied for the chromatographic analysis of the mixture, and the λmax are reported in Table 10[23]

Table 10 Standard concentration in the mixture whose chromatogram is reported in Figure 32[23]

Standard	Compound	Concentration µg mL^{-1}	Lambda Max
1	Gallic Acid	28.125	280
2	Protocatechuic acid	11.250	250
3	(+)-Catechin	65.500	280
4	Chlorogenic acid	9.375	320
5	Caffeic acid	3.750	320
6	(-)-Epicatechin	28.125	280
7	P-coumaric acid	1.875	320
8	Ferulic acid	5.625	320
9	Hyperoside	37.500	250
10	Isoquercitrin	37.500	250
11	(+)-Rutin Trihydrate	18.750	250
12	Phloridzin dihydrate	28.125	280
13	Trans-cinnamic acid	7.500	280
14	Quercitrin	56.250	250
15	Myricetin	19.800	370

16	Daidzein	17.000	250
17	Naringenin	7.500	280
18	Genistein	14.580	250
19	Hesperetin	28.200	280
20	Naringenin chalcone	16.500	370
21	Kaempferol	12.000	370
22	Apigenin	13.200	320

2.2.1.7 HPLC-DAD Quali/Quantitative Analysis

In the first step of UAE extracts characterization, a qualitative analysis was conducted by comparing the retention times of standard compounds with the chromatographic peaks identified in the UAE extract analysis. The identification was further confirmed by overlaying the UV-Vis absorption spectrum (ranging from 210 to 500 nm) of each peak in the samples, acquired at the maximum absorption wavelength, with those of the standard compounds. *Figure* 33 presents all the UV-Vis absorption spectra recorded for each reference standard, which are characteristic of the different phenolic subclasses. It is important to note that many compounds within the same class exhibit similar UV-Vis spectral profiles (as illustrated in *Figure* 33). These distinct spectral features were utilized to categorize unknown substances into phenolic subclasses and identify them as derivatives of the phenolic standards that were analyzed[83].

Comprehensive details regarding the retention times of the peaks in all analyzed UAE extracts can be found in *Table* 11 and *Table* 17. The chromatograms for each fruit UAE extracts are presented in *Figure* 34 (apple samples), *Figure* 36 and *Figure* 37 (peach samples), *Figure* 39 and *Figure* 40 (apricot samples), and *Figure* 42 and *Figure* 43 (tomato samples).

The qualitative analysis was followed by a quantitative analysis. The latter involved applying the linear regression equation obtained for each standard and interpolating the corresponding peak area of each substance or derivative found in the UAE extracts (*Table* 10). By combining the information from both the qualitative and quantitative analyses, we were able to create a profile of the polyphenolic content for each UAE extract analyzed. This comparison of the polyphenolic composition between the corresponding fruit by-products and final products is essential for achieving the primary goal of this study: to determine the potential of using by-products as a source of bioactive ingredients[23].

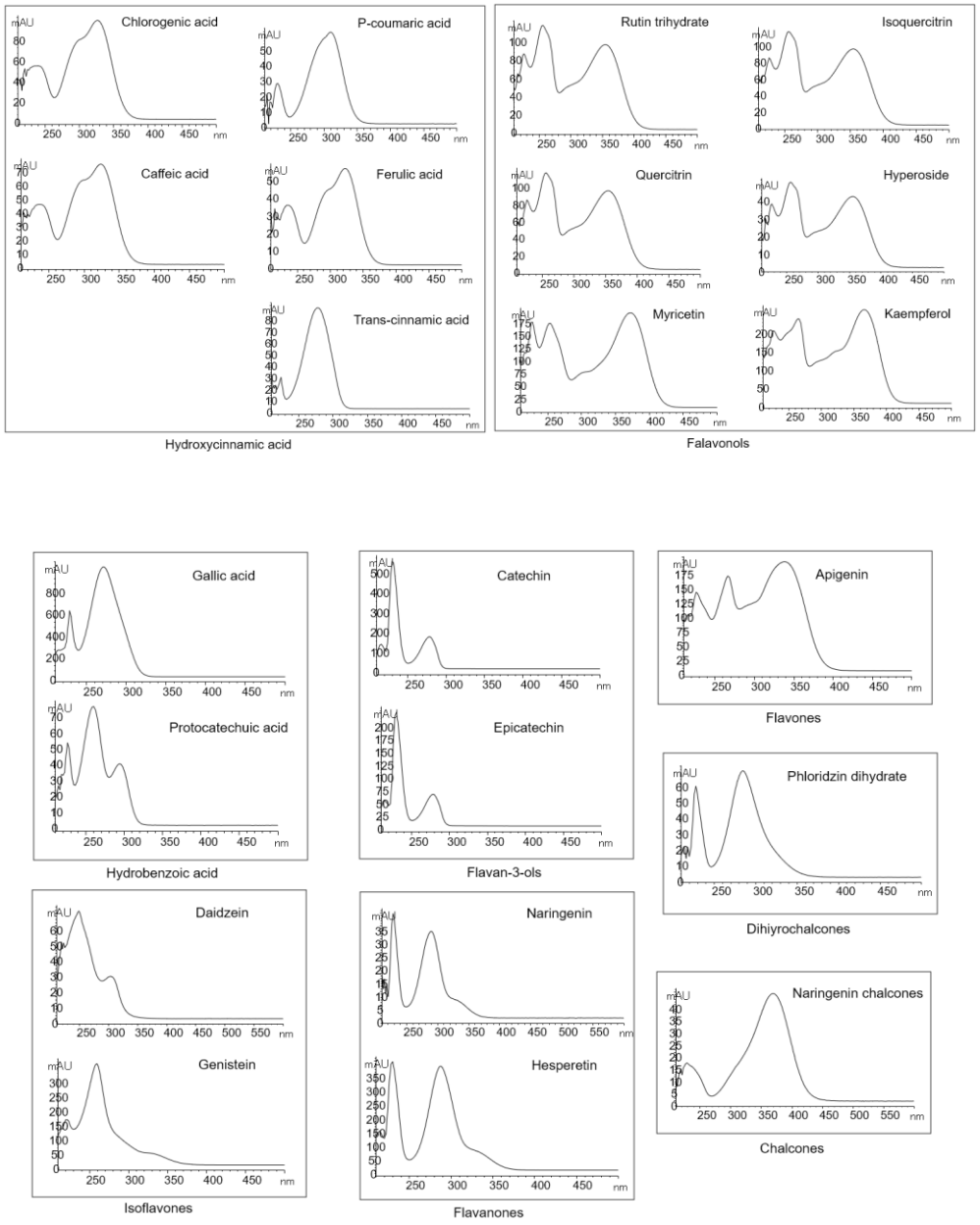
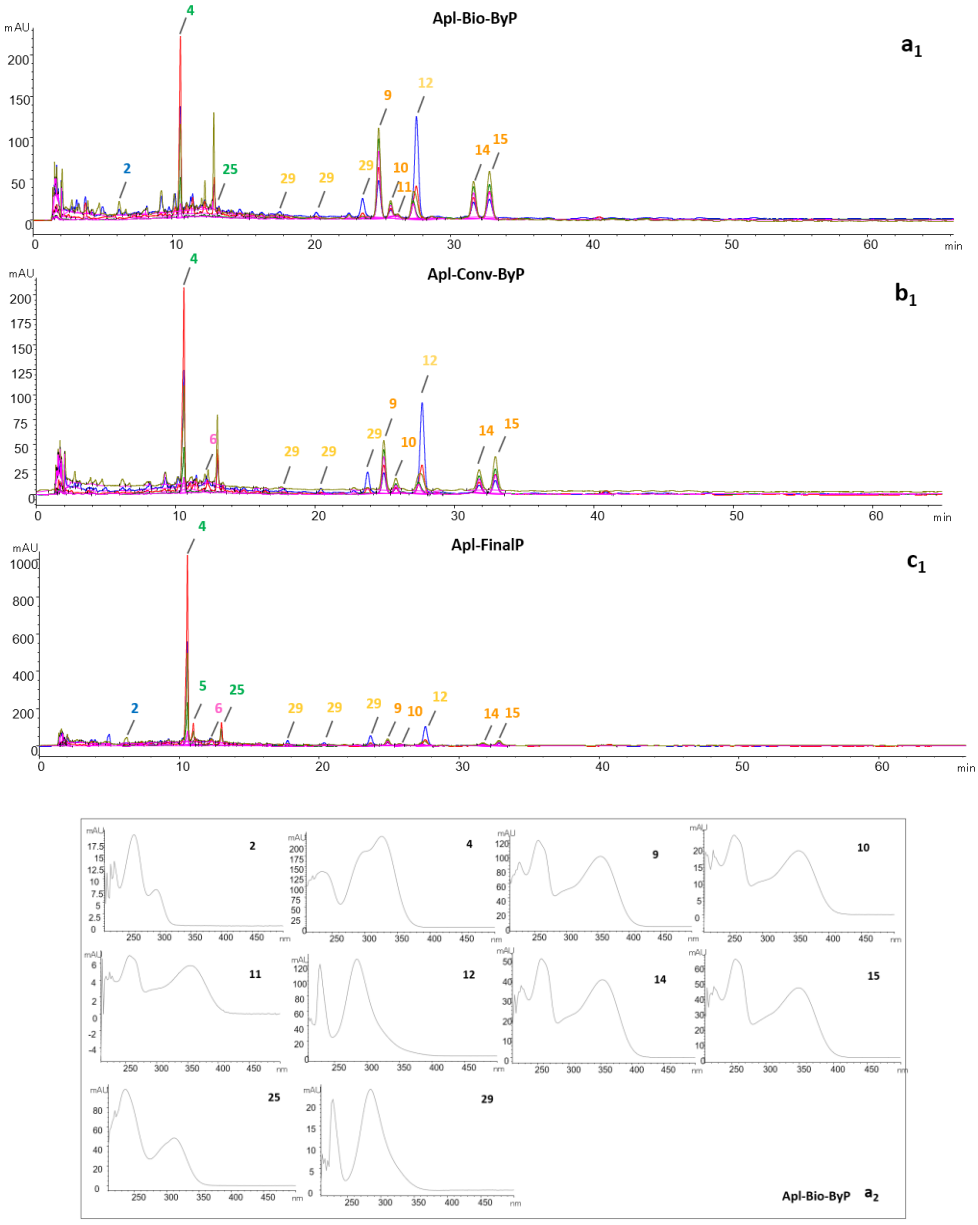


Figure 33 Characteristic UV-Vis spectra of standards classified according to their phenolic classes/families[23]

Apple Products

The quantitative phenolic characterization of apple UAE extracts (*Figure* 34) indicates that phloridzin is the polyphenol found in the highest concentration in apple by-products, with a comparable amount in Apl-FinalP (28.80 ± 2.86 mg 100 g-1 DW). Interestingly, flavonols, including hyperoside, quercitrin, myricetin, and rutin, are present in even higher quantities in the Apl-Bio-ByP compared to Apl-Conv-ByP and Apl-FinalP. Among these, hyperoside is the most abundant flavonol, measured at 19.38 ± 1.74 mg 100 g-1 DW, which is the highest amount recorded in all fruit UAE extracts studied. Quercitrin follows closely with a concentration of 17.09 ± 0.577 mg 100 g-1 DW. In contrast to peach and apricot UAE extracts, apple UAE extracts contain a lower amount of chlorogenic acid. *Figure* 35 illustrates the qualitative comparison between Apl-Bio-ByP and Apl-

FinalP, showing that the compounds protocatechuic acid, chlorogenic acid, p-coumaric acid, hyperoside, isoquercitrin, phloridzin, quercitrin, and myricetin are present in both UAE extracts. The notable presence of phloridzin (peak 12) and other potential glycosidic derivatives (peaks 29), which elute more quickly, highlights their significance in both apple by-products and final products. The distribution of phenolic classes in apple UAE extracts is more heterogeneous, with a predominance of flavonols, dihydrochalcones, and hydroxycinnamic acids[23].



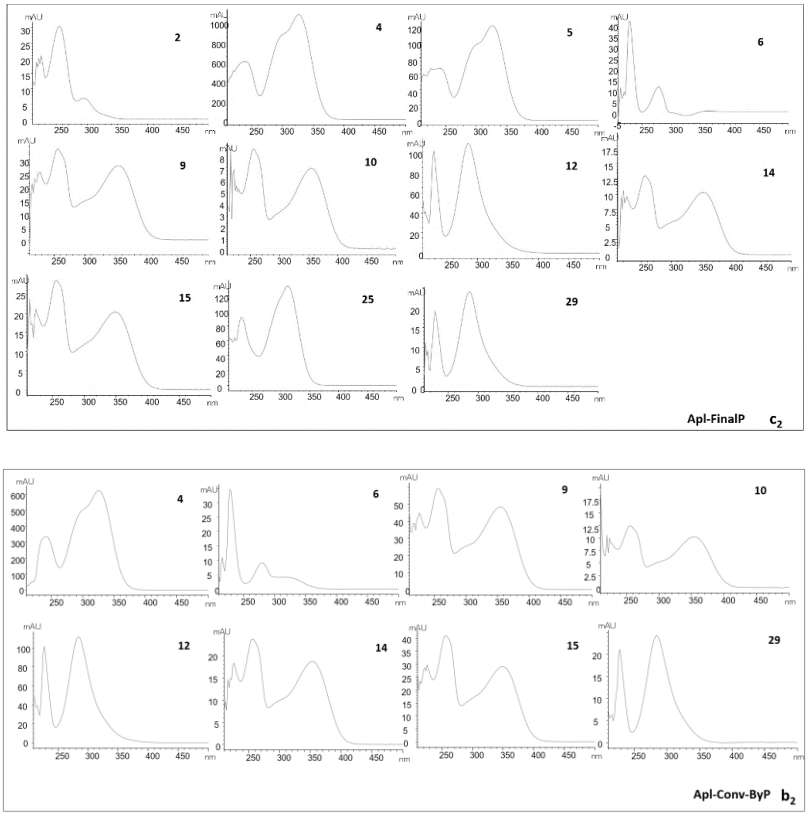


Figure 34 Overlaid HPLC chromatograms of Apl-Bio-ByP (a1), Apl-Conv-ByP (b1) and Apl-FinalP (c1) samples registered at λ= 280 nm; λ =320 nm; λ= 370 nm; λ =360 nm; λ =250 nm; the original chromatogram reports Y axis in 0-250 mAU and 0-1000 mAU range respectively; UV-Vis spectra of detected peaks of Apl-Bio-ByP (a2), Apl-Conv-ByP (b2) and Apl-FinalP (c2); 2= Protocatechuic acid; 4 = Chlorogenic acid; 5 = Caffeic acid; 6= (-)-Epicatechin; 9= Hyperoside; 10= Isoquercitrin; 11= (+)-Rutin trihydrate; 12= Phloridzin dihydrate; 14= Quercitrin; 15= Myricetin; 25 = Hydroxycinnamic acid derivatives; 29 = dihydrochalcone (phloridzin) derivative[23]

Table 11 Retention times of polyphenols identified in Apl-Bio-ByP, Apl-Conv-ByP, and Apl-FinalP[23]

Peak	**Compound**	**Reference Standard**	**Apl-Bio-ByP (a1)**	**Apl-Conv-ByP (b1)**	**Apl-Bio-FinalP (c1)**
		RT	**RT**	**RT**	**RT**
2	Protocatechuic acid	6.175	6.178	/	6.182
4	Chlorogenic acid	10.597	10.574	10.585	10.574
5	Caffeic acid	11.522	/	/	11.013
6	(-)-Epicatechin	12.204	/	12.204	12.199
25	Hydroxycinnamic acid derivatives	/	13.367	/	13.380
29	Phlorizin dihydrate derivative	/	17.738	17.792	17.741
29	Phlorizin dihydrate derivative	/	20.361	20.424	20.365
29	Phlorizin dihydrate derivative	/	23.677	23.765	23.674
9	Hyperoside	24.975	24.853	24.963	24.940
10	Isoquercitrin	25.828	25.726	25.826	25.807

11	(+)-Rutin trihydrate	26.301	26.203	/	/
12	Phloridzin dihydrate	27.814	27.577	27.692	27.679
14	Quercitrin	32.934	31.665	31.801	31.776
15	Myricetin	33.915	32.818	32.956	32.930

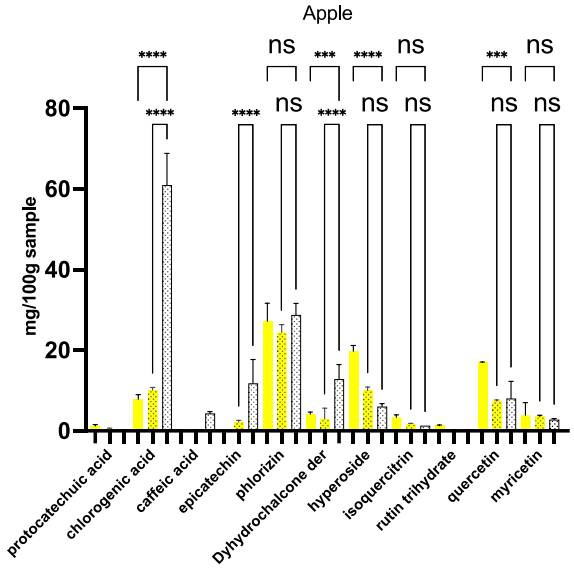


*Figure 35 Bar charts reporting the phenolic profile of the studied samples. The amount of phenolic acids and flavonoids, characterized in both the biological (colored columns) and conventional (colored/dotted columns) by-products, are compared with those found in the biological final products (colored/ribbed columns) and the conventional final ones (uncolored/dotted columns), respectively. The results are the mean value carried out from 3 analyses ± SD of the same sample. **** $p < 0.0001$; *** $p < 0.005$; ** $p < 0.01$; * $p < 0.05$. ns = not significant*[23]

Peach Products

The chromatographic analysis of Pch-Bio-ByP and Pch-Bio-FinalP, combined with UV-Vis spectral analysis of the identified peaks and their related peaks (*Figure* 36 and *Figure* 37), allowed us to determine the relative distribution of various classes of polyphenols in the UAE extracts. Several phenolic compounds were unambiguously identified, including chlorogenic acid, caffeic acid, hyperoside, naringenin, and isoquercitrin. However, many unassigned peaks, particularly those with high intensity (e.g., peaks 23, 24, and 27), were still classified as members of the polyphenol families due to their UV-Vis absorption spectra similarities to the standards. Peak 24 in both chromatograms displayed UV-Vis spectra identical to those of chlorogenic acid (*Figure* 36, a2 and b2), eluting at nearly the same retention time, 7.302 minutes for Pch-Bio-ByP and 7.296 minutes for Pch-Bio-FinalP. According to the literature, this peak may correspond to neochlorogenic acid (NCHA), an isomer of chlorogenic acid that is present in high concentrations in peaches[89]. The study shows an elution time for NCHA at around 7 min and chlorogenic acid at around 10 min (HPLC-DAD)[89], which supports the idea that this compound is NCHA. The results from the qualitative and quantitative phenolic characterization of peach UAE extracts (*Figure* 38) indicate that peach by-products can retain significant

amounts of chlorogenic acid and its derivatives. Among the tested by-products, Pch-Bio-ByP had the highest concentrations of these hydroxycinnamic acids, measuring 34.29 ± 9.71 × 10−1 mg and 34.26 ± 3.15 mg 100 g-1 DW, respectively. Interestingly, some flavonoids, such as isoquercitrin and flavanone derivatives, were only detectable in the by-product UAE extracts. This suggests that after juice pressing, these flavonoids may become more concentrated and retained in the by-products[23].

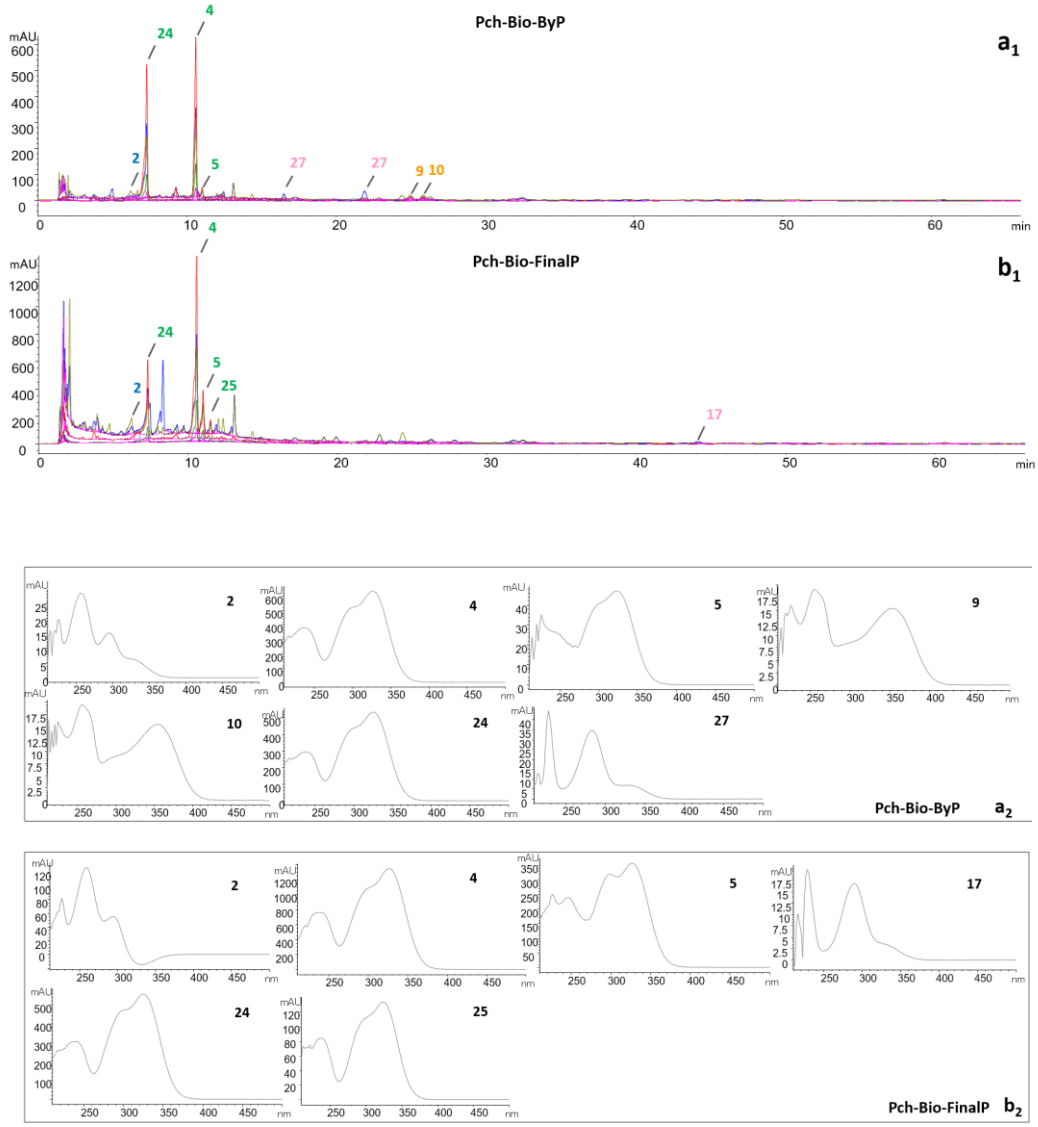


Figure 36 Overlaid HPLC chromatograms of Pch-Bio-ByP (a1) and Pch-Bio-FinalP (b1) samples registered at λ= 280 nm; λ =320 nm; λ= 370 nm; λ =360 nm; λ =250 nm; the original chromatogram reports Y axis in 0-600 mAU and 0-1200 mAU range respectively; UV-Vis spectra of detected peaks of Pch-Bio-ByP (a2) and Pch-Bio-FinalP (b2); 2 = Protocatechuic acid; 4= Chlorogenic acid; 5= Caffeic acid; 9= Hyperoside; 10= Isoquercitrin; 17= Naringenin; 24 = chlorogenic acid derivative; 25 = Hydroxycinnamic acid derivative; 27 = Flavanone derivative[23]

Table 12 Retention times of polyphenols identified in Pch-Bio-ByP and Pch-Bio-FinalP[23]

Peak	Compound	Reference Standard	Pch-Bio-ByP	Pch-Bio-FinalP
			(a1)	(b1)
		RT	RT	RT
2	Protocatechuic acid	6.175	6.202	6.201
24	Chlorogenic acid derivative	/	7.302	7.296
4	Chlorogenic acid	10.597	10.593	10.577
5	Caffeic acid	11.522	11.037	11.017
25	Hydroxycinnamic acid derivative	/	/	11.504
27	Flavanone derivative	/	16.509	/
27	Flavanone derivative	/	21.914	/
9	Hyperoside	24.975	24.995	/
10	Isoquercitrin	25.828	25.849	/
17	Naringenin	44.200	/	44.083

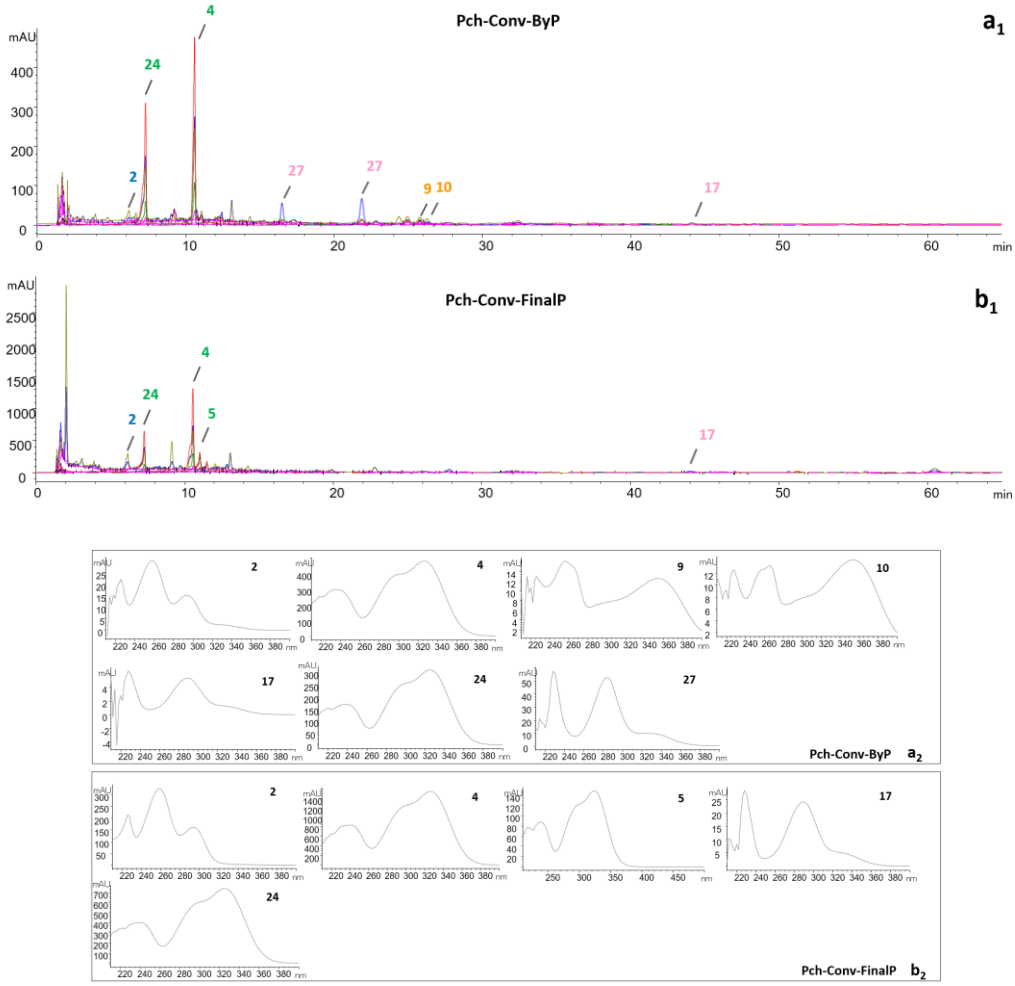


Figure 37 Overlaid HPLC chromatograms of Pch-Conv-ByP (a1) and Pch-Conv-FinalP (b1)

samples registered at λ= 280 nm; λ =320 nm; λ= 370 nm; λ =360 nm; λ =250 nm; the original chromatogram reports Y axis in 0-600 mAU and 0-1200 mAU range respectively; UV-Vis spectra of detected peaks of Pch-Conv-ByP (a2) and Pch-Conv-FinalP (b2); 2 = Protocatechuic acid; 4= Chlorogenic acid; 5= Caffeic acid; 9= Hyperoside; 10= Isoquercitrin; 17= Naringenin; 24 = chlorogenic acid derivative; 25 = Hydroxycinnamic acid derivative; 27 = Flavanone derivative[23]

Table 13 Retention times of polyphenols identified in Pch-Conv-ByP and Pch-Conv-FinalP[23]

Peak	Compound	Reference Standard RT	Pch-Conv-ByP (a1) RT	Pch-Conv-FinalP (b1) RT
2	Protocatechuic acid	6.175	6.186	6.181
24	Chlorogenic acid derivative	/	7.297	7.295
4	Chlorogenic acid	10.597	10.585	10.575
5	Caffeic acid	11.522	11.027	11.022
27	Flavanone derivative	/	16.494	/
27	Flavanone derivative	/	21.880	/
9	Hyperoside	24.975	24.947	/
10	Isoquercitrin	25.828	25.797	/
17	Naringenin	44.200	44.104	44.032

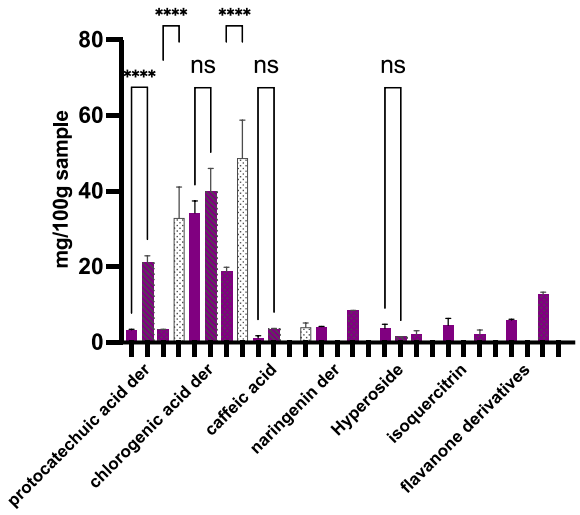


*Figure 38 Bar charts reporting the phenolic profile of the studied samples. The amount of phenolic acids and flavonoids, characterized in both the biological (colored columns) and conventional (colored/dotted columns) by-products, are compared with those found in the biological final products (colored/ribbed columns) and the conventional final ones (uncolored/dotted columns), respectively. The results are the mean value carried out from 3 analyses ± SD of the same sample. **** p < 0.0001; *** p < 0.005; ** p < 0.01; * p < 0.05. ns = not significant*[23]

Apricot Products

The quali-quantitative phenolic characterization of apricot UAE extracts (*Figure* 39 and *Figure* 40) indicates that apricot by-products tend to retain higher amounts of chlorogenic acid and its derivative, NCHA, compared to peach UAE extracts. Among the by-products studied, the Ac-Bio-ByP exhibits the highest concentrations

of these compounds, with levels of 49.95 ± 1.51 mg 100 g-1 DW for chlorogenic acid and 38.30 ± 5.92 mg 100 g-1 DW for NCHA, as shown in *Figure* 45. In terms of flavonoids, the results demonstrate that isoquercitrin is significantly present in apricot by-products, with the highest amounts recorded at 47.37 ± 4.63 mg 100 g-1 DW for Ac-Bio-ByP and 25.07 ± 8.20 mg 100 g-1 DW for Ac-Conv-ByP. Additionally, quercitrin was found in notable amounts in apricot by-products, measuring 4.80 ± 4.39 × 10^{-1} mg 100 g-1 DW for Ac-Bio-ByP and 5.09 ± 1.76 mg 100 g-1 DW for Ac-Conv-ByP. As illustrated in *Figure* 41, the phenolic profile of apricot UAE extracts is primarily characterized by a high concentration of hydroxycinnamic acids, with chlorogenic acid and its derivatives being the main components, followed by flavonols, where isoquercitrin is the dominant compound[23].

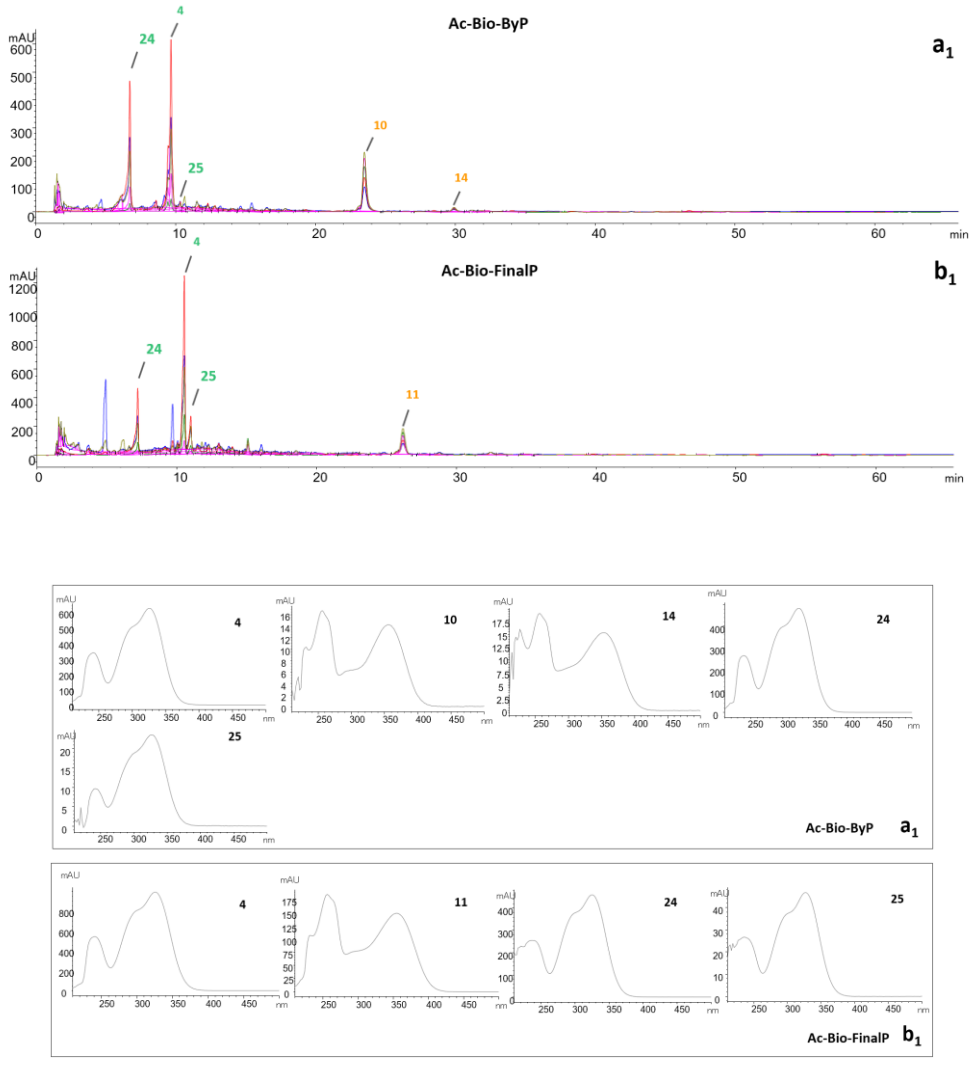


Figure 39 Overlaid HPLC chromatograms of Ac-Bio-ByP (a1) and Ac-Bio-FinalP (b1) registered at λ= 280 nm; λ =320 nm; λ= 370 nm; λ =360 nm; λ =250 nm; the original chromatogram reports Y axis in 0-600 mAU and 0-1200 mAU range respectively; UV-Vis spectra of detected peaks of Ac-Bio-ByP (a2) and Ac-Bio-FinalP (b2); 4= Chlorogenic acid; 10= Isoquercitrin; 11= (+)-Rutin trihydrate; 14= Quercitrin; 24 = Chlorogenic acid derivative; 25 = Hydroxycinnamic acid derivative[23]

Table 14 Retention times of polyphenols identified in Ac-Bio-ByP and Ac-Bio-ByP[23]

Peak	Compound	Reference Standard	Ac-Bio-ByP (a1)	Ac-Bio-FinalP (b1)
		RT	RT	RT
24	Chlorogenic acid derivative	/	6.798	6.897
4	Chlorogenic acid	10.597	9.703	10.551
25	Hydroxycinnamic acid derivative	/	12.340	13.980
10	Isoquercitrin	25.828	23.784	/
11	(+)-Rutin trihydrate	26.301	/	26.194
14	Quercitrin	32.934	30.269	/

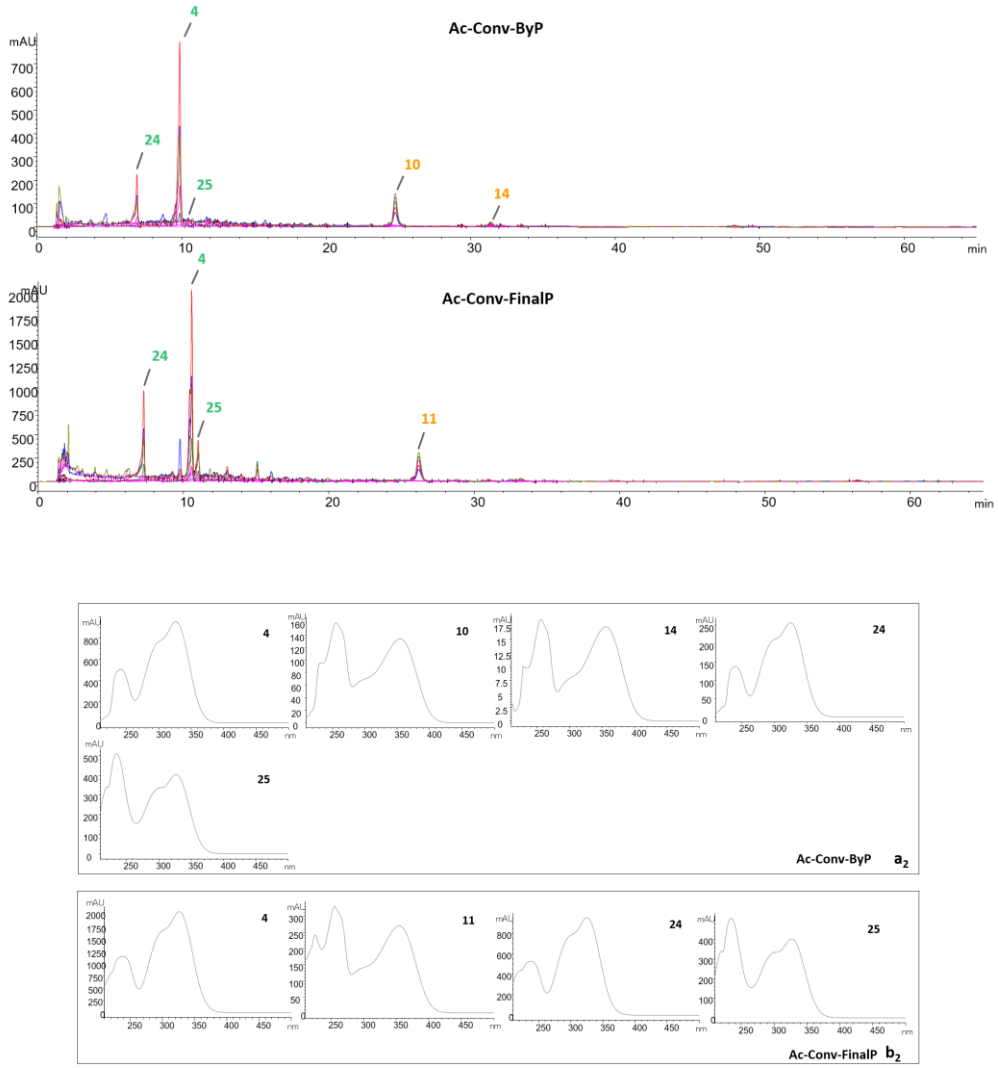


Figure 40 Overlaid HPLC chromatograms of Ac-Conv-ByP (a1) and Ac-Conv-FinalP (b1) registered at λ= 280 nm; λ =320 nm; λ= 370 nm; λ =360 nm; λ =250 nm; the original chromatogram reports Y axis in 0-600 mAU and 0-1200 mAU range respectively; UV-Vis spectra of detected peaks of Ac-Bio-ByP (a2) and Ac-Bio-FinalP (b2); 4= Chlorogenic acid; 10= Isoquercitrin; 11= (+)-Rutin trihydrate; 14= Quercitrin; 24 = Chlorogenic acid derivative; 25 = Hydroxycinnamic acid derivative[23]

Table 15 Retention times of polyphenols identified in Ac-Conv-ByP and Ac-Conv-ByP[23]

Peak	Compound	Reference Standard	Ac-Conv-ByP (a1)	Ac-Conv-FinalP (b1)
		RT	RT	RT
24	Chlorogenic acid derivative	/	6.984	7.298
4	Chlorogenic acid	10.597	9.969	10.580
25	Hydroxycinnamic acid derivative	/	11.934	11.029
10	Isoquercitrin	25.828	23.360	/
11	(+)-Rutin trihydrate	26.301	/	26.285
14	Quercitrin	32.934	30.053	/

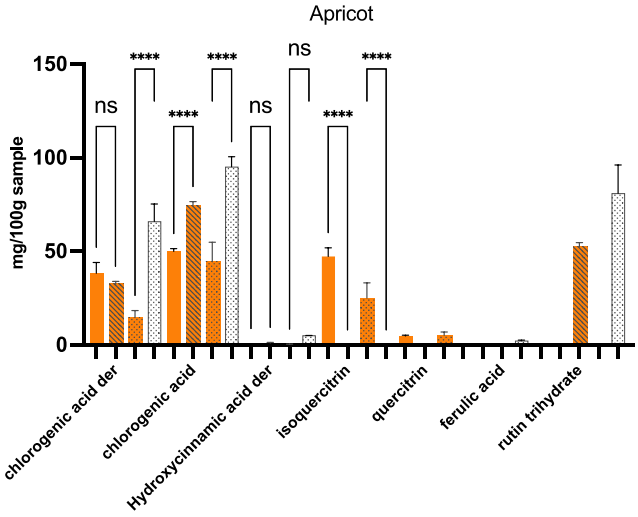


*Figure 41 Bar charts reporting the phenolic profile of the studied samples. The amount of phenolic acids and flavonoids, characterized in both the biological (colored columns) and conventional (colored/dotted columns) by-products, are compared with those found in the biological final products (colored/ribbed columns) and the conventional final ones (uncolored/dotted columns), respectively. The results are the mean value carried out from 3 analyses ± SD of the same sample. **** p < 0.0001; *** p < 0.005; ** p < 0.01; * p < 0.05. ns = not significant*[23]

Tomato Products

The quali-quantitative phenolic characterization of tomato UAE extracts (*Figure* 42 and *Figure* 43) revealed that naringenin was the predominant polyphenol, present in higher amounts than in all the other fruit UAE extracts studied (*Figure* 45). Notably, tomato byproducts contained significant levels of naringenin, with measurements of 16.83 ± 1.71 mg 100 g-1 DW (DW) for T-Bio-ByP and 15.57 ± 0.24 mg 100 g-1 DW for T-LI-ByP. These amounts were even higher than those found in the final tomato products. Additionally, rutin was also present in considerable amounts (11.31 ± 0.27 mg 100 g-1 DW for T-Bio-ByP and 15.37 ± 4.44 mg 100 g-1 DW for T-LI-ByP), along with other unidentified flavonol derivatives. However, overall, tomato UAE extracts were found to be the poorest source of polyphenols compared to the other fruit UAE extracts (Figure 45). This finding aligns with the results obtained from Total Polyphenol Content (TPC) and Total Antioxidant

Status (TAS) analyses. The tomato UAE extracts exhibited a distinct qualitative profile, with flavonoids (specifically flavanones and flavonols) being widely distributed throughout the UAE extracts (*Figure* 42 and *Figure* 43). Furthermore, similar to the peach UAE extracts, naringenin and its derivatives were also detected in the final tomato product UAE extracts[23].

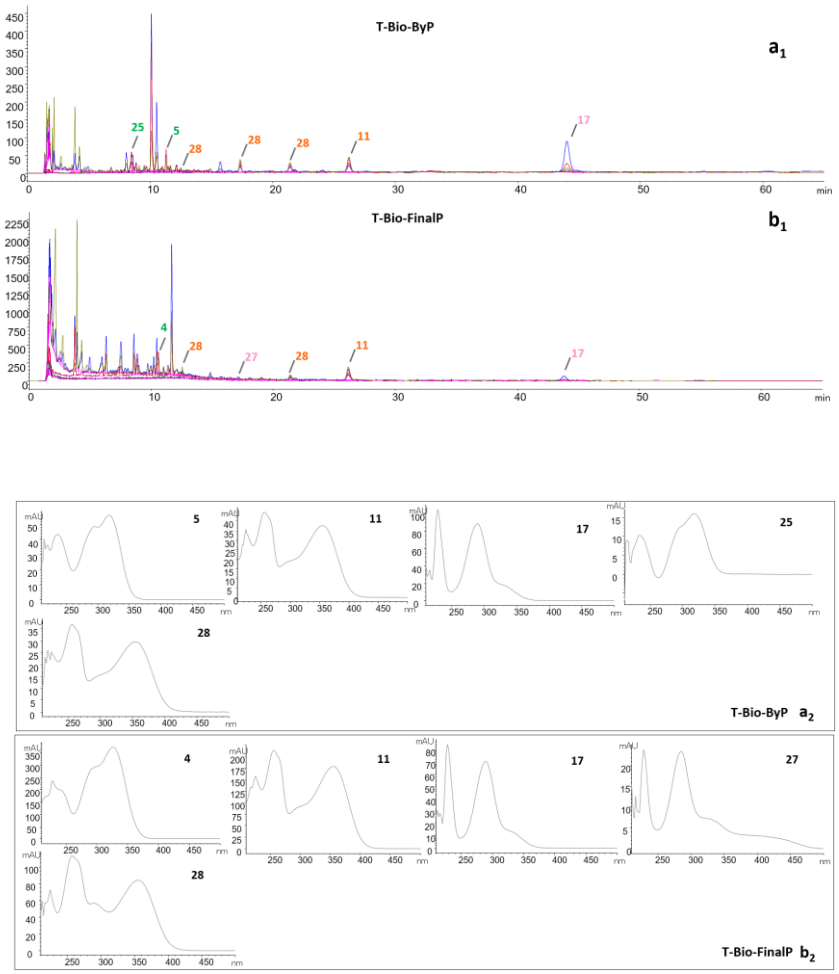


Figure 42 Overlaid HPLC chromatograms of T-Bio-ByP (a1) and T-Bio-FinalP (b1) samples registered at λ= 280 nm; λ =320 nm; λ= 370 nm; λ =360 nm; λ =250 nm; the original chromatogram reports Y axis in 0-400 mAU and 0-2000 mAU range respectively; UV-Vis spectra of detected peaks of T-Bio-ByP (a2) and T-Bio-FinalP (b2); 4= Chlorogenic acid; 5= Caffeic acid; 11= (+)-Rutin trihydrate; 17= Naringenin; 25 = Hydroxycinnamic acid derivative; 27= Flavanone derivative; 28 = Flavonol derivative[23]

Table 16 Retention times of polyphenols identified in T-Bio-ByP and T-Bio-FinalP[23]

Peak	**Compound**	**Reference Standard RT**	**T-Bio-ByP (a1) RT**	**T-Bio-FinalP (b1) RT**
25	Chlorogenic acid derivative	/	9.558	/
4	Chlorogenic acid	10.597	/	10.564
5	Caffeic acid	11.522	11.331	/

28	Flavonol derivative	/	12.527	/
27	Flavanone derivative	/	/	16.448
28	Flavonol derivative	/	17.457	/
28	Flavonol derivative	/	21.602	21.575
11	(+)-Rutin trihydrate	26.301	26.270	26.214
17	Naringenin	44.200	43.709	43.673

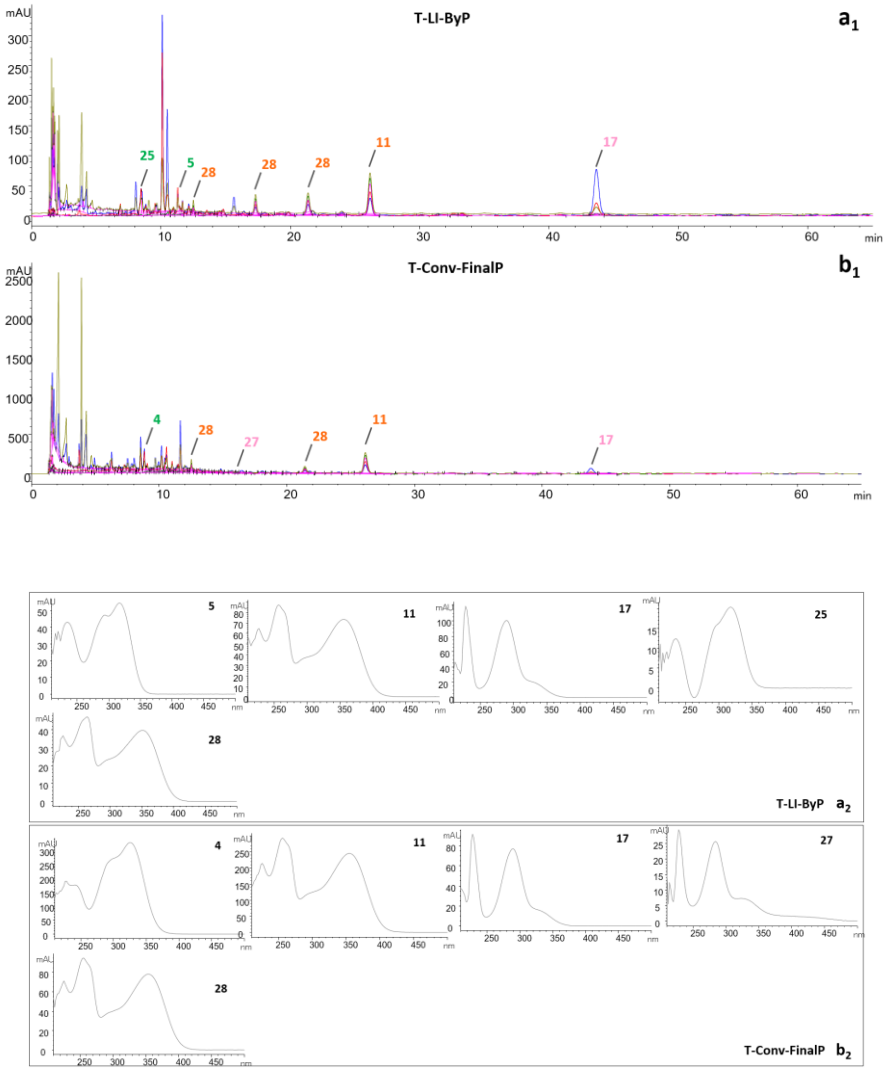


Figure 43 Overlaid HPLC chromatograms of T-LI-ByP (a1) and T-Conv-FinalP (b1) samples registered at λ= 280 nm; λ =320 nm; λ= 370 nm; λ =360 nm; λ =250 nm; the original chromatogram reports Y axis in 0-400 mAU and 0-2000 mAU range respectively; UV-Vis spectra of detected peaks of T-LI-ByP (a2) and T-Conv-FinalP (b2); 4= Chlorogenic acid; 5= Caffeic acid; 11= (+)-Rutin trihydrate; 17= Naringenin; 25 = Hydroxycinnamic acid derivative; 27= Flavanone derivative; 28 = Flavonol derivative[23]

Table 17 Retention times of polyphenols identified in T-LI-ByP and T-Conv-FinalP[23]

Peak	Compound	Reference Standard	T-LI-ByP (a1)	T-Conv-FinalP (b1)

		RT	RT	RT
25	Chlorogenic acid derivative	/	9.552	/
4	Chlorogenic acid	10.597	/	10.556
5	Caffeic acid	11.522	11.317	/
28	Flavonol derivative	/	12.523	/
27	Flavanone derivative	/	/	16.444
28	Flavonol derivative	/	17.349	/
28	Flavonol derivative	/	21.431	21.277
11	(+)-Rutin trihydrate	26.301	26.219	26.073
17	Naringenin	44.200	43.861	43.707

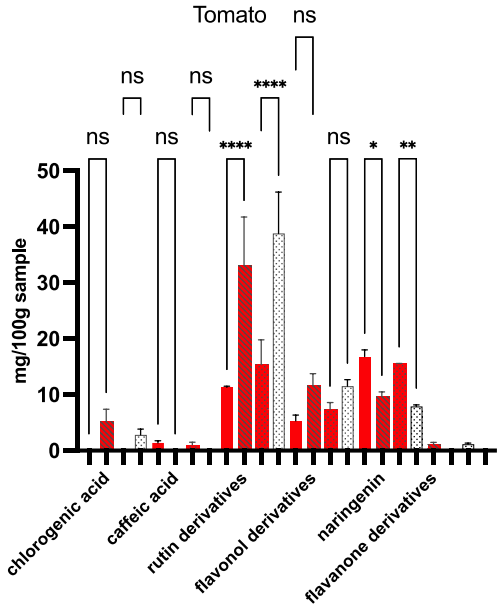


*Figure 44 Bar charts reporting the phenolic profile of the studied samples. The amount of phenolic acids and flavonoids, characterized in both the biological (colored columns) and conventional (colored/dotted columns) by-products, are compared with those found in the biological final products (colored/ribbed columns) and the conventional final ones (uncolored/dotted columns), respectively. The results are the mean value carried out from 3 analyses ± SD of the same sample. **** p < 0.0001; *** p < 0.005; ** p < 0.01; * p < 0.05. ns = not significant*[23]

The results can be summarized as follows: All peach by-products exhibit chlorogenic acid and its derivatives as the most abundant compounds. However, the concentration of these compounds is lower in the by-products (ByP) compared to the final products (FinalP). Notably, the concentration of chlorogenic acid derivatives in Pch-Bio-ByP is higher than that found in Pch-Conv-ByP and is almost comparable to that of Pch-Bio-FinalP. Interestingly, the flavonoid derivatives are more abundant in the by-products than in the final products. Similar findings were observed in apricot by-products, where a significant amount of isoquercitrin was detected in both Ac-ByP UAE extracts. This trend also applies to Apl-ByP, where the quantities of hyperoside, quercitrin,

and myricetin are greater than those in Apl-FinalP. In contrast, the concentration of chlorogenic acid in Apl-FinalP is six times higher than that in Apl-ByP. On the other hand, tomato by-products contain a very low amount of chlorogenic acid. However, T-ByP demonstrates a consistent profile of flavonoid derivatives in terms of type and concentration. In particular, the level of naringenin in T-ByP exceeds that found in T-FinalP[23].

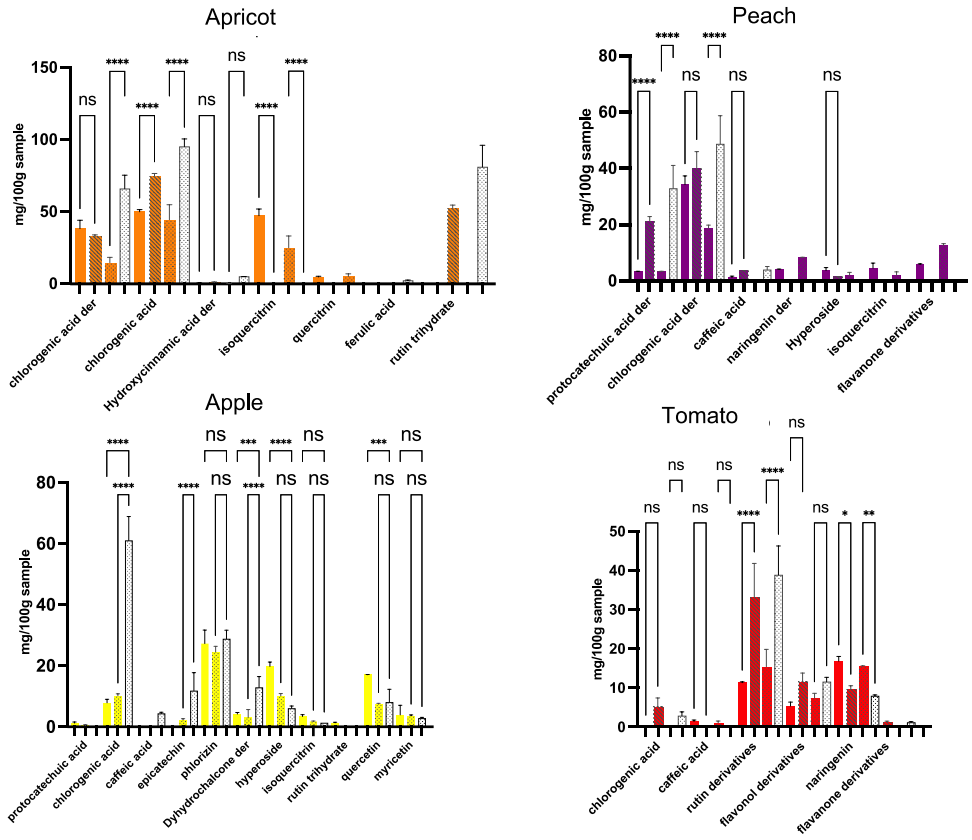


*Figure 45 Bar charts reporting the phenolic profile of the studied samples. The amount of phenolic acids and flavonoids, characterized in both the biological (colored columns) and conventional (colored/dotted columns) by-products, are compared with those found in the biological final products (colored/ribbed columns) and the conventional final ones (uncolored/dotted columns), respectively. The results are the mean value carried out from 3 analyses ± SD of the same sample. **** $p < 0.0001$; *** $p < 0.005$; ** $p < 0.01$; * $p < 0.05$. ns = not significant*[23]

2.2.1.8 From Waste to Wellness: Revealing the Potential of Fruit By-products

Plant polyphenols have been studied for their numerous benefits on human health. Their activities are especially related to the treatment and prevention of several chronic diseases[90]. Moreover, their beneficial interactions with the gut microbiota and prebiotic modulation have been assessed recently as well[91]. From a circular economy perspective, the possibility of keeping materials in circulation has pointed out the opportunity to valorize wastes from the agri-food industry. Studies carried out with this aim have evidenced the recovery of phenolic compounds from byproducts, showing the potential therapeutic applications in the nutraceutical

field due to their benefits as antiproliferative, antioxidant, and anti-inflammatory agents[92,93]. To optimize the reuse of fruit by-products as alternative sources of polyphenols, we carried out this study that ultimately confirmed that the investigated industrial wastes can retain significant amounts of several phenolic subclasses present in their traditional fruit counterparts (final products), as summarized in Figure 45. In particular, the quali/quantitative analyses evidenced phloridzin as a distinctive polyphenol found in apples[94]. It was shown to be equally retained in the apple by-product extracts studied when compared to their respective final product (apple juice). This supposes the possibility of using these by-products in the recovery of this valuable compound. Indeed, extracts rich in phloridzin could be promising candidates to develop formulations destined for managing metabolic disorders, such as diabetes, due to their reported hypoglycemic activity[95,96]. Apple by-products under study were shown to be rich in different phenolic subclasses. Particularly, the biological apple by-product was found to concentrate higher quantities of flavonols after juice processing. Among them, hyperoside (quercetin-3-O-galactoside) and quercitrin (quercetin 3-rhamnoside) add greater value to this by-product due to the well-known structurally related antioxidant/antiradical profile of quercetin and its derivatives[97–98]. This could further explain the higher TAS value obtained for Apl-Bio-ByP than that expected regardless of its low TPC value. The results also depicted a significant antioxidant activity through the TAS assay for peach and apricot by-products, seemingly related to the high amounts of chlorogenic acids quantified in these samples. Indeed, chlorogenic acids were found to be the main phenolic compounds in peach and apricot by-products, remaining in considerable amounts also after juice processing. This result could open up the opportunity to valorize the extracts from these by-products and take advantage of the antioxidant activity related to these compounds[99]. At the same time, the high and unique presence of the flavonol isoquercitrin (quercetin-3-O-β-d-glucopyranoside), found exclusively in the apricot byproducts, could increase the value of extracts obtained from these upcycled materials even more. Regarding tomato by-product extracts, although they showed the lowest TPC and TAS values, the quali-quantitative analyses of their composition, obtained by HPLC-DAD, evidenced these materials as valuable sources of flavonoids too. In particular, the concentration of the flavanone naringenin in these waste materials surpassed that found in their traditional tomato counterparts (final products). In this sense, tomato by-products can be considered alternative sources of naringenin. That is a significant aspect since naringenin is a polyphenol with promising supplement applications for cardiovascular protection[100]. Nonetheless, the main valorization of tomato by-products might be directed to the extraction of proteins since these materials were found to contain the highest values of proteins among fruit by-products. Then high-quality proteins could be recovered and/or used to produce supplements rich in bioactive peptides[28]. All these results give the possibility of designing and carrying out new strategies for the further valorization of biomass from the agri-food industry in a circular economy approach. The characterized residues may also retain other chemical compounds of interest, such as carbohydrates, carotenoids (ß-carotene and lycopene), and lipids (especially in tomato by-products[28]), which may be exploited for added health-promoting properties[23].

2.2.1.9 Assessment of Pesticide Residues in Fruit By-product Extracts for Safe Valorization

The pesticide levels in the analyzed sample extracts, using the method described in *Section* 2.1.10, were assessed to clarify the safety of their usage.

Analyzing pesticide residues in fruit extracts from agricultural by-products is essential for valorization studies and plays a crucial role in incorporating these extracts into pharmaceutical and nutraceutical practices[101]. This evaluation is a crucial safety assessment that aligns with strict regulatory standards to assess potential health risks. Meeting these safety criteria is essential for ensuring compliance with regulations that govern pesticide levels in food products, especially before considering these extracts for commercial use. Additionally, disclosing and understanding pesticide levels in by-product extracts promotes transparency, which is vital for market acceptance and consumer trust in pharmaceutical and nutraceutical products derived from agri-food by-products[102].

Table 18 presents the pesticide levels found in the tested fruit UAE extracts. The results indicate that all extracts from biological fruit by-products (apricot, apple, and peach) contained no detectable pesticides, except for biological tomato peels, which showed small traces of pesticides. In biological farming, pesticide use is minimal compared to conventional agriculture, and the findings confirm that the majority of these extracts are free from pesticides. For the biological tomato by-products, only negligible traces of pesticides were detected, well within the legal limit of less than 0.01 ppm[86].

Table 18. Pesticide content in analysed fruit UAE extracts providing (n=1).

Sample	Pesticide Content mg/kg (ppm)
Ac-Bio-ByP	*None detected*
Ac-Conv-ByP	Boscalid 0.0042 Cyprodinil 0.0009 Deltamethrin 0.0016 Difenoconazole 0.0008 Etofenprox 0.0065 Fenbuconazole 0.0003 Fluopyram 0.0002 Tebuconazole 0.0018
Pch-Bio-ByP	*None detected*
Pch-Conv-ByP	Difenoconazole 0.0004 Fludioxonil 0.0006

Apl-Bio-ByP	*None detected*
Apl-Conv-ByP	Boscalid 0.0013 Deltamethrin 0.0009 Difenoconazole 0.0004 Etofenprox 0.0087 Fludioxonil 0.0006 Triflumuron 0.0016 Tetrahydrophthalimide 0.0008 = Captan* 0.0016
T-Bio-ByP	Chlorpyriphos ethylene 0.0002 Pendimethalin 0.00025
T-LI-ByP	*None detected*

** Tetrahyrdopthalimide is a metabolite of captan*[103].

Consequently, when valorizing these extracts for pharmaceutical, nutraceutical, cosmetic, or food applications, those derived from biological by-products can be superior to conventional ones due to the lack of pesticides that could pose potential safety issues[104].

2.2.1.10 Long-Term Stability of Polyphenols in UAE By-product Extract

The stability of polyphenols in liquid UAE extracts from fruit by-products was evaluated using apple biological by-product (Apl-Bio-ByP) extract as a representative sample. To assess polyphenol stability, the quantification of polyphenols was performed using the HPLC-DAD method, as described in par 2.1.6. The extract was analyzed at the beginning of the study (time zero, t=0) and again after 15 months of storage (t=15 mth) under the same conditions outlined in *Section* 2.1.11.1. *Figure* 46 shows a remarkable consistency between the two polyphenolic profiles, as indicated by nearly identical bar charts. The difference was found to be non-significant (ns) as can be observed from the results of the statistical t-test (*Figure* 46). This consistency confirms that the polyphenols in the extracts remain stable over time when stored according to the specified conditions in *Section* 2.1.11.1. This assessment is crucial for validating optimal storage conditions that prevent the degradation of polyphenols. Understanding polyphenol stability is essential due to its thermolabile and photosensitive nature, which is important for preserving both the quantity and chemical structure of the extracts[105]. In addition to analytical purposes, preserving polyphenol integrity is important in various fields, including pharmaceuticals and nutraceuticals.

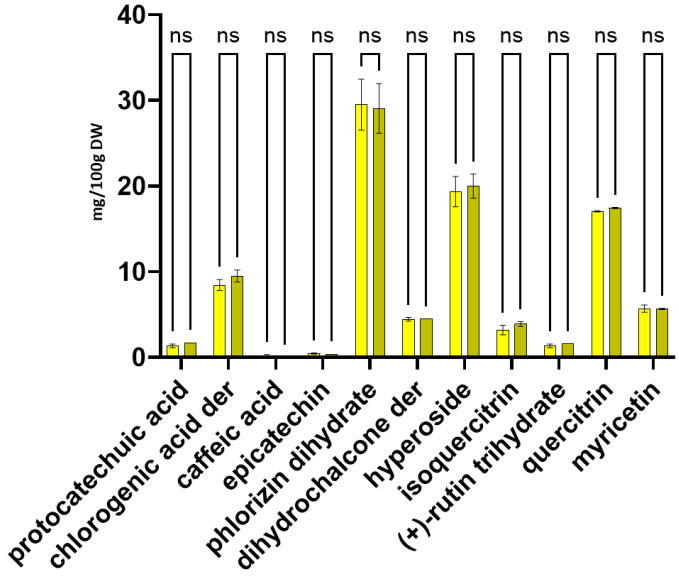


Figure 46 Bar graph reported the stability of polyphenols in the Apl-Bio-ByP UAE extract analyzed by the HPLC-DAD method (see Section 2.1.8). The quantification of polyphenols in the Apl-Bio-ByP UAE extract was measured at two time points: at t=0 (full light-yellow bar) and at t=15 mth (full dark-yellow bar). The UAE extracts were injected twice (n=2), and both the mean and standard deviation (SD) are reported. ns=not significant

2.2.1.11 Stability of Polyphenols in Stored By-product Powder

The results of the polyphenol quantification in two UAE extracts of the same powdered apple by-product (Apl-Bio-ByP) chosen as a representative sample, were compared. The first extract was obtained and analyzed immediately after the sample powder was produced, while the second extract was obtained and analyzed after 15 months of powder storage, as detailed in *Section* 2.1.11.2. The quantitative determination was performed using the HPLC-DAD method (described in *Section* 2.1.8.1) immediately after UAE extraction. *Figure* 47 illustrates the quantity of polyphenols extracted during both time periods, showing that the polyphenolic amounts remained significantly the same. The difference was found to be non-significant (ns) as can be observed from the results of the statistical t-test (see *Figure* 47). Repeating the same extraction methodology further validated the reliability of the method and assessed the consistency of the data generated. The similarity in the bar charts reinforces the reliability of the UAE process (refer to *Section* 2.1.4), demonstrating its capability to consistently produce comparable and trustworthy results.

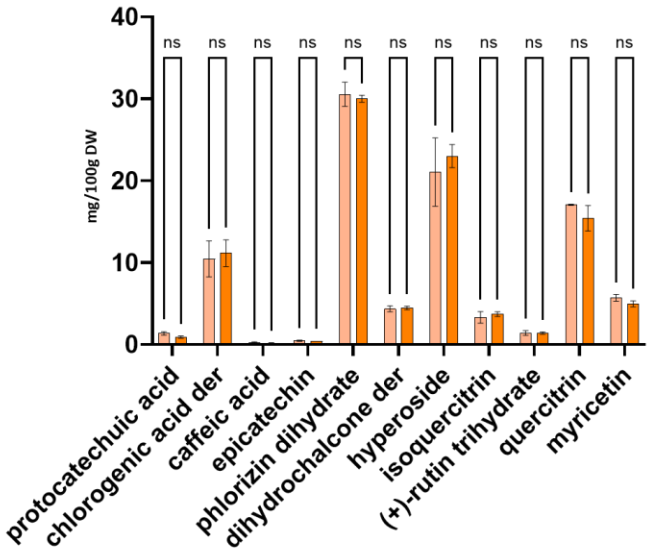


Figure 47 Bar chart showing long-term stability of polyphenols extracted from the powdered sample Apl-Bio-ByP at time zero (t=0) of storage (full light-orange bar) and after 15 months (t=15 mth) of storage (full dark-orange bar). The UAE extracts were injected twice (n=2), and both the mean and standard deviation (SD) are reported. ns=not significant

2.2.2 Characterization of Legume samples

All the characterization of UAE extracts of legumes has been reported in literature[80].

2.2.2.1 Gravimetric Determination—Extract Yield (%) of UAE legume UAE extracts

Figure 48 shows a comparison of extract yield values (%) obtained (reported in *Table* 19) from bean by-products (brawn full bars) and the respective final product (brawn striped bar). For soy, the graph compares the extract yield values (%) between the soy by-product (pink full bar) obtained after pressing during the industrial process and the seeds (RM-raw material in pink striped bars). In the case of green beans, a comparison is reported between the different by-products analyzed in the project (all green full bars). Regarding bean samples, the results demonstrate that the amount of extracts obtained from the by-products is only slightly lower than that of the corresponding final product. Moreover, the fresh biological by-product (B-Bio-Fr-ByP) even shows a higher yield value (%). This suggests that the extractable portion from the by-product and the final product is similar. As for the green beans by-products, no significant differences are observed except for the cooked biological by-product (GB-Bio-Co-ByP), which has the lowest yield values (%) among green beans. This difference could be attributed to the composition of this sample. Similar results were observed for pea samples, with both by-products (P-Fr-Bio-ByP and P-Fr-Conv-ByP) exhibiting lower percentage yield values compared to the final product (P-FinalP). Lastly, soy seeds (S-Bio-RM and S-Conv-RM) were found to have higher yield values (%) than the by-product. In this case, both the industrial procedure for obtaining soy milk and the composition of the sample itself may have affected the % yield of dry extract

obtained[80].

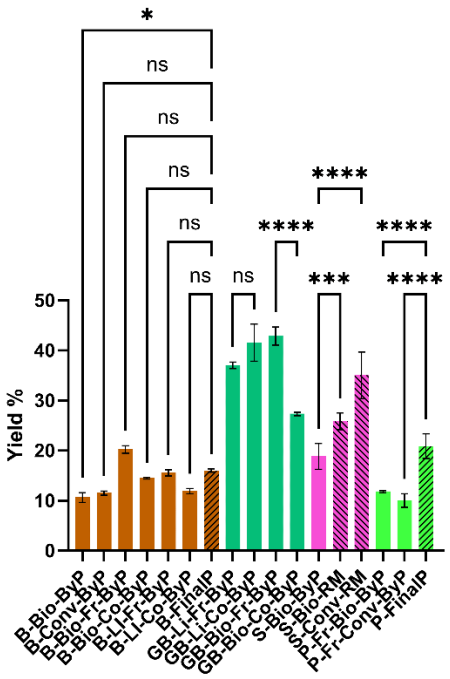


*Figure 48 The yield (%) of the extracts obtained by following the method reported by Terenzi et. al[56] of legume by-products (designated as ByP) and final products (designated as FinalP) achieved via ultrasound-assisted extraction (UAE). The results represent the mean value of three extractions, with standard deviation (SD) values for the same sample. **** p < 0.0001, ** p < 0.01, *p<0.05. ns=not significant[80]*

Table 19 Extract Yields (%) of UAE Legume extracts[80]

Samples analyzed	Gravimetric Determination Yield (%) ±SD (n=3)
B-Bio-ByP	10.6±9.77E-01
B-Conv-ByP	11.5±4.33E-01
B-FinalP	20.2±7.52E-01
B-Fr-Bio-ByP	14.5±1.60E-01
B-Co-Bio-ByP	15.6±6.26E-01
B-Fr-LI-ByP	11.9±5.14E-01
B-Co-LI-ByP	16.0±3.30E-01
GB-Fr-LI-ByP	37.0±6.57E-01
GB-Co-LI-ByP	41.6±3.72E+00
GB-Fr-Bio-ByP	42.9±1.82E+00
GB-Co-Bio-ByP	27.3±3.14E-01
S-Bio-RM	18.8±2.59E+00
S-Conv-RM	25.9±1.67E+00
S-Bio-ByP	35.1±4.64E+00
P-Fr-Bio-ByP	11.80±2.02E-01

P-Fr-Conv-ByP	10.03±1.36E+00
P-FinalP	21.57±2.97E+00

2.2.2.2 Total Phenolic Content (TPC) of UAE legume samples

This study investigated the TPC of by-products from various legumes (beans, green beans, peas and soy) and compared it to the final product for trade (see *Figure* 49, *Table* 20). Our findings revealed considerable variation in polyphenolic content among legume UAE extracts. Interestingly, the TPC found for bean by-products, ranging from 26.27±1.49E+00mg GAE/1g Ext to 43.49±1.07E+00 mg GAE1/g Ext, generally equaled or exceeded that of the final product (33.01±3.08E+00mg GAE/1g Ext). This suggests significant retention of polyphenols within the by-products after processing. These results are consistent with previous studies on black bean by-products employing conventional solid-liquid extraction methods, albeit with different solvents[106]. For example, Moreno-García et al. (2022) reported a TPC of 38.41 and 38.60 mg GAE/g Ext for black bean by-products, supporting the trends observed in our bean by-product extracts.

Further analysis revealed a slight decrease in TPC of cooked by-products compared to their fresh counterparts. This reduction is likely attributable to thermal processing during cooking, which can degrade polyphenols[107]. Similar observations have been reported in the literature, with Shu-Cheng Duan et al. (2021)[107] demonstrating a decrease in TPC of faba leaves and seeds after wet heat treatment. Notably, moist heat treatments, such as boiling, have been shown to significantly reduce TPC across various food types[107]. This observation was further supported by the decrease in TPC of biological green bean by-products after cooking, from 17.68±1.61E+00 to 13.60±5.26E-01mg GAE/g Ext and LI green bean by-products, from 28.68±3.52E+00 to 11.51±1.43E+00 mg GAE/g Ext.

In contrast, Okara (S-Bio-ByP) exhibited a significantly higher TPC, 309.6 ± 0.13 mg GAE/100 g DW, compared to the value reported by Ana Carolina Pelaes Vital et al. (2018) which is 130.50 ± 10.20 mg GAE/100 g DW (result was expressed as mg GAE/100 g dry weight (DW) to facilitate comparison with literature data) obtained by conventional extraction with a 50% acetone mixed for 30 minutes[108]. This discrepancy may be attributed to the use of an ultrasonic bath for the extraction in our study. Compared to conventional solid-liquid mixing, ultrasonic extraction facilitates cell wall rupture and disintegration of the solid matrix, thereby increasing the contact surface between solvent and target compounds and potentially enhancing polyphenol yield.

Regarding the TPC values of pea samples, it is noteworthy that by-products, both biological and conventional, exhibited higher TPC levels than the final products. These obtained results align with the literature, as reported by Gazwi et al.[109], where the total polyphenol content in pea by-products, including the seed coat, was determined to be 27.04 ± 0.94 mg GAE/g extract.

Concerning different cultivation methods, the data reported in *Figure* 49 show that for the three types of samples analyzed—green beans, beans, peas and soy—biological or LI methods generally result in higher amounts of polyphenols compared to conventional ones. This could be explained by the fact that reduced pesticide use might induce polyphenol production as part of the plants' defense mechanisms[80].

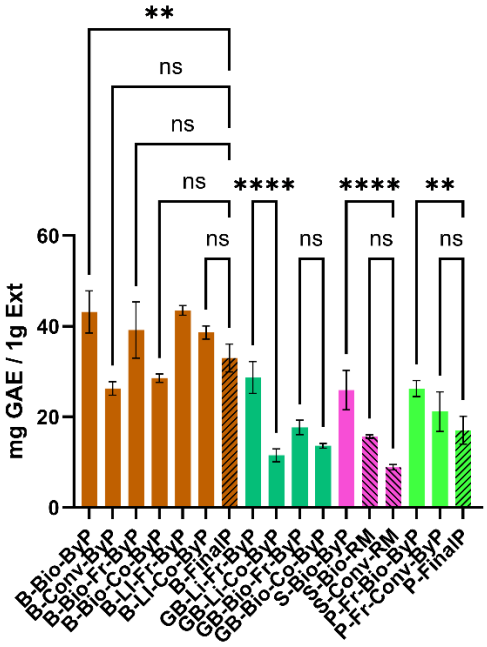


*Figure 49 TPC (mg GAE/1 g Ext) of ByP (full bars) and FinalP (stripped bars) extracts obtained via UAE. Data represent mean ± SD (n=3, each analysed in duplicate). **** p < 0.0001, ** p < 0.01*[80]

Table 20 TPC of UAE Legume extracts[80]

Samples analyzed	Total Phenolic Content (TPC) Average mg GAE g-1 Ext ±SD (n=3)
B-Bio-ByP	43.18±4.66E+00
B-Conv-ByP	26.27±1.49E+00
B-FinalP	39.17±6.19E+00
B-Fr-Bio-ByP	28.55±9.30E-01
B-Co-Bio-ByP	43.49±1.07E+00
B-Fr-LI-ByP	38.63±1.46E+00
B-Co-LI-ByP	33.01±3.08E+00
GB-Fr-LI-ByP	28.68±3.52E+00
GB-Co-LI-ByP	11.51±1.43E+00
GB-Fr-Bio-ByP	17.68±1.61E+00
GB-Co-Bio-ByP	13.60±5.26E-01
S-Bio-RM	25.94±4.35E+00
S-Conv-RM	15.66±3.68E-01
S-Bio-ByP	8.89±6.03E-01
P-Fr-Bio-ByP	26.23±1.78E-01
P-Fr-Conv-ByP	21.06±4.36E+00
P-FinalP	17.04±3.08E+00

2.2.2.3 Total Antioxidant Status Assay (TAS) of UAE legume samples

The antioxidant activity of the bean by-products (reported in *Table* 21), ranging from 162.50±2.75E+00 to

336.33±3.06E+00 µmol TE/g Ext), was comparable to that of the final product (334.33±7.64E-01 µmol TE/g Ext). Notably, B-Bio-ByP exhibited slightly higher antioxidant activity than the final product (B-FinalP), 336.33±3.06E+00 µmol TE/g Ext vs 334.33±7.64E-01 µmol TE/g Ext respectively. These findings align with previous research. For instance, Moreno-García et al.[106] reported antioxidant activity values of 345.48 and 360.81 µmol TE/g Ext for black bean by-products extracted with conventional method using 80% methanol and 70% ethanol, respectively. Our results further confirm the potential of bean by-products as a valuable source of antioxidants.

A comparative analysis of fresh and cooked bean by-products revealed a surprising trend: the antioxidant activity was consistently higher in the cooked UAE extracts, regardless of whether they were derived from biological (B-Co-Bio-ByP: 312.00±6.08E+00 µmol TE/g Ext vs B-Fr-Bio-ByP: 162.50±2.75E+00 µmol TE/g Ext) or LI (B-Co-LI-ByP: 264.25±1.68E+00 µmol TE/g Ext vs B-Fr-LI-ByP: 169.83±6.34E-01 µmol TE/g Ext) cultivation methods, as reported in *Figure* 50. Several factors may contribute to this unexpected outcome. Firstly, cooking processes, such as stewing, can inactivate certain substances that inhibit the absorption of antioxidants in fresh beans, thereby enhancing their bioavailability. Secondly, thermal treatment can disrupt the cell wall structure, facilitating the release of antioxidants into the medium. However, it is crucial to acknowledge that the impact of thermal processing on antioxidant activity can vary significantly depending on factors such as processing time, temperature, and the specific compounds present in the food matrix.

For instance, Ng et al.[110] have demonstrated that boiling, in some cases, can significantly enhance the antioxidant activity of certain vegetables, such as broccoli, bitter gourd, and water convolvulus. This highlights the importance of considering the specific vegetable type and cooking method to optimize the preservation or enhancement of its nutritional value.

These findings are further supported by the observed antioxidant activity in green bean by-products (see *Figure* 50). Consistent with previous observations, cooked green beans exhibited higher antioxidant activity compared to their fresh counterparts. This suggests that, in the specific case of green beans, steaming may enhance their antioxidant potential.

In the case of soy, in order to validate our findings, a comparative analysis of the antioxidant activity of S-Bio-ByP, a by-product of soy milk production, was conducted with previously published data. The antioxidant activity was assessed using the ABTS radical scavenging assay, and the results were expressed as inhibition percentage (I%) to facilitate comparison with literature data. Our study yielded an I% of 24.54, which is slightly lower than the value of 37.84 ± 3.14 reported by Vital et al.[108]. However, this difference is considered minor and does not significantly deviate from the general trend reported in the literature.

Interestingly, our results indicate that okara (S-Bio-ByP) exhibits higher antioxidant activity than the original soy seeds (196.61±5.01E+00 µmol TE/g Ext vs 134.86±2.93E+00 and 123.97±6.16E+00 µmol TE/g Ext respectively). This unexpected result can be attributed to the concentration of bioactive compounds that occur during the processing of soy milk. Polyphenols, known for their potent antioxidant properties, tend to accumulate in the solid fraction (okara) during the extraction process. This concentration effect likely

contributes to the enhanced antioxidant activity observed in okara.

To further support this hypothesis, it is possible to observe that TPC results revealed a lower concentration of polyphenols in soy seeds, S-Bio-RM and S-Conv-RM, (15.66±3.68E-01 mg GAE/1g Ext and 8.89±6.03E-01 mg GAE/1g Ext), compared to okara (S-Bio-ByP) (25.94±4.35E+00 mg GAE/1g Ext), in line with the observed higher antioxidant activity in the latter[80].

Interesting results were observed for the TAS values of pea by-products, which were significantly higher (218.66±4.21E-01 and 375.19±4.99E+00 µmol TE/g Ext) than those of the final product (136.15±2.40E+00 µmol TE/g Ext). Comparing the obtained data with literature values for Inhibition %[111], to facilitate cross-study comparisons, it can be detailed that the obtained TAS values for pea by-products, specifically 16.3% for the biological by-product (P-Fr-Bio-ByP) and 25.3% for the conventional by-product (P-Fr-Conv-ByP), are comparable to those reported in the literature for pea seed flour extracted with 80% ethanol[111].

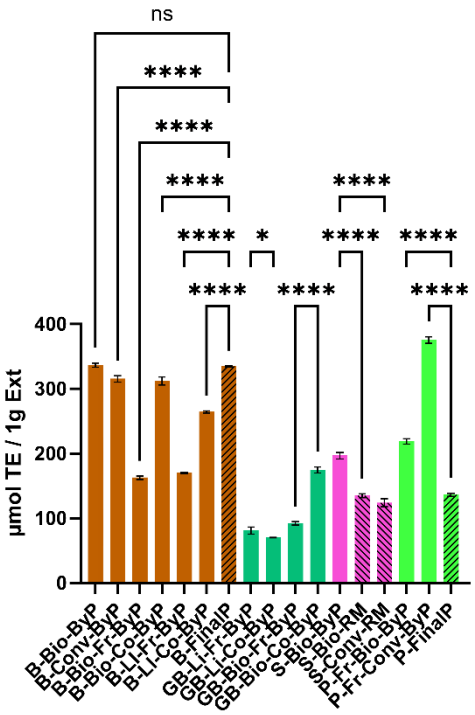


*Figure 50 TAS (µmol TE/1 g Ext) of ByP (full bars) and FinalP (stripped bars) extracts obtained via UAE. Data represent mean ± SD (n=3, each analysed in duplicate). **** p < 0.0001, * p < 0.05. ns = not significant[80]*

Table 21 TPC of UAE Legume extracts[80]

Samples analyzed	Total Antioxidant Status (TAS) Average µmol TE g-1 Ext ±SD (n=3)
B-Bio-ByP	336.33±3.06E+00
B-Conv-ByP	315.00±5.00E+00
B-FinalP	162.50±2.75E+00

B-Fr-Bio-ByP	312.00±6.08E+00
B-Co-Bio-ByP	169.83±6.34E-01
B-Fr-LI-ByP	264.25±1.68E+00
B-Co-LI-ByP	334.33±7.64E-01
GB-Fr-LI-ByP	81.08±5.45E+00
GB-Co-LI-ByP	70.20±3.87E-01
GB-Fr-Bio-ByP	92.13±2.72E+00
GB-Co-Bio-ByP	174.47±4.74E+00
S-Bio-RM	196.61±5.01E+00
S-Conv-RM	134.86±2.93E+00
S-Bio-ByP	123.97±6.16E+00
P-Fr-Bio-ByP	218.66±4.21E-01
P-Fr-Conv-ByP	375.19±4.99E+00
P-FinalP	136.15±2.40E+00

2.2.2.4 Characterization of Legume UAE Extracts by UHPLC-DAD-ESI-MS^n

Many methods are already employed for the separation and identification of bioactive compounds, such as polyphenols, including Ultra-High Performance Liquid Chromatography (UHPLC), Gas Chromatography-Mass Spectrometry (GC-MS) and Attenuated Total Reflection Infrared Spectroscopy (ATR-FTIR)[5]. In our study, we optimized and applied the UHPLC-DAD-ESI-MS^n method. The combination of Ultra-High-Performance-Liquid-Chromatography and an MS detector allowed rapid chromatographic runs with minimal solvent and energy consumption while maintaining high resolution. Furthermore, the MS detector enabled the identification of many polyphenolic compounds present in all UAE bean, soy and green bean extracts.

A reversed-phase gradient elution was optimized to separate and identify 21 standard polyphenols (*Figure* 51, *Table* 22). The standard mixture used was composed of gallic acid (1), protocatechuic acid (2), catechin (3), caffeic acid (4), chlorogenic acid (5), epicatechin (6), p-coumaric acid (7), ferulic acid (8), isoquercitrin (9), rutin trihydrate (10), hyperoside (11), phloridzin dihydrate (12), myricetin (13), quercitrin (14), daidzein (15), naringenin (16), genistein (17), hesperetin (18), naringenin chalcone (19), kaempferol (20) and apigenin (21). This LC-MS chromatographic method was applied to determine the qualitative profile and the relative amount of polyphenols in the legume UAE extracts under investigation (green beans, beans and soy).

Qualitative analysis of polyphenols in legume UAE extracts was performed using a combination of retention time, UV-Vis spectra, and MS fragmentation patterns. The MS analysis, in both positive and negative ion modes, facilitated the identification of compounds by comparing their MS spectra to those of standards and the literature data.

A semi-quantitative analysis (see *Section* 2.1.9) was then performed to estimate the relative amount of individual polyphenols. Since a comprehensive quantitative analysis was not conducted, standard deviations (SDs) cannot be provided for the data presented in the bar graphs (*Figure* 53, *Figure* 55, *Figure* 57). This analysis enabled a comparative assessment of different sample types, including fresh and cooked products, as well as samples from various cultivation methods (biological, LI, and conventional)[80].

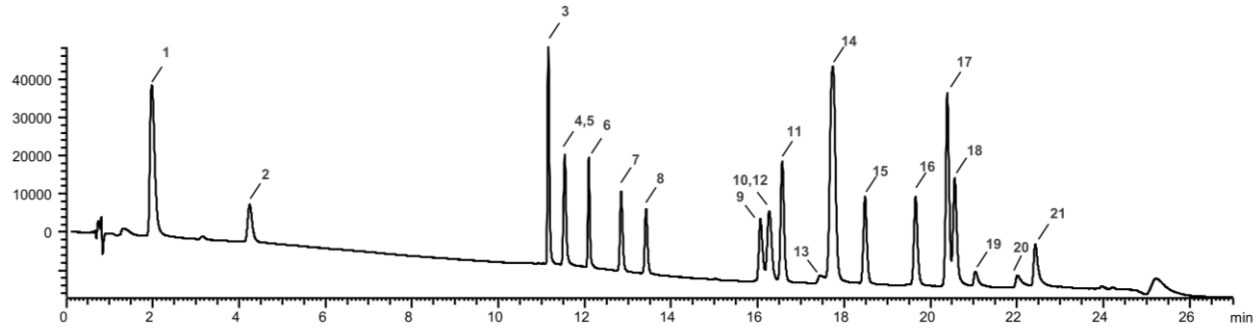


Figure 51 Chromatographic separation (λ = 280 nm) of the 21-standard polyphenol mixture[80]

Table 22 shows the retention times, mass, precursor ion, and concentration used in the analysis of each standard polyphenol[80]

Substance	RT [min]	Molecular Ion [m/z]	MS^n [m/z], rel. int. (%)	Molecular Weight [g/mol]	Compound	µg mL-1
1	1.98	169 $[M-H]^-$	MS^2[169]: 125 (100) MS^3[125]: 81 (100), 125 (65), 97 (60)	170	Gallic acid	28.13
2	4.25	153 $[M-H]^-$	MS^2[153]: 109 (100) MS^3[109]: 109 (100), 112 (55)	154	Protocatechuic acid	11.25
3	11.16	289 $[M-H]^-$	MS^2[289]: 245 (100), 205 (35), 179 (15) MS^3[245]: 203 (100), 227 (30), 187 (20)	290	(+)-Catechin	62.50
4	11.51	179 $[M-H]^-$	MS^2[179]: 135 (100) MS^3[135]: 90 (100)	180	Caffeic acid	9.38
5	11.57	353 $[M-H]^-$	MS^2[353]: 191 (100), 179 (48), 173 (30) MS^3[191]: 127 (100), 173 (90), 85 (60), 93 (45), 110 (35)	354	Chlorogenic acid	3.75
6	12.09	289 $[M-H]^-$	MS^2[289]: 245 (100), 205 (35), 179 (15) MS^3[245]: 203 (100), 227 (30), 187 (20)	290	(-)-Epicatechin	28.13
7	12.84	163 $[M-H]^-$	MS^2[163]: 119 (100)	164	p-coumaric acid	3.50
8	13.42	193 $[M-H]^-$	MS^2[193]: 149 (100), 178 (55), 134 (20) MS^3[149]: 134 (100)	194	Ferulic acid	5.63
9	16.08	463 $[M-H]^-$	MS^2[463]: 301 (100) MS^3[301]: 179 (100), 273 (15)	464	Isoquercitrin	18.75
10	16.29	609 $[M-H]^-$	MS^2[609]: 301 (100) MS^3[301]: 179 (100), 273 (15)	610	(+)-Rutin trihydrate	37.50
11	16.57	463 $[M-H]^-$	MS^2[463]: 301 (100) MS^3[301]: 179 (100), 273 (15)	464	Hyperoside	37.50
12	16.79	481 $[M+HCOO]^-$ 435 $[M-H]^-$	MS^2[435]: 273 (100)	472	Phloridzin dihydrate	28.13
13	17.47	317 $[M-H]^-$	MS^2[317]: 179 (100), 191 (15) MS^3[179]: 151 (100)	318	Myricetin	19.80
14	17.78	447 $[M-H]^-$	MS^2[447]: 301 (100)	448	Quercitrin	56.25

			MS^3[301]: 179 (100), 151 (65)			
15	18.49	253 $[M-H]^-$	MS^2[253]: 253 (100), 209 (25), 197 (10) MS^3[253]: 253 (100), 209 (30), 181 (25), 169 (10)	254	Daidzein	17.00
16	19.65	271 $[M-H]^-$	MS^2[271]: 151 (100) MS^3[151]: 107 (100)	272	Naringenin	7.50
17	20.39	269 $[M-H]^-$	MS^2[269]: 225 (100), 269 (50), 201 (35), 149 (25) MS^3[225]: 181 (100), 197 (40), 169 (15), 225 (10)	270	Genistein	14.58
18	20.56	301 $[M-H]^-$	MS^2[301]: 286 (100), 242 (45), 257 (35), 125 (20), 199 (10) MS^3[286]: 242 (100), 258 (85), 199 (40), 174 (15), 268 (15)	302	Hesperetin	28.20
19	21.08	271 $[M-H]^-$	MS^2[271]: 151 (100), 176 (20) MS^3[151]: 107 (100)	272	Naringenin chalcone	16.50
20	22.05	285 $[M-H]^-$	MS^2[285]: 285 (100), 243 (55), 151 (40)	286	Kaempferol	12.00
21	22.43	269 $[M-H]^-$	MS^2[269]: 225 (100), 269 (55), 201 (35), 149 (25) MS^3[225]: 181 (100), 197 (40), 169 (15)	270	Apigenin	13.20

Green bean samples (Phaseolus vulgaris)

The green bean samples include fresh and cooked by-products taken before and after cooking during the industrial production of frozen legumes. As the varieties grown are the same in both LI and biological systems, with the difference that the seeds are not treated at all in the latter case, we can affirm that both cooked and fresh by-products have been subjected to biological and LI cultivation.

The pattern of mass spectra was analyzed in negative mode $[M-H]^-$. The results of the UHPLC-DAD-MS^n qualitative analysis (see *Table* 23) demonstrate that green beans are rich in flavonols, a finding that is consistent with previous studies found in the literature. Indeed, as reported by Abu-Reidah et al[112], flavonoids and their derivatives were identified as the most prominent compounds in all green bean UAE extracts analyzed, underscoring the significance of this class of phenolic compounds in the phytochemical profile of this legume. From a qualitative perspective, no notable differences were observed between the fresh and cooked by-products derived from biological cultivation (*Figure* 52, A-B) and fresh and cooked by-products derived from LI cultivation (*Figure* 52, C-D).

However, a notable distinction was observed between green bean by-products derived from biological cultivation (GB-Co-Bio-ByP and GB-Fr-Bio-ByP) and green bean by-product from LI cultivation (GB-Co-LI-ByP and GB-Fr-LI-ByP). Indeed, both fresh and cooked LI by-products (A-B samples of *Figure* 52) are devoid of the presence of a compound whose precursor ion is m/z 593, which would appear to be Kaempferol 3-O-rutinoside (peak 7), as previously reported in the literature[112].

In contrast, both biological by-products (C-D samples of *Figure* 52) are devoid of the presence of peak 4, which is identified as Quercetin 3-O-glucuronide, as it has a parent ion of m/z 477[112] and peak 8, which is identified as Kaempferol 3-O-glucuronide, as it has a parent ion of m/z 461.

However, regarding all other compounds, the MS spectra have revealed the presence of the parent ion at m/z 741 (peak 1), which is identified as Quercetin 3-O-xylosylrutinoside. Peak 2 at m/z 595 has been demonstrated to be Quercetin 3-O-vicianoside, which is present even in the biological fresh by-product but absent in the cooked one. The compound with m/z 725 (peak 3) has been identified as Kaempferol 3-O-xylosylrutinoside, as previously described[112]. The identification of Quercetin 3-O-rutinoside (rutin), corresponding to peak 5, was confirmed by comparison with the reference molecule available in our laboratories. According to the literature[112], this compound was identified in all green bean UAE extracts, with the exception of the LI-cooked by-product. The parent ion of m/z 579 observed for compound 6 has been proposed to be Kaempferol 3-O-sambubioside[80].

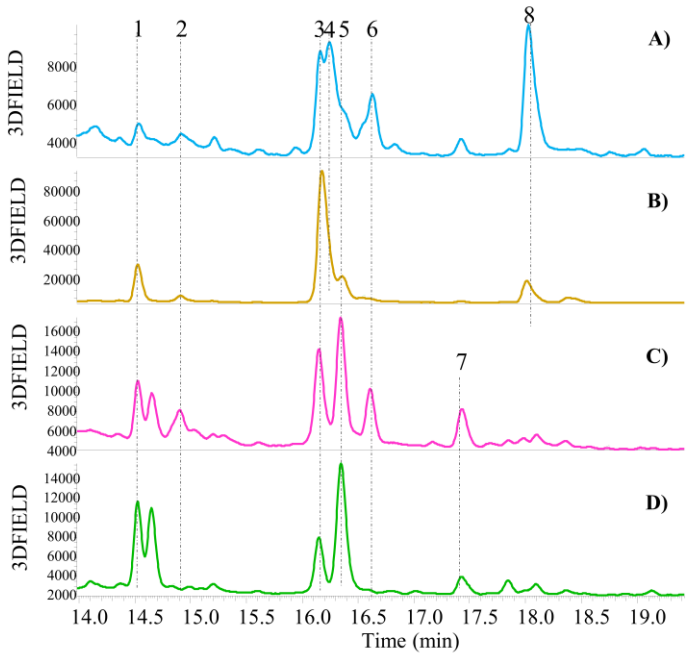


Figure 52 Qualitative UHPLC-DAD analysis (chromatograms recorded at λ = 330 nm) of green bean by-products (method described in Section 2.1.9). Zoomed-in view of the chromatogram between 13.98 and 19.42 min; A) GB-Fr-LI-ByP; B) GB-Co-LI-ByP; C) GB-Fr-Bio-ByP; D) GB-Co-Bio-ByP; 1= Quercetin 3-O-xylosylrutinoside; 2= Quercetin 3-O-vicianoside; 3= Kaempferol 3-O-xylosylrutinoside; 4= Quercetin 3-O-glucuronide; 5= Quercetin 3-O-rutinoside (Rutin); 6= Kaempferol 3-O-sambubioside; 7= Kaempferol 3-O-rutinoside; 8= Kaempferol 3-O-glucuronide[80]

Table 23 Qualitative LC-MS analysis of polyphenols in green beans by-products[80]

Substance	RT [min]	Molecular Ion [m/z]	MS^n [m/z], rel. int. (%)	Molecular Weight [g/mol]	Tentative Identification

1	14.53	741 [M-H]$^-$	MS^2[741]: 253 (100) MS^3[253]: 235 (100)	742	Quercetin 3-O-xylosylrutinoside [112]
2	14.91	595 [M-H]$^-$	MS^2[595]: 300 (100), 445 (40), 463 (25), 475 (15), MS^3[300]: 271 (100), 255 (50)	596	Quercetin 3-O-vicianoside [112]
3	16.16	725 [M-H]$^-$	MS^2[725]: 575 (100), 285 (40), 593 (35) MS^3[575]: 339 (100), 393 (90), 429 (70), 547 (25)	726	Kaempferol 3-O-xylosylrutinoside [112]
4	16.27	477 [M-H]$^-$	MS^2[477]: 301 (100) MS^3[301]: 179 (100), 150 (60)	478	Quercetin 3-O-glucuronide [112]
5	16.38	609 [M-H]$^-$	MS^2[609]: 301 (100) MS^3[301]: 179 (100), 273 (15)	610	Quercetin 3-O-rutinoside
6	16.63	579 [M-H]$^-$	MS^2[579]: 285 (100), 429 (60), 447 (20), 257 (15) MS^3[285]: 257 (100), 151 (50), 267 (45)	580	Kaempferol 3-O-sambubioside [112]
7	17.43	593 [M-H]$^-$	MS^2[593]: 285 (100) MS^3[285] 257 (100), 267 (55), 241 (45), 213 (25)	594	Kaempferol 3-O-rutinoside [112]
8	18.04	461 [M-H]$^-$	MS^2[461]: 285 (100) MS^3[285]: 257 (100), 267 (55), 229 (40), 213 (25), 197 (20)	462	Kaempferol 3-O-glucuronide [112]

A semi-quantitative analysis of the green bean by-products was also carried out and the results are shown in *Figure* 53. The bar graph illustrates a comparative analysis of the polyphenol content in fresh and cooked green bean by-products from biological and LI cultivation.

A marked increase in specific polyphenols was observed in cooked by-products when compared to fresh by-products. The most striking increase was observed for Kaempferol 3-O-xylosylrutinoside (compound 3, *Figure* 53) in the cooked LI by-product (GB-Co-LI-ByP) compared to the fresh counterpart (GB-Fr-LI-ByP). A similar pattern is observed for compound 1 (Quercetin-3-O-xylosylrutinoside) and compound 5 (rutin) in higher amounts in both cooked by-products (GB-Co-LI-ByP and GB-Co-Bio-ByP) compared to their fresh counterparts (GB-Fr-LI-ByP and GB-Fr-Bio-ByP). The elevated concentration of polyphenols in cooked green bean by-products relative to their fresh counterpart can be ascribed to the structural alterations induced by cooking, which are likely to disrupt cell wall integrity and facilitate the diffusion of these compounds. These observations are consistent with findings of previous research, which have demonstrated that the cooking methods can influence the nutritional composition of vegetables, including green bean. This can result in alterations to the level of specific compounds such as polyphenols[113].

On the contrary, some compounds are no longer present in the cooked by-products, including Quercetin-3-O-glucuronide (peak 4, *Figure* 53) and Kaempferol 3-O-sambubioside (peak 6, *Figure* 53) in the GB-Co-Bio-

ByP UAE extract. This outcome is to be expected, given that the cooking process is even likely to result in the degradation or hydrolysis of the sugar chains of these two compounds, in direct opposition to what we observed before. Indeed, previous research has demonstrated that various processing conditions can significantly impact the degradation of phenolic compounds in common beans, with increased soaking time and water temperature accelerating polyphenol degradation[114].

Thus, there are several mechanisms involved in thermal processes that can influence the increase or decrease of certain compounds, such as polyphenols, in vegetables.

A significant finding from our semi-quantitative analysis of green bean by-products was the presence of some polyphenols in all UAE extracts studied, particularly Quercetin-3-O-rutinoside, which has been demonstrated to possess cardioprotective effects[115]. Additionally, the presence of Quercetin glucuronide, known for its anti-vascular properties[116], further highlights the potential health benefits of these by-products. Moreover, the crucial role of lipid peroxidation in various human diseases, the inhibitory effects of Quercetin-3-O-glucuronide and Quercetin-3-O-rutinoside, had been already demonstrated by Geoffrey, highlight their potential therapeutic significance[80].

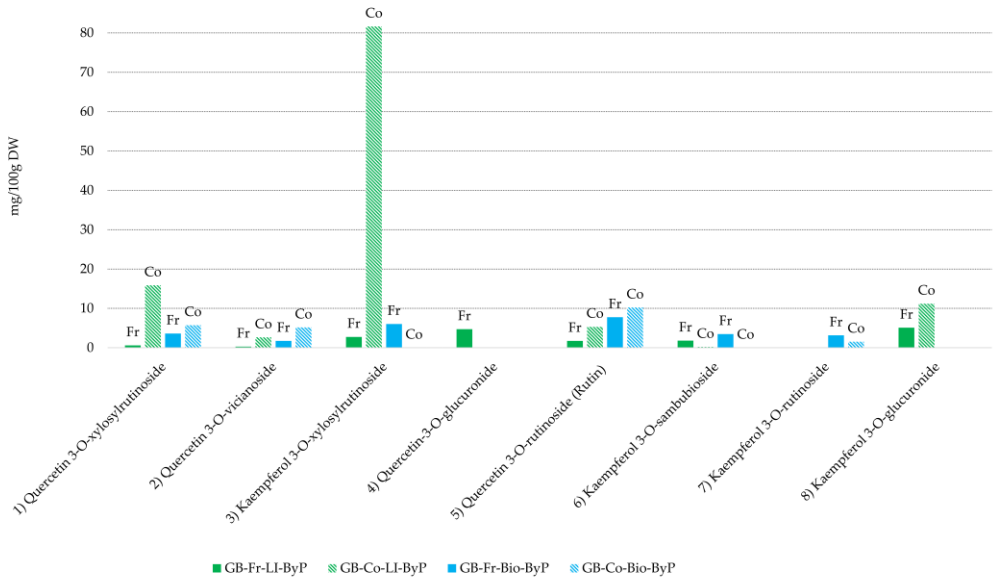


Figure 53 Polyphenol content (semi-quantitative UHPLC-DAD analysis) in green bean by-products from biological (blue) and LI (green) cultivation. Co = Cooked, Fr = Fresh[80]

Beans samples (Phaseolus vulgaris)

In order to evaluate the potential of bean by-products, qualitative and semi-quantitative analyses were conducted to compare bean by-products derived from different production stages with the final product.

In the case of bean by-products, the most rudimentary waste material obtained from the initial stages of the industrial process, defined as B-Bio-ByP and B-Conv-ByP, underwent UHPLC-DAD-MS^n analysis. This material is characterized by the presence of a multitude of foreign bodies, including branches and leaves.

Furthermore, also by-products from collection points close to the final product were analyzed, which therefore appear to be less contaminated by foreign bodies than those previously mentioned. Among them, there are the biological by-products (B-Fr-Bio-ByP and B-Co-Bio-ByP), and the LI by-products (B-Fr-LI-ByP and B-Co-LI-ByP), while the final product (B-FinalP) corresponds to frozen beans.

A comparison of the chromatograms of all the bean UAE extracts is shown in *Figure* 54. From a qualitative standpoint, the phenolic composition of all bean UAE extracts is comparable, including both by-products and the final product. Specifically, the presence of five distinct main compounds, with retention times of 9.47, 9.96, 10.95, 11.14, and 14.69 minutes (see *Table* 24), was identified in all bean UAE extracts. The peaks exhibit the same absorption spectra (λ = 326 nm) and identical mass spectra patterns (see *Table* 24), with a parent ion of m/z 385 in negative mode $[M-H]^-$. It has been postulated that these compounds are derivatives of ferulic acid coupled with glucaric or galactaric acid, as previously described by Nguyen et al[117].

The qualitative analysis revealed a promising polyphenol profile in bean by-products, notably the presence of feruloylglucaric acid derivatives. As demonstrated by Zbigniew Walaszek et al.[118], these compounds have shown significant potential in lowering serum cholesterol levels in rats. Thus, bean by-products could serve as a valuable source of natural anti-cholesterolemic agents, transforming waste into a resource with significant health benefits[80].

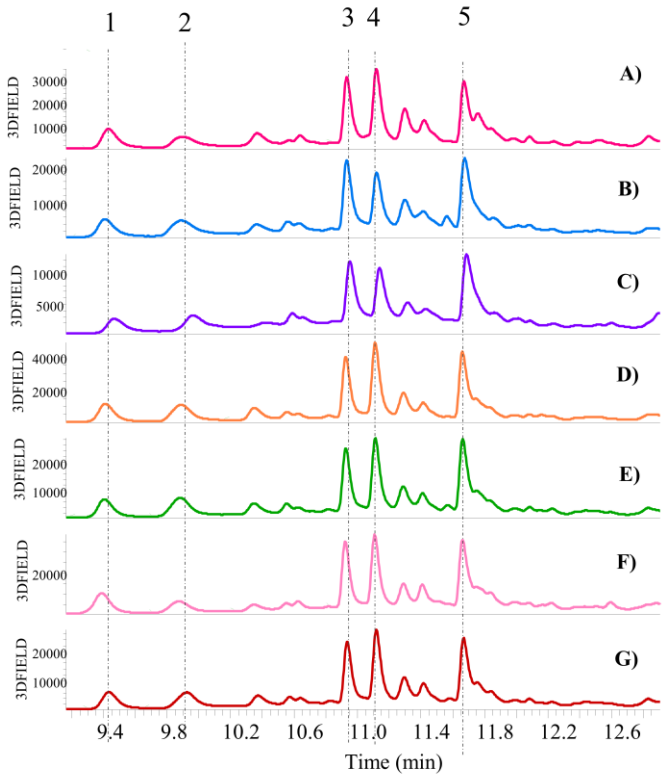


Figure 54 Qualitative UHPLC-DAD analysis (chromatograms recorded at λ = 330 nm) of green bean by-products (method described in Section 2.1.9). Zoomed-in view of the chromatogram between 9.16 and 12.91 min; A) B-FinalP; B) B-Bio-ByP; C) B-Conv-ByP; D) B-Fr-Bio-ByP; E)

B-Co-Bio-ByP; F) B-Fr-LI-ByP; G) B-Co-LI-ByP; 1, 2, 3, 4, 5= Feruloyl glucaric/galactaric acid derivative[80]

Table 24 Qualitative LC-MS analysis of polyphenols in bean products[80]

Substance	RT [min]	Molecular Ion [m/z]	MS^n [m/z], rel. int. (%)	Molecular Weight [g/mol]	Tentative Identification
1	9.47	385 $[M-H]^-$	MS^2[385]: 191 (100) MS^3[191]: 84 (100), 146 (30), 173 (10)	386	feruloyl glucaric/galactaric acid derivative [119]
2	9.96	385 $[M-H]^-$	MS^2[385]: 191 (100) MS^3[191]: 84 (100), 146 (30), 173 (10)	386	feruloyl glucaric/galactaric acid derivative [119]
3	10.58	385 $[M-H]^-$	MS^2[385]: 191 (100) MS^3[191]: 84 (100), 146 (30), 173 (10)	386	feruloyl glucaric/galactaric acid derivative [119]
4	11.14	385 $[M-H]^-$	MS^2[385]: 191 (100) MS^3[191]: 84 (100), 146 (30), 173 (10)	386	feruloyl glucaric/galactaric acid derivative [119]
5	11.69	385 $[M-H]^-$	MS^2[385]: 191 (100) MS^3[191]: 84 (100), 146 (30), 173 (10)	386	feruloyl glucaric/galactaric acid derivative [119]

As previously mentioned, five compounds, all derivatives of ferulic acid coupled with galactaric or glucaric acid, were identified across all bean UAE extracts. *Figure* 55 presents a bar graph illustrating the relative amount of these compounds in the analyzed UAE extracts. Despite the qualitative similarity in polyphenol composition, the semi-quantitative analysis highlighted significant variations in the amount of these compounds across the different bean by-products.

A comparative analysis was conducted between fresh and cooked bean by-products and unlike green bean by-products, no significant differences were observed between the two forms. In fact, fresh by-products consistently exhibited higher concentrations of polyphenols than their cooked counterparts (is the case of peaks 1, 2, 3 and 5, *Figure* 55). This finding suggests that the cooking process may lead to the extraction of certain polar compounds into the cooking water. This result is in line with previous studies on common beans, which demonstrated that various processing conditions, particularly extended soaking times and high-water temperatures, can significantly accelerate polyphenol degradation[114].

Conversely, peak 4 exhibits a different trend, with cooked bean by-products displaying slightly higher concentrations than their fresh counterparts. This finding aligns with previous research demonstrating that specific cooking conditions can lead to an increase in certain polyphenol compounds within plant matrices[113].

In conclusion, similar to green bean by-products, the thermal processing of bean by-products significantly impacts their polyphenol profile, leading to both increases and decreases in specific compounds. A detailed quantitative analysis will be assumed in future studies to further elucidate these findings.

Furthermore, the study undertook a comparative analysis of the three distinct cultivation methods: biological, conventional, and LI. *Figure* 55 reveals that peak number 4 (which is one of the feruloylglucaric acid derivatives) is consistently the most abundant across all analyzed by-products. This finding suggests that cultivation practices may influence the accumulation of specific polyphenols. Previous research, such as that conducted by Kalinowska et al.[120], supports this hypothesis, demonstrating that different cultivation methods can significantly impact polyphenol content. It is plausible that reduced pesticide uses in biological, LI or sustainable cultivation could contribute to variations in polyphenol profiles. A closer examination of the *Figure* 55 reveals a notable trend: LI and biological by-products exhibit higher polyphenol amount compared to conventional by-products, highlighting the potential of sustainable agriculture to produce crops with enhanced nutritional value.

A significant finding from this study is the elevated polyphenol content in by-products compared to the final product, confirmed from the *Figure* 55. This observation underlines the potential of plant-based waste as a valuable source of bioactive compounds, aligning with the primary objective of this research[80].

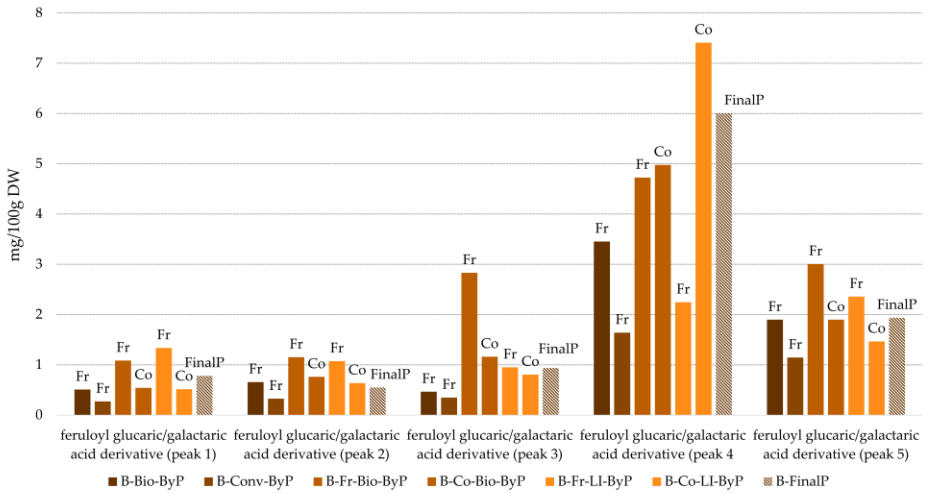


Figure 55 Polyphenol content (semi-quantitative UHPLC-DAD analysis) in bean samples from biological, conventional and LI cultivation. Co = Cooked, Fr = Fresh. By-product = full bars; Final product = stripped bars[80]

A comparative analysis of green beans and mature bean by-products revealed distinct polyphenol profiles. Green beans, representing the 'youngest' stage in bean ripening, were characterized by the presence of glycosylated flavonols, as detailed in *Table* 23. In contrast, mature beans predominantly contained ferulic acid derivatives, as shown in *Table* 24. According to literature[121],[122], this difference can be attributed to the plant's developmental stage and its response to environmental stress. During early development, plants often accumulate flavonoids to protect themselves from UV radiation and oxidative stress. As the plant matures and becomes more resilient, it may shift its metabolic focus towards other defense mechanisms.

Indeed, the existing literature confirms that heat affects the degradation of flavonols[123]. For instance, it has

been demonstrated that the application of heat can result in the degradation of rutin, leading to the formation of a mixture comprising Protocatechuic acid and other degradation derivatives with a reduced antioxidant activity[123],[124]. In light of these results, it can be hypothesized that in green beans, which represent the earliest and therefore most vulnerable stage of ripening, polyphenols with higher antioxidant activity, such as flavonols, are present. Conversely, beans exhibit a greater presence of polyphenols with lower antioxidant activity, such as phenolic acids. Once they have reached maturity, beans have a reduced need to actively defend themselves and therefore produce fewer substances. Additionally, it is essential to consider that the mature plant will have endured adverse weather conditions, such as drought or extreme temperatures, which may have caused a degradation of the polyphenol structure, leading to the formation of phenolic acids[80].

Soy samples

In the case of soy samples, the qualitative LC-MS analysis (see *Figure* 56, *Table* 25) was performed on raw materials which are seeds, including both biological (S-Bio-RM) and conventional (S-Conv-RM) seeds, as well as biological okara which is the soy by-product (S-Bio-ByP). The pattern of mass spectra was analyzed in positive mode $[M-H]^+$.

According to literature[125], soy samples are well-established sources of isoflavones and their derivatives. A key distinction of our research is the comparative analysis of soy by-product (okara) and its corresponding raw materials (seeds). Among the by-products investigated in this research, okara stands out as a particularly promising candidate due to its relative purity. The closed-loop nature of the soy-milk production process, which involves minimal exposure to external contaminants like stones, branches, or insects, contributes to the cleanliness of the resulting okara.

The qualitative analysis of soy, comprising both seed and waste samples, revealed a comparable isoflavone composition. Among the isoflavones identified, all soy UAE extracts showed the presence of daidzin m/z 417 (peak 1), glycitin m/z 447 (peak 2), genistin m/z 433 (peak 3), malonyl daidzin m/z 503 (peak 4), malonyl glycitin m/z 533 (peak 5), and malonyl genistin m/z 519 (peak 6). The identification of these compounds in our soy UAE extracts was confirmed through a comparison with previously published LC-MS data by Lee et al.[126]. While, the identification of daidzein and genistein, with respective parent ions of m/z 255 and m/z 271 (peaks 7 and 8) was confirmed by comparison with the standard molecules available in our laboratories.

The qualitative analysis revealed good similarity in the isoflavone profiles of okara (S-Bio-ByP) and soy seeds (S-Conv-RM and S-Bio-RM). This result underlines the potential of okara as a valuable source of bioactive compounds, mainly isoflavones. Given the well-documented health benefits of isoflavones, including their role in bone health, okara-derived isoflavones may offer promising therapeutic applications for conditions such as osteoporosis [127],[80].

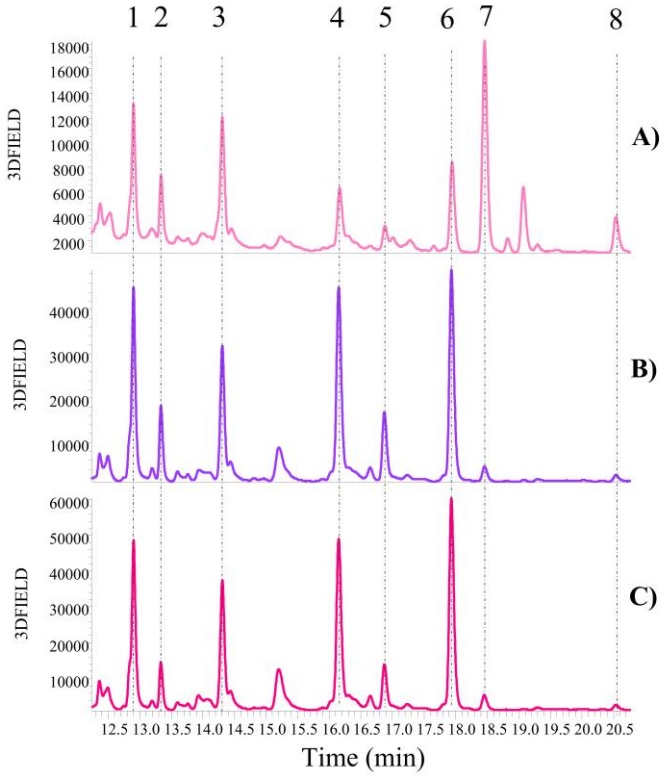


Figure 56 Qualitative UHPLC-DAD analysis (chromatograms recorded at λ = 330 nm) of soy samples (method described in Section 2.1.9). Zoomed-in view of the chromatogram between 12.24 and 20.77 min; A) S-Bio-ByP; B) S-Bio-RM; C) S-Conv-RM; 1= Daidzin; 2 = Glycitin; 3=Genistin; 4= Malonyl daidzin; 5= Malonyl glycitin; 6= Malonyl genistin; 7= Daidzein; 8= Genistein[80]

Table 25 Qualitative LC-MS analysis of polyphenols in soy products[80]

Substance	RT [min]	Molecular Ion [m/z]	MS^n [m/z], rel. int. (%)	Molecular Weight [g/mol]	Tentative Identification
1	12.9	417 [M+H]+	MS^2[417]: 255 (100) MS^3[255]: 199 (100), 136 (70), 227 (55), 237 (30), 255 (15)	416	Daidzin [126]
2	13.3	447 [M+H]+	MS^2[447]: 285 (100) MS^3[285]: 270 (100), 229 (20), 144 (10), 285 (5)	446	Glycitin [126]
3	14.3	433 [M+H]+	MS^2[433]: 271 (100) MS^3[271]: 152 (100), 215 (80), 243 (70), 253 (40), 271 (10)	432	Genistin [126]
4	16.1	503 [M+H]+	MS^2[503]: 255 (100) MS^3[255]: 199 (100), 136 (70), 237 (30), 255 (15)	502	Malonyl daidzin [126]
5	16.8	533 [M+H]+	MS^2[533]: 271 (100), 285 (60) MS^3[271]: 215 (100), 152 (70), 243 (45), 253 (30), 271 (10)	532	Malonyl glycitin [126]
6	17.9	519 [M+H]+	MS^2[519]: 271 (100) MS^3[271]: 215 (100), 152 (50), 243 (35), 253 (25),	518	Malonyl genistin [126]

			271 (10)		
7	18.4	255 [M+H]+	MS^2[255]: 199 (100), 136 (70), 227 (55), 237 (30), 255 (15) MS^3[199]: 191 (100), 171 (30), 153 (15)	254	Daidzein [126]
8	20.5	271 [M+H]+	MS^2[271]: 152 (100), 215 (75), 243 (65), 253 (55), 271 (10) MS^3[152]: 153 (100), 110 (45), 66 (40)	270	Genistein [126]

Figure 57 shows a comparative analysis of the semi-quantitative data for soy UAE extracts. The qualitative analysis demonstrated a comparable polyphenol profile across the seeds (S-Conv-RM and S-Bio-RM) and okara (S-Bio-ByP), whereas the semi-quantitative analysis revealed notable discrepancies in the quantity of specific isoflavones.

A comparative analysis of the amount of individual isoflavone revealed a distinct pattern: glucoside forms (peaks 1-6, *Figure* 57) were more abundant in seeds (S-Conv-RM and S-Bio-RM), while aglycone forms, such as daidzein (peak 7, *Figure* 8) and genistein (peak 8, *Figure* 57), were more concentrated in okara (S-Bio-ByP). This observation is consistent with the existing literature, which has established that soy primarily contains isoflavone glycosides, such as malonyl glucosides and β-glucosides[65]. The increased aglycone content in the by-product can be attributed to thermal processing, which can facilitate the hydrolysis of glycosides into aglycones. Additionally, the extraction of glycosides into soy milk during processing may further contribute to the enrichment of aglycones in the by-product.

Thus, the qualitative and semi-quantitative analyses demonstrated the significant polyphenol content of okara (S-Bio-ByP), including a comparable isoflavone profile to soy seeds. These results highlight the potential of okara as a valuable source of bioactive compounds, transforming a previously not-used by-product into a resource with significant health benefits[80].

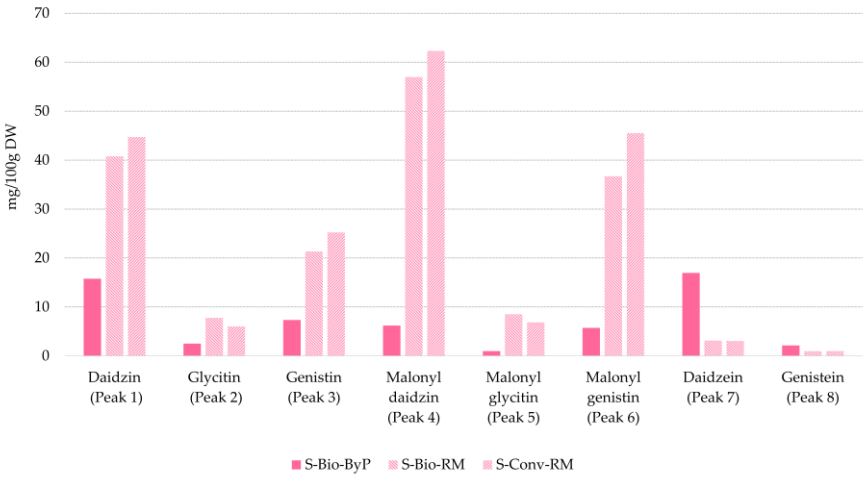


Figure 57 Polyphenol content (semi-quantitative UHPLC-DAD analysis) in soy samples. By-

product (okara) = full bars; Raw material (seeds) = stripped bars[80]

Pea samples

Regarding pea samples, including fresh biological and conventional by-products (P-Fr-Bio-ByP and P-Fr-Conv-ByP) and the final product (P-FinalP), preliminary studies suggest further investigation. Indeed, qualitative LC-MS analysis (see *Section* 2.1.9) revealed a similar polyphenolic profile of all pea UAE extracts. Two prominent recurring peaks were observed at retention times of 11.7 and 12.0 minutes with m/z ratios of 278 and 308 [M-H]-, respectively (see *Figure* 58). These compounds were also detected in positive ion mode with m/z ratios of 280 and 310 [M-H]+, respectively.

A subsequent literature review aimed to identify these compounds. Notably, the compound with m/z of 280 [M-H]+ could correspond to (+)-N-[4′-Hydroxy-(E)-cinnamoyl]-L-aspartic acid[128], while the compound with an m/z of 310 in positive ion mode potentially corresponds to either (-)-N-[3′,4′-Dihydroxy-(E)-cinnamoyl]-L-glutamic acid or (+)-N-[4′-Hydroxy-3′-methoxy-(E)-cinnamoyl]-L-aspartic acid, both exhibiting the same parent ion of m/z 310 [M-H]+[128].

Therefore, literature analysis[128] suggests that these two peaks belong to the class of N-phenylpropenoyl-L-amino acids. This result is significant as this class of polyphenols has demonstrated inhibitory activity against Helicobacter pylori adhesion to the human gastric mucosa[129].

Given that coriander fruit (Coriandrum sativum L.) and elderberry flower (Sambucus L.) are known to contain such molecules[130], an extract from these plants was prepared (in a 50:50 water:ethanol solution, 15 minutes using ultrasound) to be exploited as a reference. Analysis of the coriander extract revealed the presence of two compounds with m/z ratios of 278 [M-H]- at 14.79 minutes and 308 [M-H]- at 16.42 minutes. However, these compounds exhibited different retention times compared to the peaks observed in the pea UAE extracts (14.96 and 16.60 minutes, respectively). These differences suggest that the pea UAE extracts may contain isomers of these molecules or that the amino acid moiety is bound to a different region of the molecule.

Thus, it was considered necessary to isolate the two compounds of interest. A semi-preparative HPLC fractionation followed by NMR analysis was chosen for this purpose. A stirring overnight extraction method was established using a 1:10 sample-to-solvent ratio with a 50:50 methanol-water mixture. Subsequently, a liquid-liquid extraction using ethyl acetate in a separating funnel was performed to remove unwanted compounds. The methanol portion was then evaporated to dryness, yielding a pure aqueous extract. This extract, which contains the two compounds of interest, is currently being subjected to semi-preparative HPLC analysis and NMR characterization at the *Department of Pharmacognosy, University of Graz, Austria*.

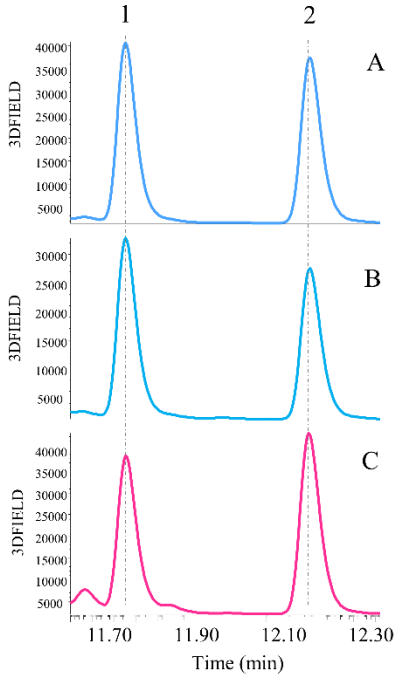


Figure 58 Qualitative UHPLC-DAD analysis (chromatograms recorded at λ = 330 nm) of pea samples (method described in Section 2.1.8). Zoomed-in view of the chromatogram between 11.65 and 12.36 min; A) P-Fr-Bio-ByP; B) P-Fr-Conv-Byp; C) P-FinalP; 1= 278 m/z [M-H]$^{-1}$; 2= 308 m/z [M-H]$^{-1}$

2.2.2.5 Polyphenol profile and Antioxidant activity of legume by-products

Green beans represent the 'youngest' stage in bean ripening and for this reason, it was interesting to observe any similarities or differences in the type of polyphenols they contain. Comparing the two species shows that the polyphenol content is totally different. Indeed, green bean by-products contain glycosidic flavonols (see *Table* 23), whereas beans do not contain flavonols, but ferulic acid derivatives (see *Table* 24). This difference could be attributable to the fact that plants produce various substances, including polyphenols, during ripening to protect themselves from environmental stresses and pathogens[131]. Indeed, the existing literature confirms that heat affects the degradation of flavonols[124]. For instance, it has been demonstrated that the application of heat can result in the degradation of rutin, leading to the formation of a mixture comprising protocatechuic acid and other degradation derivatives with a reduced antioxidant activity[124]. In light of these results, it can be hypothesized that in green beans, which represent the earliest and therefore most vulnerable stage of ripening, polyphenols with higher antioxidant activity, such as flavonols, are present. Conversely, beans exhibit a greater presence of polyphenols with lower antioxidant activity, such as phenolic acids. Once they have reached maturity, beans have a reduced need to actively defend themselves and therefore produce fewer and fewer substances. Additionally, it is essential to consider that the mature plant will have endured adverse weather conditions, such as drought or extreme temperatures, which may have caused a degradation of the polyphenol structure, leading to the formation of phenolic acids.

Still following the results obtained for green bean by-products, the comparison between cooked and fresh by-products was carried out in order to observe whether the cooking process affected the polyphenol content. The most striking example, both qualitatively and semi-quantitatively, concerns green beans. Since green beans generally contain predominantly glycosidic flavonols, including the cooked by-product, it was expected to find the corresponding aglycosidic flavonols in the fresh by-product. In fact, chemical reactions generally occur during cooking that can transform some polyphenols into other forms, making them more stable or more easily absorbed. For example, cooking may favor the formation of compounds such as glycosylated flavonoids, which are more easily absorbed than free flavonoids. In reality, the results obtained disproved this hypothesis. Glycosylated flavonols are not present in either fresh or cooked by-products. However, one difference emerged from the semi-quantitative analysis: some glycosidic flavonols, see *Figure* 53, were found to be higher in the cooked by-product. It was therefore hypothesized that probably in fresh green beans, polyphenols are bound to other molecules or trapped within the plant cells. Probably, cooking altered the cell structure, releasing these compounds and making them more available[113]. In contrast, in the case of bean by-products, which do not contain glycosidic flavonols but derivatives of ferulic acid, a different pattern was observed between cooked and fresh by-products. Indeed, no notable dissimilarities were identified in the semi-quantitative analysis between cooked and fresh bean by-products, as was observed for green beans.

Moreover, in general all the legume UAE extracts showed no significant differences in terms of antioxidant activity. In the case of the bean and soy samples, it was interesting to observe that the by-products present a TAS value comparable to that of the final products, for beans, and seeds, for soy. This is in accordance with the extract yield (%) and the TPC values. In contrast, in the case of the bean by-products, a slight trend difference was observed between the TAS and TPC values. For example, GB-Li-Fr-ByP is the sample with the highest TPC compared to the others of the same species, but this was not confirmed by the TAS values. This could be due to the different antioxidant capacities of the individual polyphenols. Indeed, the chemical structure of polyphenols is linked to a higher free radical scavenging capacity[57]. Furthermore, according to the results obtained and discussed, even a higher extraction yield (%) does not necessarily correlate with a proportional increase in the content of phenolic compounds. This observation applies to all by-products of green beans, which have a high yield but no correlated TPC, and to conventional soy seeds[80].

2.2.3 Comparative Analysis of UAE Extracts from Fruits and Legumes

All samples, both fruits and legumes, underwent the same UAE extraction procedures (as detailed in *Section* 2.1.4) and were subjected to the same analytical procedures, including total phenolic content (TPC) and total antioxidant activity (TAS) assays. Even gravimetric analysis was applied to both fruit and legume samples. A significant disparity emerged upon comparing the yield % obtained: fruit UAE extracts exhibited approximately 50% higher yield values compared to legume UAE extracts. Notably, the apple final product (Apl-FinalP) achieved a remarkable yield of nearly 90%. Analyzing the fruit samples specifically, a consistent

trend was observed: by-products generally yielded lower amounts of extractable material compared to their respective final products. However, this trend was not consistently observed in the legume samples. While soy by-product (S-Bio-ByP) demonstrated a lower yield compared to the seeds (S-Bio-RM and S-Conv-RM), bean by-products exhibited no significant differences in yield values when compared to their corresponding final products. Furthermore, biological peach by-products (Pch-Bio-ByP) demonstrated yield values comparable to those of its final product. These observed variations in yield % across different sample types may be attributed to several factors, including inherent differences in the composition and cellular structure of the raw materials, the impact of processing techniques on the physical and chemical properties of the samples, and the varying levels of bioactive compounds present in each sample type.

A significant disparity in Total Phenolic Content (TPC), expressed as mmol GAE/100 g DW, was also observed when comparing fruit and legume UAE extracts. While less pronounced than the differences observed in yield values, all fruit UAE extracts consistently exhibited higher TPC compared to legume UAE extracts. Notably, Pch-Bio-FinalP emerged as the sample with the highest TPC among all samples analyzed. Fruit by-products with high pulp content, such as apricot and peach, demonstrated high TPC values comparable to their respective final products. A similar trend was observed in bean by-product UAE extracts, which generally exhibited comparable or even higher TPC than their corresponding final products, suggesting substantial retention of polyphenols within these by-products. Conversely, tomato by-product UAE extracts showed significantly lower TPC compared to their final product UAE extracts. This disparity can likely be attributed to their characteristically low pulp content and the observed low extraction yields.

A significant disparity in Total Antioxidant Activity (TAS), expressed as mmol Trolox/100 g DW, was also observed when comparing fruit and legume UAE extracts. Fruit UAE extracts consistently exhibited substantially higher TAS values, exceeding those of legume UAE extracts by more than double. Within the fruit UAE extracts, final products generally exhibited comparable TAS values to their respective by-products, with the exception of Pch-Bio-ByP. This observation is particularly noteworthy considering that TPC values exhibited significant differences between by-products and final products for most fruit UAE extracts, with the exception of apricots. In line with fruit UAE extracts results, even bean by-product UAE extracts demonstrated comparable or slightly higher antioxidant activity than their corresponding final products. Furthermore, okara (soy by-product, S-Bio-ByP) exhibited significantly higher antioxidant activity compared to seeds (S-Bio-RM and S-Conv-RM).

Regarding the application of chromatographic methods, it is important to note that fruit and legume UAE extracts were analyzed by distinct analytical approaches. The HPLC-DAD chromatographic method (detailed in *Section* 2.1.8) proved to be fast and effective for the qualitative and quantitative analysis of all fruit UAE extracts, as it was validated. In contrast, the analysis of legume UAE extracts required more sophisticated techniques as it was not possible to identify the polyphenols without the MS detector. The employed UHPLC-DAD-ESI-MS^n chromatographic analysis (*Section* 2.1.9), while allowing the identification of polyphenols in all legume UAE extracts, currently provides qualitative and semi-quantitative data. More comprehensive

investigations, including full quantitative analyses, will be carry out in future studies.

The chromatographic analyses were necessary for clarifying the unique phenolic composition of each UAE extract, particularly emphasizing the valuable polyphenols within the by-products, which are intended for incorporation as active ingredients into new nutraceutical formulations. Each by-product UAE extract demonstrated a distinct and abundant polyphenol profile, offering promising potential for many applications. Specifically, the biological apple by-product (Apl-Bio-ByP) exhibited high concentrations of phlorizin, hyperoside, and quercetin, comparable to or even greater those found in the corresponding final product. This by-product UAE extract presents significant interest due to the well-documented anti-diabetic and anti-inflammatory properties of phloridzin[94]. Conversely, the biological peach and apricot by-product UAE extracts (Pch-Bio-ByP and Ac-Bio-ByP) were characterized by high levels of chlorogenic acid and its derivatives, again comparable to or exceeding their respective final products. These compounds are recognized for their potential in preventing various chronic diseases, including diabetes, cardiovascular diseases, obesity, cancer, and hepatic steatosis[132]. Finally, tomato by-product UAE extracts (T-Bio-ByP and T-LI-ByP) were found to be rich in naringenin, with concentrations even exceeding those observed in the final products. Naringenin is a particularly noteworthy compound due to its established cardioprotective effects[133].

In contrast to fruit by-products, legume UAE extracts exhibited a distinct polyphenol profile. This observation underlines the significant compositional differences and distinct end-use applications between these two food categories. Notably, bean by-product UAE extracts were found to be rich in ferulic acid derivatives, compounds well-known for their cholesterol-lowering properties[118]. On the other hand, whole bean by-product UAE extracts were characterized by a higher abundance of flavonols, which possess a diverse range of beneficial health effects. Finally, the soy by-product UAE extract (S-Bio-ByP), rich in aglycone isoflavones, presents significant potential for applications in the prevention of menopause-related pathologies[127].

2.3 CONCLUSIONS

This research widely investigated the polyphenol content of different agri-food by-product derived from fruits (peach, apricot, apple and tomato) and legumes (peas, beans, green beans and soy) coming from *Fruttagel*® (an agri-food industry of Italy). The main objective was to assess their potential as sustainable sources of polyphenols for their application as active ingredients in food supplements and nutraceutical formulations following a circular economy perspective.

Primarily, the developed Ultrasound-Assisted Extraction (UAE) proved to be an effective method for achieving high yields of polyphenols from all lyophilized vegetal matrices analyzed. The preliminary Total Phenolic Content (TPC) UV-vis spectrophotometric assay for total polyphenol content provided a rapid screening, effectively differentiating between by-products and their respective final products. In some cases, the by-products exhibited equal or even higher polyphenol content than the final products.

More sophisticated analytical techniques were employed for detailed chemical characterization of polyphenols

in UAE extracts analyzed. The HPLC-DAD chromatographic analysis was used to qualitatively and quantitatively determine the polyphenol profiles of UAE fruit by-product extracts, revealing the presence of many compounds, including hydroxycinnamic acids and various flavonoids. Notably, many fruit by-products demonstrated comparable or greater polyphenol content to their corresponding final products, highlighting their potential as valuable sources of polyphenols.

The HPLC-DAD chromatographic method proved to be a robust and versatile technique for characterizing polyphenols in a wide range of fruit matrices. Furthermore, this method was successfully applied to evaluate the stability of polyphenols in both UAE extracts and the original plant powders. Results showed that polyphenols remained stable for at least 15 months when stored at -20°C.

Additionally, safety assessments were performed at *Fruttagel*® to determine pesticide residues in both conventional and biological fruit by-product UAE extracts. These analyses established the safety of using these UAE extracts as active ingredients in nutraceutical formulations, minimizing the risk of pesticide contamination.

In the case of legume by-product UAE extracts, advanced UHPLC-DAD-ESI-MS^n analysis, developed at the *University of Graz, Austria*, revealed different and interesting phenolic profiles. Key results included for examples the presence of ferulic acid derivatives in bean by-products and isoflavones in okara (soy by-product). Even in this case, some by-products exhibited higher polyphenol content compared to their corresponding final products. Concerning cultivation practices, it has been demonstrated that their significantly influenced the polyphenol content of legume by-products. Indeed, sustainable cultivation methods, such as biological and “Lotta Integrata” (LI) farming, were associated with increased polyphenol levels in bean by-products compared to conventional methods. Additionally, thermal processing applied variable effects on polyphenol content in legume samples.

Another interesting aspect that emerged from the research was the marked differences in phenolic profiles between green beans (young stage of ripening) and mature beans (Phaseolus vulgaris L.). Indeed, green beans exhibited higher levels of glycosylated flavonoids, reflecting their role in defense against environmental stresses.

Finally, interesting results were obtained for pea by-products, with the identification of two compounds potentially belonging to the N-phenylpropenoyl-L-amino acid family. However, further analysis, such as NMR spectroscopy, is required to confirm their identity.

In conclusion, these results demonstrate the significant potential of agri-food by-products as valuable sources of bioactive compounds, particularly polyphenols, for the development of innovative nutraceuticals and food supplements. The use of by-products aligns with a circular economy approach, minimizing waste and maximizing resource use within the agri-food sector.

3 PROTEIN CHARACTERIZATION IN AGRI-FOOD SAMPLES

3.1 MATERIAL AND METHODS

3.1.1 Agri-food samples

The analyzed samples are reported in *Section* 2.1.3, *Table* 1 and *Table* 2.

3.1.2 Quantification of Protein Content in Agri-Food Samples via Kjeldahl Method

The Kjeldahl method was performed in accordance with the guidelines set forth by the Association of Official Agricultural Chemists International (AOAC)[134]. Approximately 1 g of agri-food sample powder was lyophilized and subsequently hydrolyzed with 15 mL of concentrated sulfuric acid containing two copper catalysts at 420 ◦C for two hours. Following this, H_2O was added before proceeding with the neutralization and titration. The total nitrogen content of the samples was multiplied by 6.25 as the conversion factor, as reported by the protocol.

3.1.3 Statistical Evaluation

All results were expressed as the mean ± SD of 3 independent experiments. Statistical analyses were performed using ordinary one-way ANOVA and Sidak's multiple comparison tests. The statistical software GraphPad 10.0 version (GraphPad Prism, San Diego, CA, USA) was used, and p-values < 0.05 were considered statistically significant.

3.2 RESULTS AND DISCUSSION

The protein content results and discussion of fruit and legume samples has been reported in literature[23],[80].

3.2.1 Protein Content of Fruit By-products

The protein content of each sample was determined by the Kjeldahl method (see *Section* 3.1.2). The results are presented in *Figure* 59. The protein content values are expressed in grams per 100 grams of dry weight (DW). It is notable that the majority of fruit by-products display a protein content that is comparable to that of their final products. It is noteworthy that both the apple and tomato by-products exhibited a markedly elevated protein content, which was comparable to or exceeded that of their final products. Indeed, the protein content

of the biological tomato by-products (T-Bio-ByP) was found to be two to three times higher than that of the other fruit by-products. These findings can be attributed to the fact that both apple and tomato by-products are primarily composed of their corresponding seeds, which have been described as rich sources of protein, accounting for up to 49.55% and 32% of their nutritional value, respectively[28].

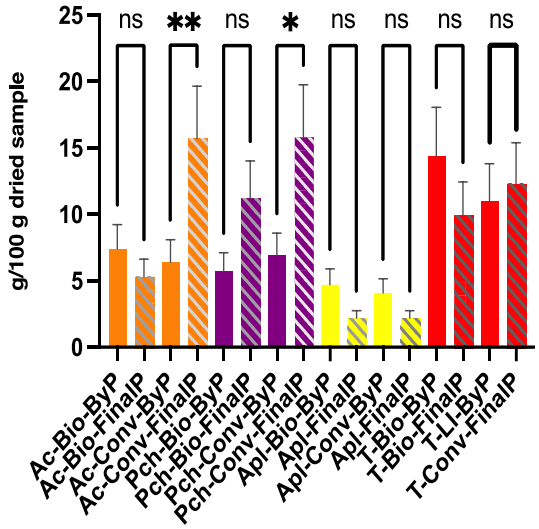


*Figure 59 Protein content obtained from fruit ByP and FinalP by the Kjeldahl method. The results are the mean value of 2 experiments ± SD. ** p < 0.01; * p < 0.05. ns = not significant*[23]

Table 26 Protein Content of fruit samples[23]

Samples analyzed	Kjeldahl method: protein content Average g 100g-1 DW ±SD
Ac-Bio-ByP	7.40±1.30E+00
Ac-Conv-ByP	6.40±1.20E+00
Ac-Bio-FinalP	5.30±9.50E-01
Ac-Conv-FinalP	15.70±2.80E+00
Pch-Bio-ByP	5.70±1.00E+00
Pch-Conv-ByP	6.90±1.20E+00
Pch-Bio-FinalP	11.20±2.00E+00
Pch-Conv-FinalP	15.80±2.80E+00
Apl-Bio-ByP	4.70±8.50E-01
Apl-Conv-ByP	4.10±7.40E-01
Apl-FinalP	2.20±4.00E-01
T-Bio-ByP	14.40±2.60E+00
T-LI-ByP	11.00±2.00E+00
T-Bio-FinalP	9.90±1.80E+00
T-Conv-FinalP	12.30±2.20E+00

The protein content of apricot (*Ac-Bio-ByP and Ac-Conv-ByP*) and peach biological by-products (*Pch-Bio-*ByP) is significantly comparable to that of their final products, with the exception of conventional peach by-products (Pch-Conv-ByP), which have a lower protein content. The observed correlation between conventional apricot and peach final products and the highest protein contents can be explained by considering the association between conventional farming and the use of pesticides, which have been shown to contain nitrogen that can interfere with this method. Nevertheless, despite the fact that the final products were obtained through conventional farming, no significant levels of pesticides were detected. Consequently, the observed results can be attributed to the actual protein content. The protein content detailed above has been reported in the literature[23].

3.2.2 Protein Content of Legume By-products

The protein content of each sample was determined using the Kjeldahl method, and the results expressed as g of protein in 100 g of dry weigh (DW) are presented in *Figure* 60[80].

All bean by-products revealed a wide range, going from 11.52±2.17E-01 g/100g DW to 23.40±4.20E+00 g/100g DW of protein (see *Table* 27). Notably, some bean by-products exhibited protein content lower than, equal to, or even exceeding that of the corresponding final product (19.80±3.60E+00 g/100g DW of protein). To validate these findings, a literature review was conducted. A study by Mariscal-Moreno et al. (2021)[135] reported a protein content of 23.94% in black bean (*Phaseolus vulgaris L.*) flour. This value aligns well with the higher end of the observed protein content range, further supporting the potential of bean by-products as valuable protein sources.

Concerning green bean by-products, obtained results revealed a protein content ranging from 10.30±1.90E+00 g/100 g DW of protein to 30.70±5.50E+00 g/100 g DW, comparable to that of bean by-products (11.60±2.10E+00 g/100g DW to 23.40±4.20E+00 g/100g DW of protein). Especially, the cooked biological by-product (GB-Co-Bio-ByP) exhibited the highest protein content among all bean and green bean samples, including the bean final product, reaching 30.70±5.50E+00 g/100 g DW. The current results validate those of previous research. For instance, published studies[136] have reported protein content in green beans by-product of 22 g/100 g DW, exceeding that of beans (Phaseolus vulgaris) of 20.9 g/100 g DW. This comparison with established literature further confirms the protein-rich nature of the examined green bean by-products.

Among all investigated legume by-products, okara (soy by-product) demonstrated the highest protein content, reaching 34.50 ± 6.20 g/100g DW. This is supported by previous research. Asghar et al. (2023)[137] reported a protein content of 34.15% in dry okara, further validating our result. These results underscore the potential of soy by-products as a valuable source of protein, potentially offering greater nutritional benefits than the seeds themselves.

Concerning the cultivation method, no significant differences were observed between B-ByP and B-Final-P. However, for green beans and soy, bio cultivation resulted in a higher protein content in the cooked GB-Bio-

Co-ByP and S-Bio-RM samples.

Analysis of total protein content in pea samples revealed no significant differences between biological and conventional by-products when compared to the final product. Moreover, the obtained protein content values, 29.20±5.30E-01 g/100g DW and 25.50±4.60E+00 g/100g DW, were comparable to those reported in the literature, which correspond to 23.7 g/100g[111].

The legume by-products studied, especially okara (S-Bio-ByP), could be widely used in the cosmetics industry due to their high protein content. Indeed, proteins contribute to skin hydration by increasing water content in the stratum corneum, reducing dehydration, and improving barrier function. They also hydrate the scalp, making them suitable for hair care products[33],[138].

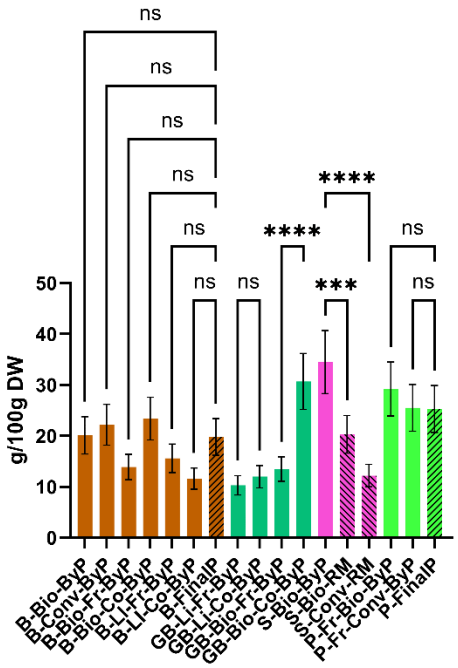


*Figure 60 Protein content (g/100 g DW) of ByP (full bars) and FinalP (stripped bars) determined by the Kjeldahl method. Data represent mean ± SD (n=2). **** p < 0.0001, *** p < 0.005, ns: not significant[80]*

Table 27 Protein content of Legume samples[80]

Samples analyzed	Kjeldahl method: protein content Average g 100g-1 DW ±SD
B-Bio-ByP	20.10±3.60E+00
B-Conv-ByP	22.20±4.00E+00
B-FinalP	13.90±2.50E+00
B-Fr-Bio-ByP	23.40±4.20E+00

B-Co-Bio-ByP	15.60±2.80E+00
B-Fr-LI-ByP	11.60±2.10E+00
B-Co-LI-ByP	19.80±3.60E+00
GB-Fr-LI-ByP	10.30±1.90E+00
GB-Co-LI-ByP	12.00±2.20E+00
GB-Fr-Bio-ByP	13.50±2.40E+00
GB-Co-Bio-ByP	30.70±5.50E+00
S-Bio-RM	34.50±6.20E+00
S-Conv-RM	20.30±3.70E+00
S-Bio-ByP	12.20±2.20E+00
P-Fr-Bio-ByP	29.20±5.30E-01
P-Fr-Conv-ByP	25.50±4.60E+00
P-FinalP	25.30±4.60E+00

3.2.3 Comparative Analysis of Fruits and Legumes protein content

A significant disparity in protein content, expressed as grams per 100 grams of dry weight, was observed when comparing fruit and legume samples. Legume samples exhibited evidently higher protein content, with values nearly double those of fruit samples. Notably, okara (S-Bio-ByP) emerged as the by-product with the highest protein content among all samples analyzed, exceedingly even the protein content of the all-final products and soy seeds (S-Bio-RM and S-Conv-RM). Within the legume category, bean by-products demonstrated a wide range of protein content, with some samples exhibiting values exceeding those of their final products. Similarly, green bean by-products showed a considerable range, with the cooked biological by-product exhibiting the highest protein content among all legume and fruit samples. In the case of fruits, most by-products exhibited protein content comparable to their respective final products. However, notable exceptions included apple and tomato by-products, which demonstrated significantly higher protein content, likely attributable to their high seed content.

3.3 CONCLUSIONS

In conclusion, the analysis of protein content, determined using the Kjeldahl method (see *Section* 3.1.2), provided valuable insights. The obtained results revealed that protein content in many fruit by-products was comparable to that of their corresponding final products. Similarly, a comparable trend was observed in legume samples. Significantly, the analysis demonstrated that all analyzed by-products, particularly soy by-product (S-Bio-ByP), exhibited high protein content. This observation underscores the substantial potential for the utilization of these by-products in various applications, including the nutraceutical and cosmetic industries.
Indeed, proteins derived from food waste hold significant potential for application in the nutraceutical field, particularly for athletes. It has been reported by Zare et al[139] that protein supplementation is crucial for optimal athletic performance, aiding muscle repair and growth. Soy protein has emerged as a viable alternative to

animal proteins, demonstrating comparable efficacy in increasing lean mass[139]. Furthermore, soy protein consumption has been shown to reduce exercise-induced metabolic stress, enhance antioxidant capacity, and potentially improve exercise performance by enhancing high-intensity exercise capacity, delaying fatigue, and improving endurance[139]. Additionally, protein derivatives find large application in various skin care and makeup formulations[75]. Soluble proteins are versatile and suitable for a broad range of formulations, while insoluble proteins have niche applications, such as fibrous collagen in face masks for hydration and silk powder in anhydrous preparations[75]. Native proteins and high molecular weight hydrolysates are preferred for their film-forming properties, creating a continuous film on the skin and providing a soft and smooth feel[75]. Therefore, proteins are typically added to formulations after emulsification and cooling to minimize heat-induced denaturation[75]. Consequently, the high protein content of agri-food by-products presents significant potential for valorization across different sectors.

4 APPLICATION OF FRUIT BY-PRODUCT UAE EXTRACTS AS ACTIVE INGREDIENT IN NEW FORMULATION

The final phase of this research was performed at *Valpharma S.p.A.*, where I spent a six-month internship to investigate the formulation of a new food supplement incorporating polyphenols derived from the UAE extracts of agri-food by-products.

4.1 MATERIAL AND METHODS

4.1.1 Chemicals and Reagents

Methanol HPLC grade ≥ 99.9%, gallic acid ≥ 95%, protocatechuic acid ≥ 97%, trans-cinnamic acid ≥ 98.0%, caffeic acid ≥ 98.0%, p-coumaric acid ≥ 98.0%, ferulic acid ≥ 99.5%, (+)-catechin ≥ 99.0%, isoquercitrin ≥ 98.0%, daidzein ≥ 97.0%, genistein ≥ 97.0%, and phloridzin dihydrate ≥ 98.5%, Ethyl acetate ≥ 99.5%, anhydrous sodium sulfate powder ≥ 99.0% were purchased from Sigma-Aldrich (Taufkir-chen, Germany); chlorogenic acid ≥98.0% was purchased from Apollo Scientific (Bredbury, UK); and Quercitrin ≥ 98% was purchased from Cayman Chemical (Ann Arbor, MI, USA). Folin–Ciocalteu reagent was purchased from VWR Chemicals (Darmstadt, Germany); copper (II) sulfate pentahydrate and potassium sodium tartrate tetrahydrate EMSURE® were purchased from Merck (Darmstadt, Germany); (−)-epicatechin ≥ 97.0%, hesperetin ≥ 97.0%, and apigenin ≥ 97.0% were purchased from TCI (Zwijndrecht, Belgium); hyperoside ≥ 92.0% was purchased from HWI group (Rulzheim, Germany); (+)-rutin trihydrate ≥ 97% was purchased from Alfa Aesar (Haverhill, MA, USA); myricetin ≥ 98%, naringenin ≥ 97% and kaempferol ≥ 98.0% were purchased from ThermoFisher (Kandel, Germany); and naringenin chalcone ≥ 95% was purchased from PhytoLab (Vestenbergsgreuth, Germany). Ultrapure (type 1). VWR Syringe Filter, PTFE, 0.22 μm, was purchased from VWR International Srl (Milan, Italy). Kudzu, Linophenol, red clover vegetal matrices from *Valpharma S.p.A.* 0.45 μm RC filters, Ø 25 mm.

4.1.2 Equipment

The analytical UHPLC-DAD system employed was Agilent Technologies 1290 Infinity II, equipped with Empower software (Agilent, Santa Clara, CA, USA), a Kinetex XB-C18 column (4.6 mm × 150 mm, 5 μm, 100 Å) (Phenomenex, Torrance, CA, USA). The Spectrophotometer: Agilent Technologies Cary 60 UV-vis (Agilent, Santa Clara, CA, USA). Ultrasound Bath: ARGOLab Sweep technologies (Sinergica Soluzioni S.r.l., Milan, Italy). The centrifuge: VWR Imac CT 6 (VWR International Srl, Milan, Italy). Rotatory evaporator: IKA Rotary Evaporators RV 10 basic (IKA-Werke GmbH & Co. KG, Staufen im Breisgau, Germany). Dissolution Tester PerkinElmenr (Shelton, USA).

4.1.3 Agri-food samples

The final part of this project was focused on developing a new formulation that incorporates polyphenols derived from the by-products of *Fruttagel*®'s UAE extracts as active ingredients. Biological and conventional fruit samples, reported in ***Table*** 28, have been selected for inclusion in the formulation, as they were found to exhibit a higher concentration of polyphenols compared to the by-products derived from legumes.

Table 28 Fruit sample by-products included in Part 4

Sample		Acronym
Apricot	Biological by-product	Ac-Bio-ByP
	Conventional by-product	Ac-Conv-ByP
Peach	Biological by-product	Pch-Bio-ByP
	Conventional by-product	Pch-Conv-ByP
Apple	Biological by-product	Apl-Bio-ByP
	Conventional by-product	Apl-Conv-ByP

4.1.4 UAE extract treatment: a Purification and Concentration procedure

In this *Section*, fruit extracts obtained by the Ultrasound-Assisted extraction method (UAE) reported in *Section* 2.1.4 have been discussed. A small portion (approximately 4 mL) of the UAE fruit extracts was set aside for subsequent analysis, comparing the pure extracts with the concentrated and purified extracts. All fruit UAE extracts were purified and concentrated using a separating funnel and ethyl acetate. The UAE extract from each fruit by-product sample was transferred to a rotavapor flask to evaporate the organic solvents (methanol and acetone). Once an aqueous extract (about 10 mL) was obtained, the following procedure was applied: 10 mL of the extract was mixed with 10 mL of ethyl acetate in a separating funnel 4 times. After mixing, a small amount of anhydrous sodium sulfate was added to the ethyl acetate extract (approximately 40 mL) to remove excess water. The anhydrous sulfate was then removed by filtering through filter paper directly into a rotavapor flask. The ethyl acetate extract was completely dried in the rotavapor for weighing. The weight of the dry extract obtained was utilized for the gravimetric analysis mentioned in *Section* 2.1.5. Finally, the dried extract was dissolved in 2 mL of a 50:50 mixture of a 2% aqueous solution of acetic acid and 100% methanol. The process is reported in ***Figure*** 61. The Total Phenolic Content (TPC) assay was then performed on this mixture by following the method reported in *Section* 2.1.6.

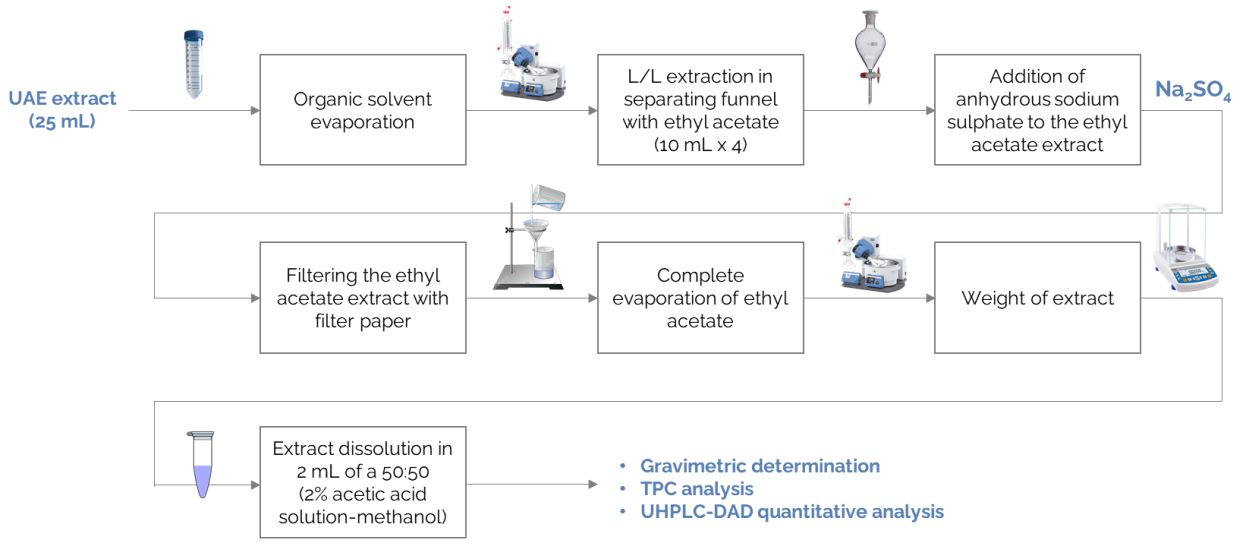


Figure 61 Extract Treatment

4.1.5 Purified and Concentrate UAE Extract Analysis: TPC assay and HPLC-DAD analysis

The % yield (*Section* 2.1.5) of both untreated and treated fruit UAE extracts (concentrated and purified extracts) was performed and compared. Additionally, a Total Phenolic Content (TPC) assay (*Section* 2.1.6) was carried out on both untreated and treated UAE extracts to assess their polyphenol content. Finally, quantitative analysis using the chromatographic HPLC-DAD analysis (*Section* 2.1.8) was performed to confirm or disprove the findings from the TPC assay.

4.1.6 Determination of the titre of active ingredients in solid formulation

The spectrophotometric method for determining the average titer of a mixture of lifenol®, red clover, and kudzu in Tablets was studied by *Valpharma S.p.A.* To perform the analysis, select and weigh 10 doses of the mixture. Grind the doses finely in a mortar and weigh out an amount of powder equivalent to 285 mg of the active ingredient in a 200 ml volumetric flask. Add approximately 120 ml of methanol to the flask, mix it for 1 hour at 800 RPM, and then sonicate for 5 minutes. Make up the volume to 200 ml with methanol. Centrifuge an aliquot at 4000 RPM for 5 minutes and dilute 2 ml of the supernatant to 100 ml with water, achieving a final concentration of 0.0285 mg/ml. Read the absorbance in a spectrophotometer using a 1 cm cell at a wavelength of 250 nm, ensuring to zero the instrument with a blank solution of water. Filter all solutions using 0.45 µm RC filters with a diameter of 25 mm, discarding a waste volume of 7 ml.

For the average title, calculate the mg of Active Ingredient with the following formula:

$$mg/dose = Abs\ Sample \times Dilution\ of\ Sample\ (mL) \times Weight\ of\ 10\ dose\ (grams)$$

$$E1\% \times Weight\ of\ sample\ (grams) \times 10\ (n°\ dose) \times 100$$

E1%=329 (already calculated from *Valpharma S.p.A*)

4.1.7 In Vitro Dissolution Test of Tablet

The spectrophotometric method is used to determine the average titer of a mixture of lifenol®, red clover, and kudzu in Tablets, as studied by *Valpharma S.p.A*. To begin, transfer 1000 ml of a dissolution medium consisting of 0.75% sodium lauryl sulfate (SLS) into the jars and maintain the temperature of the liquid at 37°C ± 0.5°C. Insert 1 mm cells and configure the software to collect data at designated time points. Place the Tablets into each jar and initiate stirring at 50 rpm. The dissolution test apparatus should be equipped with a peristaltic pump and a spectrophotometer with a paddle apparatus. The time points for measurements are set at 6 hours, 12 hours, and 18 hours (with additional informative measurements at 2 hours and 4 hours) using a 10 µm filter and a wavelength of 250 nm.

Calculate the % dissolution with the following formula:

mg/dose = [Abs Sample x Dilution sample (mL)] / [E1% x 1 cm x Dosage x F.C.]

Where:

E1%; 1 cm: 309 (already calculated from *Valpharma S.p.A*)

F.C.: Optical path correction factor (0.1)

4.2 RESULTS AND DISCUSSION

4.2.1 Impact of Purification on fruit by-product UAE Extract

The gravimetric analysis and subsequent TPC assay were conducted to determine whether the UAE extract treatment method (outlined in the previous *Section* 4.1.4) enabled the purification and concentration of the extracts. Indeed, as previously mentioned in *Section* 2.2.1.1, the yield percentage of the UAE fruit extracts was significantly higher than the total polyphenol content (TPC). This discrepancy may be due to the UAE extraction process (*Section* 2.1.4), which is not selective for polyphenols. For this reason, it has been hypothesized that the dry extract also contains other components, such as proteins and carbohydrates.

4.2.1.1 Gravimetric analysis

The bar graph in *Figure* 62 illustrates the comparison of the % yield (n=1) between the untreated and treated fruit by-product UAE extracts. The bar graph indicates that the amount of the treated extract (stiped bars) was significantly lower than that of the untreated extract (full bars). This observation suggests that polyphenols from the aqueous extract transferred to the ethyl acetate during the separation in the funnel, leaving behind

less relevant compounds in the aqueous layer. Therefore, the obtained results confirm that the purification and concentration process (*Section* 4.1.4) effectively concentrated the extract while removing undesirable components.

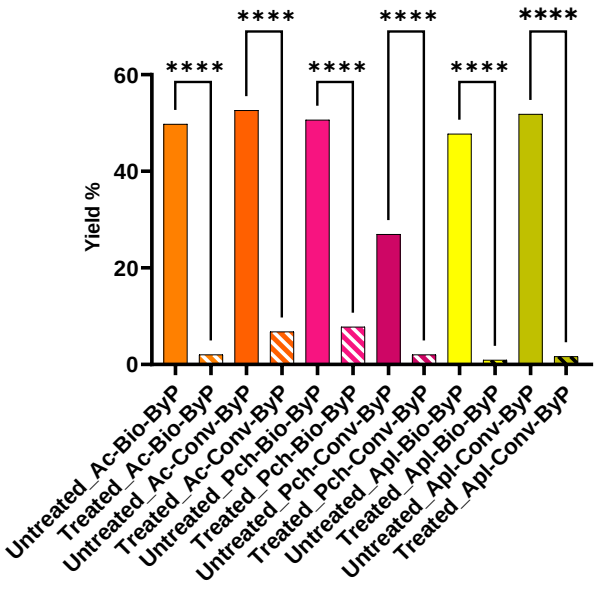


*Figure 62 Comparison of the % yield (n=1, see Section 2.1.5) between untreated (full-colored bars) and treated (striped bars) fruit by-product UAE extracts (peach, apricot, apple). The samples defined as "treated" are obtained using the specified method reported in Section 4.1.4. **** $p < 0.0001$*

4.2.1.2 TPC analysis

Following the Gravimetric Analysis, which confirmed that the treated UAE extracts exhibited a lower percentage yield (see *Figure* 63) than the untreated UAE extracts, the Total Phenolic Content assay (TPC) was conducted. The TPC assay (outlined in *Section* 2.1.6) was performed to determine the total polyphenol content of both the untreated and treated UAE fruit extracts. The aim was to confirm that the most concentrated extract, which underwent the specified treatment described in *Section* 4.1.4, exhibited a higher concentration of polyphenols. As illustrated in the bar graph in *Figure* 63, the hypothesis was validated. The total polyphenol content of all treated extracts (stiped bars) was found to be significantly higher than that of the corresponding untreated extracts (full bars).

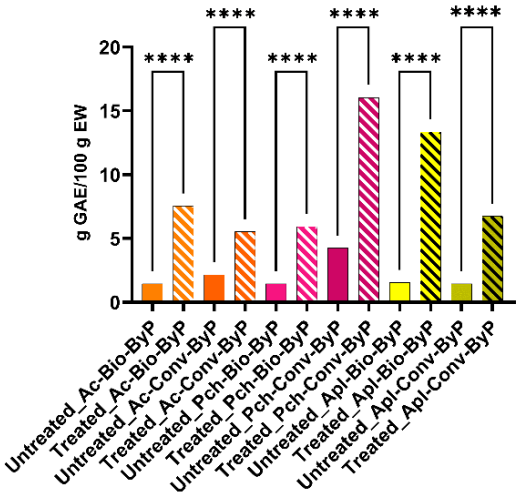


*Figure 63 Comparison of the % TPC (n=1, see Section 2.1.5) between untreated (full-colored bars) and treated (striped bars) fruit by-product UAE extracts (peach, apricot, apple). The samples defined as "treated" are obtained using the specified method reported in Section 4.1.4. **** $p < 0.0001$*

4.2.1.3 Semi-Quantitative HPLC-DAD analysis: a comparative study of untreated and treated fruit UAE extracts

The final step in confirming the hypothesis that the treated extracts are more concentrated than the untreated ones involved the application of the HPLC-DAD chromatographic method (see *Section* 2.1.8), which is essential for conducting a semi-quantitative comparative analysis. The semi-quantitative HPLC-DAD analysis was conducted by comparing the peak areas of the reference standards with the peak areas of all extracted UAE fruit by-products, by following the formula:

$$Area\ (std) : Conc\ \mu g/mL(std) = Area\ (peak\ sample) : Conc\ \mu g/mL\ (peak\ sample)$$

This enabled the known concentrations of the standards to be related to the determination of the unknown concentrations of the peaks in the UAE extracts.

This procedure was applied for all fruit UAE extracts, both biological and conventional. *Figure* 64, *Figure* 65, and *Figure* 66 present a comparative analysis of the phenolic profiles of untreated and treated UAE extracts of apricot, peach, and apple by-products, respectively. The full bars represent the profiles of the untreated UAE extracts, while the striped bars illustrate the profiles of the treated UAE extracts. It has been observed that the concentration of polyphenols is significantly higher in all treated extracts of biological and conventional cultivated apricots, peaches, and apples in comparison to their untreated counterparts. This final analysis confirmed the hypothesis that the treated extracts are indeed more concentrated in terms of polyphenols. As a result, the formulation of a Tablet containing the dry extracts from the treated UAE extracts was initiated, thereby ensuring patient compliance. Indeed, the preliminary Tablet formulation, illustrated in *Table* 29, has been reassessed with the objective of developing a Tablet with a minimum weight of less than 1.0 g, representing a significant reduction from the initial 1.841 g (≈ 2g).

Apricot By-products

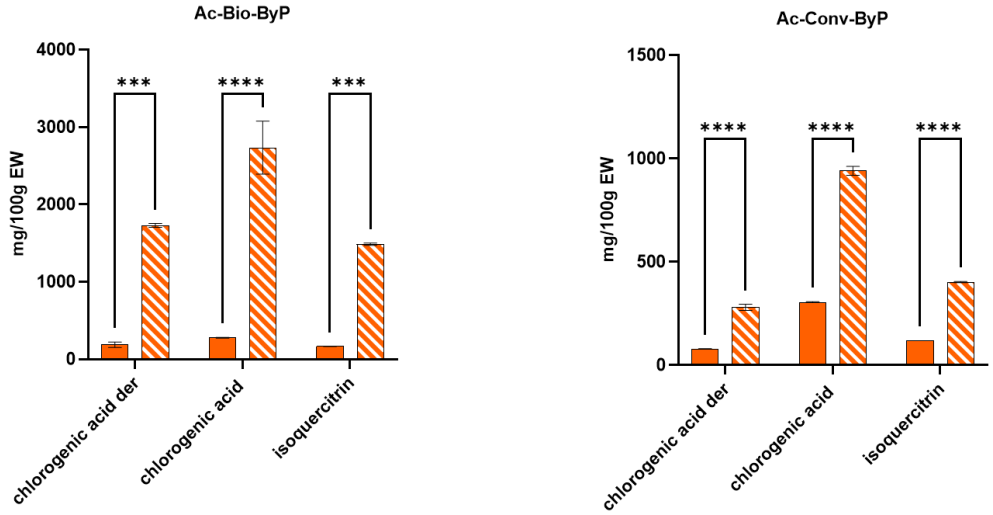


*Figure 64 Phenolic profiles of UAE extracts of apricot by-products: biological on the left (Bio), conventional on the right (Conv). The comparison is made between the untreated samples (orange full bar) and treated samples (orange striped bar). Treated samples are obtained by the specified method reported in Section 4.1.4. Results are expressed as the mean values from 2 analyses ± SD of the same sample. Statistical significance is indicated as follows: **** p < 0.0001; *** p < 0.005*

Peach By-products

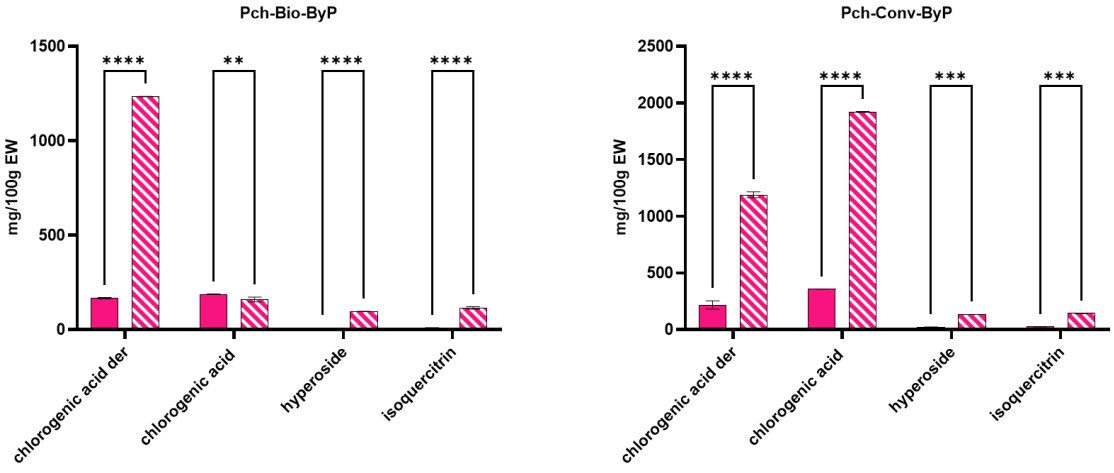


*Figure 65 Phenolic profiles of UAE extracts of peach by-products: biological on the left (Bio), conventional on the right (Conv). The comparison is made between the untreated samples (pink full bar) and treated samples (pink striped bar). Treated samples are obtained by the specified method reported in Section 4.1.4. Results are expressed as the mean values from 2 analyses ± SD of the same sample. Statistical significance is indicated as follows: **** p < 0.0001; *** p < 0.005*

Apple By-products

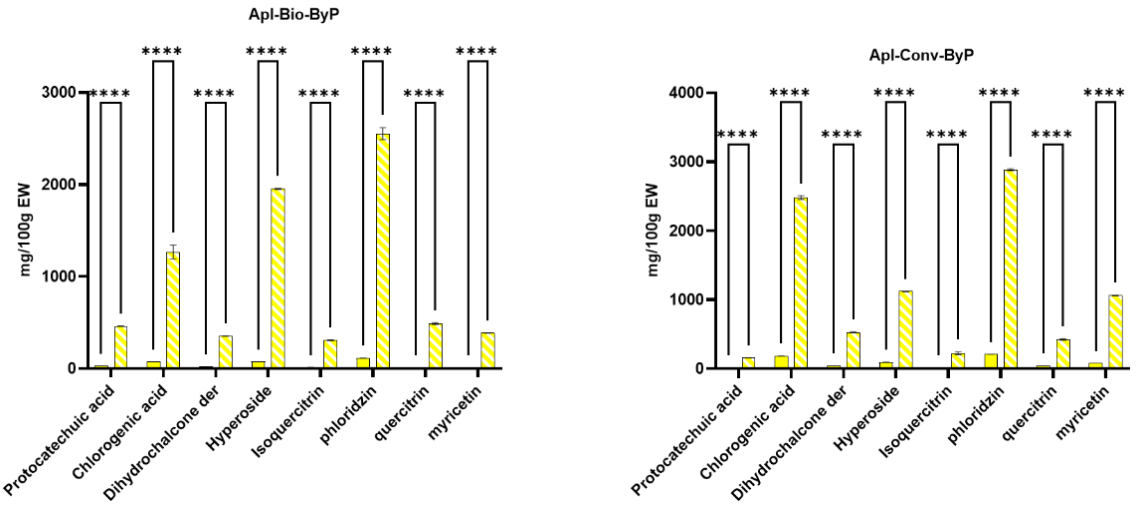


*Figure 66 Phenolic profiles of UAE extracts of apple by-products: biological on the left (Bio), conventional on the right (Conv). The comparison is made between the untreated samples (yellow full bar) and treated samples (yellow striped bar). Treated samples are obtained by the specified method reported in Section 4.1.4. Results are expressed as the mean values from 2 analyses ± SD of the same sample. Statistical significance is indicated as follows: **** $p < 0.0001$*

4.2.2 From Fruit by-product UAE extracts to Food supplement: focus on Tablet formulation

4.2.2.1 Development of a new dietary supplement: optimization of the formulation for improved patient compliance

This study focused on a formulation incorporating polyphenols extracted from fruit by-product UAE extracts as active ingredients. This decision was driven by the successful application of HPLC-DAD analysis, which allowed the precise quantification of individual polyphenols within UAE fruit by-product extracts (see *Section* 2.1.8), demonstrating their significantly higher polyphenol content compared to UAE legume by-product extracts (*Section* 2.2.3). To maximize polyphenol yield, the study arranged the use of UAE fruit by-product extracts from conventional cultivation (Apl-Conv-ByP, Pch-Con-ByP, and Ap-Conv-ByP), known for their higher concentration of these compounds (see *Section* 2.2.1.6).

Furthermore, the analysis of *Fruttagel*® revealed pesticide residue levels to be within low limits, despite the fruits being cultivated by conventional approaches (see results *Section* 2.2.1.9).

While UAE extracts of polyphenols from legume by-products exhibited unique polyphenolic profiles (see *Section* 2.2.2.4), as revealed by UHPLC-DAD-ESI-MS^n analysis, they were temporarily excluded from further formulation development. A more comprehensive quantitative analysis of legume by-product extracts using HPLC-DAD will be carry out in the future.

This study started with the theoretical formulation of a controlled-release and gastro-resistant Tablet intended for the food supplement market. The Tablet was designed to incorporate polyphenol extracts from conventional

fruit by-products as active ingredients (Apl-Conv-ByP, Pch-Con-ByP, and Ap-Conv-ByP). The gastro-resistant property of the Tablet was strategically incorporated to target the release of polyphenols directly into the intestinal tract, bypassing the acidic environment of the stomach. This approach was motivated by the well-documented prebiotic potential of polyphenols, as evidenced in the literature[6]. Indeed, preclinical and clinical studies strongly suggest that polyphenols exhibit prebiotic properties and exert antimicrobial effects against pathogenic members of the gut microbiota[6]. These beneficial effects contribute to the modulation of gut microbiota composition and function, influencing bacterial quorum sensing, membrane permeability, and sensitizing bacteria to xenobiotic compounds[6]. Furthermore, polyphenols have been shown to impact intestinal metabolism, enhance immune function, and exert anti-inflammatory properties within the gut[6].

The new dietary supplement developed at *Valpharma S.p.A* is shown in *Table* 29. A combination of Surelease E-7-19040 and NS-Enteric 29Z19241 in water was selected for the gastro-resistant coating of the tablet (see *Table* 29). Surelease E-7-19040, as documented in the literature[140], is a film-forming material designed to delay drug release. It consists of ethylcellulose, a natural polymer derived from cellulose. This polymer exhibits pH-dependent solubility, being insoluble in the acidic environment of the stomach but soluble in the alkaline environment of the intestine. This characteristic enables the Surelease coating to protect the active ingredient within the stomach and facilitate its release exclusively in the intestinal tract, where absorption occurs. The term E-7-19040 indicates a specific formulation with defined characteristics, including viscosity and particle size distribution. Concerning the NS-Enteric 29Z19241, it is a widely used component of enteric coatings in pharmaceutical formulations and it primarily includes sodium alginate. Sodium alginate, a natural polymer derived from brown algae, exhibits unique gel-forming properties in the presence of calcium ions[141]. This interaction leads to the formation of a robust barrier that effectively resists the acidic environment of the stomach while dissolving in the alkaline environment of the intestine, where calcium ion concentration is significantly lower. Other added excipients include Microcrystalline Cellulose PH102 (see *Table* 29) which acts as a binder, enhancing powder cohesiveness by effectively agglomerating individual particles[142]. Then, magnesium stearate acts as a lubricant and anti-adherent in tablet formulation[143]. It reduces friction between powder particles facilitating tablet ejection and preventing sticking. Finally, Colloidal Silica acts as an anti-caking agent and a disintegrant[144]. It prevents powder agglomeration, ensuring homogenous powder blending and facilitating efficient tablet compression.

This project (*Table* 29) employed polyphenols extracted from untreated conventional fruits via ultrasound-assisted extraction (UAE), Apl-Conv-ByP, Pch-Con-ByP, and Ap-Conv-ByP, as active ingredients (see purity data in *Table* 29, corresponding to TPC assay results, in *Section* 4.2.1.1). A target dosage of 40 mg of polyphenols per tablet was established based on market research and an analysis of existing product lines within the *Valpharma S.p.A.* manufacturing portfolio[145]. However, to reach this polyphenol total dosage, the preliminary Tablet formulation resulted in a final weight of 1.841 g, potentially compromising patient compliance due to the excessive size of the Tablet. To address this issue, a subsequent investigation focused on purifying and concentrating the polyphenol extracts was carried out (see *Section* 4.1.4).

Table 29 Preliminary project: Tablet *formulation*

Active ingredient	Dosage (mg)	Ingredient	Purity	Overdose	mg
	Tablet				
Ac-Conv-ByP	5	Polyphenols	1.80%	20%	333.333
Pch-Conv-ByP	30	Polyphenols	3.20%	20%	750.000
Apl-Conv-ByP	5	Polyphenols	1.30%	20%	461.538
Vitamin A Acetate 500000 UI ACEF	0.400	Vitamin A	15.00%	20%	3.200
Microcrystalline Cellulose PH102			100.00%	0%	0.000
Glycerol Dibeenate Nourishes	150.000		100.00%	0%	150.000
Magnesium Stearate	1.500		100.00%	0%	1.50
Anhydrous Colloidal Silica	1.500		100.00%	0%	1.50
	Coat				
Surelease E-7-19040 Clear (25% dry solids)	119.000		100.00%	0%	119.00
NS-Enteric 29Z19241 Clear	21.000		100.00%	0%	21.00
Water	1540.000		100.00%		1540.00
	Total				
	323.400				1841.07

Building upon the purification and concentration studies described in *Section* 4.1.4, a reformulated Tablet with reduced weight was developed to enhance patient compliance. *Table* 30 presents the composition of this revised formulation. Notably, while the overall formulation remains consistent, the "purity" column, indicating the total polyphenol content per UAE extract of fruit by-product, has increased. This increase in extract purity has allowed the reduction in the overall extract weight required to achieve the target dosage of 40 mg polyphenols per Tablet. Therefore, the new Tablet weighs approximately 0.641 g.

Table 30 Final project: Tablet *formulation*

Active ingredient	Dosage (mg)	Ingredient	Purity	Overdose	mg
	Tablet				
Ac-Conv-ByP	5	Polyphenols	5.60%	20%	107.143
Pch-Conv-ByP	30	Polyphenols	16.0%	20%	150.000
Apl-Conv-ByP	5	Polyphenols	6.80%	20%	88.235
Vitamin A Acetate 500000 UI ACEF	0.400	Vitamin A	15.00%	20%	3.200
Microcrystalline Cellulose PH102			100.00%	0%	0.000
Glycerol Dibeenate Nourishes	150.000		100.00%	0%	150.000
Magnesium Stearate	1.500		100.00%	0%	1.50
Anhydrous Colloidal Silica	1.500		100.00%	0%	1.50
	Coat				
Surelease E-7-19040 Clear (25% secco)	119.000		100.00%	0%	119.00
NS-Enteric 29Z19241 Clear	21.000		100.00%	0%	21.00
Water	1540.000		100.00%		1540.00
	Total				
	323.400				641.58

4.2.2.2 The development of a "marker Tablet" strategy

Before starting the Tablet formulation process, a comprehensive assessment of the available quantities of UAE extracts derived from conventional peach, apricot, and apple by-products was led to ensure the production of Tablets containing a target dosage of 40 mg polyphenols per Tablet.

To produce a batch size of approximately 2000 Tablets, estimated to be suitable for processing on *Valpharma S.p.A*'s pilot-scale equipment, an estimated 400 g of total dry UAE extracts from the above-mentioned fruit by-products was required. However, insufficient quantities of these extracts were available.

To overcome this limitation, a "marker Tablet" strategy was employed. This approach involved formulating a Tablet that imitated the original formulation using a substitute active ingredient with comparable physicochemical properties. A mixture of plant extracts, including kudzu, lifenol®, and red clover (see *Figure* 67), available in Valpharma S.p.A, containing various isoflavones was selected as the marker active ingredient. Specifically, the red clover (Trifolium pratense L.), employing a dry flower extract containing formononetin, daidzein, biochanin A, and genistein. Kudzu (Pueraria lobata (Willd.) Ohwi.) was included as a dry root extract standardized to contain isoflavones expressed as the sum of puerarin, daidzein, and daidzin. Lastly, lifenol®, derived from Humulus lupulus L., was included as a standardized extract containing 8-prenylnaringenin[145]. This selection allowed the rapid replication of the original Tablet formulation (see *Table* 29) and facilitated subsequent analytical procedures, such as Tablet titre determination and in vitro release studies.

Figure 67 Active ingredients

Table 31 shows the final formulation of the Tablet which is also enriched with Vitamin E compared to the two previous projects (see *Table* 29 and *Table* 30). Indeed, the combined presence of vitamin E and vitamin A is frequently associated with product claims (HR ID 160, 162, 1947 for Vitamin E and HR ID 206 for Vitamin A) emphasizing antioxidant properties, skin health, eye health, and anti-aging benefits. This synergistic combination is particularly beneficial due to their complementary roles in cellular defense. Both vitamins function as potent antioxidants, protecting cells from oxidative damage by free radicals, which are implicated in cellular aging and the pathogenesis of many chronic diseases[146],[147].

The *Table* 31 also includes the comparison between the original Tablet formulation containing UAE extracts from fruit by-products (Ac-Conv-ByP, Pch-Conv-ByP, and Apl-Conv-ByP) and the "marker Tablet". To

achieve a target polyphenol dosage of 40 mg per Tablet, a total of 483.489 mg of the combined dried UAE fruit by-product extracts was required.

To maintain the target polyphenol dosage while following the established dosages for the "marker Tablet" active ingredients (kudzu, lifenol®, and red clover extract), a total of 285 mg of the marker extract mixture was incorporated into the "marker Tablet" formulation. Thus, to maintain the overall Tablet weight of 926.416 mg, 200 mg of microcrystalline cellulose was added to the "marker Tablet" to compensate for the weight difference between the fruit by-product UAE extracts and the marker extract mixture.

It is important to note that the only difference between the original tablet and the "marker Tablet" formulations is the substitution of the fruit by-product UAE extracts with the marker extract mixture. All subsequent analytical procedures, including Tablet titre and release assessments, were performed on the "marker Tablet" to evaluate the relevant parameters.

Figure 68 and *Figure* 69 illustrates the “marker Tablet”, before and after the application of the enteric coating.

Table 31 Comparison between the original Tablet *and the “marker* Tablet*” formulation*

Ingredients	**Composition based on a theory developed using fruit by-products**					**Marker-Tablet**	
	Composition	**Purity**	**Surdose**	**Final composition**	***Percetage***	**Final composition**	***Percetage***
	mg/dose	**%**	**%**	**mg/dose**	***%***	**mg/dose**	***%***
Ac-Conv-ByP	7.500	5.50%	20%	163.636	*17.66*	-	-
Pch-Conv-ByP	25.000	16.00%	20%	187.500	*20.24*	-	-
Apl-Conv-ByP	7.500	6.80%	20%	132.353	*14.29*	-	-
Vitamin A Acetate 500000 UI ACEF (Retinyl acetate)	0.400	15.00%	20%	3.200	*0.35*	3.200	*0.35*
Natural Vitamin E Acetate (5 DL-alpha-Tocopherol acetate)	30.000	46.92%	20%	76.726	*8.28*	76.726	*8.28*
Microcrystalline Cellulose PH102	110.000	100.00%	0%	110.000	*11.87*	308.489	*33.30*
HPMC K4 MCR (5 (Hydroxypropyl)methyl cellulose)	200.000	100.00%	0%	200.000	*21.59*	200.000	*21.59*
Lifenol® (IMCD) (Humulus lupulus L)	-	100.00%	0%	-	-	85.000	*9.18*
Red Clover e.s. 40% isoflavon (Trifolium pratense L.)	-	100.00%	0%	-	-	100.000	*10.79*
Kudzu e.s. 40% isoflavon (Pueraria lobata (Willd.) Ohwi.)	-	100.00%	0%	-	-	100.000	*10.79*
Magnesium Stearate ($Mg(C_{18}H_{35}O_2)_2$)	1.500	100.00%	0%	1.500	*0.16*	1.500	*0.16*
Silica Colloidal Anhydrous (Silica gel Inorganic Sorbent)	1.500	100.00%	0%	1.500	*0.16*	1.500	*0.16*
Total	-	-	-	***876.416***	-	***876.416***	-
Coat							
Surelease E-7-19040 Clear (25% solid)	42.5	100.00%	0%	42.500	*4.59*	42.500	*4.59*

Water Surelease E-7-19040 Clear	*127.5*	*100.00%*	*0%*	*127.500*	-	*127.500*	-
NS-Enteric 29Z19241 Clear	7.5	100.00%	0%	7.500	*0.81*	7.500	*0.81*
Water	*322.5*	*100.00%*	*0%*	*322.500*	-	*322.500*	-
Total	-	-	-	***926.416***	*100.000*	***926.416***	*100.000*

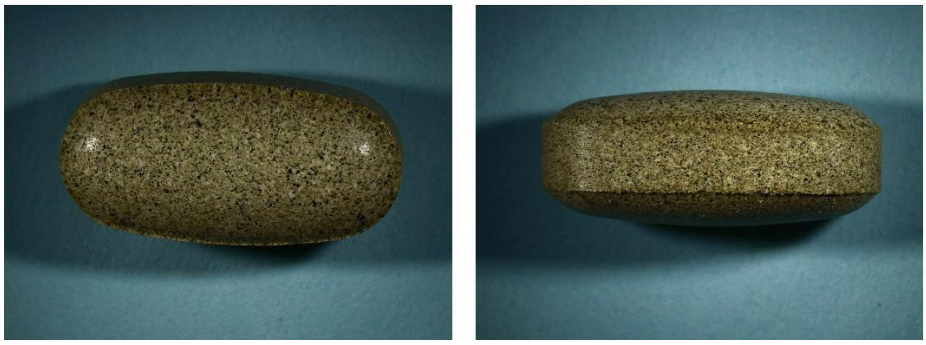


Figure 68 Uncoated Tablet

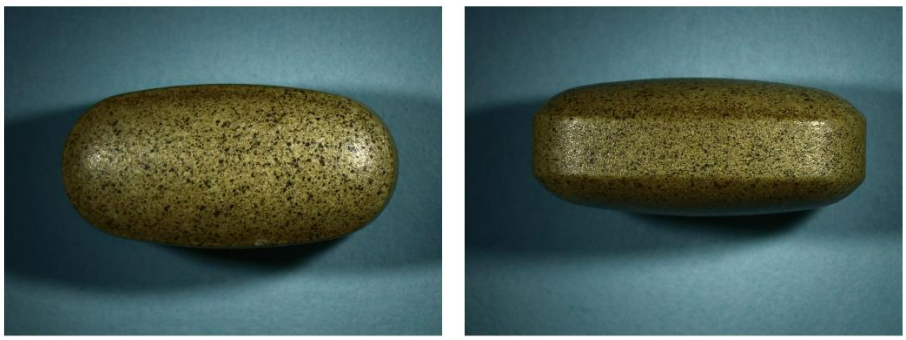


Figure 69 Coated Tablet

4.2.3 Development of spectrophotometric methods for titer and dissolution determination

All analytical methods for determining Tablet titer and in vitro dissolution were performed in *Valpharma S.p.A.*

4.2.3.1 Development of spectrophotometric titer

Before the titer determination, the solubility of each individual component (kudzu, lifenol®, and red clover extract, *Figure* 67) was assessed. Stock solutions (0.25 mg/mL) were prepared by dissolving 10 mg of each vegetal matrix in 25 mL of ethanol. Solubility analysis revealed that lifenol® exhibited complete solubility in ethanol, while red clover and kudzu exhibited partial solubility.

Subsequently, the stock solutions were diluted 1:10, centrifuged, and their absorption spectra were recorded. The wavelength of maximum absorbance was determined to be 250 nm.

To establish a reference standard for titer determination, a mixture of kudzu, lifenol®, and red clover powders was prepared, reflecting the component ratios in the Tablet formulation. This standard mixture was analyzed

in triplicate in 100% methanol and dilution in water, with absorbance readings recorded at 250 nm. The obtained absorbance values were used to calculate the E1% value for the mixture, which was used as the basis for determining the average Tablet titer.

4.2.3.2 Determination of the titre of active ingredients in solid formulation

The titer analysis was performed on the Tablet without the enteric coating. The experimentally determined average titer was found to be 304.1 mg/dose, with a relative standard deviation (RSD) of 0.6%. This value is 6.8% higher than the nominal titer of 285 mg/dose. However, this difference falls within the acceptable tolerance limit of ±10% for this specific product.

4.2.3.3 Development of in Vitro Dissolution Test

Initial solubility studies of the marker Tablet in various media revealed inadequate dissolution profiles. To enhance solubility, the influence of sodium lauryl sulfate (SLS) as a surfactant was investigated. Dissolution studies were conducted in unbuffered water containing 0.20% and 0.75% SLS, respectively. Based on these findings, a 0.75% SLS solution in unbuffered water was selected as the optimal dissolution medium for subsequent in vitro studies.

4.2.3.4 In Vitro Dissolution Test of delayed-release Tablet

Before the coating, preliminary dissolution studies were performed on the uncoated "marker Tablet" (*Figure* 68) in two media separately: the first test only in 0.1 M HCl to assess gastro-resistance and the second test only 0.75% SLS solution to simulate intestinal conditions. These studies provided a baseline for subsequent dissolution studies on the coated Tablets (*Figure* 69) in the same media, enabling a direct comparison of the impact of the enteric coating on drug release.

The *Table* 32 shows release obtained in the 0.1 M HCl medium of the uncoated Tablet which exhibits significant drug release, indicating a lack of gastro-resistance. This observation underlines the critical importance of the enteric coating in achieving the desired controlled and gastro-resistant release profile. While *Table* 33 shows the dissolution profile of the uncoated Tablet in 0.75% SLS.

Table 32 Dissolution of uncoated Tablet in HCl 0.1M

Time point HCL 0.1M	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Mean	DSR
2h	2.8	3.1	2.9	3.1	3.4	3.4	3.1	8.3

Table 33 Dissolution of uncoated Tablet in SLS 0.75%

Time point SLS 0.75%	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Mean	DSR
2h	10.7	10.6	13.5	10.7	11.1	13.2	11.6	11.7
4h	20.7	20.1	23.6	20.2	21.4	23.7	21.6	7.7

6h	30.4	29.2	33.1	29.4	31.3	34.0	31.2	6.3
12h	57.3	52.7	57.8	53.6	57.4	62.2	56.8	6.0
18h	80.8	74.6	79.4	75.8	81.6	82.0	79.0	3.9

Following observation of the dissolution profile of the uncoated Tablet, the coated Tablet was subjected to a comprehensive dissolution test (*Table* 34). The dissolution test consisted of two consequential stages: an initial 2-hour dissolution in 0.1 M HCl to simulate gastric conditions, followed by an 18-hour dissolution in 0.75% SLS solution to simulate intestinal conditions.

The dissolution results reported in *Table* 34 demonstrated that the enteric coating effectively prevented drug release in the acidic 0.1 M HCl medium, confirming its gastro-resistant properties.

However, the observed drug release in the 0.75% SLS medium (57% at 18 hours, *Table* 34) was lower than that observed for the uncoated Tablet under the same conditions (79% at 18 hours, *Table* 33).

Table 34 Dissolution test of the coated Tablet

Time point HCL 0.1M	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Mean	DSR
2h	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Time point SLS 0.75%	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Mean	DSR
2h	1.8	2.1	2.1	2.0	2.1	1.5	1.9	12.5
4h	6.5	7.4	7.6	6.9	7.2	5.0	6.8	14.0
6h	11.7	14.1	14.4	13.5	13.4	8.6	12.6	17.3
12h	31.6	36.8	37.8	38.3	34.1	21.2	33.3	19.3
18h	57.3	63.5	62.9	64.9	57.1	37.0	57.1	18.2

This discrepancy in release profiles could be attributed to several factors, including excessive compression force resulting in Tablet hardness, or an excessively robust coating that deters drug release. Indeed, as illustrated in the *Figure* 70, the Tablet's coating appears to be divided into two halves while remaining intact, which physically obstructs the active ingredient from dissolving.

Figure 70 Coated Tablet *dissolution test (3° time point)*

To address these potential issues, further investigations are necessary to optimize Tablet formulation parameters, such as compression force, and to refine the enteric coating composition to achieve the desired controlled-release profile. Following these optimization studies, the formulation of the original Tablet

containing UAE extracts of fruit by-products can be resumed, using the knowledge gained from the “marker Tablet” studies.

4.3 CONCLUSIONS

In conclusion, this research carried out in *Valpharma S.p.A* successfully demonstrated the possibility of developing a dietary supplement from fruit by-product UAE extracts. The purification and concentration of fruit by-product UAE extracts effectively removed undesirable components, as the initial UAE extracts, while rich in yield, exhibited low Total Phenolic Content (TPC), indicating the presence of non-polyphenolic compounds. This was confirmed by the Gravimetric analysis, which demonstrated a reduction in the overall dry mass of the treated UAE extracts. The TPC analysis revealed a significant increase in the TPC of the treated UAE extracts compared to the untreated UAE ones. Furthermore, the HPLC-DAD analysis confirmed the higher concentration of polyphenols in treated UAE extracts across the different fruit by-products (apricots, peaches, and apples). These results supported the development of a dietary supplement using the purified fruit by-product UAE extracts (Ac-Conv-ByP, Pch-Conv-ByP, and Apl-Conv-ByP). Therefore, a "marker Tablet" strategy was implemented using a substitute active ingredient (a mixture of plant extracts) to optimize the formulation and assess Tablet properties. Analytical methods, such as spectrophotometric titer determination and in vitro dissolution tests, were developed and validated to achieve the desired controlled-release and gastro-resistant properties. These efforts yielded several key benefits: enhanced extract quality due to the purification process significantly increased the concentration of polyphenols, improving their efficacy; improved patient compliance was achieved through the optimized Tablet formulation, resulting in a smaller and more convenient size; and targeted delivery was ensured by the gastro-resistant coating, maximizing the prebiotic potential of polyphenols by releasing them in the intestine and promoting gut health. Furthermore, the "marker Tablet" strategy provides a valuable framework for future formulations using fruit by-product UAE extracts as active ingredients.

5 GENERAL CONCLUSIONS

5.1 FINAL CONSIDERATIONS AND FUTURE RESEARCH

This PhD project successfully achieved its purposes by comprehensively investigating the potential of agri-food by-products from *Fruttagel*® as sustainable sources of bioactive compounds. The scientific research focused on fruit (apricots, peaches, apples, tomato) and legumes (beans, green beans, peas, soy) by-products generated during juice production, tomato puree processing, and frozen legume manufacturing and soy milk production and their respective final product intended for trade.

The first step in the research was a thorough on-site analysis of *Fruttagel*®'s production chains provided a detailed understanding of the physical characteristics of each by-product.

The second step of this study included the development of an Ultrasound-Assisted Extraction (UAE) method, classified among the latest generation, for the high-yield extraction of polyphenols from these by-products. Then, the chemical analysis started with a fast and functional screening to determine the total content of polyphenols in the UAE extracts by using the Total Phenolic Content (TPC) UV-vis spectrophotometric assay. The chemical analysis proceeded with the development and optimization of two sophisticated chromatographic methods for comprehensive profiling of polyphenols in all UAE extracts. The HPLC-DAD chromatographic analysis revealed that fruit by-products, including apple, apricot, and peach, were abundant sources of different polyphenols, in some case exceeding the levels found in the corresponding final products. The UHPLC-DAD-ESI-MS^n chromatographic analysis, entirely developed at the *Department of Pharmacognosy at the University of Graz (Austria)*, demonstrated that legume by-products exhibited high polyphenol content.

The chemical analyses performed, as described above, were key for satisfying a primary objective of this project: to demonstrate the polyphenol-rich nature of the by-products, with contents often exceeding or equaling those of the corresponding final products which makes them worthy of being valorized. Furthermore, these analyses revealed the unique polyphenol profiles of the different by-products, suggesting their potential for specific applications within the nutraceutical sector.

These analytical methods were also applied in assessing the stability of polyphenols within the UAE extracts and in the original starting vegetal powders. The stability studies demonstrated that polyphenols within the UAE extracts and the vegetal powders maintained their integrity for at least 15 months when stored at -20°C.

Furthermore, collaboration with *Fruttagel*® allowed a detailed examination of individual pesticides within the UAE extracts, specifically focusing on fruit by-products selected for Tablet formulation. The results of this investigation were highly encouraging: pesticide levels were found to be low or even non-detectable, consistently remaining below legal limits.

Regarding functional test, the Total Antioxidant Status (TAS) assay proved to be a highly efficient and rapid method. This UV-vis spectrophotometric and colorimetric assay allowed the rapid determination of the

antioxidant activity of all UAE extracts, facilitating comparisons with the relative final products. This assay highlighted the significant valorization of fruit and legume by-products.

Furthermore, Kjeldahl protein analysis revealed that many by-products analyzed in the project exhibited high protein content, often comparable to or exceeding that of the corresponding final products, with soy by-product (okara) demonstrating particularly high levels. These results underline the potential of agri-food by-products as valuable sources not only of polyphenols but also of high-quality proteins. This is particularly significant given the increasing demand for plant-based protein sources within the nutraceutical and food industries. Proteins derived from food waste hold considerable promise for varied applications, including nutraceutical supplements for athletes to support muscle repair and growth, and in the cosmetic sector for the development of innovative skincare formulations.

A significant achievement of this research was the successful demonstration of the possibility of developing a food supplement using UAE extracts from fruit by-products, thus reaching a key project objective. This final project step was conducted in collaboration with the pharmaceutical company *Valpharma S.p.A*. Purification and concentration steps effectively removed undesirable components, resulting in a substantial increase in the Total Phenolic Content of the fruit by-product UAE extracts. A “marker Tablet” strategy, employing a substitute active ingredient, was effectively realized to optimize the formulation and assess critical Tablet properties, including controlled release and gastro-resistance.

Future research directions will include the investigation and application of new green extraction techniques, such as Microwave-Assisted Extraction (MAE) coupled with Deep Eutectic Solvents, to further enhance the circular economy approach. Subsequent research will focus on completing the quantitative analysis of polyphenols in legume UAE extracts to validate the results obtained from semi-quantitative LC-MS analysis. A crucial next step will involve the incorporation of UAE by-product extracts as active ingredients in Tablet formulations for commercialization as dietary supplements. This transition is possible given the successful development of the Tablet formulation in this study. Additionally, future perspectives include the in vitro dissolution studies to comprehensively characterize the release profile and gastro-resistance of the developed formulations.

In conclusion, these obtained results provide the basis for the development of innovative and sustainable nutraceutical products that effectively exploit the bioactive compounds present in agri-food by-products, thereby promoting the principles of the circular economy.

ABBREVIATION

ABTS 2,2'-Azino-Bis(3-Ethylbenzothiazoline-6-Sulfonic Acid) Diammonium Salt
AOAC Association of Official Agricultural Chemists International
ATR-FTIR Attenuated Total Reflectance Infrared Spectroscopy
ByP By-Products
CEAP Circular Economy Action Plan
DES Deep Eutectic Solvents
DPPH 1,1-Diphenyl-2-Picrylhydrazyl
DW Dry weight
EAE Enzyme-Assisted Extraction
EU European Union
Ext Dry extract
FAO Food and Agriculture Organization
FinalP Final Products
FRAP Ferric Reducing Antioxidant Power
GAE Gallic Acid Equivalent
GC-MS Gas Chromatography-Mass Spectrometry
HPLC-DAD High-Performance Liquid Chromatography–Diode Array Detector
LI Lotta Integrata
LoD Limit Of Detection
LoQ Limit Of Quantification
MAE Microwave-Assisted Extraction
NCHA Neochlorogenic Acid
ORAC Oxygen Radical Absorbance Capacity
PEF Pulsed Electric Fields
pI Isoelectric Point
PLE Pressurized Liquid Extraction
RM Raw Material
RT Retention Time
SD Standard Deviation
SFE Supercritical Fluid Extraction
SPC Sum of the individual phenolic compounds
SS Stock Solutions
TAS Total Antioxidant Status
TE Trolox Equivalent

TEAC Trolox Equivalent Antioxidant Capacity

TPC Total Phenolic Content

TPCC Tetrakis(4-chlorophenyl)carbohydrate

UAE Ultrasound-Assisted Extraction

UHPLC-DADESI-MS^n Ultra High-Performance Liquid Chromatography-Diode Array Detector-Electrospray Ionization-Mass Spectrometry LC-MS Liquid Chromatography-Mass Spectrometry

UN United Nations

UNEP United Nations Environment Programme

UNI Ente di Normazione Italiano

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