UNIVERSITAT ROVIRA i VIRGILI

EXTRACTION AND CHARACTERIZATION OF (POLY)PHENOL-RICH EXTRACTS FROM FRUITS

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FINAL DEGREE PROJECT

Biotechnology

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Jo, Jorge Martínez García, amb DNI 53791548E, sóc coneixedor de la guía de prevenció de plagi de la URV *Prevenció, detecció i tractament del plagi en la docencia: guía per a estudiants* (aprobada el juliol 2017) (https://www.crai.urv.cat/ca/serveis/suport-aprenentatge/plagi/) i afirmo que aquet TFG no constitueix cap de les conductes considerades com a plagi per la URV.

París, 30 de maig de 2023

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1. CENTER INFORMATION

The Nutrigenomics Research Group of the Universitat Rovira i Virgili aims to generate general knowledge oriented to the design of functional foods able to prevent, delay or alleviate metabolic diseases such as obesity, diabetes, hypertension, and metabolic syndrome. These studies also allow to increase the value of by-products from the food industry by the obtaining of functional ingredients.

The research group has several research lines that are supported by grants from national and international public and private entities. The group always has at least a competitive national project or international.

The main research lines that we currently follow are:

- Search of dietary bioactive compounds with beneficial effects against pathologies related with metabolic syndrome, obesity, diabetes mellitus and hypertension.
- Development of a functional multi-ingredient mix able to simultaneously treat different disorders.
- Generation and purification of bioactive peptides from different protein sources such as food by-products.
- Evaluation of the impact of biological rhythms on the effectivity of bioactive compounds.

2. ABSTRACT

Fruits' beneficial effects have been attributed to their nutritional profile, including (poly)phenols. (Poly)phenols are secondary metabolites of plants that reduce the risk of chronic human diseases by protecting cell constituents against oxidative damage acting as antioxidants. The main dietary polyphenols are flavonoids, two thirds of the total consumed, more precisely, flavanols and anthocyanins.

The bioavailability of these compounds is usually low and differs between some internal host factors but also in the food matrix they are included in. Because of this, (poly)phenol extracts have been gaining popularity, as their concentration is higher, and the matrix effect is reduced.

In this study, (poly)phenol-rich extracts were obtained when using optimized maceration protocols from different fruits (apricot, cherry, pomegranate, grape, persimmon, orange, strawberry and plum). Moreover, macro- and micronutrients were characterized, being able to reduce the so-called matrix effect, increasing their bioavailability when comparing it to consuming the whole fruits. However, a more detailed (poly)phenol characterisation and an *in vivo* study should be made to see the health benefits these extracts give.

KEY WORDS: (poly)phenols, fruit, extraction methods, bioavailability.

3. BACKGROUND

Fruit consumption awareness has been rising all over the world and each time, there are more studies to prove its beneficial effects in health (Ballesteros et al. 2022; Ang et al. 2019; Stangierska et al. 2022; Nyanchoka et al. 2022). In Spain, 2-3 daily portions of fruit are recommended, prioritising fresh and seasonal fruit. This is the equivalence to around 120g of a medium fruit ('Food-Based Dietary Guidelines in Europe - Table 4 | Knowledge for Policy' n.d.). However, most of the population is below this recommended daily servings.

Fruit is known to reduce the risk of many chronic diseases, including cardiovascular diseases, type 2 diabetes, some cancers, and obesity. All these benefits are added to their excellent nutritional profile (Bhaswant et al. 2023). Fruit-derived food components include water, fibres, proteins, fats, digestible carbohydrates, minerals and vitamins, as well as various additional bioactive phytochemicals that prevent diseases such as phytosterols or antioxidants (Vincente et al. 2014).

Antioxidants are present in all plant organs and include compounds such as ascorbic acid, carotenoids, tocopherols, and phenolic compounds. These compounds are one of the three levels of the antioxidant defence system, protecting cells from free oxygen atoms and ensuring their physiological levels (Mucha et al. 2021).

3.1. Phenolic compounds

Phenolic compounds are secondary metabolites of plants that play a vital role in growth and reproduction, providing protection against pathogens and predators, and contributing to colour characteristics of vegetables and fruits. They have a structure based on an aromatic ring containing one or more hydroxyl groups, occurring as conjugates with mono- or polysaccharides and one or more phenolic groups (**Figure 1**). Over 10,000 compounds have been identified, classified into different groups based on the number of phenolic rings and the structural elements that bind them (Vuolo, Lima, and Maróstica Junior 2019; Pandey and Rizvi 2009). The most important classes found in human diets are flavonoids, phenolic acids, and tannins.

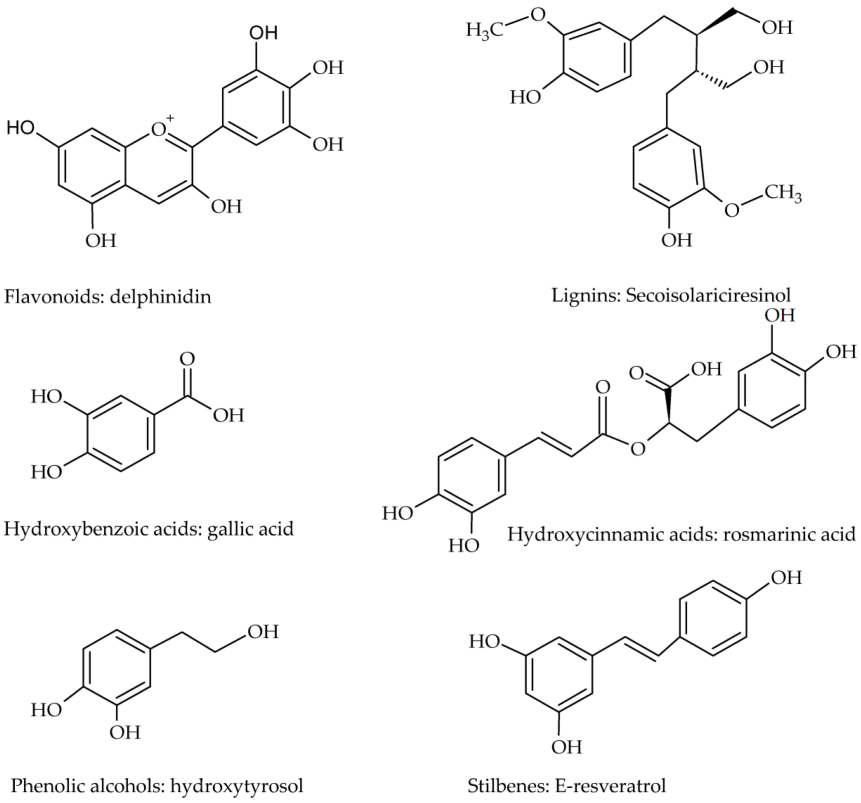

Figure 1. Basic structure of phenolic compounds. *Represented with an example of each (Bié et al. 2023).*

(Poly)phenols have shown to modulate signalling pathways, reducing the risk of degenerative diseases such as Alzheimer's and cardiovascular disease (CVD) by anti-inflammatory properties. Inflammation is associated with the development of obesity and the onset of age-related diseases such as CVD and type 2 diabetes mellitus. The consumption of a diet rich in (poly)phenols may reduce the risk of chronic human diseases by protecting cell constituents against oxidative damage acting as antioxidants (Pandey and Rizvi 2009; Joseph, Edirisinghe, and Burton-Freeman 2015). These beneficial effects are associated to the consumption of flavonoids, which consist of two thirds of the total (poly)phenol intake. Flavonoids can be classified into six different subclasses (**Figure 2**): flavonols, flavones, isoflavones, flavanones, anthocyanins and flavanols; being the last two the most important ones in terms of beneficial effects (Bié et al. 2023).

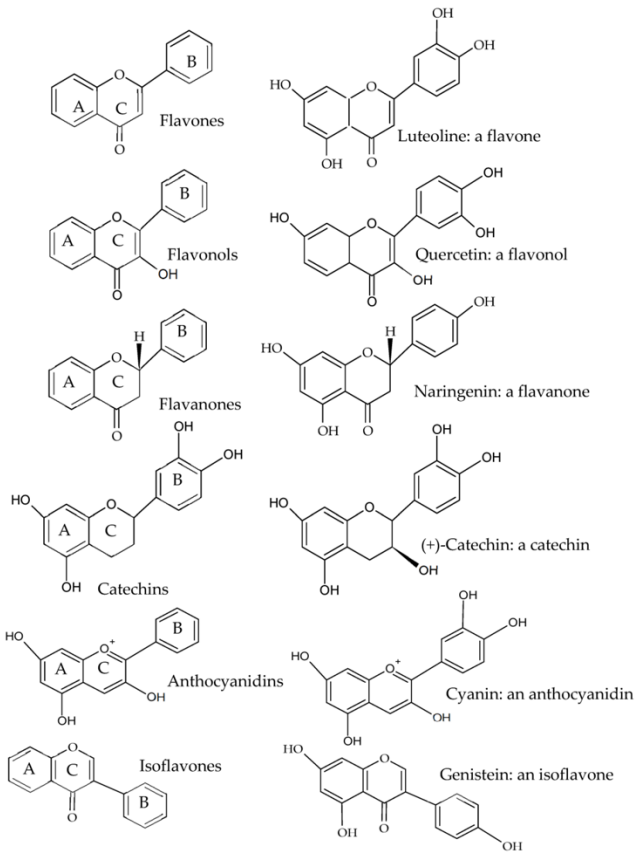

Figure 2. Subclasses of flavonoids. *Basic structure and a representative example (Bié et al. 2023).*

3.1.1. Anthocyanins

Anthocyanins are pigments soluble in water that give the tints of red, violet and blue in fruits. Red fruits and red wine are some of the important sources of these pigments in our diet. Moreover, they are one of the phenolic compounds present in a higher concentration in foods (Di Lorenzo et al. 2021).

Anthocyanins have various properties such as antioxidant, anti-inflammatory and anti-tumour, as well as other activities as protection of vision, anti-diabetic effects and inhibition of lipid peroxidation. Their antioxidant activities include removing free radicals, reducing oxidase activity or reducing cholesterol levels by inhibiting its absorption, or reducing plasma levels of low-density lipoproteins (LDLs), protecting the cardiovascular system (Wallace, Slavin, and Frankenfeld 2016). Anthocyanins can reduce intestinal permeability and change the metabolism of intestinal bacteria to prevent inflammation. They also have anti-diabetic effects as the reduction of blood glucose. Finally, some of these compounds can regulate tumour suppressor genes, such as p53, increasing apoptosis of cancer cells and repairing the integrity of genomic DNA. However, the diversity of the structures

of their different forms and derivatives makes their effects different (Xue et al. 2023).

3.1.2. Flavanols

Flavanols can be classified depending on their structure in flavan-3-ols, flavan4-ols, isoflavan-4-ols and flavan-3,4-ols, being flavan-3-ols the most common ones (Luo et al. 2022). Cocoa, tea and apple are rich in flavanol compounds, but berries, in particular strawberry, and red wine also contain a high level of catechins and proanthocyanidin dimers (Di Lorenzo et al. 2021).

The principal benefits of flavanols studied have been associated with antioxidant, anti-inflammatory, anti-cancer, anti-viral and protective cardiovascular properties (Luo et al. 2022). Flavan-3-ols show three main mechanisms of antioxidant activity: free radical scavenging, chelation of transition metals and inhibition of enzymes (Cos et al. 2004). Epigallocatechin-3-gallate, a flavanol monomer, has shown strong effects on the induction of apoptosis and regulation of cell cycle by formatting internucleosomal DNA fragments. Some flavanols can inhibit viral infections of viruses as hepatitis C virus, or HIV (human immunodeficiency virus), and they also showed good docking scores with the possible targets of COVID-19 (Mhatre, Naik, and Patravale 2021). Lastly, some monomers, as catechin and epicatechin, are able to inhibit angiotensin I-converting enzyme activity which is linked to cardiovascular and cerebrovascular diseases. Moreover, they can inhibit LDL oxidation, resulting in a cardioprotective property (Luo et al. 2022).

3.2. (Poly)phenol bioavailability

The health implications linked to (poly)phenols, depend on the bioavailability of their metabolite's derivatives (Frolinger et al. 2019). This is defined as how (poly)phenols are released from the food matrix, metabolised, absorbed and carry out its bioactivity on the target cells or tissues. This depends on the food content, its matrix and processing but also differs in everyone from internal host factors as gut microbiota composition, genetic polymorphisms, sex, age, and the individual's health status (Arfaoui 2021).

Most (poly)phenols are released from the food matrix during the gastro-intestinal digestion similarly to the other food components which can affect the breakdown of (poly)phenols. Apart from interfering with digestion, fruit constituents can

modulate (poly)phenol absorption, metabolism, and bloodstream transport in various ways (Bohn 2014; Čepo et al. 2020).

3.2.1. Proteins and nitrogen compounds

Protein represents less than 1% of the fresh mass of most fruit and contribute a 1% of protein daily intake (Wu 2016).

(Poly)phenols can form complexes with brush-border membrane proteins or plasma proteins via hydrogen bonds with their hydroxyl groups reducing their absorption. Moreover, the formation of longer peptide chains in fruits is associated with an increased fat content that results in more of these (poly)phenol complexes being formed. Therefore, it appears that proteins affect negatively to (poly)phenol bioavailability, however, additional effects of matrix components and the different types of protein and phenolic compounds involved cannot be excluded (Bohn 2014).

3.2.2. Lipids

The fat concentration in fruit varies with the species but most of them have less than a 1% of lipids (Vincente et al. 2014).

The physical and chemical properties of lipids are defined by their constituent fatty acids that are usually aliphatic or monocarboxylic. Thanks to this, dietary lipids increase transit time altering the absorption rate of (poly)phenols. However, there are not many studies investigating this effect in (poly)phenol assimilation. In the single human study, it was found that the AUC of an aglycon supplement in plasma was higher when consuming a fat-rich meal (Arfaoui 2021; Bohn 2014).

3.2.3. Carbohydrates

After water, carbohydrates are the most abundant constituents in fruits, reaching up to 80% of dry weight. Carbohydrates can be classified as glycaemic carbohydrates (digested and absorbed in the small intestine) and non-glycaemic carbohydrates (dietary fibre) (Englyst and Englyst 2005) .

There is few evidence that look for the impact of carbohydrates on (poly)phenol absorption, however it appears that the addition of carbohydrates enhances the uptake of (poly)phenol glucosides. A proposed mechanism is the activation of

transporters as SGLT1 that are involved in the absorption of these compounds (Bohn 2014).

Dietary fibres include very diverse macromolecules exhibiting a variety of physical-chemical properties. The main components are cellulose, cross-linking glycans, pectins, lignin, resistant starch, and non-digestible oligosaccharides. Fruits average 1-3% fibre on a fresh weight basis. Fibre consumption can modulate the intestinal function but also has been inversely related to obesity, type two diabetes, cancer, and cardiovascular disease (CVD) (Lattimer and Haub 2010).

Many (poly)phenols are associated with dietary fibres, forming fibre-(poly)phenol complexes that may not be available in the small intestine but may be released into the colon by microbiota fermentation. Fermentable fibres act as prebiotics, enhancing this fermentation and thus, changing the (poly)phenol profile. However, free of (poly)phenol-fibre can have a negative effect on (poly)phenol absorption due to an entrapment with hydrophobic interactions. Both soluble and insoluble dietary fibres increase the intestinal bulk, but they increase and reduce, respectively, transit time. A larger bulk with less transit time results in a loss of metabolites obtained at a colonic level and in an alteration in the absorption rate in the small intestine (Bohn 2014).

3.3. (Poly)phenol extraction methods

Due to the importance of (poly)phenols in health and their important role in the pharmaceutical and nutraceutical sector, the interest on (poly)phenol-rich extracts usage has been growing to increase their bioavailability and to ensure their beneficial activities (Kiyimba et al. 2023).

Several extraction techniques have been used to increase the (poly)phenol recovery yield from different plant foods, being commonly classified as simple methods and advanced methods (**Figure 3**). Simple methods usually are more time and energy consuming when compared to advanced extraction techniques but generally they are easier to use and the sample sizes that can be used are considerably larger (Sridhar et al. 2021). Although the quality of the resulting extract depends on the technique used and the optimization of the main factors that affect the extraction procedure (temperature, solvent, or time), generally the

more modern techniques give a higher extraction yield and a higher selectivity of (poly)phenol (Anand et al. 2005; Chuo et al. 2020).

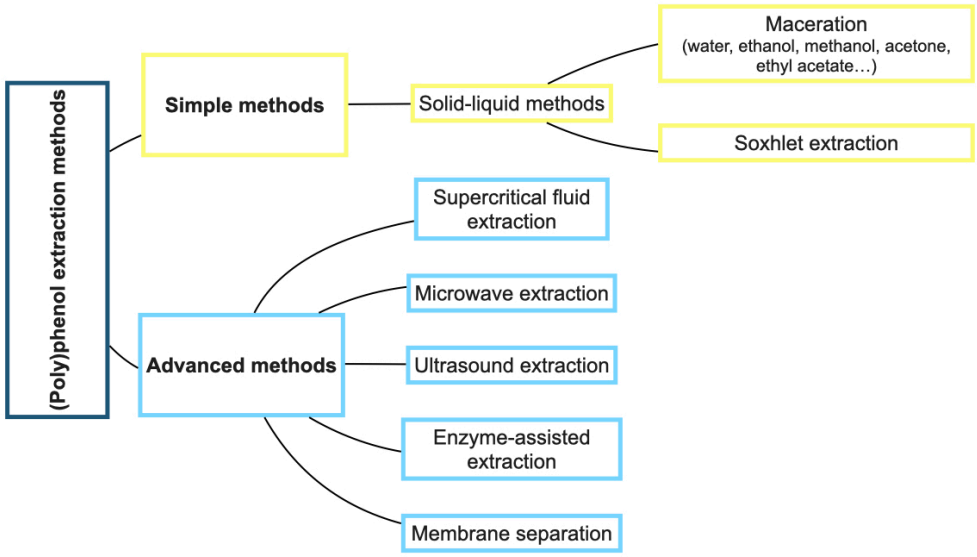

***Figure 3. (Poly)phenol extraction methods**. Adapted from (Jauhar et al. 2018).*

3.3.1. Advanced extraction techniques

Advanced extraction technologies include ultrasound, microwaves, supercritical fluid, enzyme-assisted extraction, or membrane separation (Cai et al. 2021). Ultrasound-assisted extraction is based on the disruption of cells by the acoustic cavitation effect, resulting in a quick, simple, and efficient extraction. Microwave-assisted extraction transfers heat to the whole system through the solvent to extract aqueous samples reducing extraction time and solvent volumes. Supercritical fluid extraction uses supercritical fluids as carbon dioxide that is easy to remove but the phase equilibrium, pressure and temperature need to be precisely considered to increase the yield and selectivity (Ahmad et al. 2022). In enzyme-assisted extractions, an enzyme is used as a catalyst to help soften the cell wall and release the bioactive compounds (Nadar, Rao, and Rathod 2018). Membrane separation includes techniques as micro-, ultra- and nanofiltration that can be coupled to membrane systems to enhance (poly)phenol recovery (Castro-Muñoz et al. 2021).

3.3.2. Simple extraction methods

At a laboratory level, conventional extraction techniques are generally used due to their low cost and simplicity. The most used techniques are solid-liquid

extractions (SLE). These techniques result in crude extracts that are a pool of different compounds soluble in the solvent used, that is typically alcohols (methanol, ethanol), acetone, diethyl ether or ethyl acetate mixed with different proportions of water (Oluwaseun Ruth Alara, Abdurahman, and Ukaegbu 2021; Brglez Mojzer et al. 2016). Choosing the solvent is a critical step on the design as it needs to provide extraction capacity, selectivity, and a high solute mass transfer rate. Methanol has been found to give higher yields in lower molecular weight (poly)phenols, while aqueous acetone extracts more of the higher molecular weight ones. However, choosing a GRAS solvent, as ethanol (EtOH), is key if the resulting extracts are thought to be consumed (Silva, García, and Ottens 2018).

SLE includes different methods as maceration, distillation, Soxhlet or alcohol extraction among others and their extraction capacity depends on factors as the extraction solvent, its respective concentration, time, and temperature (Liberal et al. 2022). The most used ones are Soxhlet and maceration.

Soxhlet extraction

Soxhlet extraction is a well-established technique where the sample is placed in a thimble-holder that is gradually filled with condensated fresh solvent from a distillation flask. Then, a siphon aspirates the solute of the thimble-holder, unloading it to the distillation flask carrying the extracted (poly)phenols into the bulk liquid (Luque de Castro and García-Ayuso 1998; Oluwaseun R. Alara, Abdurahman, and Ukaegbu 2018). However, the duration of the extraction can be up to 50h, there is a lot of energy loss by heat and there is a reasonably loss of flavonoids.

Maceration

Maceration is typically the preferred traditional extraction method as its simple, cheap, requires less material and is also environmentally friendly.

The samples are placed in a bottle with the chosen solvent and then stirred at a constant temperature. The most important factors are the speed of agitation and the total duration as they increase the mass transfer rate (Abubakar and Haque 2020; Sridhar et al. 2021).

3.3.3. Optimizing simple extraction methods

Due to the big variety of (poly)phenol structures and polarity, it is difficult to develop a single method to optimally extract all this compounds from different fruits (Kelly, Kelly, and O'Mahony 2019). For this reason, several studies have tried to optimize the different maceration protocols to increase the quality of the resulting extracts. In these, different variables as the time of extraction, solvent/water ratio, temperature and stirring speed are set to increase the total (poly)phenol profile in the resulting extract (Iglesias-Carres, Mas-Capdevila, Bravo, Mulero, et al. 2019; Iglesias-Carres et al. 2018; Iglesias-Carres, Mas-Capdevila, Bravo, Bladé, et al. 2019; Borges et al. 2011; Iglesias-Carres, Mas-Capdevila, Bravo, Aragonès, et al. 2019; Makris et al. 2016; Klongdee and Klinkesorn 2022).

4. HYPOTHESIS AND OBJECTIVES

The main objective of this project is to **obtain (poly)phenol-rich extracts from fruits (cherry, plum, apricot, strawberry, persimmon, grape, orange and pomegranate)**.

The specific objectives (SO) are:

- **SO1**: Demonstrate the increase in concentration of (poly)phenols in the extracts.
- **SO2**: Characterize the different macronutrients (protein, lipids, and carbohydrates) and micronutrients (minerals) in the resulting extracts.

5. MATERIALS AND METHODS

5.1. Plant material

Fruits were purchased from a local market in their corresponding season.

Cherry (*Prunus avium*), apricot (*Prunus armeniaca*), plum (*Prunus domestica*), and persimmon (*Diospyros* kaki) stones were manually removed, and their peel and flesh were grounded, separately, until reaching homogeneity. Oranges (*Citrus sinensis* L) were peeled and ground until reaching homogeneity. Strawberries (*Fragaria x ananassia*) had their calyx removed and were ground until reaching homogeneity. Pomegranate (*Punica granatum*) grains were extracted manually and ground until reaching homogeneity. Grapes (*Vitis*

vinifera) were ground until reaching homogeneity. All homogenates were lyophilized and ground to a fine powder.

5.2. Extraction procedure

Extractions were made following the conditions set by their optimized method of extraction, summarised in **Table 1**.

(Poly)phenols were extracted from cherries following their optimized method of extraction described in (Iglesias-Carres, Mas-Capdevila, Bravo, Mulero, et al. 2019). This method consists of stirring 20 mL of solvent (72% EtOH and 1% formic acid) per gram of fruit at 250rpm during 20 minutes at 55ºC. As plum also belongs to the *Prunus* family, its (poly)phenols were extracted following the same method, extending the extraction time to 30 minutes after doing some testing. Also, as strawberry has a high content of anthocyanins, the same protocol was used except that this time, a larger volume of solvent per gram of fruit was used, being 40 mL/g.

The (poly)phenolic extractions on apricot and persimmon were made following the same method, described in (Iglesias-Carres, Mas-Capdevila, Bravo, Bladé, et al. 2019) as they have a similar (poly)phenol profile. This method consists of an agitation of 20 mL of solvent (72% EtOH and 1% formic acid) per gram of fruit at 250 rpm during 30 minutes at 38ºC.

The (poly)phenolic extractions on grape and pomegranate were made following the same method as described in (Iglesias-Carres et al. 2018). This method consists of an agitation of 80mL of solvent (65% EtOH and 1% formic acid) per gram of fruit at 250rpm during 100 minutes at 72ºC.

(Poly)phenols were extracted form orange's pulp following its optimized method of extraction described in (Iglesias-Carres, Mas-Capdevila, Bravo, Aragonès, et al. 2019). This method consists of an agitation of 30mL of solvent (70% EtOH and 1% formic acid) per gram of fruit at 250rpm during 40 minutes at 55ºC.

Table 1. Extraction conditions for each fruit.

Fruit	Volume of solvent (mL)	EtOH concentration (%)	Extraction time (min)	Temperature (ºC)	Agitation speed (rpm)
Cherry	20	72	20	55	250
Plum	20	72	30	55	250
Apricot	20	72	30	38	250
Strawberry	40	72	30	55	250
Persimmon	20	72	30	38	250
Grape	80	65	100	72	250
Orange	30	70	30	38	250
Pomegranate	80	65	100	72	250

All extractions were cooled at room temperature. After a 10-minute centrifugation at 4ºC and 7.871 G, supernatants were collected and rotary vacuum evaporated at 40ºC in the dark until all the EtOH is removed. Next, extracts were lyophilized using the Telstar LyoQuest lyophilizer (Thermo Fisher Scientific, Barcelona, Spain) to obtain a fine powder.

5.3. Protein quantification

Protein quantification was performed following the Kjeldahl method. First, 200mg of sample were digested with 10mL of H2SO4 99% with potassium sulphate and copper sulphate at 420ºC for 2 hours. Afterwards, the samples were distilled using NaOH and boric acid and titrated with HCl 0,1N. By using the volume of HCl used, the percentage of N present in the samples was calculated and, by using the conversion factor 6.25, protein percentages in the samples were calculated. The results were expressed as a percentage (w/w) of g of protein per gram of dry weight.

5.4. Lipid quantification

Lipids were quantified using an adapted version of the Folch protocol (Folch, Lees, and Sloane 1957; El-Sohaimy and Hafez 2010; Saini et al. 2021).

Lipids were extracted from 1g of sample by a magnetic agitation (600 rpm) in 20 mL of chloroform and methanol (2:1) of 2 hours. After filtration, 4 mL of a solution of NaCl (17,5 mg/mL) was added to form two phases with the help of a 10-minute centrifugation (3.000 rpm at room temperature). The organic phase was recovered in a pre-weighted falcon tube, and the chloroform was dried with

nitrogen. The extracted fat mass was weighted, and the results were expressed as a percentage (w/w) of g of fat per gram of dry weight.

5.5. Carbohydrates quantification

The carbohydrate content was determined with a simple and rapid colorimetric method, the phenol-sulfuric acid method (Dubois et al. 1951). A seven-point standard curve (0, 0.032, 0.062, 0.125, 0.25, 0.5, and 0.8 mg/mL of D-glucose in MQ water) was used for experimental samples.

Briefly, 25µL of sample and 25µL of water are added, by triplicate, in a 96-well plate. Then, 25µL of 5% phenol and 125µL of H_2SO_4 are added in a fume hood before covering and sealing the plate. After an incubation of 30 minutes at 80ºC, the absorbance of the plate is read at a wavelength of 490nm in an Eon BioTek spectrophotometer (Izasa, Barcelona, Spain). The results were expressed as a percentage (w/w) of g of carbohydrates per gram of dry weight.

5.6. Mineral and inorganic compounds quantification

Minerals and other inorganics compounds in food are measured using an ash test, removing moisture, volatiles, and organics in the samples.

The ash quantification test involved taking 1g of sample, placing it unto a dried/pre-weighed porcelain crucible, burning away the sample in a stove at temperatures above 500ºC, and weighing the crucible after being cooled at room temperature in a desiccator. The results were expressed as a percentage (w/w) of g of mineral and inorganic compounds (MIC) per gram of dry weight.

5.7. Fibre quantification

The total dietary fibre (TDF) content was determined following an enzymatic-gravimetric treatment described in (McCleary et al. 2012).

In a 100mL Erlenmeyer, 1g of sample was mixed with 40mL of MES-TRIS 0.05M (pH 8.2) buffer and pre-heated to 95ºC with agitation to add later 50µL of α-amilase from *Bacillus licheniformis* (Sigma-Aldrich, Spain). After 15 minutes of incubation with agitation, the solution was cooled to 60ºC and 300µL of alcalase from *Bacilus* licheniformis (Sigma-Aldrich, Spain) were added and then incubated for 30 minutes at 60ºC. Then, 5mL of HCl 0.5N were added and the pH was adjusted to a value between 4.2 and 4.7 at 60ºC. Once the pH was between these

values, 300µL of amyloglucosidase from *Aspergillus niger* (Sigma-Aldrich, Spain) were added and the mixture was incubated with agitation for 30 minutes at 60ºC.

5.7.1. Insoluble fibre quantification

The resulting liquid was filtered with a pre-weighted filter paper. The residue was washed twice with 10mL of distilled water and the resulting liquid was kept for soluble fibre quantification. The residue was washed again twice with 10mL of 96% ethanol and twice with 10mL of acetone.

After being dried overnight at 80ºC and cooled in a desiccator, the filter with the residue was weighted. The results were expressed as a percentage (w/w) of g of insoluble fibres per gram of dry weight.

5.7.2. Soluble fibre quantification

The filtered liquid kept was mixed with 4 times its volume of 96% ethanol previously heated at 70ºC and left overnight. This mixture was filtered with a pre-weighted filter paper. The residue was washed twice with 15mL of 78% ethanol, twice with 10mL of 96% ethanol and twice with 10mL of acetone.

After being dried overnight at 80ºC and cooled in a desiccator, the filter with the residue was weighted. The results were expressed as a percentage (w/w) of g of soluble fibres per gram of dry weight.

5.8. (Poly)phenol profile quantification

5.8.1. Total (poly)phenol quantification

Total (poly)phenols were quantified following the Folin-Ciocalteau method adapted from (Iglesias-Carres, Mas-Capdevila, Bravo, Bladé, et al. 2019). Gallic acid at different concentrations was used as a standard compound to construct the calibration curves.

Briefly, 50 µL of the extract and 50 µL of the Folin-Ciocalteu (Fluka/Sigma-Aldrich, Spain) reagent were consecutively added to an Eppendorf tube containing 500 µL of water. After 3 min, 100 µL of Na_2CO_3 (25%) were added to the mixture. Milli-Q water was added until reaching a final volume of 1 mL. The absorbance was read at 725 nm using an Eon BioTek spectrophotometer (Izasa, Barcelona, Spain) against a water sample that underwent equal treatment after 1 hour of

incubation in the dark. The results were expressed as milligram gallic acid equivalents per gram of dry weight (mg GAE/g dw).

5.8.2. Flavanol quantification

Total flavanol content in the samples was determined by the DMAC method (Borges et al. 2011). A seven-point standard curve (concentrations 0, 5, 10, 25, 50, 75 and 100 mg/mL catechin in methanol) was used for quantification of experimental samples. Strawberry, grape and plum extract samples were diluted in water 1/4 in order to be within the working range.

Briefly, 500µL of DMACA Reagent (consisting of DMAC (Fluka/Sigma-Aldrich, Spain) in MetOH and 1N HCl) were added to 100µL of sample, and the mixture is left in the dark for 10 minutes. Afterwards, absorbance of the samples was measured at a wavelength of 640nm and room temperature using an Eon BioTek spectrophotometer (Izasa, Barcelona, Spain). The results were expressed as milligrams of catechin equivalents per gram of dry weight (mg catechin/g dw).

5.8.3. Anthocyanidin quantification

Anthocyanidin content was analysed by a pH differential method adapted from (Iglesias-Carres et al. 2018).

Samples were diluted with sodium acetate buffer (0.4M, pH 4.5) and potassium chloride buffer (0.025M, pH 1) to be able to work at reasonable spectrophotometric ranges (0.4-0.6). Absorbance was read at 515 nm and 700nm using an Eon BioTek spectrophotometer (Izasa, Barcelona, Spain) and results were expressed as milligrams of malvidin 3-O-glucoside equivalents per gram of dry weight (mg Mv3G/g dw).

5.9. Statistical analysis

The type of fruit and the extraction type (extract or fruit) were considered the independent variables and the components quantified were considered dependent variables.

Analysis was performed using the appropriate procedures of the Prism 9 (GraphPad). A significance level of $\alpha=0.05$ was used. Results were analysed by two-way ANOVA (fruit type + extraction type). In addition, pair-wise comparison of means (Tukey's T-test) was used to determine the significance between

extraction means ($p<0.05$) for the type I error. Effects were considered significant at the level of $p<0.05$. Correlation matrixes were performed using Pearson's correlation test.

6. RESULTS AND DISCUSSION

6.1. Nutrient profile

6.1.1. Fat

The extraction appeared to concentrate fat content in apricot, strawberry, and persimmon ($p<0.05$) (**Figure 4.A**). However, a tendency to its concentration could be seen, increasing around 5% in almost every fruit (**Table 2**). This general concentration can be due to the capacity of EtOH to solubilize apolar compounds as fats, by dissolving them into micelles that are stabilized with the presence of water (Fagerberg et al. 2012). Moreover, some studies have investigated the use of EtOH as a solvent for green fat extraction, reaching up to a 23% of fat extraction yield (Soares, Okiyama, and Rodrigues 2020; Li et al. 2014). In (Lucci et al. 2015), an ethanolic extraction from pomegranate seeds was performed and the extract was rich in lipidic compounds as glycolipid-linoleic or a-linoleic acid phospholypids.

Due to the increase on fat content, apolar (poly)phenols could be absorbed by their micellization in the intestine (Guo et al. 2013; Arfaoui 2021).

6.1.2. Proteins

Protein content was seen to generally decrease in all fruit extracts, except in grape where there was no significant change ($p<0.05$) (**Table 2**). The biggest change in protein content was observed in strawberry, followed by cherry and apricot, while the smallest variation was seen in persimmon ($p<0.05$) (**Figure 4.B**). As described in (Bose et al. 2019; Tahmasian et al. 2022), protein is extracted more efficiently by using different polar solvents, being more unstable when compared to non-polar solvents (Dongmo et al. 2020). However, protein concentration was not the goal of these extractions, so the loss in protein content is not only not relevant but also expected.

As a result of the negative effect proteins have in (poly)phenol bioavailability due to their interaction and binding, this reduction of the total protein content in the

extracts would translate in an increased free (poly)phenol level (Bohn 2014; Lund 2021).

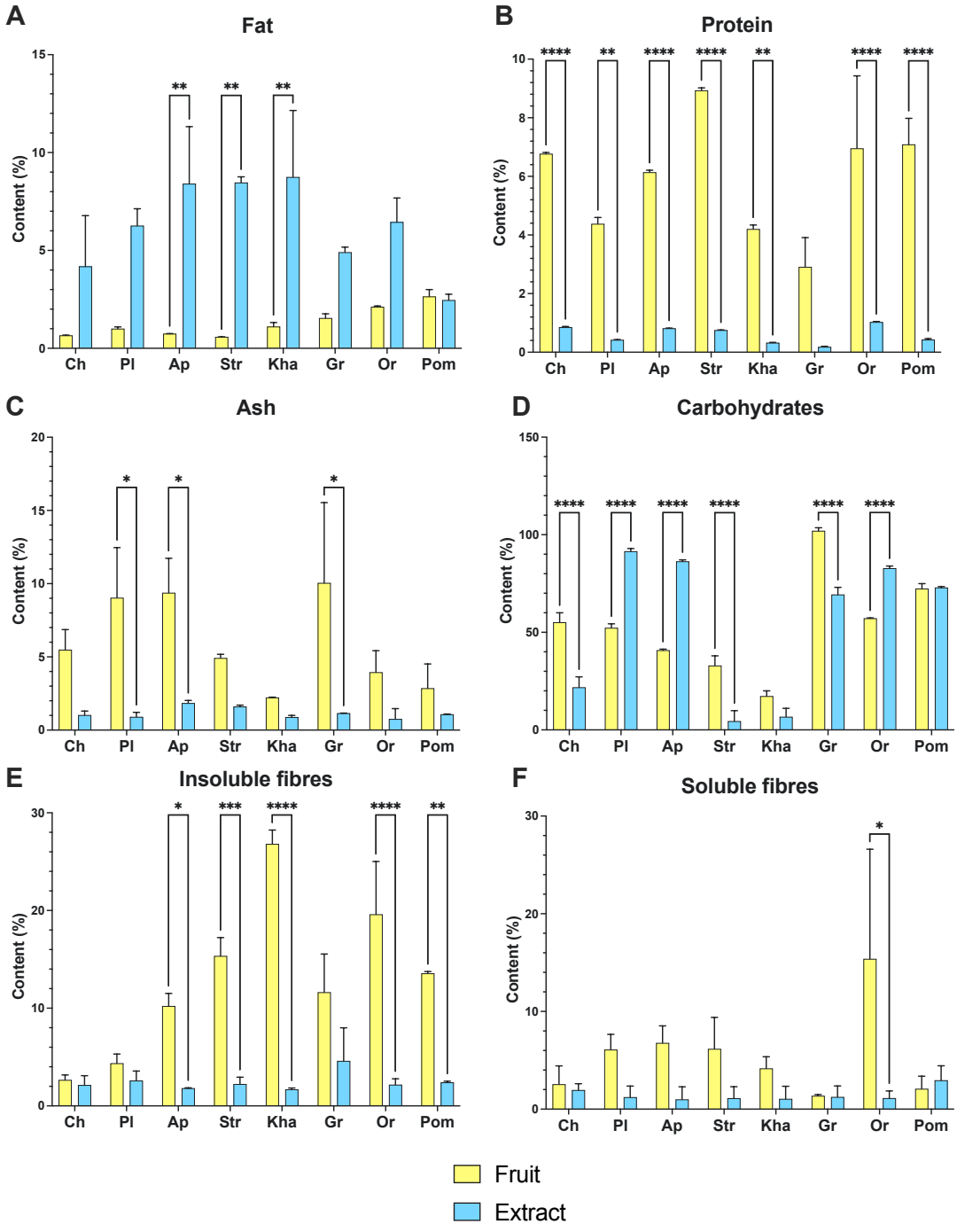

Figure 4. Nutrient profile in fruit and extracts. *Expressed as percentage (g of component/g of dry sample). (**A**) Fat. (**B**) Protein. (**C**) Ash, representing minerals and inorganic compounds. (**D**) Carbohydrates. (**E**) Insoluble fibres. (**F**) Soluble fibres. Ch: cherry; Pl: plum; Ap: apricot; Str: strawberry; Kha: persimmon; Gr: grape; Or: orange; Pom: pomegranate. P estimated by a two-way ANOVA (Tukey's t-test). Different * indicate statistical differences between groups being * p≤0.05; ** p≤0.01; *** p≤0.001; **** p≤0.0001.*

***Table 2. Summary of nutrient differences**. Diff. (%) being the average of each nutrient difference (n=4). SD being standard deviation. P value was estimated by using a one-way ANOVA (Tukey's t-test). Different letters indicate statistical differences between groups.*

Fruit	Fat			Insoluble fibres			Soluble fibres			Protein			Ash			Carbohydrates		
	Diff. (%)	SD		Diff. (%)	SD		Diff. (%)	SD		Diff. (%)	SD		Diff. (%)	SD		Diff. (%)	SD	
Cherry	3,53	2,12	b	-0,53	0,89	a	-0,59	1,63	a	-5,92	0,04	c	-4,46	1,15	abc	-33,29	6,13	e
Plum	5,26	0,71	ab	-1,77	1,11	ab	-4,87	1,58	a	-3,96	0,18	ab	-8,14	2,81	bc	39,22	2,16	a
Apricot	7,66	2,38	a	-8,41	1,05	cd	-5,78	1,78	ab	-5,33	0,06	bc	-7,53	1,93	bc	45,54	0,82	a
Strawberry	7,88	0,24	a	-13,12	1,64	de	-5,06	2,81	a	-8,18	0,07	d	-3,32	0,22	ab	-28,48	6,37	de
Persimmon	7,63	2,79	a	-25,12	1,17	f	-3,13	1,43	a	-3,88	0,11	ab	-1,34	0,09	a	-10,63	4,42	cde
Grape	3,37	0,28	bc	-7,03	4,23	bc	-0,12	0,92	a	-2,73	0,81	a	-8,9	4,48	c	1,36	51,11	bc
Orange	4,33	1,01	ab	-17,44	4,45	e	-14,26	9,19	b	-5,93	2,02	c	-3,19	1,33	ab	25,65	1,01	ab
Pomegranate	-0,19	0,38	c	-11,17	0,18	cd	0,87	1,61	a	-6,66	0,73	cd	-1,79	1,35	a	0,54	2,26	bcd

6.1.3. Minerals and inorganic compounds

Minerals and inorganic compounds systematically tended to decrease (**Table 2**); however, the only significant changes were found in plum, apricot and grape extracts, with an 8.41%, 7.52% and a 8.90% of variation respectively (**Figure 4.C**). The non-significant change follows the line as the ethanolic extraction of (poly)phenols in seaweeds performed by (Múzquiz De La Garza et al. 2019), where ash content remained basically the same in all the extracts.

Although there are some studies revealing the impact of (poly)phenols in mineral absorption (Duan et al. 2021), there is a lack of evidence on how this affects (poly)phenol bioavailability, so it is difficult to say how the presence of these minerals would affect the absorption of (poly)phenols in the resulting extracts.

6.1.4. Carbohydrates

An increase in the total carbohydrate content can be seen in plum, apricot, and orange, while a decrease on its concentration is present in cherry, strawberry, and grape extracts (**Table 2**). Although persimmon extract shows a tendency to decrease, this change is non-significant ($p>0.05$) (**Figure 4.D**). The types of sugars present in the different fruits might explain the variability present in this quantification, however the extraction conditions used should also be considered, as pH, temperature or time affects the carbohydrates extracted (Méndez et al. 2022).

Using high concentrations of EtOH in the extraction method has been studied that can decrease the extraction of carbohydrates, especially polysaccharides, that precipitate in this solvent (Liu and Huang 2019). In previous studies performed in the group, it was seen how grape and persimmon were rich in these type of carbohydrates and, strawberry, even if it had a low sugar content, had a high proportion of them. This could explain the reduction on this macronutrient content in these fruits. However, there are some studies that report no impact in the use of high concentrations of EtOH in carbohydrate extraction (Hoehnel et al. 2022; Navarro et al. 2016).

In terms of dietary fibres, a general reduction of their content can be seen all over the different extracts (**Table 2**). Nevertheless, these changes are only significant ($p<0.05$) for apricot, strawberry, persimmon, orange and pomegranate in the

insoluble fibres and in orange in the soluble ones (**Figure 4.E** and **Figure 4.F**). As described by (Spinei and Oroian 2021), the use of different acid or basic solvents in combination with high temperatures and sometimes, digestive enzymes, are used to extract dietary fibres. As our method was not designed for this, it is not surprise that these nutritional components are not concentrated in the resulting extracts.

As for the effect in (poly)phenol bioavailability, as both types of dietary fibres are reduced, globally there should be no effect on the absorption.

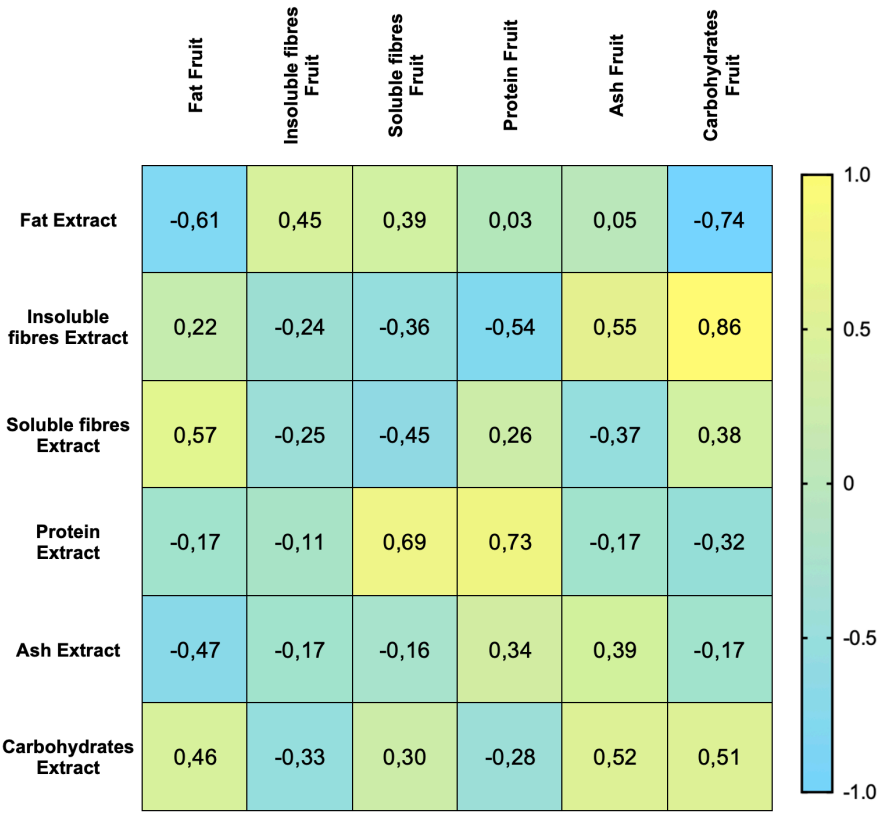

Figure 5. Correlation matrix, using Pearson's correlation of fruit nutrients and extract nutrients. *-1: negative correlation; 0: no correlation; 1: positive correlation.*

Fats and fibres have a negative correlation between the fruits and the extracts, this shows how fats are really concentrated when using the extractions methods while fibres are practically not extracted (**Figure 5**). Meanwhile, protein, ash and carbohydrates have a positive correlation between fruit and extract, indicating that the extraction method used does not really affect these nutrients.

Seen the changes of each individual nutrient, it is also interesting to see if there is any correlation with their variation after the extraction process, as nutrients commonly are subjected to a matrix effect.

In this correlation matrix, it can be seen how the fruit carbohydrate content has a positive relation with the resulting content in fat and insoluble fibres in the extracts. The strong positive correlation between carbohydrates and insoluble fibres could mean that a part of the carbohydrates present in fruits are polysaccharides that are part of the insoluble fibre content.

Also, soluble fibres in fruits appear to have a positive correlation with the resulting proteins in the extracts while insoluble ones have almost no correlation, indicating that there is a great content of polar proteins present in fruits or even that the conditions used denaturalize them, making them more polar.

This shows that a different food matrix will not only affect (poly)phenol bioavailability but also the extraction of the matrix itself, varying depending on the nutrient content in fruits.

To conclude, as changes in nutrient concentration may have an effect in (poly)phenol absorption, it is necessary to know the concentration of each of them in the resulting extracts while seeing the variation when comparing it to their respective fruits. Protein, fibres (both soluble and insoluble) and minerals tend to decrease its concentration in the extracts, while fats were more concentrated. In terms of carbohydrates there is a variation between fruits, in some they are concentrated while in others are decreased. Also, as the protocols used are optimized for (poly)phenol extraction, a general loss was expected. The concentrations of protein, minerals and fibres were reduced to concentrations lower than 5% each. This translates in the matrix effect mainly being affected by fats and carbohydrates, which their concentration could help with the absorption of apolar (poly)phenols and glucoside derivatives. Although, the bioavailability could be increased, the absorption of the different compounds could differ depending on the sugar they have attached and the host gut microbiota profile, among other internal factors (Tian et al. 2019).

6.2. (Poly)phenolic profile

(Poly)phenol contents were concentrated in all fruits when making the extracts (**Figure 6.A**), being strawberry the fruit that showed a major difference in (poly)phenol content (**Table 3**)**.** However, a general loss of anthocyanins can be seen, except in cherry, orange and, specially, plum extract where they have been

greatly concentrated (**Table 3**). The loss could be explained by the temperatures used to perform the extraction, as temperatures over 70ºC lead to rapid anthocyanin degradation (Brglez Mojzer et al. 2016). Moreover, anthocyanins are water soluble, therefore, using a higher concentration of ethanol would decrease the extraction of these compounds. Flavanol content in the other hand, mostly has not been affected by the extraction ($p>0.05$), except in plum, strawberry, and grape extracts where they have been decreased (**Table 3**).

***Table 3. Summary of (poly)phenol differences**. Diff. (%) being the average of each nutrient difference (n=9). SD being standard deviation. P value was estimated by using a one-way ANOVA (Tukey's t-test). Different letters indicate statistical differences between groups.*

Fruit	Total (Poly)phenols			Flavanols			Anthocyanins		
	Diff. (mg/g)	SD		Diff. (µg/g)	SD		Diff. (µg/g)	SD	
Cherry	4,33	1,21	d	-50,33	1,58	a	77,59	8,48	b
Plum	6,29	1,86	c	-1146,67	89,44	d	243,69	4,96	a
Apricot	2,93	0,28	d	-63,67	1,87	a	-6,46	0,83	d
Strawberry	31,87	1,46	a	-664,67	33,92	c	-85,00	32,40	f
Persimmon	3,20	0,19	d	-46,33	5,29	a	-34,01	3,23	e
Grape	7,34	1,76	bc	-258,00	138,61	b	-75,76	6,11	f
Orange	8,86	0,39	b	-36,33	6,86	a	14,03	3,77	c
Pomegranate	3,06	0,42	d	-66,67	2,65	a	-157,30	1,88	g

More precisely, for example, the (poly)phenol concentration in the orange extract was 12.48mg/g (**Figure 6.A**), following the same line as the extraction performed by (Iglesias-Carres, Mas-Capdevila, Bravo, Aragonès, et al. 2019), where it was around 14mg/g. Also, it was seen that the total (poly)phenol concentration in the strawberry extract was 54.79mg/g, an increase compared to the extraction performed by (Salas-Arias et al. 2023), where the concentration was 0.89mg/g. This could be due to the use of 40mL of solvent per gram of fruit compared to the 1:10 ratio in that study. Furthermore, compared to the extraction made in (Iglesias-Carres, Mas-Capdevila, Bravo, Bladé, et al. 2019), the total (poly)phenol concentration in apricot extract was also increased, but the flavanol concentration was lower, obtaining 0.03mg/g compared to the 0.3mg/g obtained in that study (**Figure 6.B**).

On top of that, in the grape extraction, (poly)phenol concentration was increased up to 17.44mg/g, but in the extraction made in (Iglesias-Carres et al. 2018), the

resulting concentration was 25mg/g. The same goes to the flavanol concentration, where our resulting extract was reduced to 1.67mg/g when in that study the concentration was 4mg/g. Still, even if the extraction method is the same, a different (poly)phenolic profile can be found depending on crop conditions (Pinasseau et al. 2017).

Finally, in the cherry extract, as seen in (Popovic et al. 2022), ethanolic (poly)phenol extraction was performed, obtaining similar values to total (poly)phenol content and flavanols. However, a decrease in anthocyanidins could be observed. The reduction could be due to our usage of 72% ethanol compared to the 50% and the solubility in water of anthocyanins (**Figure 6.C**).

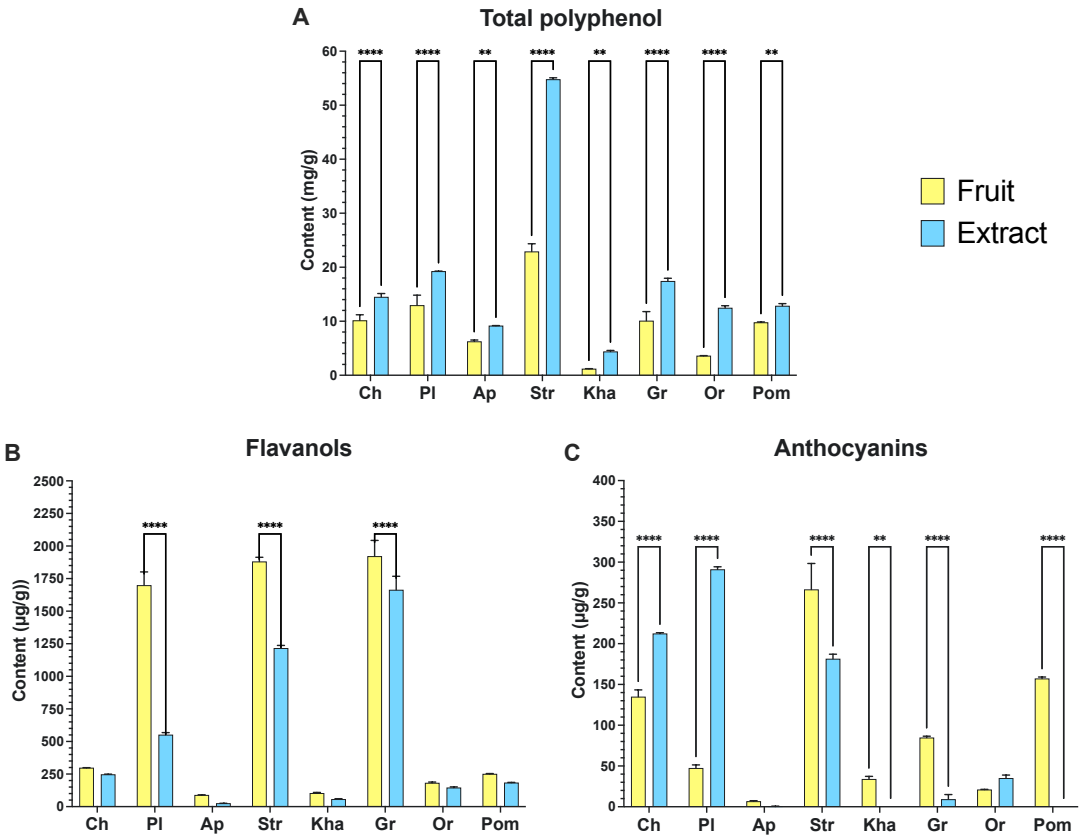

Figure 6. (Poly)phenolic profile in fruits and extracts. *(**A**) Total (poly)phenol concentration. (**B**) Flavanol concentration. (**C**) Anthocyanin concentration. Ch: cherry; Pl: plum; Ap: apricot; Str: strawberry; Kha: persimmon; Gr: grape; Or: orange; Pom: pomegranate. P estimated by a two-way ANOVA (Tukey's t-test). Different * indicate statistical differences between groups being * p≤0.05; ** p≤0.01; *** p≤0.001; *** p≤0.0001.*

Although in (Domínguez-Rodríguez et al. 2021) it can be seen how the use of advanced extraction techniques can increase the total concentration of (poly)phenols in the resulting extracts, and flavanols and anthocyanins concentrations were lower compared to other studies, the concentration in total (poly)phenols means that other type of these compounds have been

concentrated. To know more precisely which compounds are present in each extract, a characterization of (poly)phenols should be made, having more detailed information about their composition and the health benefits they would provide.

It is interesting to see how the nutritional and the (poly)phenolic profiles in fruits could affect the (poly)phenol concentration in the extracts due to the matrix effect. In the following correlation matrix, it can be seen the different relations between the fruit components with the extract (poly)phenols (**Figure 7**).

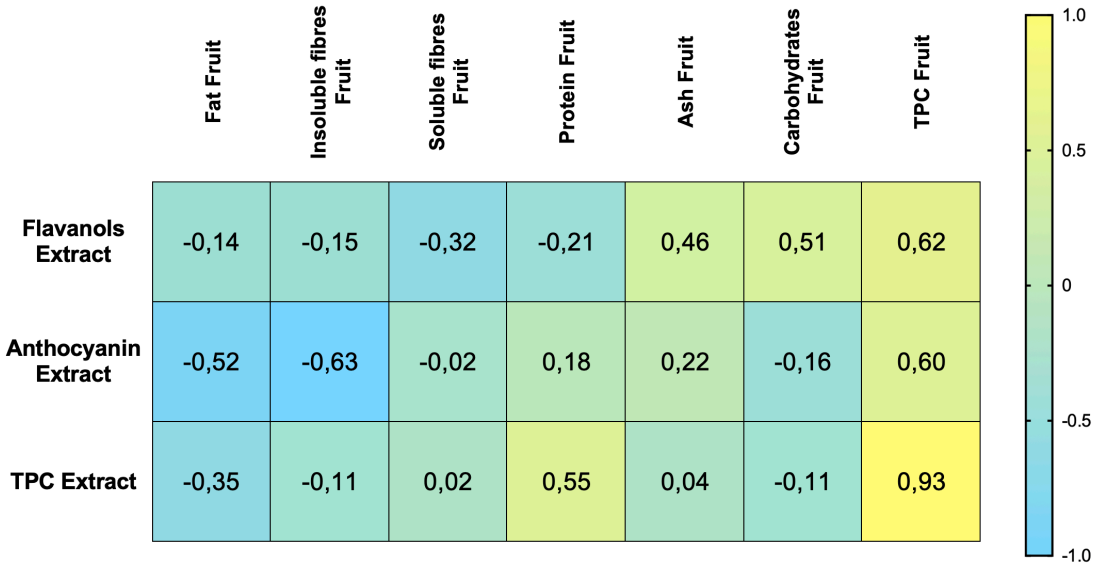

Figure 7. Correlation matrix, using Pearson's correlation of fruit nutrients and (poly)phenol content in extracts. *-1: negative correlation; 0: no correlation; 1: positive correlation.*

The matrix shows a positive relation between total (poly)phenols and proteins, showing that part of them may be forming insoluble complexes that can be extracted (Bandyopadhyay, Ghosh, and Ghosh 2012). It is also shown the polar nature of anthocyanins, as they have a negative relation with fat and insoluble fibres from fruit. This can be seen in (Soares, Okiyama, and Rodrigues 2020) where the extraction of lipids using alcoholic solvent favoured the simultaneous extraction of carotenoids, a lipophilic group of antioxidants, and hindered the extraction of polar (poly)phenols.

It can be seen how the total (poly)phenol content in fruits has a similar relation with extract flavanols and anthocyanins, seeing that these two families are extracted similarly using this method. The fact that this correlation is positive, even if this compounds' concentrations are decreased in the extracts, is due to

the more (poly)phenols present in fruits, more compounds can be extracted, even if its extraction yield is low.

With these correlations the food matrix is seen not only to have an effect in the absorption of (poly)phenolic compounds but also in their extraction.

7. CONCLUSIONS

In conclusion, (poly)phenol-rich extracts were obtained when using an optimized a maceration as extraction method from different fruits, even in the ones that no optimized protocol was described. The increase was demonstrated by quantifying the (poly)phenolic profile both in the extracts and the fruits. Moreover, the macro- (proteins, lipids, and carbohydrates) and micronutrients (minerals) present in these extracts were characterized, being able to increase the bioavailability of (poly)phenols by reducing the so-called matrix effect, when comparing it to consuming the whole fruits. However, a more detailed (poly)phenol characterisation and an *in vivo* study should be made to see the true health benefits these extracts give.

8. FUTURE PERSPECTIVES

It has been seen how the difference in nutrient concentrations in the extracts and the increase in their (poly)phenol concentration could help with a higher (poly)phenolic absorption. However, to see how the bioavailability of these extracts has changed, *in vitro* digestion models or absorption models in Caco-2 cells could be used. Moreover, the elaboration of these extracts was the first step of the project “Seasonal phenol-based multi-ingredients for the prevention of pathologies associated with obesity”. The objective of this project is to develop specific multi-ingredients based in (poly)phenols from fruits of different seasons (spring-summer and autumn-winter) that are able to palliate obesity-associated pathologies. First, the more efficient extracts for each season have been selected by performing an *in vivo* experiment and now, they are going to be tested in their corresponding season to see the beneficial effects in body fat accumulation, adiposity, main metabolic processes in liver and adipose tissue, local and systemic proinflammatory biomarkers, and endothelial function, along with the impact in central biological rhythms.

The increase on fruit consumption has led to higher production, totalling almost 890 million tons per year (Ritchie, Rosado, and Roser 2023). However, 21,6% of this production is lost in post-harvest processes on average, for example, up to 50% is discarded in orange production and up to 42% is wasted in total (Lipinski et al. 2013). This creates significant waste management challenges, necessitating new strategies for waste prevention and utilization of by-products and coproducts (Despoudi et al. 2021). The method used for these extractions was performed with this necessity in mind, as it can be used not only in the edible parts of fruits but also in their wasted products (seeds, peels, discarded fruits…) (Méndez et al. 2022). First, the use of a conventional extraction method enables the use of a higher volume of sample, keeping up with all the waste products produced. Second, the conditions used are easily achievable with not big facilities, not involving a huge inversion for companies. Finally, the chosen solvent was EtOH, a cheap and safe solvent that enables the consumption of the resulting extracts and also can be recovered after the rotary evaporation used to obtain the extracts.

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10. AUTOEVALUATION

Doing this project has not been easy. There were a lot of long experimental procedures, each of them for each different fruit and extracts. It is true that the volume of work helped me learn how to manage time efficiently. Also, the trust that the group put on me made me gain more confidence when working in the lab.

However, the most difficult part was thinking how I could transform these results in a final degree project, as they were meant to be supplementary for the research group's project. Initially, the group only needed the results as they are required for any publication in this field, so, I had to think how I could give them an added value to write this project. This is why I had decided to link the nutrient content with (poly)phenol bioavailability and then how the nutrient profile could affect the extraction.

In the end, I am happy with the results I have obtained and the work I have done, as it is the part of biotechnology I liked the least and this way it started growing in me. Also, I think I was able to give the results a new perspective while trying to reason everything accordingly, even when bibliography in this specific topic was scarce.

11. ANNEX

11.1. Annex 2

Normativa de Treball Fi de Grau Facultat d'Enologia
Aprovada per Junta de Facultat d'Enologia del dia 30 d'octubre de 2014

ANNEX 2

FITXA DE SEGUIMENT DEL TUTOR/A del TFG

Nom i Cognoms de l'Alumne/a: Jorge Martínez García

Nom i Cognoms del Tutor/a: Begoña Muguerza Marquínez

Data de la entrevista amb l'alumne: 17/03, 30/03, 15/05

Recomanacions durant el seguiment:
Dado que el estudiante ha llevado a cabo la investigación incluida en su TFG en mi grupo de investigación, además de las fechas de entrevistes indicadas en el apartado anterior, ha habido un contacto muy estrecho con Jorge en el que hemos ido discutiendo la organización de los resultados.
En cuanto a las recomendaciones llevadas a cabo durante las entrevistas de seguimiento se han centrado en la memoria, principalmente en modificaciones de escritura en el texto.

Observacions:
Me gustaría destacar la actitud del estudiante ya que ha sido una persona muy proactiva con muchas ganas por aprender y con gran organización para la preparación de su TFG

Observacions Darrera revisió:
Considero que el TFG està llisto para presentar.

Signatura del Tutor/a

Maria Begoña Muguerza Marquínez - DNI 33420551X (TCAT)

Firmado digitalmente por Maria Begoña Muguerza Marquínez - DNI 33420551X (TCAT)
Fecha: 2023.05.29 11:59:21 +01'00'

Signatura del Alumne/a

Tarragona a 29 de Mayo 2023