MODERN CHANGES IN FRUIT INTAKE PATTERNS MODULATE THE BIOAVAILABILITY AND METABOLISM OF FRUIT PHENOLIC COMPOUNDS IN RATS

Lisard Iglesias Carres

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Doctoral Thesis
Tarragona 2018

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Modern changes in fruit intake patterns modulate the bioavailability and metabolism of fruit phenolic compounds in rats

DOCTORAL THESIS

Supervised by Dr. Anna Arola Arnal and Dr. Begoña Muguerza Marquínez

Nutrigenomics Research Group

Departament of Biochemistry and Biotecnology

UNIVERSITAT ROVIRA i VIRGILI

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FEM CONSTAR que aquest treball titulat **"Modern changes in fruit intake patterns modulate the bioavailability and metabolism of fruit phenolic compounds in rats"** que presenta Lisard Iglesias Carres per a l'obtenció del títol de doctorat ha estat realitzat sota la nostra direcció al Departament de Bioquímica i Biotecnologia de la Universitat Rovira i Virgili i que compleix els requisits per a l'obtenció de la Menció Internacional de Doctorat.

HACEMOS CONSTAR que este trabajo titulado **"Modern changes in fruit intake patterns modulate the bioavailability and metabolism of fruit phenolic compounds in rats"** que presenta Lisard Iglesias Carres per a la obtención del título de doctorado ha estado realizado bajo unestra dirección en el Departament de Bioquímica y Biotecnologia de la Universitat Rovira i Virgili y que cumple con los requisitos para la obtención de la Mención Internacional de Doctorado.

WE STATE that the present study entitled "**Modern changes in fruit intake patterns modulate the bioavailability and metabolism of fruit phenolic compounds in rats"** presented by Lisard Iglesias Carres for the award of the degree of Doctor has been carried out under our supervision at the Departament de Bioquímica i Biotecnologia from the Universitat Rovira i Virgili and that is eligible to apply for the International Doctoral Mention.

Tarragona, 5 Setembre 2018
Les directores de la tesi doctoral
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Dr. Anna Arola Arnal

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Aquesta tesi està emmarcada dins del projecte FRUITOBES (AGL2013-49500-EXP), que té com a objectiu avaluar si el consum de fruites fora de temporada està associat a una major predisposició a desenvolupar obesitat així com aquelles patologies que hi estan associades. Lisard Iglesias Carres ha estat guardonat amb una beca pre-doctoral (2015PMF-PIPF-50) per a la realització d'aquesta tesi doctoral atorgada per la Universitat Rovira i Virgili dins del programa Matrí i Franqués.

This thesis is framed within the FRUITOBES (AGL2013-49500-EXP) project, which evaluates whether the consumption of fruits out of season involves a higher risk to develop obesity and their associated pathologies. Lisard Iglesias Carres is the recipient of a pre-doctoral grant (2015PMF-PIPF-50) to carry out this doctoral thesis awarded by Universitat Rovira i Virgili within the Matrí i Franqués program.

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Als meus

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Juntarem les mans en gran rotllana
sorgirà un fort clam de la plaça entera
mentre abraça l'arbre la gran sardana:
Això és Catalunya, i Visca Colera.

Sardana *La vall de Puig d'Esquers*, Lluís Duran i Masseguer, 1992.

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LIST OF ABBREVIATIONS:

ADME	Absorption, distribution, metabolism and excretion
BMAL	Brain and muscle ARNT-like protein
BMI	Body mass index
CLOCK	Circadian locomotor output cycles kaput
COMT	Catechol-*O*-methyl transferase
CRY	Cryptochrome
CVD	Cardiovascular disease
CVR	Cardiovascular risk
EU-28	European Union countries
HLD	High density lipoprotein
LDL	Low density lipoprotein
LSR	Liquid-to-solid ratio
MetS	Metabolic syndrome
NAFLD	Non-alcoholic fatty liver disease
Per	Transcription of the period
RSM	Response surface methodology
SAD	Seasonal affective disorder
SCN	Suprachiasmatic nucleus
SGLT	Sodium-glucose transporter
SULT	Sulfotransferase
UGT	UDP-glucuronosyltransferase
WHO	World Health Organisation

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LIST OF PUBLICATIONS

A) Published papers:

Margalef, M., Pons, Z**., Iglesias-Carres, L.**, Bravo, F.I., Muguerza, B., Arola-Arnal, A., 2017. Flavanol plasma bioavailability is affected by metabolic syndrome in rats. Food Chem. 231, 287–294.

Margalef, M., Pons, Z., **Iglesias-Carres, L.**, Quiñones, M., Bravo, F.I., Arola-Arnal, A., Muguerza, B., 2017. Rat health status affects bioavailability, target tissue levels, and bioactivity of grape seed flavanols. Mol. Nutr. Food Res. 2, 1–9.

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Margalef, M., Pons, Z., **Iglesias-Carres, L.**, Bravo, F.I., Muguerza, B., Arola-Arnal, A., 2015. Lack of Tissue Accumulation of Grape Seed Flavanols after Daily Long-Term Administration in Healthy and Cafeteria-Diet Obese Rats. J. Agric. Food Chem. 63, 9996–10003.

B) Submitted papers:

Iglesias-Carres, L., Mas-Capdevila, A., Bravo, F.I., Bladé, C., Arola-Arnal, A., Muguerza, B. Optimization of extraction method for characterization of phenolic compounds from apricot fruit (*Prunus armeniaca*). [Submitted to J. Food Comp. Anal.].

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Iglesias-Carres, L., Mas-Capdevila, A., Sancho-Pardo, Lucía., Bravo, F.I., Muelro, M., Muguerza, B., Arola-Arnal, A. Optimized extraction by response surface methodology, characterization and quantification of phenolic compounds in whole red grapes (Vitis vinifera). [Submitted to J. Agric. Food Chem.].

Iglesias-Carres, L., Mas-Capdevila., Bravo, F.I., Aragonès, G., Arola-Arnal, A., Muguerza, B. Organic red grapes have a particular phenolic bioavailability in rats. [Submitted to Nutrients].

Iglesias-Carres, L., Mas-Capdevila., Bravo, F.I., Bladé, C., Muguerza, B., Arola-Arnal, A. The bioavailability and metabolism of ornge phenolic compounds is conditioned by orange growing region and photoperiod exposure. [Submitted to J. Nutr. Biochem.].

C) <u>In preparation papers:</u>

Iglesias-Carres, L., Mas-Capdevila., Bravo, F.I., Arola, L., Arola-Arnal, A., Muguerza, B. The bioavailability of red grape polyphenols is higher in winter-like conditions [In preparation for Mol. Metab.].

Iglesias-Carres, L., Mas-Capdevila., Bravo, F.I., Mulero, M., Muguerza, B., Arola-Arnal, A. The bioavailability and metabolism of red grape polyphenols is conditioned by its consumption season in metabolically-altered Fischer 344 rats. [In preparation for J. Photochem. Photobio. B.].

D) <u>Poster communications:</u>

Iglesias-Carres, L., Mas-Capdevila, A., Bravo, F.I., Arola-Arnal, A., Muguerza, B. Administration of pure epicatechin or as part of a flavanol-rich extract does not influence flavanol metabolism and body distribution. The 2nd Conference on Food Bioactives & Health. Lisbon, 2018.

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Mas-Capdevila ,A., **Iglesias-Carres, L.,** Pons, Z., Arola-Arnal, A., Bravo, F.I., Muguerza, B. Relationship between dose and antihypertensive effect of an ACE inhibitor chicken feet hydrolysate. XVII Congreso de la Sociedad Española de Nutrición. Barcelona, 2018.

Iglesias-Carres, L., Mas-Capdevila, A., Bravo, F.I., Muguerza, B., Arola-Arnal, A. Photoperiod exposure modulates sweet orange polyphenol bioavalability and metabolism. X Seminario sobre alimentación y estilos de vida saludables. Palma de Mallorca, 2018.

Mas-Capdevila, A., **Iglesias-Carres, L.,** Laza, R.,Arola-Arnal, A., Muguerza, B., Bravo, F.I., Antihypertensive effect of 3-weeks supplementation with chiken-feet hydrolysate (Hpp11) in an animal model of metabolic syndrome. X Seminario sobre alimentación y estilos de vida saludables. Palma de Mallorca, 2018.

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Iglesias-Carres, L., Sancho-Pardo, L., Arola-Arnal, A., Muguerza, B. Optimization of a specific method to extract whole red grape polyphenols using Response Surface Methodology. The 1st International Conference on Food Bioactives & Healt. Norwich, 2016.

Margalef, M., **Iglesias-Carres, L.,** Bravo, F.I., Muguerza, B., Arola-Arnal, A. Flavanol metabolism and plasma bioavailability is affected by metabolic syndrom in rats. The 1st International Conference on Food Bioactives & Healt. Norwich, 2016.

E) <u>Oral communications.</u>

Iglesias-Carres, L., Mas-Capdevila, A., Bravo, F.I., Muguerza, B., Arola-Arnal, A. Differences in the serum kinetic behavior of organic and conventional whole red grape phenolic metabolites. V Reunión de Jóvenes Investigadores. Santa Coloma de Gramanet, 2018.

Mas-Capdevila ,A., **Iglesias-Carres, L.,** Pons, Z., Arola-Arnal, A., Bravo, F.I., Muguerza, B. Relationship between dose and antihypertensive effect of an ACE inhibitor chicken feet hydrolysate. V Reunión de Jóvenes Investigadores. Santa Coloma de Gramanet, 2018.

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RESUM

El patró de consum d'alguns aliments com les fruites ha variat considerablement. Actualment es poden consumir fruites fora de temporada, de diferents orígens geogràfics o cultivades mitjançant diferents sistemes (p. ex. orgànic o convencional). Les fruites són riques en polifenols i el seu consum és considerat beneficiós per a la prevenció de patologies metabòliques com l'obesitat, la incidència de la qual ha augmentat considerablement. No obstant, els patrons dietètics anteriorment exposats i l'obesitat *per se* poden afectar a la biodisponibilitat i metabolisme d'aquests compostos, modificant d'aquesta manera els seus metabòlits, que són els compostos potencialment responsables de la seva bioactivitat.

L'objectiu d'aquesta tesi és avaluar si els canvis actuals del patró de consum de fruites influeixen a la biodisponibilitat i metabolisme dels seus polifenols en rates. Primerament es va optimitzar l'extracció dels polifenols de diferents fruites de temporada (albercocs, cireres, raïms i taronges), per caracteritzar detalladament el seu perfil fenòlic. Posteriorment es va estudiar en rates si l'origen geogràfic i l'època de consum de les taronges afectaven la biodisponibilitat i metabolisme dels seus polifenols. Finalment es va avaluar si la biodisponibilitat i metabolisme dels polifenols dels raïms es afectada pel seu sistema de cultiu, època de consum i estat metabòlic de l'animal.

Els resultats d'aquesta tesi han permès posar a punt mètodes d'optimització per a l'extracció i caracterització dels polifenols de les fruites estudiades. Així mateix, s'ha evidenciat que la biodisponibilitat i metabolisme dels polifenols de la taronja són afectats pel seu origen geogràfic i l'època de l'any en la qual es consumeixen, i s'ha comprovat que el sistema de cultiu, l'època de consum i l'estat metabòlic de l'hoste influeixen en la biodisponibilitat i metabolisme dels polifenols del raïm.

RESUMEN

El patrón de consumo de algunos alimentos como la fruta ha variado considerablemente. Actualmente se pueden consumir frutas fuera de su temporada, de diferentes orígenes geográficos o cultivadas mediante diferentes sistemas (p. ej. orgánico o convencional). Las frutas son ricas en polifenoles y su consumo es considerado beneficioso para la prevención de patologías metabólicas como la obesidad, cuya incidencia ha aumentado considerablemente. No obstante, los patrones dietéticos anteriormente expuestos y la obesidad *per se* pueden afectar a la biodisponibilidad y metabolismo de estos compuestos, modificando de este modo sus metabolitos, que son los compuestos potencialmente responsables de su bioactividad.

El objetivo de esta tesis es evaluar si los cambios actuales del patrón de consumo de fruta influyen en la biodisponibilidad y metabolismo de sus polifenoles en ratas. Primeramente se optimizó la extracción de polifenoles de diferentes frutas de temporada (albaricoques, cerezas, uvas y naranjas), con el fin de caracterizar detalladamente su perfil fenólico. Posteriormente se estudió en ratas si el origen geográfico y la época de consumo de las naranjas afectaban a la biodisponibilidad y metabolismo de sus polifenoles. Finalmente se evaluó si la biodisponibilidad y metabolismo de los polifenoles de uvas es afectada por su sistema de cultivo, época de consumo y estado metabólico del animal.

Los resultados de esta tesis han permitido poner a punto métodos de optimización para la extracción y caracterización de polifenoles de las frutas estudiadas. Asimismo se ha evidenciado que la biodisponibilidad y metabolismo de los polifenoles de la naranja son afectados por su origen geográfico y la época del año en que se consumen, y se ha comprobado que el sistema de cultivo, la época de consumo y el estado metabólico de huésped influyen en la biodisponibilidad y metabolismo de los polifenoles de uva.

SUMMARY

The consumption pattern of some foods like fruits has considerably changed. Currently, fruits can be consumed out of their season, from different geographical origins or cultivated under different systems (i.e. organic or conventional). Fruits are rich in polyphenols and its consumption is considered beneficial for the prevention of metabolic pathologies such as obesity, whose incidence has considerably increased. However, the dietary patterns previously mentioned and obesity *per se* can affect the bioavailability and metabolism of these compounds, modifying that way their metabolites, which are the compounds potentially responsible for their bioactivity.

The aim of this thesis is to evaluate whether the current changes in fruit consumption patterns influence the bioavailability and metabolism of their polyphenols in rats. First, the extraction of different seasonal fruits (apricots, cherries, grapes and oranges) was optimised to characterise their phenolic profile in detail. Then, the study of whether geographical origin and season consumption of oranges affected the bioavailability and metabolism of their polyphenols in rats followed. Finally, the evaluation of whether the bioavailability and metabolism of grape polyphenols was affected by grape culture system, season consumption and host's metabolic state was conducted.

The results of this thesis have allowed the development of optimised methods for the extraction and characterisation of the polyphenols from the studies fruits. Likewise, the bioavailability and metabolism of orange polyphenols was evidenced to be affected by the orange geographical origin and consumption season, and it has been evidenced that growing system, season consumption and host's metabolic state affect the bioavailability and metabolism of grape polyphenols.

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INTRODUCTION:

The World Health Organisation (WHO) recommends the consumption of 400 g of fresh vegetables and fruits per day to improve health status and reduce the risk to develop chronic non-communicable diseases. This recommendation is translate into the consumption of 5 portions of fruit and vegetables a day, and about half of this recommended quantity should proceed from fruits. Moreover, dietary guidelines recommend the consumption of whole fruits over their juices. In this sense, health organisations recommend limiting the consumption of fruit juices and smoothies under 150 mL a day (1).

1. Fruit intake patterns in modern occidental societies:

In the last decades, occidental societies have experienced changes on their population lifestyle, and a major factor that has changes is dietary patterns (2–4). For example, in the last five decades, in Spain, dietary patterns have evolved from a typical Mediterranean diet, rich in fruits and vegetables, towards an American-similar diet (5). Remarkably, societies are increasing their consumption of fat and sugar, which has led to an increase of obesity (which is described as body mass index (BMI) equal or higher than 30 Kg/m^2) worldwide (3,4,6). For example, in the European Union countries (EU-28) and in 2014, obesity affected a considerable percentage of their population (Figure 1) (7). Importantly, obesity is associated with different altered physiological conditions, which include type II diabetes, hypertension, gut dysbiosis, low high-density lipoprotein (HDL) cholesterol and high triglycerides, and non-alcoholic fatty liver disease (NAFLD), among other changes. These pathologies are clustered together and are then referred to as metabolic syndrome (MetS) (8).

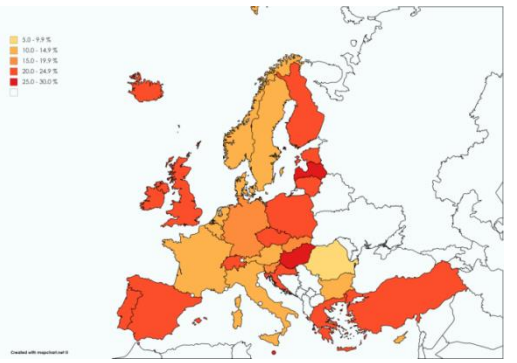


Figure 1: Percentage of population affected by obesity in the European Union countries in 2014. Data source (7).

Due to the global marked in which modern occidental societies are enmeshed, lifestyle changes have also affected the fruit intake patterns (9). In this sense, fruits were traditionally consumed during their natural harvest season. As a consequence, the consumption of a given fruit was limited to a certain period of the year and/or season. However, due to fruit importation, there is now commercial availability and consumption of a wide variety of fruits outside their traditional consumption season (9). For example, in the specific case of citrus fruits, although their consumption is reduced outside their traditional consumption season, it still occurs at considerable amounts (Figure 2) (10).

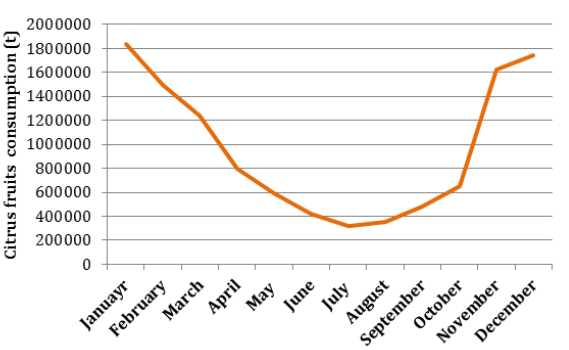


Figure 2: Consumption of citrus fruits (t) in the European Union countries in the year 2016. Data source (10)

Similarly, the consumption of fruits was traditionally restricted to the fruits produced in near regions to the production/harvest region. Now, however, there is commercial availability of fruits produced at far regions,

countries and even hemispheres of the consumption region (11,12). This is easily seen in the commercial production and importation of oranges within the EU-28 frame (Figure 3). In this sense, although Spain is the major producer of oranges in the EU-28, which represents around 55 % of all orange production in the EU-28, the EU-28 also imports substantial quantities of oranges from different countries, and these are mainly South Africa, Egypt, Morocco, Argentina and Uruguay (12).

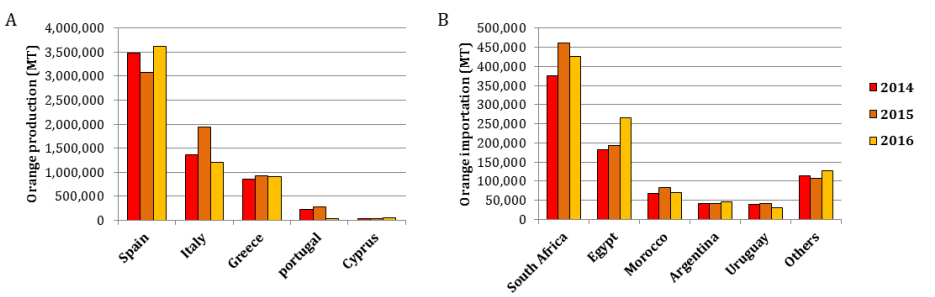


Figure 3: Production of oranges by the most relevant European Union producers (A) and import origin and amount in the European Union. Data source (12).

Moreover, fruits were typically produced without any chemical treatment of the plant (organically) (13). Currently, conventionally produced foods are more consumed over those produced organically (14–16). However, and although strict regulations on their cultivation (prohibited use of synthetic pesticides, growth hormones, antibiotics, modern genetic engineering techniques, chemical fertilizers, or sewage sludge), their production is currently increasing (17). In this regard, certain fruits are produced more commonly in organic systems than others. This is the case of grapes, which in 2015 represented a 21 % of the total hectares dedicated to organic crop production in the EU-28. In this sense, the amount of hectares dedicated to organic grape production is increasing fast in the EU-28, and especially in major production countries such as Spain, France and Italy (Figure 4). Precisely, in 2015, Spain was the country from the EU-28 with the most hectares dedicated to organic grape production (18).

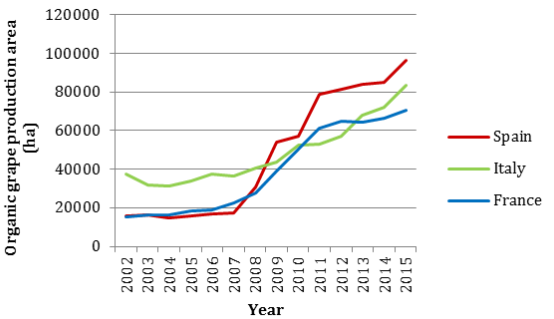


Figure 4: Increase of cultivation area designated to organic grape production in the principal production countries of the European Union. Data source (18).

In the year 2016, the consumption per capita of fresh fruits in the Spanish population was of 99.54 Kg/year and person. This consumption of fruits supposes a 9.23 % of the total domestic budget destined for food and beverages shopping, which represents a mean of 141.12 €/month and person (19). Although the consumption of fruits and vegetables has increased in the last decades, the consumption does not reach the WHO recommendations (1,3). The consumption of fruits is of interest due to their capacity to prevent and/or attenuate the development of chronic diseases such as diabetes, hypertension, obesity and MetS. This has been linked with the wide content of bioactive phytochemicals present in fruits, such as vitamins and polyphenols (20–22). As a matter of fact, many studies correlate the health associated effects of fruit consumption to their phenolic content (22–24). In this sense, phenolic compounds are plant secondary metabolites produced in response to environmental conditions such as light exposure, water and mineral availability, bacterial infection or injury by herbivores (25–29). Importantly, phenolic compounds are widely present in human diets. As a matter of fact, important human dietary sources of phenolic compounds include fruits and vegetables, cocoa, tea and wine (24,30).

2. Phenolic compounds:

Phenolic compounds are naturally-occurring compounds that, at least, present one aromatic ring with one or more hydroxyl group attached to

their main structure. According to their structure, they can be classified in two major families: the flavonoids and the non-flavonoids (24).

2.1. Flavonoids:

Flavonoids are 15-carbone structures composed by 2 aromatic rings connected by a 3-carbone bridge. According to their chemical modifications of the main C6-C3-C6 structure, flavonoids can be further divided into several subclasses. Flavanols, flavonols, flavanones, flavones, isoflavones and anthocyanidins are the most representative families. Other flavonoids subclasses found in minor quantities in dietary sources include chalcones, dihydrochalcones, dihydroflavonols, flavan-3,4-diols, coumarins and aurones (24). The main structures of each flavonoid families can be found in Figure 5. In the nature, flavonoids can be found in a wide range of dietary components and medicinal plants such as red wine, tea, fruits and vegetables (31). Moreover, their consumption has been associated with several health effects (Table 1). Typically, when discussed in the literature, flavonoids are the most described class of phenolic compounds since they account for approximately to two thirds of the dietary phenolic compounds (32).

Table 1: Flavonoid families and their main aglycone representatives, dietary sources and biological effects.

Flavonoids	Main aglycone representatives	Dietary sources	Bioactivity	Ref.
Flavanols	(+)-Catechin (-)-Epicatechin	Tea Wine Grapes Cocoa Apples	Anti-oxidant Anti-hypertensive Anti-diabetic Anti-inflammatory	(24,33)
Flavonols	Quercetin Kaempferol	Onions Grapes Apples Apricots	Anti-oxidant Anti-cancer Anti-diabetic Anti-inflammatory	(24,34–37)
Flavanones	Hesperetin Naringenin	Citrus fruits	Anti-oxidant Anti-inflammatory Anti-cancer Cardioprotective	(24,37–40)
Flavones	Apigenin	Celery Parsley	Anti-oxidant Anti-cancer Anti-inflammatory Cardioprotective	(24,41,42)
Isoflavones	Genistein Daidzin	Soy Soy-derived products	Anti-oxidant Anti-cancer	(24,43,44)
Anthocyanidins	Cyanidin Malvidin	Berries Red wine Cherries	Anti-oxidant Anti-obesity Anti-inflammatory	(24,45–48)

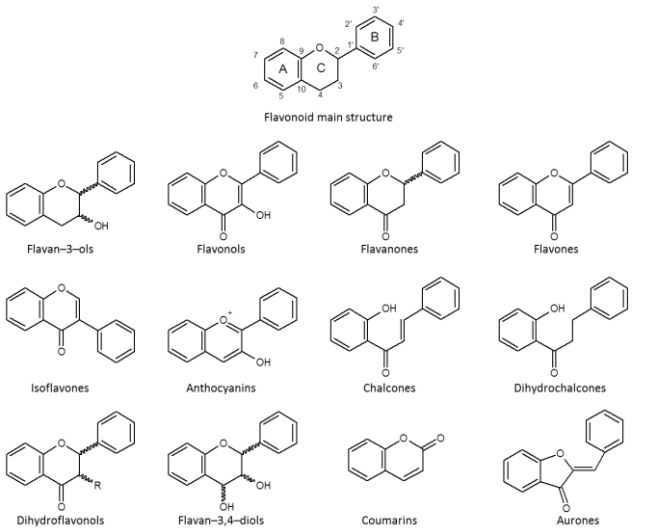


Figure 5: Flavonoid families and their basic chemical structures.

2.1.1. Flavanols:

Among the different flavanol representatives, (+)-catechin and (-)-epicatechin are the most widespread compounds in the nature (24). Unlike other flavonoids, flavanols are rarely conjugated with sugars (24,49). However, conjugation with gallic acid is much more frequent. Those forms are referred to as gallate flavanols, and include epicatechin gallate, catechin gallate, epigallocatechin gallate and gallocatechin gallate (24). Free flavanols occur in grapes, wine, apples, apricots, tea, cocoa and chocolate products and other food stuffs (36,50–54). It is worth to note that, in general, the main representative flavanols in fruits are catechin and epicatechin, whilst gallocatechin, epigallocatechin and epigallocatechin gallate are specially abundant in tea (49).

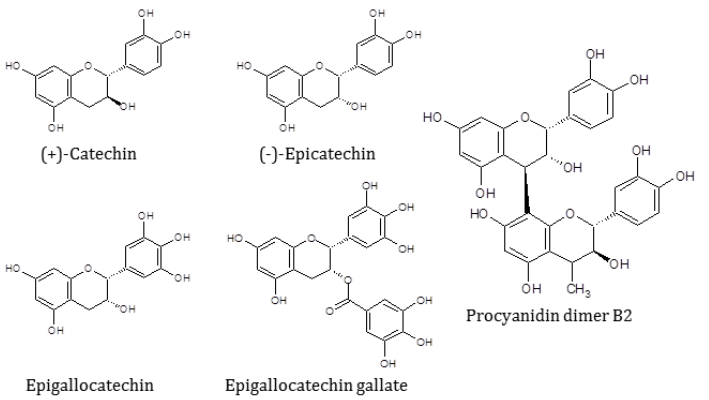


Figure 6: Chemical structures of relevant flavanol representatives.

Flavanol polymeric forms are very common in edible vegetal sources (24). Condensed tannins, also known as proanthocyanidins, are oligomeric and polymeric compounds consisting of coupled flavanol units (55). The number of monomeric constituents in proanthocyanidins can range from a few monomeric units up to 50 (24). Depending on the type of monomeric units, proanthocyanidins can be sub-classified into two different sub-groups. (56). Proanthocyanidins containing exclusively (epi)catechin units are referred as to procyanidins, and these are the most abundant class of proanthocyanidins (24). Some relevant oligomeric procyanidins in foods include procyanidin dimer B1, B2 and procyanidin trimer C1 (55,57). Dietary sources of proanthocyanidins include grapes, grape seeds and wines, cocoa and cocoa-derived products, apples, apricots and cherries, among other foodstuffs (51,52,58). The structure of the main representative of the flavanol family can be found in Figure 6.

2.1.2. Flavonols:

About 450 different flavonol aglycones have been reported in plants (59), but the most common ones are kaempferol and quercetin (Figure 7) (24). However, flavonols in plants usually occur as O-glycosides (60). In this sense, there exist more than 200 glycosyl derivates of kaempferol (24). Flavonols occur largely in the plant kingdom with the exception of fungi

and algae (24), and onions, tomatoes, apricots, grapes, cherries and berries are significant dietary sources of flavonols (36,37,58,61–63).

2.1.3. Flavanones:

Around 350 different flavanone aglycones have been found in the nature (59). Some representatives include hesperetin, naringenin, eriodictyol and isosakuranetin (64,65), and hesperetin and naringenin are the most widespread aglycones of all (Figure 7). However, the majority of flavanones occur as *O*-glycosides at C7 by rutinose or neohesperidose (59,66). Some representatives of this family include hesperidin (hesperetin 7-*O*-rutinoside), narirutin (naringenin 7-*O*-rutinoside) and naringin (naringenin 7-*O*-neohesperidoside) (65). In most of the plant kingdom, flavanones occur is small amounts compared with other flavonoids (66). An exception are citrus fruits, where flavanones are the typical flavonoid (39,64,65,67). Although several hundred milligrams per litre can be found in citrus juices, the highest concentration is found in solid tissues (39).

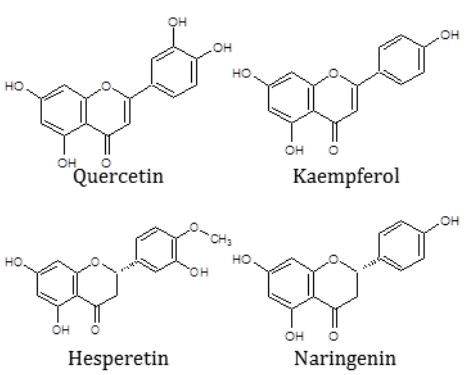
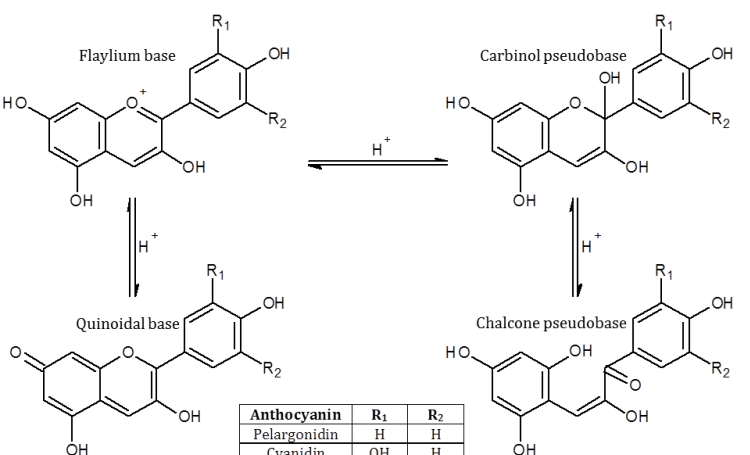


Figure 7: Chemical structures of relevant flavonols and flavanones.

2.1.4. Anthocyanidins:

Anthocyanidins are water-soluble pigments that give red, blue and purple colours to plant tissues (46). Cyanidin, malvidin, delphinidin, peonidin, petunidin and pelargonidin are the most commonly distributed anthocyanidins in the nature (45). These compounds exist in equilibrium

of four different species: the colourful predominant flavylium base, the quinoidal base, the carbinol pseudobase and the chalcone pseudobase (Figure 8) (68). This equilibrium is highly influenced by the pH at which anthocyanidins are exposed, switching the predominant form towards one or another form (69,70). In the nature, anthocyanidins normally occur as *O*-glycosides and are then called anthocyanins (71). Other chemical modifications such as the acylation with acetic and p-coumaric acid can also occur in anthocyanins (72). Anthocyanins are present in significant amounts in foods such as red grapes, red wine, cherries, berries and other food stuffs (63,73,74).



Anthocyanin	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

Figure 8: Chemical structure in equilibrium of relevant anthocyanidins.

2.2. Non-flavonoids:

Non-flavonoid phenolic compounds are all those structures that do not fit into the main flavonoid structure (Table 2). If classified depending on major structural characteristics, no-flavonoids can be divided into three major families: phenolic acids, that include hydroxybenzoic and hydroxycinnamic acids, stilbenes and lignans (75). Besides, another family can be included, which is the tyrosol group, mainly found in olive oil (75).

Table 2: Non-flavonoid families and their main representatives, dietary sources and biological effects.

No-flavonoids	Main representatives	Dietary sources	Bioactivity	Ref.
Hydroxybenzoic acids	Gallic acid	Wine Berries Tea	Anti-oxidant Anti-cancer Anti-diabetic Cardioprotective	(76–81)
Hydroxycinnamic acids	Chlorogenic acid Caffeic acid p-Coumaric acid	Coffee Stone fruits Grapes Spinaches	Anti-oxidant Anti-inflammatory Cardioprotective	(82–86)
Stilbenes	Resveratrol	Grapes Wine	Anti-oxidant Anti-cancer Anti-inflammatory Anti-diabetic Anti-obesity Cardioprotective	(87–89)
Lignans	Ringaresinol Lariciresinol Hydroxymatairesinol	Whole grains Flaxseed	Anti-oxidant Anti-cancer Anti-inflammatory	(44,90).
(Hydroxy)tyrosols	Tyrosol Hydroxytyrosol	Olives Olive oil	Anti-oxidant Anti-inflammatory Cardioprotective	(49,91,92)

2.2.1. Phenolic acids:

Phenolic acids are widespread in the nature (93). As a matter of fact, phenolic acids, either in free or bound forms, constitute around one-third of the dietary phenolic compounds (94). Dietary sources of phenolic acids include fruits such as apricots, cherries and peaches, as well as other vegetal sources such as eggplant, cereals and spinaches (94,95). Depending on their structure, phenolic acids can be sub-divided into two different categories: the derivates of benzoic acid and the derivates of cinnamic acid (Figure 9) (49).

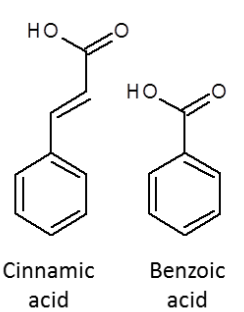


Figure 9: Basic chemical structure of cinnamic and benzoic acids.

2.2.1.1. Hydroxybenzoic acids:

The main representatives of hydroxybenzoic acids are gallic acid, *p*-hydroxybenzoic acid, protocatechuic acid, vanillic acid and syringic acid (94). Hydroxybenzoic acids can naturally occur as free compounds, bound to sugars and organic acids or bound cell wall components such as lignin. However, only a small fraction exist as free compounds (32). Indeed, hydroxybenzoic acids are mainly found glycosylated (96), being the only exception gallic acid (97). Instead, gallic acid is mainly found esterified with quinnic acid, flavanols or the products form their condensation (96). Hydroxybenzoic acids are found in very few dietary sources consumed by humans. Hence, their poor received nutritional interest (49). The content of hydroxybenzoic acids in edible plant sources is generally low with the exception of some red fruits, onions and teas where they are especially abundant (49,95).

2.2.1.2. Hydroxycinnamic acids:

Hydroxycinnamic acids are much more common than benzoic acids (42,98), and most their representatives derive from *p*-coumaric, caffeic or ferulic acids (96). Indeed, caffeic acid is the most abundant phenolic acid, representing between 75 % and 100 % of the total hydroxycinnamic acids in most fruits, whilst ferulic acid is the most abundant in cereals (42,49,95). However, free hydroxycinnamic acids are rarely found in dietary sources with the exception of foods that have undergone freezing, sterilisation or fermentation during their processing (95). In the nature, hydroxycinnamic occur mostly as simple esters with quinnic acid or glucose (96). The most common hydroxycinnamic acids are chlorogenic (3-caffeoylquinic acid) and neochlorogenic (5-caffeoylquinic acid) acids (96), and these are abundant in drupes (stone fruits) like cherries (63) and apricots (36) as well as coffee (99). Hydroxycinnamic acids can also be linked to flavonoid glycosides in the form of esters or glycosides (96). For

example, anthocyanins have been found to be linked to different hydroxycinnamic acids in grapes (100).

2.2.2. Stilbenes:

Structurally, stilbenes are phenolic compounds comprised by two aromatic rings linked by an ethene bridge (Figure 10) (73). The main representative is resveratrol, which exists in both *cis* and *trans* isomeric forms, and occurs mostly as glycosylated derivates (49). Unlike other phenolic compounds, stilbenes are not widespread in food plants (101). Significant sources of resveratrol include grapes and wine (89,102,103), and red wine is the most important dietary source of resveratrol (88). However, stilbenes occur in low concentrations in fruits and other foodstuffs (95,101).

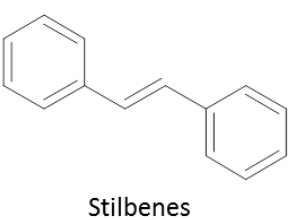


Figure 10: Basic chemical structure of stilbenes.

3. Seasonal fruits and phenolic compounds:

The presence or absence of specific genes involved in the biosynthesis of phenolic compounds allows each fruit to present a differentiated phenolic profile. Moreover, different varieties of the same fruit can present higher or lower expression rates of key genes involved in phenolic compounds biosynthesis, and this modulates the concentration of specific polyphenol representatives in fruit varieties (104–106). For example, while anthocyanin biosynthetic genes are present in red grapes and blood oranges, these genes are not expressed on their white and blond varieties (105). Thus, fruit variety is the major factor contributing into the variability of phenolic profile of a given fruit type. However, environmental

conditions also modulate the phenolic compounds of fruits. In this sense, water availability, soil mineral content, light exposure and temperature can modify the phenolic profile of fruits, and this is mainly mediated by modulating the expression of key biosynthetic genes (98,107–111). It has been reported that cultivation system (conventional vs organic) and geographical growing region can modulate the phenolic profile of several fruits (17,112,113).

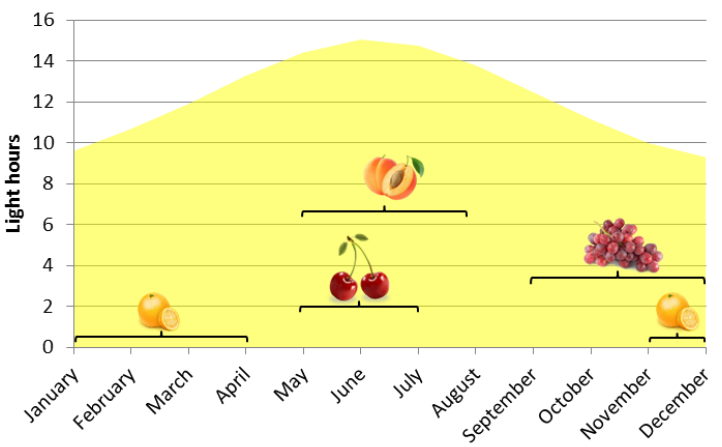


Figure 11: Amount of light hours a day within a year and traditional consumption season of apricots, cherries, grapes and oranges. Data source (114) and (115)

Fruits are typically produced in a certain season or time of the year (114,116–120). Thus, fruits are exposed to certain environmental conditions (i.e. light exposure). This seasonality in their production involved also a seasonality on their consumption. Therefore, the traditional consumption of fruit was marked by different day light (photoperiod) exposures (9). For example, apricots, cherries, grapes and oranges are fruits with a traditionally marked consumption seasonality and a high fresh consumption rate (19). In this sense, apricots are traditionally available between May and August; cherries between May and July; grapes between September and December; and oranges between November and April (Figure 11) (114). Thus, the consumption of phenolic compounds varied depending on the season of the year given the wide variability on the phenolic profile between fruits and vegetables (24,121).

For instances, apricots, sweet cherries, red grapes and sweet oranges present a different phenolic profile (Table 3). However, due to globalisation, there is now commercial availability fruits out of their traditional consumption season (9). Consequently, fruit consumption occurs out of their traditional consumption season. Other changes triggered by globalisation also include commercial availability of fruits produced in different regions, countries and even hemispheres, and a commercial predominance of fruits produced under controlled conditions and chemically treated with pesticides (9–12,14–16,18). As a result, the consumption of fruits produced at far geographical locations and a higher consumption of non-organic (conventional) fruits takes place in modern occidental societies (9,14,16).

Table 3: Presence (+) or absence (-) of phenolic families in apricots, sweet cherries, red grapes and sweet oranges.

	Apricot	Sweet cherry	Red grape	Sweet orange
Flavanols	+	+	+	–
Flavonols	+	+	+	–
Flavanones	–	–	–	+
Flavones	–	–	–	+*
Isoflavones	–	–	–	–
Anthocyanins	–	+	+	–
Phenolic acids	+	+	+	–
Stilbenes	–	–	+	–

* In peels.

3.1. Apricots:

Apricots are an edible fruit mainly cultivated in the Mediterranean area. Indeed, more than 80 % of the world's apricot production belong to a few countries, which include France, Iran, Italy, Pakistan, Turkey and Spain (123). Remarkably, apricots are one of the most important commercial crops (124). For example, the consumption *per capita* of apricots in the Spanish population was of 0.97 Kg/year and person in the year 2016 (19).

Apricots are a rich nutritional source, containing several minerals, organic acids, vitamins and phenolic compounds (125–131). Phenolics in apricots occur both in the pulp and the peel. However, peels present higher concentrations (130,132). Like cherries, grapes and oranges, apricots are amongst the richest dietary sources of phenolic compounds (30).

In more detail, apricots are characterised by the presence of both flavonoid and non-flavonoid phenolic compounds. Like in other stone fruits, hydroxycinnamic acids have been described to stand out (85). In this sense, chlorogenic acid and neochlorogenic acid have been reported as the most abundant hydroxycinnamic acids occurring in apricots (126,127). Also, other phenolic acids (i.e. gallic acid, caffeic acid and *p*-coumaric acid) have been reported in apricots (126). According to different studies in the literature, the flavonoid profile of apricots is mainly dominated by flavanols and flavonols (126,133). Among flavanols, catechin and epicatechin are described the most abundant representatives (50,126,134). Other flavanol derivates (i.e. epigallocatechin gallate or epigallocatechin) have been quantified in apricots, but at lower concentrations (134). Similarly, oligomeric procyanidins have also been detected in apricots though in small amounts (51,132). As for flavonols, apricots are reported to be rich in quercetin and kaempferol glycosides (36,134,135), and rutin and kaempferol glycosides have been found at abundant concentrations (126).

3.2. Cherries:

Cherries are one of the most consumed fresh fruits (136). For example, in Spain, the consumption *per capita* of cherries was 1.11 Kg/year and person in the year 2016 (19). Cherries can be classified as sweet or sour cherries, and while sour cherries are mostly used to produce jam, wine, juice, dried fruit candy or other processed products, sweet cherry varieties are mainly consumed fresh (136–138).

Cherries are known for their rich nutritional content and their wide range of bioactive compounds such as vitamins carotenes, melatonin and phenolic compounds (136,139–141). Indeed, cherries have been identified as one of the 100 richest dietary sources of phenolic compounds (30). The phenolic profile in sweet cherries is characterised by the presence of different anthocyanin, hydroxycinnamic acid, flavanol and flavonol representatives (48,142). Hydroxycinnamic acids are an abundant family of phenolics in cherries, and have been reported to represent between 32.65 and 57.67 % of all phenolics sweet cherries (48). Among them, neochlorogenic acid has been reported as the most abundant representative of all (143–145). Anthocyanins have also been identified as one of the most representative phenolic compounds in sweet cherries (48,63,139,146), and, among all representatives of this family, cyanidin 3-*O*-rutinoside has been reported to be the most abundant one (145,147). According to different studies in the literature, epicatechin is usually the most abundant flavanol in sweet cherries, followed by catechin (48,142,147–150). Other flavanols, such as procyanidin B2, and epicatechin gallate, have also been reported in sweet cherries (51,148). Flavonols in cherries have been reported to mainly occur as rutin (48,142,147,149,150), and other flavonols (i.e. other quercetin glycosides and kaempferol glycosides) can yield to significant amounts in several sweet cherry varieties (48,63,148).

3.3. Red Grapes:

Grapes are one of the essential foods of the Mediterranean diet, which has been associated with a reduced risk of chronic disease development (151,152). Grapes are a fruit highly consumed in Spain, and the consumption *per capita* of table grapes in the year 2016 was of 2.1 Kg/year and person (19). There is a wide variety of grape varieties, which include coloured and colourless representatives (153). Grapes can be divided into two major groups depending on their skin colour, and those are the coloured and the colorless grape varieties. Although varieties can

be further classified according to the colour of their skin (i.e. white, green, rosé pink, noir or purple) (154), in this thesis all coloured grapes will be referred as to red grapes.

Grapes are a rich source of bioactive compounds, which include phenolic compounds, vitamins, minerals and fibre (155–157). Indeed, red grapes have been identified as one of the richest dietary sources of phenolic compounds (30). Importantly, the total polyphenol content of red grapes varies depending on variety (52,156,158), and the main difference between red and white grapes is the presence of anthocyanins in the first (159–163). As a consequence, coloured varieties usually have a higher phenolic content than colourless varieties (160,162). The main phenolic compounds present in red grapes have been reported to be anthocyanins, flavanols, flavonols, hydroxycinnamic acids and stilbenes, and their distribution in grape parts (i.e. seeds, pulp and pel) is not homogeneous (Figure12) (73,161,164,165).

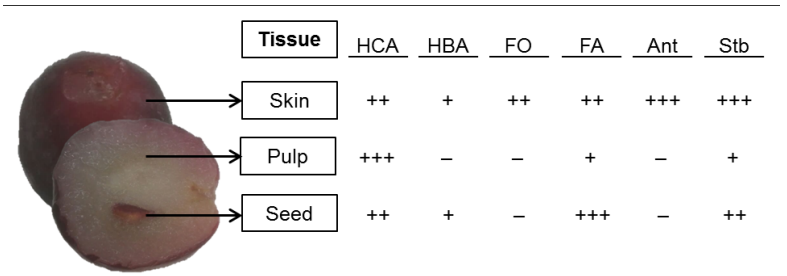


Figure 12: Phenolic families present in red grape tissues.
Abbreviations: HCA, hydroxycinnamic acids; HBA, Hydroxybenzoic acids, FO, flavonols; FA, flavanols; Ant, Anthocyanins; and Stb, stilbenes.

The most abundant anthocyanin in red grapes has been reported to depend on the grape variety, but most grape varieties present malvidin-based anthocyanins as the main anthocyanin (89,100,166–169). However, other anthocyanins as well as acylated anthocyanins have been found in red grapes (74,100,164,167,168,170). A multitude of flavanol representatives coexist in red grapes. Those include free monomeric,

gallate, oligomeric and polymeric flavanols (51,171,172). Free monomeric flavanols in red grapes are mainly catechin and epicatechin (58,173), and most varieties report catechin as the most abundant free flavanol (173–175). Epigallocatechin and epicatechin gallate have also been described in red grapes, but at much reduced concentrations (50,51,165,176). As for dimeric flavanols, procyanidin dimer B1 and B2, and other representatives such as procyanidin C1 have also been described in red grapes, and specially, on their seeds (51,176,177). Different studies corroborate that flavonol representatives in red grapes are mainly quercetin- and kaempferol-glycosyl derivates, and that rutin is the dominating representative (58,161,166,167,178). Phenolic acids have been found in abundant concentrations in red grapes in the form of hydroxycinnamic acids (52,164,179), and caffeoyl and coumaroyl tartaric ester derivates have been described as the most abundant representatives (52,166,180–182). Free phenolic acids have also been described in red grape varieties, and, among them, gallic acid stands out (89,156,165,175,183). Stilbenes occur mainly in grape skins as resveratrol glycosyl derivates, and their concentrations are low when considering the whole fruit (162,166,174,184,185).

3.4. Oranges:

Oranges are a very freshly consumed fruit in Spain. Out of the total consumption *per capita* of 99.54 Kg fresh fruit/year and person in the Spanish population in the year 2016, consumption of fresh oranges represented the 19.68 %, which corresponds to a consumption *per capita* of 19.59 Kg fresh oranges/year and person (19). Oranges can be classified into two main classes: Sweet oranges, which are the most widely grown and consumed orange type, and sour oranges, which is rarely used to produce foodstuffs rather than marmalade (186,187).

Flavanones have been described as the most abundant family of polyphenols in sweet oranges and their juices (188,189). Particularly,

different studies describe hesperidin as the most abundant phenolic compound occurring in sweet oranges. Other flavanones (i.e. narirutin) have also been described in sweet oranges and their juices (64,188–192). Besides flavanones, flavones have also been quantified in sweet oranges (189,193). However, flavones have been described at much more abundant concentrations in citrus peels (189,194,195). Indeed, different studies report flavones as abundant phenolic compounds of citrus peels (196,196,197).

3.5. Factors affecting phenolic composition in fruits:

Fruit phenolic content and profile is highly dependent on different parameters (Figure 13) such as fruit variety (104–106), type and origin of cultivation (62,198), environmental conditions (107–111), maturity/ripeness (112,126,143,199), and processing treatments (93,130,200).

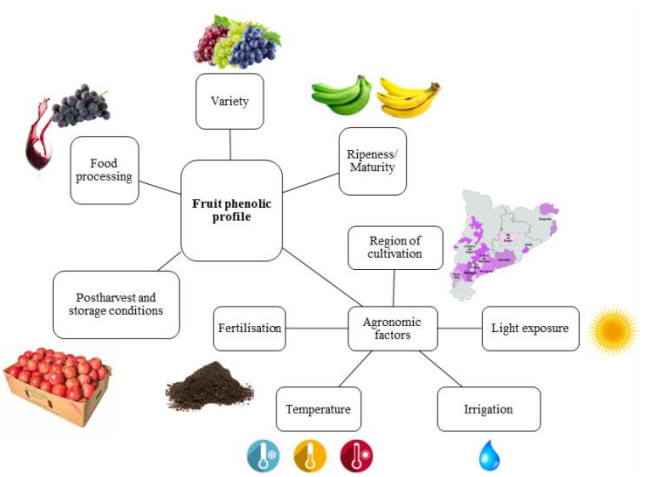


Figure 13: Principal factors affecting the phenolic profile of fruits.

Fruit type and variety mostly determine the phenolic profile of fruits. Importantly, changes in phenolic profile between varieties can be attributed to different expression rates of genes involved in phenolic biosynthesis. For example, the gene expression of several biosynthetic enzymes is different between varieties of grapes (105,106) and cherries

(104). Specifically, the white varieties of grapes do not express UFGT genes, directly involved in the biosynthesis of anthocyanins (105). Another factor that could cause differences in phenolic content depending on fruit variety is the enzyme itself, as flavonoid biosynthetic genes have shown diversity in their sequence depending on fruit variety (106,201).

Some differences in fruits' phenolic profile arise from agronomical factors. Indeed, phenolic compounds are stress metabolites, so many factors modulate their content in fruits (110). For example, soil fertilisation, irrigation, temperature, light exposure and growing latitude can modulate the phenolic profile of fruit and other vegetables, and these effects are mediated by the regulation of the expression of relevant genes involved in phenolic compounds' biosynthetic pathways (107–111). Given that light exposure, water availability, temperature and inorganic salts can modulate the phenolic composition of fruits (107–111), there exist some differences in the phenolic profiles of fruits grown in different countries and regions. This has been reported for favorita tomatoes (62), blueberries and strawberries (198), to cite some. There is scientific controversy to determine the effect of organic cultivation as some studies report higher polyphenol content in organic fruits whilst others do not draw consistent results (17). The results reported by Faller *et al*. (202) suggest that fruits have a higher susceptibility to the agricultural practice applied than any other plant tissue. In this sense, organic grapes reported higher phenolic concentrations than conventional grapes in an immature stage. However, those differences normalised at the ripening stage (112).

4. Extraction methods for fruit phenolics:

As previously evidenced, different factors affect the phenolic composition of fruits (98). Therefore, a correct characterisation of the phenolic profile of a fruit is essential in many cases, and these include the study the factors that affect the phenolic profile of fruits; the study of the relationship between fruit consumption and the associated health benefits; and the

study of the bioavailability and metabolism of fruit phenolic compounds. When profiling the phenolic content of a fruit or any other plant material the extraction step is extremely important, as its outcome will depend the release of analytes proper of vegetable matrix into the medium, which in turn will allow the quantitative determination of extracts (203). Extraction is a separation process where the distribution of an analyte, in this case a phenolic compound, between two immiscible phases is made in order to arrive at the appropriate distribution coefficient (204). The solvent extraction of an analyte from a plant material includes two steps: First, the solid phase swells due to the sorption of the solvent, which is caused by osmotic forces, capillarity and solvation of ions in the solid matrix. As a result, a portion of the solute contained in the damaged plant material is extracted by leaching. In addition, initially insoluble compounds may also be solubilised by hydrolytic processes. The second step includes the diffusion of solutes, first inside the plant matrix and then out the surrounding layer of the plant particles (205). There are many factors that can influence this process and, thus, modulate quantification of phenolic compounds from fruit (165,206). The most relevant factors include temperature, extraction solvent, liquid-to-solid ratio (LSR), extraction time, particle size, pH, number of extraction steps and food matrix (Figure 14).

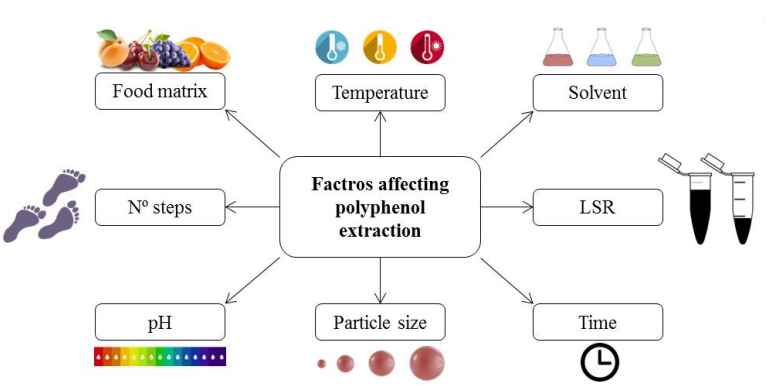


Figure 14: Principal factors affecting the extraction of phenolic compounds from fruits.

4.1. Factors affecting the extraction of phenolics in fruits:

4.1.1. Extraction temperature:

Extraction temperature can greatly modulate the extraction of phenolic compounds from fruit sources (206). Although rare, in some cases extraction temperature does not produce an increase in the extraction of phenolic compounds (207,208). Normally, an increase of temperature leads to a higher extraction of phenolic compounds (134,209). Indeed, an increase on the extraction temperature may cause an increase of phenolics' diffusion coefficient and a decrease in solvent's viscosity coefficient, which lead to an increase on phenolics' extraction (210,211). In addition, it can also decrease the interfacial tension in the pores of the fruits (210), further facilitating the extraction process. Nevertheless, too high extraction temperatures may lead to thermal degradation of phenolic compounds (212,213). It has been suggested that the main mechanism leading to the reduction in phenolic compounds involves a reduction in their stability due to chemical and enzymatic degradation and thermal losses (214). This is especially relevant in fruits with a high content in anthocyanins and/or flavonols, which are the most thermosenistive flavonoids (205,211,215). In addition, high temperatures result in increased solvent losses, which is not desirable in an industrial point of view (216,217). Thus, given the potential thermodegradation of phenolic compounds, many authors have fixed an upper temperature limit when developing their extraction methods (205,210,218).

4.1.2. Extraction solvent:

Type of solvent can have a great impact on the extraction of phenolic compounds from vegetal sources, including fruits. Different organic solvents can be used to extract phenolic compounds from fruits (94). Commonly used extraction solvents include methanol and ethanol (94,219,220). Normally, methanol achieves better extraction rates

(209,221,222). However, ethanol is used in the food industry due to methanol's toxicity (94).

Normally, pure organic solvents are not used as extraction solvents. Instead, water mixtures are preferred (94,219,220). In fact, the addition of water in extraction solvents promotes fruit particle's swelling. This increases the contact area between fruit particles and the extraction solvent, which allows the solvent to penetrate more easily into the food matrix, leading to increased phenolic extraction yields (209,221). The typical behaviour of phenolic compounds extracted under different proportions of water mixtures of organic compounds is characterised by an increase on their extraction as the proportion of the organic solvent increases until reaching a maximum at a certain proportion after which their extraction starts to decrease (216,223,224). However, optimal water organic mixtures depend on the phenolic being extracted and the food matrix. For example, the optimal extraction solvent for phenolics from sun-dried apricots was methanol 35 % (223), ethanol 51 % for sour cherries (224), and ethanol 74.63 % for *Phyllanthus emblica L*. bark (216).

4.1.3. Liquid-to-solid ratio:

The ratio between the extraction solvent and the plant matrix matter, defined as LSR, has a huge impact on the extractability of phenolic compounds (206). Nevertheless, this factor is usually overlooked since a wide range of LSR have been used in various plant matrixes (225). However, it has been reported that increasing the LSR increases the extraction yield of phenolic compounds (165,206). Remarkably, the driving force in the mass transfer process is believed to be the gradient concentration, which is greater under higher LSRs (211). Put simply, higher LSRs lead to larger concentration difference between the interior of cells and the solvent, which promotes faster extractions (209). In fact, high LSR enable more solvent to enter cells and more phenolic compounds can permeate into the solvent (226). On top of that, too low LSR are not

suitable as inhibition of enzymatic degradation of phenolic compounds may not occur (227).

Although sufficiently high LSRs must be reached to prevent solvent saturation (227), very diluted solutions of fruits may not lead to a sufficient increase in phenolics concentration and their extraction yield (209). For example, Li *et al*. (209) reported that after a SLR of 25 mL/g hesperidin, nobiletin and tangeretin did not increase their extraction yield in *Citrus reticulata* peels. On top of that, high LSR may lead to a reduction in the extraction of phenolic compounds due the solubilisation of polysaccharides and proteins, which hinders the dissolution of phenolic compounds (228). Moreover, working at a high LSR involves higher costs and production of solvent waste (165). Therefore, a compromise between working at a high and a low LSR must be made.

4.1.4. Extraction time:

Some phenolic compounds are bound to cellular structures through hydrophobic interactions and hydrogen bonds (165). Exposure to optimal extraction solvent and temperature during a certain period of time weakens interactions between cell walls and polyphenols, leading to more polyphenols transferring into the solvent and an increase on their extraction yield (216,222). Indeed, extraction time can significantly increase the extraction rate of phenolic compounds in fruits and other plant materials (211,224,229). In addition, the selection of an adequate extraction time is an important issue when considering efficiency, labour and cost (224). In this sense, a large extraction time increases the solvent loss by evaporation (217) and may even lead to degradation of those phenolic compounds that are easily solubilised in the extraction solvent (212).

4.1.5. Multistep:

Some extraction methods include more than one extraction step (230,231). As a matter of fact, increasing the number of extraction steps can lead to a greater extraction of phenolic compounds (216,217). For example, Chirinos *et al*. (231) only extracted 69.5 % of the total phenolic content from *Tropaeolum tuberosum* in one extraction step whilst they reached 98.1 % in the third extraction step. However, increasing the number of extractions does not always lead to an increase in the extraction of phenolics compounds (206,227). For example, in grape seeds and pulp a single extraction extracted the same flavanol content as a four multi-step extraction (227).

4.2. Strategies to optimise an extraction method:

The complete characterisation of the phenolic profile of a fruit is a pre-requisite to understand the bioavailability and metabolism of fruit phenolic compounds. Moreover, this is also essential to stablish clear relationships between their consumption and the health associated effects of their consumption. As previously reviewed, many factors can affect the extraction of phenolic compounds. Importantly, only those compounds that have been extracted from the food matrix will be detectable and, thus, quantifiable. Therefore, polyphenol extraction from fruits is the main limiting factor for a proper characterisation of the fruit phenolic profile. Hence, extractions must be performed using the proper extraction conditions (i.e. solvent and temperature). In this sense, and given the wide range of food matrixes and polarities of phenolic compounds, it is difficult to develop a universal extraction method for all food matrixes. As a consequence of this diversity, specific and optimised extraction methods must be developed to completely extract and fully characterise the phenolic profile of each specific fruit (204).

Optimising means to improve the performance of a system, process or product to obtain a maximum benefit from it. In analytical chemistry, the

term *optimisation* has been widely used as a means of discovering a set of conditions that result in the best possible response (203). It is important to improve the performance of a system as well as to increase the yield of processes whilst not increasing costs (232), which is what optimisation studies target. As for the extraction of phenolic compounds from food matrixes, there are two strategies that can be followed for their optimisation: the classical optimisation studies or the extraction optimisation by Response Surface Methodology (RSM).

4.2.1. Classical optimisation:

Classical optimisation studies vary one independent factor at a time whilst the rest are kept constant. This type of approach is called one-variable-at-a-time and results time-consuming and expensive. Moreover, the interactions between independent variables cannot be evaluated, which may result in drawing misleading conclusions (205). Currently, this type of approach is used to evaluate whether an independent parameter influences the extraction of phenolic compounds or to narrow its range in the RSM design (207,208,216).

4.2.2. Response Surface Methodology:

RSM is a set of statistical and mathematical techniques useful for optimising, improving or developing a process in which a targeted response, influenced by several independent variables, is to be optimised (233). This methodology is based on the fit of a polynomial equation to the experimental data which must describe the behaviour of a data set with the objective of making statistical predictions (203). Indeed, RSM defines the effect of independent variables, alone or in combination, in a process and generates a mathematical model which describes it (233). Being developed during the 50s, the term RSM originated from the graphical perspective generated after fitness of the mathematical model (203). Put simply, RSM is used to design statistical experiments, model processes, verify the statistical significance of the independent variables and to obtain

the optimal conditions of an entire process (223). This type of approach has been very popular in the recent years for several optimisation studies (232). In the field of phenolic compounds, it has been used for the optimisation of their extraction in several fruit matrixes (211,223,224,234) and other vegetable sources (205,216,235,236).

4.2.2.1. Stages for RSM optimisation:

The application of RSM to optimise a process includes 3 different stages (223,232). The first stage involves the selection of the independent variables and their experimental range. Chemical processes are influenced by many factors and it is not possible to identify them all (232). Therefore, it is necessary to identify the ones presenting a major effect on the response variable (203). To do so, screening studies involving the one-variable-at-a-time approach are usually used (207,208,216,232). When optimising the extraction of phenolic compounds from vegetable sources, representative independent variables include extraction temperature, solvent concentration, extraction time, LSR, particle size and pH among others (205,211,213,222,223,237–241). Once identified, the levels of the independent variables must be selected very carefully as the selection of unsuitable levels lead to unsuccessful optimisation (232).

The second stage involves the selection of the experimental design and performing it (232). The different designs available differ from one another in number of experimental points, and number of runs and blocks (203,232). In this sense, central composite designs are widely used to optimise the extraction of phenolic compounds from vegetal sources (207,211,223,224,234,242,243). Coding the variables is also a key aspect during design selection (203,232). The units of the independent variables usually differ from one another, and even when some of them share the same units, they are not tested over the same experimental range. As a consequence, regression analysis should not be performed. To solve this drawback, variables must be normalise before performing the regression

analysis. Thus, each variable is forced to range from -1 to +1 so they all affect the response variable more evenly and the units of the parameter become irrelevant (232).

The third stage involves the mathematical and statistical treatment of the data. To do so, it is necessary to fit a mathematical equation which describes the behaviour of the response according to the different studied levels (203). Then, the response surface and contour plots of the responses as a function of the independent variables and the determination of the optimal points are generated (232). The optimal point is the set of independent variable values at which the dependent values reach their maximum (203,232). Then, experimental confirmation of the predicted response follows (205,223).

5. Bioactivity of phenolic compounds and health effects associated with fruit consumption:

As previously denoted, fruits are a rich source of bioactive molecules, and this includes fibre, minerals, vitamins and phytochemicals such as phenolic compounds (130,139,156,244). Importantly, fruit consumption has been linked with a reduction of the development of chronic diseases such as diabetes, hypertension, and other pathologies associated with obesity and MetS (20–22). Some authors attribute these beneficial effects to the high phenolic content found in fruits (22–24). Indeed, phenolic compound have reported a wide range of biological functions (33,38,245). There is a consistent evidence on the relationship between polyphenol chemical structure and their bioactive effects (246,247). Thus, the consumption of different phenolic compounds, extracts or foods with significantly different phenolic profiles involves different bioactivities. In this sense, the administration of phenolic extracts has reported a wide range of bioactive functions in *in vitro* studies, animal models and humans (24,33,248). For example, apricot, cherry and orange phenolic extracts have been shown potent *in vitro* anti-oxidant activity (127,223,249,250). In the same line,

cherry polyphenols showed a protective effect on neuronal PC12 cells against cell-damaging oxidative stress (63). Grape polyphenol extracts have reported antioxidant and anti-inflammatory functions *in vitro* and *in vivo* animal and human studies (251–255), antihypertensive effects in humans (256) and rats (257,258), reduction of other cardiovascular risk factors such as low density lipoproteins (LDL) and triglycerides (248,259), effects on glucose metabolism in humans and animal models (260), and modulation of key genes involved in the circadian rhythm control (261–263), among other effects (33). Orange polyphenol extracts have reported endothelial-dependent vascular relaxing effects in aortic rings from spontaneously hypertensive rats (SHR) (264), and the potential of hesperidin to prevent cancer and cardiovascular diseases has been widely reported (38).

However, dietary guidelines recommend the consumption of whole fruits (1), and whole fruit consumption has also been linked with several health effects in animal models and humans. For example, in rats, apricot consumption has been reported to protect against alcohol-induced liver injury and oxidative and has been linked to cardio-protective effects after ischemia-reperfusion procedure (124,265). Similarly, its consumption has also been found to protect against cancer treatment associated oxidative stress (266–268). In rats, it has been demonstrated that cherry consumption reduces body weight and abdominal adiposity, regulates gene transcription in adiposites and reduces inflammation in rats consuming a high fat diet (269). In humans with recurrent gout attacks, cherry intake has been associated with a 35 % lower risk of gout attacks (270). In old adults (69 ± 4 years), cherry juice consumption has proven effective in improving antioxidant defences by increasing the capacity to resist an oxidative damage (271), whilst in early hypertensive men (systolic blood pressure= 137 ± 11 mmHg), cherry juice consumption led to a reduction in systolic blood pressure (272). Organic and conventional grape juice consumption has demonstrated neuroprotective, anti-

convulsant and anti-oxidant effects in rat animal models (273,274). Consumption of whole grape powder reduces low-grade inflammation and protects against retinal injury by oxidative stress in mice (275,276) and has demonstrated anti-coagulant functions in humans (277). In humans, red grape consumption has reported relevant ant-oxidant and hypo-lipidemic effects (278). As for orange bioactivity, a lot of attention has been focused on their effects on cardiovascular disease. Indeed, orange juice consumption has been reported to produce anti-inflammatory, anti-oxidant and blood pressure lowering effects as well as improve lipid profile and endothelial function (279). For example, consumption of 500 mL twice a day of commercial orange juice over a four-week period resulted in a reduction of over 5 % of both systolic and diastolic blood pressure in healthy humans (280). Male and female hypercholesterolemic (>230 mg/dL) patients consuming 270 mg of citrus flavonoids and 30 mg tocotrienols a day over a period of 4 weeks reported a reduction in between 20 and 30 % of the LDL blood levels and a reduction in between 24 and 34 % of the triglyceride blood levels (281). Also in hypercholesterolemic humans, consumption of 750 mL orange juice/day for a period of 4 weeks produced a 21 % increase in the HDL blood values and a reduction of 16 % on the LDL/HDL blood ratio (282). In addition, blood orange juice consumption (500 mL over two weeks with a 3-day wash period) promoted an improvement of endothelial function as well as an amelioration in the levels of inflammatory markers (283). Remarkably, some of these effects of orange juice are mediated by hesperidin (23,284).

6. Bioavailability of phenolic compounds:

As evidenced before, fruit consumption involves the promotion of health effects, and these are partially mediated by the phenolic compounds present in fruits (22). However, different authors highlight that the real bioactive molecules are the phenolic metabolites rather than the natural occurring phenolic compounds in fruits (248,285–289). Thus, the study of

polyphenol bioavailability and metabolism is of key relevance to understand the biological functions associated with fruit consumption.

In general terms, bioavailability can be defined as the fraction of a nutrient or non-nutrient that is available for the host for physiological functions and/or storage. In the specific case of phenolic compounds bioavailability involves the release of the phenolic compounds from the food matrix, chemical modifications during digestion, absorption by enterocytes, microbial metabolism of non-absorbed compounds and those compounds undergoing enterohepatic recirculation, phase-II metabolism, transport through the blood and body distribution of the generated compounds, and excretion via the kidney or the faeces. In this sense, polyphenol bioavailability and metabolism are the main limiting factors for polyphenol bioactivity (290).

6.1. Digestion, absorption, distribution, metabolism and excretion of phenolic compounds:

The digestion, absorption, distribution, metabolism and excretion of phenolic compounds is complex, and a simplified representation of these processes can be found in Figure 15. The first step relevant in the absorption, metabolism, distribution and excretion (ADME) of phenolic compounds starts in the mouth. There, saliva α-amylase is able to hydrolyse glycosides into their aglycone counterparts. However, given the low contact time, the impact of this digestion step is low (290). Remarkably, different *in vitro* simulations of polyphenol digestion avoid this step (291–293). Nevertheless, particle reduction of the food matrix takes place in the oral cavity, and this allows a better enzymatic accessibility in other digestion stages (290).

Phenolic compounds are mostly released from the food matrix in the stomachal digestion step. In this sense, pepsin activity along with reduced pH can further reduce food matrix particle size. Moreover, low pH, which

usually is between 2 – 4, enhances the diffusion of phenolic compound from the food matrix to the aqueous phase due to reduced ionic interactions (290). The chemical environment at which phenolic compounds are exposed in the stomach is harsh. This is not suitable for most of phenolic compounds, with the exception of anthocyanins, which are stable at acidic pH (294,295). Although the intestine is the principal absorption site for phenolic compounds (296,297), some phenolic families can be absorbed at a stomachal level. This is the case of anthocyanins and phenolic acids like caffeic acid (298,299).

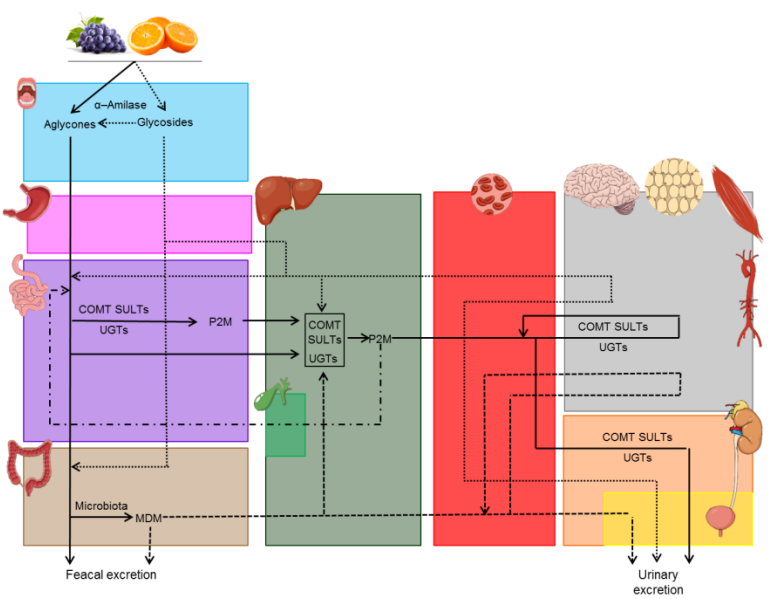


Figure 15: Schematic representation of the digestion, absorption, distribution, metabolism and excretion of phenolic compounds.
Abbreviations: COMT, catechol-*O*-methyltransferases; SULTs, sulfotransferases; UGTs, UDP-glucuronosyltransferase; P2M, phase-II metabolites; and MDM, microbial-derived metabolites.

In the small intestine pH increases up to 7, which allows the activation of pancreatic and biliary enzymes (290). This increase in pH is especially relevant for anthocyanins, which shift to chalcone pseudobase structures (69,70). Precisely, different studies show anthocyanin degradation under intestinal conditions (68,295,300). During digestion, micelles containing apolar phenolic compounds are formed, which affect their bioavailability (301).

Most phenolic compounds, with the exception of aglycones, need to be cleaved from their sugar moiety by brush-border enzymes (i.e. lactase-phlorizin hydrolase) to be absorbed. Normally, only when pharmacological doses are achieved can phenolic compounds be absorbed as glycosides (290). However, some phenolic compounds can be absorbed in their native from, which is the case of hesperidin (302,303). Moreover, anthocyanidins can also reach the bloodstream when absorbed via the stomach (295). Absorption of phenolic compounds can be via facilitated transport, active transport or passive diffusion (290,304). For example, quercetin glycosides can enter enterocytes though active transport mediated by sodium-glucose-linked transporter 1 (SGLT1) (303). Nevertheless, phenolic compounds are mostly transported by passive diffusion (304,305).

Once absorbed, phenolic compounds are recognised as xenobiotics and therefore are subjected to phase-II metabolism. Phenolic phase-II metabolism is mainly mediated by catechol-*O*-methyltransferases (COMTs), UDP-glucuronosyltransferase (UGTs) and sulfotransferases (SULTs), which respectively generate methylated, glucuronidated or sulphated phenolic derivates (286). The intestine is the first organ where phenolic compounds are subjected to those detoxifying reactions (306). The main phase-II reaction occurring in the small intestine is glucuronidation. As a matter of fact, the small intestine of several mammal species has been reported to widely express different UGT isoenzymes (307–309). In this sense, the small intestine has been described as primary source of glucuronidation for catechin (310) as well as other flavonoids such as kaempferol, quercetin, hesperetin and pelargonidin (311,312). Whilst methylation is also relevant in the intestine, sulfuration plays a poor role in the phase-II detoxification of phenolic compounds at an intestinal level. However, the capacity to metabolise phenolic compounds of the intestine is not unlimited, so high phenolic doses can lead to the lack

of glucuronidation, methylation and sulfation of phenolic compounds, and this effect arises from phase-II enzymatic systems saturation (313).

Phenolic metabolites are directed to the liver via the portal vein (314). The liver is a key organ in the phase-II metabolism of phenolic compounds. Indeed, liver produces a wide variety of phase-II enzymes in large quantities (315–317). After being metabolised by the liver, metabolites reach the systemic circulation. Several phase-II metabolites can co-exist in the plasma, including singly-conjugated metabolites like catechin-glucuronide, and multiply-conjugated metabolites, such as methylquercetin diglucuronide (318–320). However, the major phase-II metabolite occurring in plasma differs from flavonoid family to flavonoid family. For example, quercetine-3'-sulfate has been reported as the major plasmatic phase-II metabolite of quercetin in humans (318) whilst catechin-glucuronide and epicatechin-glucuronide have been reported as the major plasmatic phase-II metabolites of catechin and epicatechin in humans (320) and rats (321,322). Generally, maximum concentrations of phase-II metabolites in plasma are reached in between 1 and 4 hours after the ingestion of phenolic compounds (318,322,323). For example, the maximum plasma concentration for flavanol phase-II metabolites has been found between 1 – 2 hours after their ingestion in rats and humans (319,324). However, for some compounds, maximum plasmatic concentrations are reached at later or sooner times. For example, hesperidin reached maximum concentrations 5 hours after the consumption of hesperidin-rich foodstuffs (325).

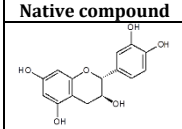
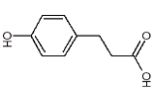
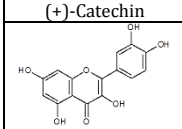
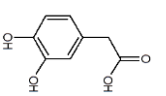
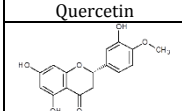
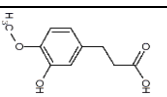
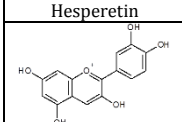
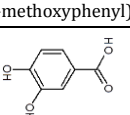
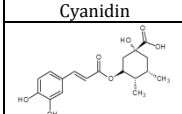
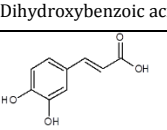
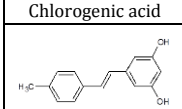
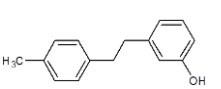
Once in the plasma, circulating flavonoid metabolites can reach different tissues and organs, including the aorta, brain, lungs, muscles, spleen, perirenal adipose tissue, and testicles, among others (313,321,326,327). For example, pelargonidin glucuronides have been found in brain and lungs of rats (312), methyl-, sulfo- and glucurono-metabolites of catechin have been found in placental homogenates from pregnant rats (328), and

methyl-metabolites of quercetin have been found in pig muscles, lungs and lymph nodes (329). Several studies have demonstrated that the profile of metabolites found in the plasma does not correspond to the one found in other tissues (327,330). Indeed, deglucuronidation at a vascular level can occur, releasing free flavonoids into target tissues (331,332). In addition, several organs and tissues can further metabolise phenolic compounds as their express several phase-II enzymes (315,317,333). It is important to highlight that phenolic compounds are not completely directed to the systemic circulation after undergoing detoxification metabolism in the liver. As a matter of fact, a small fraction of phenolic metabolites can be redirected to the small intestine though bile secretion. This rout is known as enterohepatic circulation of phenolic compounds, and although minority, it can contribute to phenolic compound bioavailability and metabolism (286,334).

Intestinal and hepatic absorption and metabolism of phenolic compounds is not the only way by which phenolic compounds are bioavailable. Indeed, it is estimated that 90 – 95 % of dietary phenolic compounds are not absorbed in the small intestine and reach the colon (286). There, phenolic compounds can be metabolised by the gut microbiota, which produces smaller molecules such as phenolic acids or valerolactones (335,336). Importantly, depending on the structure of the phenolic compound different microbial metabolites can be generated (Table 4) (337). For example, 3-(3'-hydroxy-4'-metoxy-phenyl)propionic acid is one of the major microbial-derived metabolites of hesperetin (336) and 2-(3,4-dihydroxyphenyl)acetic acid from rutin (338). Hot's microbiota diversity also plays an important role on the type and amount of microbial-derived metabolites (339). For example, healthy and obese animals, which are known to present differences in their microbiota composition and diversity (340), have reported different microbial-derived metabolite's profile after the ingestion of grape seed polyphenols (322). The generated compounds can be absorbed *in situ* and reach the liver though the portal

vein (286). Thereafter, microbial metabolites can reach different organs and tissues such as the plasma, brain and aorta (321,335,341). Unlike phase-II metabolites, microbial-derived metabolites appear in plasma at latter time points (319,323). For example, maximum concentrations of hippuric acid are found in plasma 7 hours after the consumption of grape seed flavanols in rats (335). Indeed, *in vitro* studies evaluating microbial transformation of phenolic compounds report long times for the production of these phenolic acid metabolites (336,338).

Table 4: Principal phenolic representatives and their main microbial-derived metabolites.

Native compound	Main microbial metabolite	References
 (+)-Catechin	 3-(4-Hydroxyphenyl)propionic acid	(335)
 Quercetin	 2-(3,4-Dihydroxyphenyl)acetic acid	(338)
 Hesperetin	 3-(3-Hydroxy-4-methoxyphenyl)propionic acid	(336)
 Cyanidin	 3,4-Dihydroxybenzoic acid	(342)
 Chlorogenic acid	 Caffeic acid	(343)
 *trans*-Resveratrol	 Lunularin	(344)

Both phase-II and microbial-derived metabolites reach the kidneys (313,326), where they will be eliminated via the urine (320,324). Indeed, plasma clearance of phenolic metabolites is fast. Precisely, within 24 hours, plasma is usually clear of phase-II metabolites, while microbial-derived metabolites can still be present (318,323,335,345–347).

Notoriously, chronic polyphenol supplementation studies report a lack of tissue accumulation of phenolic compound metabolites (348).

6.2. Factors affecting phenolic compound bioavailability and metabolism:

Several studies suggest that phenolic metabolites rather than the natural-occurring forms hold the key of phenolic compound bioactivity (248,285–289). Thus, all those factors that can modulate the bioavailability and metabolism of phenolic compounds could involve substantial changes in the health associated effects with fruit consumption, as previously evidenced for grape seed flavanols in hypertensive and normotensive rats (321). In this sense, many factors can affect polyphenol bioavailability and metabolism. Those include external factors, food processing factors, food factors, interactions with other compounds, polyphenol-related factors and host-related factors (Table 5) (349).

Table 5: Main factors affecting polyphenol bioavailability and metabolism.

External factors	Food availability, culture, fruit maturity…
Food processing factors	Thermal treatment, cooking, storage…
Food factors	Food matrix content in fibre, fat, protein…
Interaction with other compounds	Polyphenols with similar absorption mechanisms
Polyphenol factors	Structure, concentration in food, dose…
Host factors	Age, gender, health status…

Adapted from (349).

Different external factors (i.e. environmental factors) and food processing (i.e. storage) can modulate the phenolic profile and content of fruits as previously stated, and this subsequently affects the bioavailability and metabolism in humans (349). Food matrix can also play an important role in polyphenol bioavailability, and this includes micro and macro constituents of the food matrix (i.e. fibre, fat or minerals), food matrix processing (i.e. drying or encapsulation) as well as food matrix consistency (liquid or solid) (290). In this sense, phenolic compounds can be linked to fibre naturally or under gastrointestinal conditions, and this prevents the release of phenolic compound form the food matrix, hence modifying their

bioavailability (350). Dietary fat modulates polyphenol bioavailability by increasing passage intestinal time (290). For example, flavanone plasma behaviour was different depending on administration as juice or co-administered with fat-rich yogurt (325). Catechin, resveratrol and quercetin presented different serum kinetic profile depending on administration in water (juice), ethanol (wine) or vegetal homogenate (351). The bioavailability of phenolic compounds can also be conditioned by the interaction with other food matrix components than fibre and proteins. For example, transport competition between phenolic compounds with similar transport mechanisms can occur and modulate the bioavailability and metabolism of phenolic compounds (349). Polyphenol structure can have an important impact on their own bioavailability and metabolism (290). For example, due to their structure, anthocyanins can survive the acidic stomachal conditions (300), which affect their bioavailability. Precisely, and unlike other phenolic families, anthocyanins can be absorbed in the stomach (299). Moreover, the maximum plasma concentration after the ingestion of anthocyanins, flavanols or flavanones significantly differs (319,325,352). Oligomeric procyanidins such as procyanidin B2 are considerably less bioavailable that their free flavanol counterparts (321).

Generally, any condition that modifies the physiology of an organism can have an impact on polyphenol bioavailability and metabolism (349), and this might involve relevant changes in their bioactivity. Age (319), gender (330), and health status (321,322) are among the host-related factors that modulate phenolic compound bioavailability and metabolism. Under those conditions, relevant physiological changes occur. For example, obesity can produce important changes in gut microbiota (353), ageing produces a decrease on gastric motility (354), and sexual hormones such as estradiol can modulate the activity and expression of phase-II enzymes (333). The specific case of obesity is of key relevance. In this sense, in modern occidental societies the consumption of high-fat and high-sugar diets has

promoted the increase of metabolic diseases such as obesity and MetS (3,4). Importantly, under these altered homeostatic conditions, different physiological changes occur. For example, obese people usually develop MetS, which clusters a wide range of physiological alterations (Figure 16) (8). Importantly, many of the physiological changes triggered by MetS can modulate polyphenol bioavailability and metabolism. In this sense, we previously demonstrated that the development of obesity and MetS by the consumption of cafeteria diet has a huge impact on the bioavailability and metabolism of grape seed flavanols in rats (322).

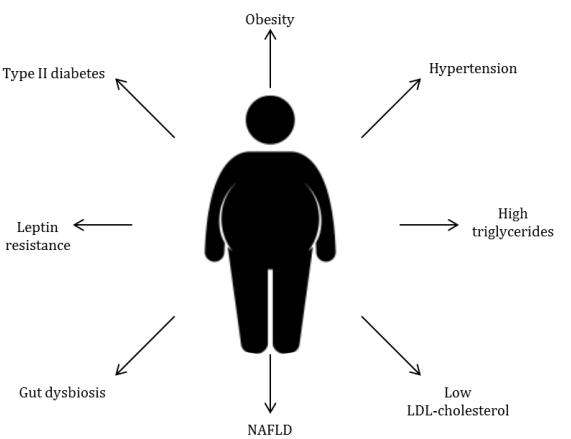


Figure 16: Hallmarks of metabolic syndrome.

6.3. Biological rhythms and polyphenols:

Almost all light-sensitive organism, including humans, display physiological changes in cycles of approximately 24 hours. These cycles are known as circadian rhythms and include the anticipation of food availability and predator avoidance (355). These biological oscillations in physiology are regulated by external environmental cues named zeitgebers, and light is the most important one of all (262). The purpose of these circadian rhythms is to optimise the metabolism and energy utilisation to sustain life, and, thus, almost any physiological parameter is influenced to a greater or lower extent by circadian rhythms (355–357). In mammals, the circadian control system is structured hierarchically, and

the master regulatory system is placed the suprachiasmatic nucleus (SCN), which is part of the hypothalamus. However, SCN is ultimately regulated by light/dark signals (358). In this sense, it is like humans had a central clock system that tells them the time of the day in which they are. Remarkably, SCN is the only region in the body able to integrate light signals, and thus is considered the central and master clock (359). Molecularly, circadian rhythmicity in physiology and behaviour is regulated by a set of genes ultimately regulated by light signals which are referred as to clock genes (356).

Besides circadian rhythms, mammals also show cyclic rhythms that approximate to a year, and these are known as circannual rhythms. Evident circannual rhythms include the migration of birds (360) and the hibernation (361) and the seasonality in reproductive traits (362) of some mammals. In the specific case of humans, since the beginning of the last century seasonal affective disorder (SAD) has been known. This disorder is characterised by regular winter depression alternated with summer hypomania. During winter, humans affected by this syndrome feel depressed and usually sleep and eat in excess and crave for carbohydrates. Importantly, day light treatments produce an anti-depressive effect in these individuals (363). Also, SAD is positively correlated with latitude, and so, in countries with a lower light exposure during winter, SAD is more prevalent (364). In this sense, day length (photoperiod) significantly contributes to seasonal patterns, but nutritional and temperature patterns also play a role (365). Indeed, organisms that live in high latitudes experience relevant changes in temperature and photoperiod within a year, and these seasonal changes play a key role in the regulation of different body functions, including reproduction, metabolism and immune response. The adaptation of these environmental changes involves intrinsic mechanism, which are called circannual clocks (366).

Circannual clocks are controlled by key genes of the circadian clock, namely circadian locomotor output cycles kaput (CLOCK), brain and muscle ARNT-like protein 1 (BMAL1), transcription of the period (Per) 1 and2 and cryptochrome (CRY) 1 and 2 genes. In peripheral organs, circannual rhythmicity is not self-sustained and requires maintenance by photoperiod signals. These signals are recognised in the retina and transmitted to the pineal gland by multi-synaptic neuronal pathways. The pineal gland integrates these photoperiod signals and regulates the production of melatonin, which is rhythmically produced at night (366). Thus, melatonin signals encode night length. Importantly, melatonin can regulate many physiological factors in humans, and those include immunity, metabolism and body temperature. Thus, melatonin controls the rhythmicity and seasonality of body physiology (Figure 17) (357).

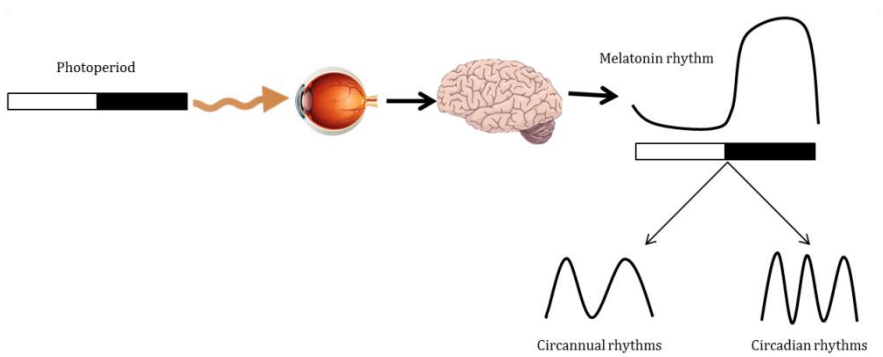


Figure 17: Schematic representation of the regulation of photoperiod on circannual and circadian rhythms.

Similarly to circadian rhythms, circannual rhythms also condition human physiology (367). Precisely, relevant physiological changes occur due to photoperiod exposure (Table 6). For example, blood pressure and body fat are higher in the low-light exposure months when compared with high-light exposure months (368). Moreover, blood lipids (i.e. total cholesterol, LDL-cholesterol and triglycerides) are increased in the winter (369). In addition, antioxidant defences are reduced in the winter (370). Also, and besides SAD, human behaviour also presents seasonal variations. For example, physical activity is reduced in low-light exposure months (371).

All these changes involve a higher risk to develop diseases in low-light exposure months, which is particularly evident for cardiovascular risk (CVR) and mortality. In this sense, deaths due to cardiovascular disease (CVD) increase in low-light exposure months such as November (368). There are also annual variations in human's physiology that can potentially affect the bioavailability and metabolism of phenolic compounds. For instances, photoperiod-associated changes in gut microbiota composition have been shown in different animals and humans (372–374). Kidney glomerular filtration rate, estradiol plasma levels and metabolic rate also present circannual variations (375–377). Moreover, the consumption nutrients, such as fat or protein (378), and the caloric intake also variate (379) depending on the season of the year.

Table 6: Physiological parameters modulated by photoperiod exposure.

System	Effect	Ref.
Human behavior	↓Physical activity Changes in nutrient intake	(371,378)
Body composition	↑Body fat ↑BMI ↑Waist circumference	(368,380–382)
Cardiovascular system	↑LDL ↑Tryglicerides ↑Systolic blood pressure	(368,369,380)
Digestive system	Changes in the gut microbiota	(372–374)
Metabolism	↓ Metabolic rate Modulation of insulin levels and glucose metabolism Modulation of the expression of metabolic enzymes	(375,380,382)
Other	↓Antioxidant defenses Modulation of hormone plasma levels Modulation of kidney filtration rate Modulation of clock genes expression Modulation of thermogenesis	(367,370,376,377,381,383)

Although scientific evidence suggests that the bioavailability and metabolism of phenolic compounds, and hence their bioactivity, could be affected by consumption season, so far, no attempts have been performed to elucidate it. However, there is scientific interest to link the consumption of phenolic compounds and their effects on mammalian physiological adaptations. Indeed, and like circadian and circannual rhythms, phenolic compounds can modulate physiological parameters (384–387). As previously stated, diets rich in fruits and vegetables are known to prevent

the development of chronic diseases, and this has been attributed to their content in phenolic compounds, at least partially (20–22). Many of the biological effects of phenolic compounds are attributed to their antioxidant properties (388). However, there is now consistent evidence that many of the effects of phenolic compounds consumption are mediated by their direct interaction with enzymes or cellular receptors (389–391). But, why are these molecules able to interact with mammalian physiology? To give answer to this question the xenohormesis theory appeared. This theory stablishes that heterotroph organisms (i.e. humans) have the capacity to sense signalling and stress-induced molecules from other species (i.e. plants) such as phenolic compounds. By this evolutionary adaptation, heterotrophs use these chemical cues from other species to gain information on the environmental status to mount a pre-emptive defence response that will increase their chances of survival (391). In this sense, and according to the xenohormesis theory, the consumption of fruits out of their natural consumption season, from far distant geographical regions or the cultivated under conditions that modulate their phenolic profile in a non-natural way (i.e. treated with pesticides), would provide chemical cues that do not correspond to the current environment situation to the consuming organism, and thus result in a non-efficient adaptation of their physiology. Precisely, the consumption of cherries out of their traditional consumption season has recently been shown to change the regulation of key clock genes (392).

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HYPOTHESIS AND OBJECTIVES:

UNIVERSITAT ROVIRA I VIRGILI
MODERN CHANGES IN FRUIT INTAKE PATTERNS MODULATE THE BIOAVAILABILITY AND METABOLISM OF FRUIT PHENOLIC COMPOUNDS IN RATS
Lisard Iglesias Carres

HYPOTHESIS AND OBJECTIVES

Important lifestyle changes are occurring in modern occidental societies, and those are clearly marked in dietary patterns. These changes include the consumption of high-fat and high-sugar foods, and this has led to an important increase of metabolic disorders such as diabetes, obesity and MetS.

In addition, changes in fruit intake pattern are also related to modern occidental societies. In this sense, fruits were traditionally cultivated with the absence of pesticides and consumed depending on seasonal availability. However, the global economy in which we now live in promotes the production of pesticide-treated fruits (nowadays conventional fruits) and allows the consumption of fruits produced in different regions, countries and even hemispheres as well as of fruits out of their traditional consumption season. In this sense, there are important physiological changes that occur with a seasonal rhythmicity, and those are mediated by day light exposure. As a consequence of these changes in fruit intake patterns, changes in the intake of fruit components are also modified in modern occidental societies. Among these fruit compounds, phenolics are of special relevance since these molecules are related to the beneficial effects associated with fruit consumption.

Nevertheless, after fruit consumption, phenolic compounds are subjected to extensive metabolism, and differences in their metabolism depending on fruit intake patterns and the metabolic state of the consumer, both of them related to the changes in modern lifestyle patterns, could occur.

Therefore, **we hypothesize that modern changes in lifestyle affect the bioavailability and metabolism of fruit polyphenols**.

Thus, the main objective of this thesis was to evaluate whether modern lifestyle patterns related to fruit intake can condition the bioavailability and metabolism of fruit polyphenols in rats.

To assess this general objective, specific objectives were proposed:

1. To extract and fully characterise the phenolic profile of fruits with a marked traditional consumption season. [**Chapter 1**].

Apricots, cherries, grapes and oranges are fruits traditionally produced and consumed at different seasons, and their consumption as fresh fruits is high in modern societies. In this sense, red grapes and sweet oranges are consumed during low-light exposure months, and sweet cherries and apricots during high-light exposure months. In order to evaluate the bioavailability and metabolism of phenolics, a complete characterisation of their phenolic profile is of mandatory requirement. However, to date, no specific and optimised extraction methods exist for the extraction of phenolic compounds from the edible parts of apricots, sweet cherries, red grapes and sweet oranges.

To assess this first objective, we proposed the development of specific and optimised polyphenol extraction methodologies in order to fully characterise the phenolic profile of apricots **[Manuscript 1]**, sweet cherries **[Manuscript 2]**, red grapes **[Manuscript 3]**, and sweet oranges **[Manuscript 4]**.

2. To evaluate whether modern changes in fruit intake patterns affect the bioavailability and metabolism of fruit phenolic compounds in normal and diet-induced metabolic-altered state. [**Chapter 2**].

Modern occidental lifestyles can lead to the development of metabolic disorders such as diabetes, obesity and MetS. In this sense, cafeteria diet, characterised by an excessive access to high-fat and high-sugar foodstuffs, is a well-stablished dietary intervention that produces obesity and MetS in

rats. Moreover, lifestyle changes also involve changes in fruit intake patters. Modern occidental fruit intake patterns include the consumption of non-ecological (conventional) fruits over ecological ones, the consumption of fruits produced in far geographical regions and the consumption of fruits out of their traditional consumption season. Importantly, these patterns could involve a different bioavailability and metabolism of fruit polyphenols. Considering that the key limiting factors for polyphenol bioactivity are their bioavailability and metabolism, the study of the modern conditions that can condition polyphenol bioavailability and metabolism is of relevance.

To assess this second objective, different goals were proposed:

2.1. To study whether organic modern cultivation system affects the bioavailability and metabolism of red Grenache grapes. **[Manuscript 5]**.

2.2. To study whether the intake of sweet oranges produced in different hemispheres influences the bioavailability and metabolism of their polyphenols. **[Manuscript 6]**.

2.3. To study whether consumption of red grapes and sweet oranges out of their traditional consumption season affects the bioavailability and metabolism of their polyphenols. **[Manuscript 6]** and **[Manuscript 7]**.

2.4. To study whether the bioavailability and metabolism of red grape polyphenols produced conventionally is affected by photoperiod exposure under a homeostatic-disrupted state induced by cafeteria diet. **[Manuscript 8]**.

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EXPERIMENTAL DESIGNS:

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EXPERIMENTAL DESIGNS

Different experimental designs were used to assess the main hypothesis and reach the experimental objectives previously stated in this thesis.

1- Extraction and quantification of phenolic compounds from seasonal fruits.

The experimental design used in the extraction optimisation step for the different fruits studied in this thesis can be found in **Figure 1**. Briefly, samples were freeze-dried and grounded to obtain a fine powder, which was then subjected to different extraction conditions. The effect of liquid-to-solid ratio (LSR), solvent, extraction temperature, extraction time and number of extraction steps were evaluated and optimised for each fruit. The phenolic profile the different selected fruits was then characterised by HPLC-ESI-MS/MS.

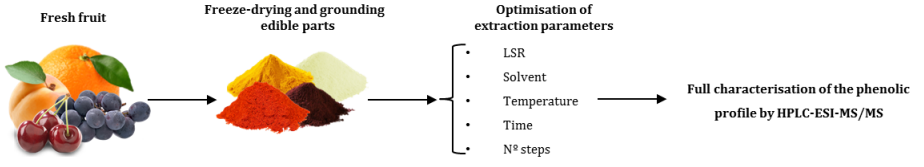

Figure 1: General procedure to optimise the extraction of phenolic compounds from fruits.

2- Acute serum profiling of the bioavailability and metabolism of red Grenache grape polyphenols.

To evaluate whether organic culture system conditions the bioavailability and metabolism of fruit polyphenols a first acute intake study was performed (**Figure 2**). Two different cultivars of red grapes of the variety Grenache were selected; one was produced organically (organic grapes, OG) and the other non-organically (conventional grapes, CG). OC and CG were produced in contiguous vineyards and were recollected at maturity the same day. A total of 65 mg GAE/Kg bw was administered to rats by

oral gavage and blood was recollected before and 2, 4, 7, 24 and 24 after the oral gavage from saphenous vein. The phenolic metabolites present in serum samples were concentrated and purified by μ-SPE. Phenolic metabolites were detected, identified and quantified by HPLC-ESI-MS/MS.

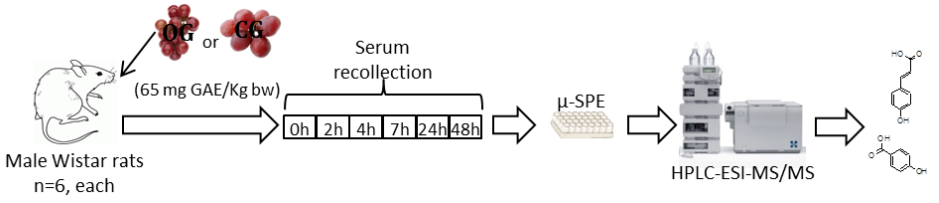

Figure 2: Experimental design to study the bioavailability and metabolism of organic (organic grapes, OG) and non-organic (conventional grapes,
CG) grape polyphenols.

3- Effect of consumption out of season of red Grenache grapes on the bioavailability and metabolism of their phenolic compounds.

To evaluate the contribution of the consumption of grapes out of their traditional consumption season on the bioavailability and metabolism of phenolic compounds a chronic fruit intake study was designed (**Figure 3**). This study was also designed to evaluate the effect on cultivation system on the bioavailability and metabolism of red Grenache grape. Fischer 344 rats were housed on three different photoperiods (n=18, each), which consisted on 18 (L18), 12 (L12), and 6 (L6) light hours/day (lights on 9:00 am). Rats were pre-conditioned to the light/dark cycles for 4 weeks. Then rats were randomly sub-divided (n=6, each) according to consumption of OG (100 mg dried fruit/Kg bw), CG (100 mg dried fruit/Kg bw) or vehicle (VH) (10 mg glucose and 10 mg fructose/Kg bw). Consumption of OG, CG or VH took place for a period of 10 weeks by voluntary liking. On the day of the last administration dose, rats were removed from food access after dose administration and sacrificed 1 hour later. Blood was recollected to obtain serum samples, which were processed as previously explained to

purify, concentrate and analyse the phenolic metabolite profile of each grapes administration group.

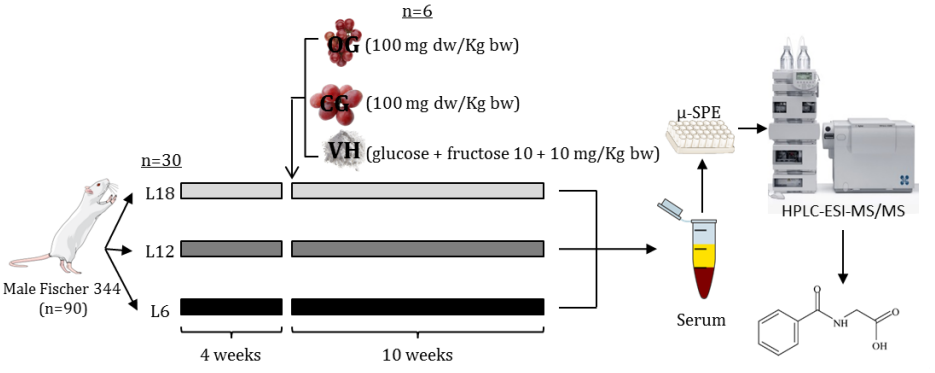

Figure 3: Experimental design to study the bioavailability and metabolism of organic (organic grapes, OG) and non-organic (conventional grapes, CG) grape polyphenols depending on photoperiod exposure.

4- Evaluation of growing site and out-of-season consumption of sweet oranges on the bioavailability and metabolism of sweet orange polyphenols.

A chronic administration study with oranges grown in the southern (southern oranges, SO) and northern (northern oranges, NO) hemispheres was designed to evaluate the effect of consumption of oranges from two different geographical regions and their consumption out of their traditional consumption season on the bioavailability and metabolism of their phenolic compounds. The experimental design is shown in **Figure 4**.

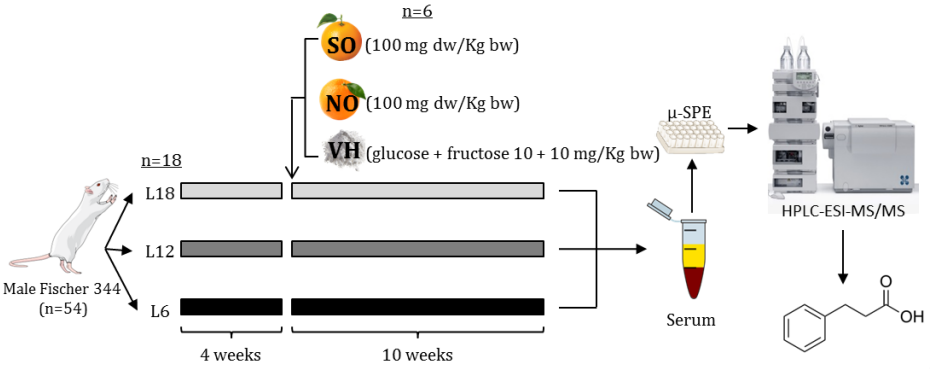

Figure 4: Experimental design to study the bioavailability and metabolism of polyphenols from oranges cultivated in the northern (northern oranges, NO) or southern (southern oranges, SO) hemispheres depending on photoperiod exposure.

5- Evaluation of host homeostatic state on the bioavailability and metabolism of Conventional red Grenache grape polyphenols.

Finally, to evaluate the contribution of the host metabolic state on the modulation of the bioavailability and metabolism of fruit phenolic compounds depending on photoperiod exposure, a chronic administration study with CG was design (**Figure 5**). After adaptation to photoperiod exposure rats were divided into CG-administration (100 mg dw/Kg bw) and VH-administration (10 mg glucose and 10 mg fructose/Kg bw) sub-groups (n=6, each). Administration of CG and VH took place for during 7 weeks in which rats consumed a cafeteria (CAF) diet which provided access to sausage, bacon, biscuits with paté, cheese, ensaïmada (sweetened pastry), carrots and sweetened milk (20% sucrose w/v) in addition to the standard chow diet. Phenolic metabolite purification, concentration and analysis were performed as previously explained.

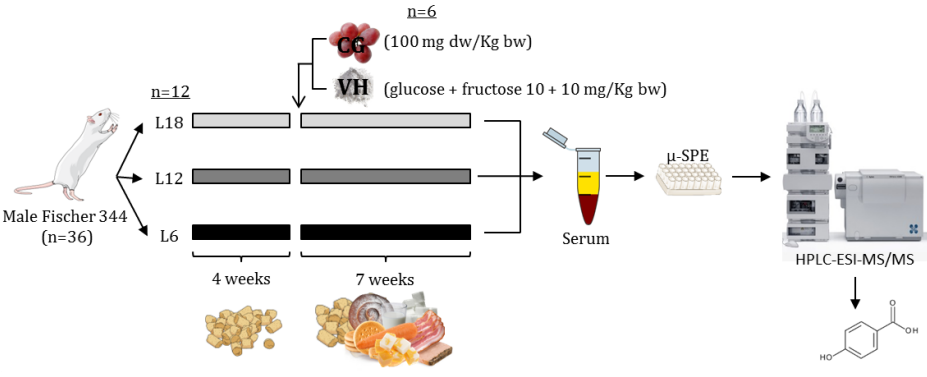

Figure 5: Experimental design to the influence of metabolic syndrome and photoperiod exposure on the bioavailability and metabolism of conventional grape (CG) polyphenols.

RESULTS:

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CHAPTER 1: EXTRACTION AND FULL QUANTIFICATION OF PHENOLIC COMPOUNDS FROM FRUITS WITH A MARKED TRADITIONAL CONSUMPTION SEASON.

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MANUSCRIPT 1:

Optimization of extraction method for characterization of phenolic compounds from apricot fruit (*Prunus armeniaca*).

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Abstract:

Fruits are rich in phenolic compounds with health-promoting activities. However, phenolic profiles vary between fruits. Hence, specific extraction methods are required for accurate profiling of the functional compounds. This study aims to develop an optimised method to extract phenolics from apricots (*Prunus armeniaca*) and correctly characterise apricot's phenolic profile. For this, the effects of solid-to-liquid ratio, temperature, extraction solvent, extraction time and sequential extraction steps on the extraction major phenolic families were investigated. Methanol- and ethanol-based extractions were suitable, although methanol was the optimal solvent. The optimised extraction conditions were 20 g/mL LSR, 38 °C and 72 % methanol (1 % formic acid). Characterization of apricot fruit by HPLC-ESI-MS/MS showed higher extraction of phenolic compounds than previously reported without specific extraction methods. In conclusion, using this fast and economically feasible method we achieve an accurate characterisation of the phenolic profile of apricot fruits, essential to study their bioactivity.

Keywords:

Apricot, extraction, flavanol, flavonol, phenolic acid, polyphenols

Chemical compound studied in this article:

Catechin (PubChem CID: 9064), Rutin (PubChem CID: 5280805), Chlorogenic acid (PubChem CID: 1794427).

Abbreviations used:

dw, dry weight; GAE, gallic acid equivalents; LSR, liquid-to-solid ratio; RSM, response surface methodology; TEC, tartaric ester content; TFaC, total flavanol content; TFoC, total flavonol content; TPC, total phenolic content.

1. Introduction:

Diets rich in fruits and vegetables are associated with a beneficial role in several human diseases, and these benefits are attributed to the phenolic content of plants (1). Indeed, in recent years, phenolic compounds have attracted interest due to their beneficial health effects. Different phenolic compounds have been reported to exert different health-promoting activities (2,3). In addition, different fruits have different phenolic profiles (4–8). Therefore, their potential roles in human health differ.

In this sense, it is important to note that when characterising the phenolic profile of a food matrix, only compounds that are extracted can be quantified. Given the diversity of food matrixes and phenolic profiles in foods, specific methods that fully extract fruit phenolics should be developed. This is essential to correlate the consumption of a mixture of phenolics with a beneficial health effect. Factors such as the liquid-to-solid ratio (LSR), type of solvent, temperature and extraction time can greatly influence phenolic extraction from food matrixes (9–11). In systems where several factors can affect the output, response surface methodology (RSM) is a useful tool to optimise extraction processes. The primary advantage of this approach is the evaluation of the effect of multiple variables and their interactions on the output variables with a reduced number of trials (12–14). Indeed, RSM has been widely used to optimise the extraction of phenolic compounds in various plant materials and fruits (4–6,8,12–20).

Apricots are one of the dietary sources with the highest polyphenol content (21) and can be considered as natural functional foods. Indeed, apricot consumption is associated with several health effects, including hepatoprotective, anti-inflammatory effects and anti-hypertensive effects, among others (22). Apricot phenolic content can vary between varieties (23–26) and depends on maturity (27) and the region and system of cultivation (26,28). Nevertheless, apricots have been widely reported to be rich in flavonoid, specially flavanols and flavonols, and non-flavonoid

phenolics, specifically hydroxycinnamic derivatives (25,27). In apricots, flavonols occur largely as quercetin and kaempferol glucosides, being quercetin-3-*O*-rutinoside (Rutin) its main component (22,27). Flavanols occur mainly as (+)-catechin and (-)-epicatechin, being one or the other the predominant form depending on the apricot variety (25). In addition, several dimeric flavanol forms can accumulate up to significant levels (24,27). Among the hydroxycinnamic derivates, chlorogenic acid is widely reported as the main compound (27). Neochlorogenic acid, p-coumaric acid, caffeic acid and gallic acid can also be present in different apricot cultivars (25,27). In addition, other phenolics from different families, such as resveratrol, can also occur in small quantities (24,28).

The phenolic composition of apricots has been widely determined by using different extraction methods (23,24,29), non-specific for apricots. For instances, many of the studies that evaluate phenolic compounds in apricots use adaptations of the method described by Bengoechea *et al*. (25–28,30,31), which was developed for the extraction of phenolic compounds form peach and apple purees and concentrates (7). Although Zitka *et al*. (11) evaluated the effect of some factors (i.e. solvent and temperature) on apricot polyphenol extraction, this study was centred in the extraction of particular apricot phenolic compounds. In addition, other authors have reported optimized methods to extract apricot polyphenols, they carried out the extraction from other apricot matrixes specifically from sun dried apricots (6) and apricot wastes (32). However, since beneficial health effect of apricot are mainly associated to the consumption of fresh fruit, a full characterization of phenolic composition of apricot fruits is required to identify the involved bioactive compounds. Hence, this study aimed to develop a specific method to extract phenolic compounds from apricot fruit by RSM to accurately characterise the phenolic composition of apricots by HPLC-ESI-MS/MS.

2. Materials and Methods:

2.1. Plant material:

Apricots (*Prunus armeniaca*, Charisma variety) were purchased from Mercabarna (Barcelona, Spain). Apricot stones were manually removed and discarded. Apricots (peel and flesh) were first frozen in liquid nitrogen and grounded. Next, homogenates were lyophilisied for a week in a Telstar LyoQuest lyophilizer (Thermo Fisher Scientific, Madrid, Spain) at -85 °C and further grounded to obtain a fine powder. The apricot powder was kept dry and protected from humidity and light exposure until extraction.

2.2. Chemicals and reagents:

Acetonitrile, methanol, ethanol (HPLC analytical grade) and glacial acetic acid were purchased from Panreac (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q Advantage A10 system (Madrid, Spain). Formic acid was purchased from Scharlab (Barcelona, Spain). The Folin-Ciocalteu and p-dimethylaminocinnamaldehyde (DMACA) reagents were purchased from Fluka/ Sigma-Aldrich (Madrid, Spain). Chlorogenic acid, eriodictyol, eriodyctiol-7-*O*-glucoside, hyperoside (quercetin-3-*O*-galactoside), isorhamnetin-3-*O*-glucoside, kaempferol, kaempferol-3-*O*-glucoside, and kaempferol-3-*O*-rutinoside were purchased from Exrtasynthese (Lyon, France). Benzoic acid, caffeic acid, (+)-catechin, epigallocatechin gallate (EGCG), p-coumaric acid, (-)-epicatechin, ferulic acid, gallic acid, procyanidin dimer B2, protocatechuic acid and quercetin were purchased from Fluka/Sigma-Aldrich (Madrid, Spain). Resveratrol was purchased from Quimivita (Barcelona, Spain) and rutin was kindly provided by Nutrafur (Murcia, Spain).

All standard compounds were individually dissolved in methanol at 2000 mg/L, with the exception of isorhamnetin-3-*O*-glucoside, dissolved at 1000 mg/L, and hyperoside, dissolved at 500 mg/L. All standard stock solutions

were newly prepared every 3 months and stored in amber-glass flasks at -20 °C. Mixed standard stock solutions were prepared with Milli-Q water to obtain the concentration needed to construct the calibration curves.

2.3. Extraction procedure:

Apricot powder was weighed to obtain the desired LSR and mixed with 1 mL of pre-heated extraction solvent (methanol:water, v:v). Different methanol proportions, temperatures, times and extraction steps were used throughout the experiment. In addition, methanol was prepared in all cases including 1 % formic acid. Extractions were performed at 500 rpm agitation under protection from light exposure. Samples were centrifuged at 9,500^{x}g for 10 min at 4 °C, and supernatants were stored at -20 °C until further analyses.

2.4. Single factor studies:

Prior to RSM, a first set of tests were performed to select experimental ranges for independent variables. Individual effects of LSR, methanol concentration and temperature were evaluated based on total phenolic content (TPC), total flavonol content (TFoC), tartaric ester content (TEC) and total flavanol content (TFaC) as the major phenolic families present in apricots (25,27,28). LSR was evaluated at the ratios of 10, 20, 40, 60 and 80 mL/g; temperature at 25, 40, 55, 70, 85 °C; and methanol at 30, 50, 60, 70 and 90 %. All extractions lasted for 30 min and LSR, temperature and methanol were kept constant at 20 mL/g, 55 °C, and 50 % when not under study.

2.5. Surface response design:

The extraction was optimised by using an experimental design by RSM. A face-centered central composite design with two factors and three levels, consisting of 11 randomised runs with 3 centre points, was selected. Independent variables used in the RSM were temperature (25-55 °C, X_i)

and methanol proportion (60-100 %, X_j). LSR (20 mL/g) and extraction time (30 min) were fixed as constant variables during the RSM experiment. Experimental data were fitted to a second polynomial response surface, which follows the equation

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X^2{}_{ii} + \sum_{i=1} \sum_{j=i+1} \beta_{ij} X_{ii} X_{ji}$$

where Y is the dependent variable, β_0 the constant coefficient, and β_i, β_{ii} and β_{ij} are the linear, quadratic and interaction regression coefficients, respectively. X_i, X_{ii} and X_{ji} represent the independent variables.

Individual phenolic compounds were quantified by the HPLC-DAD method and used in the RSM optimisation study. The results of the RSM design were analysed with Design-expert 9.0.6 software (Trial version, Stat-Ease Inc., Minneapolis, MN, USA). The single parameters that were not influenced by the extraction factors were omitted in the model.

2.6. Kinetic study:

A kinetic study was performed to evaluate the effect of time on the polyphenol extraction yield. Seven extraction times, ranging from 0 to 120 min, were selected. LSR was fixed at 20 mL/g, methanol percentage at 72 % and temperature at 38 °C. TPC, TFoC, TEC and TFaC were determined to evaluate the effect of time on the polyphenol extraction.

2.7. Sequential extractions:

Three consecutive extractions were performed to evaluate the influence of multiple extractions on the polyphenol extraction yield. LSR was fixed at 20 mL/g, methanol percentage at 72 % and temperature at 38 °C. Samples were mixed with solvent, vortexed and centrifuged (9,500^{X}g, 10 min, 4 °C). TPC, TFoC, TEC and TFaC were determined to evaluate the effect of sequential extractions on the polyphenol extraction yield.

2.8. Methanol-ethanol comparison:

To evaluate ethanol efficiency in the polyphenol extraction, apricot powder was extracted twice under the following conditions: LSR of 20 mL/g, methanol or ethanol (EtOH) proportion of 72 % (1 % formic acid), temperature of 38 °C. Samples were mixed with the solvent, vortexed and centrifuged (9,500^{x}g, 10 min, 4 °C). TPC, TFaC, TEC and TFoC were determined to evaluate the ethanol efficiency in the polyphenol extraction.

2.9. Analysis of response variables:

2.9.1. Total phenolic content:

TPC of extracts was determined by the Folin-Ciocalteu method adapted from Nenadis *et al*. (3). Briefly, 10 µL of the extract and 50 µL of the Folin-Ciocalteu reagent were successively added to an Eppendorf tube containing 500 µl of Milli-Q water and mixed. After waiting for 3 minutes in the dark, samples received 100 µL of Na_2CO_3 (25 %) and were brought to a final volume of 1 mL with Milli-Q water. Absorbance was read at 725 nm using an Eon BioTek spectrophotometer (Izasa, Barcelona, Spain) against a water sample (blank) that underwent equal treatment after 1 hour of incubation in the dark. Gallic acid was used to construct the calibration curve between 40 mg/L and 400 mg/L. The results were expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g dw).

2.9.2. Total flavonols and tartaric ester content:

TFoC and TEC were determined with the method described by Cacace *et al*. (4). Briefly, 250 µL of extracts were mixed with 250 µL of 0.1 % HCl in ethanol 95 % and 4.55 mL of 2 % HCl and were allowed to react for 15 minutes. Absorbance was then read at 360 and 320 nm for flavanols and tartaric esters quantification, respectively, using an Eon BioTek spectrophotometer (Izasa, Barcelona, Spain). Quercetin and caffeic acid

were used to construct calibration curves, and the results were expressed as milligrams of quercetin or caffeic acid equivalents per grams of dry weight (mg quercetin/g dw; mg caffeic acid/g dw).

2.9.3. Total flavanol content:

The TFaC of extracts was estimated by the DMACA method (33). Briefly, 100 µL of extract samples were mixed with 500 µL of DMACA solution (0.1 % 1 N HCl in methanol) and allowed to react at room temperature for 10 min while protected from light exposure. Next, absorbance was read at 640 nm using an Eon BioTek spectrophotometer (Izasa, Barcelona, Spain). Catechin concentrations in between 5 mg/L and 100 mg/L were used to construct a calibration curve. TFaC was expressed as milligrams of catechin equivalents per gram of dry weight (mg catechin/g dw).

2.9.4. HPLC-DAD method:

Polyphenol separation was achieved using a ZORBAX Eclipse XDB-C18 (150 mm x 2.1 mm i.d., 5 µm particle size) as the chromatographic column (Agilent Technologies, Palo Alto, CA, USA) equipped with a Narrow-Bore guard column (2.1 mm x 12.5 mm, 5 µm particle size). The mobile phase was water:acetic acid (99.8:0.2, v:v) (A) and acetonitrile (B) in a gradient mode as follows: initial conditions 0 % B; 0-30 % B, 0-18 min; 30-100 % B, 18-19 min; 100% B isocratic, 19-20 min; 100-0 % B, 20-21 min. A post-run of 6 min was required for column re-equilibration. The flow rate was set at 0.5 mL/min, and the injection volume was 10 µL for all runs.

Identification and quantification of the phenolic compounds of interest was achieved with a UV/ViS photodiode array detector (1260 Infinity, Agilent Technologies, Palo Alto, CA, USA). Chromatograms were recorded from 200 to 600 nm. Catechin was detected at 280 nm, chlorogenic acid at 320 nm, and rutin at 340 nm. The results were expressed as micrograms of equivalents per gram of dry weight (mg/g dw).

Calibration curves, linearity, intraday variability (precision), interday variability (reproducibility), detection and quantification limits were calculated in mobile phase A spiked with polyphenol standards (Supplementary table 1). The peak areas of various concentrations of standards were used to construct the calibration curves. The method's precision was calculated as the relative standard deviation (% RSD) of the concentration in a triplicate analysis of three different spiked samples (100, 50 and 1 μg/mL). Method reproducibility was calculated as the relative standard deviations (% RSD) of three different standard compound concentrations (100, 50 and 1 μg/mL) analysed in triplicate over three consecutive days. Sensitivity was evaluated by determining the limits of detection (LOD) and quantification (LOQ), which were respectively defined as the concentration corresponding to 3-fold and 10-fold of the signal-to-noise ratio.

2.9.5. HPLC-ESI-MS/MS METHOD:

The extracts were directly analysed using a 1200 LC Series coupled to a 6410 MS/MS (Agilent Technologies, Palo Alto, CA, USA). The column and mobile phases used were the same as in the HPLC-DAD method (see section 2.9.4). The gradient mode was: initial conditions 0 % B; 0-0.5 min, 0 % B; 0.5-2 min, 0-10 % B; 2-12 min, 10-30 % B; 12-16 min, 30-60 % B; 16-17 min, 60-100 % B, 17-20 min, 100 % B; 20-21 min, 100-0 % B. A post-run of 6 min was required for column re-equilibration. Flow rate was set at 0.4 mL/min, and the injection volume was 2.5 μL for all runs. Electrospray ionization (ESI) was conducted at 200 °C and 14 L/min with 20 psi of nebulizer gas pressure and 3000 V of capillary voltage. The mass spectrometer was operated in the negative mode, and MS/MS data were acquired with the dynamic mode. Optimized conditions for the analysis of the phenolic compounds studied using HPLC-ESI-MS/MS are summarized in Supplementary table 2. Data acquisition was carried out using MassHunter Software (Agilent Technologies, Palo Alto, CA, USA). The

calibration curves, coefficient of determination, linearity and detection and quantification limits of the HPLC-ESI-MS/MS method can be found in Supplementary table 3 and were evaluated following the same principles as in the HPLC-DAD method (see section 2.9.4).

2.10. Statistics:

The results of the RSM design were analysed using Design-expert 9.0.6 software (Trial version, Stat-Ease Inc., Minneapolis, MN, USA). SPSS 19 software (SPSS Inc., Chicago, IL, USA) was used for any other statistical analysis. All experiments were performed in triplicate; the statistics' significance was evaluated using One-way ANOVA or Student's t-test, and p-values less than 0.05 were considered to be statistically significant.

3. Results and Discussion:

The phenolic content of apricots has been largely studied (25–28,30,31), as well as their beneficial health effects (22). However, when apricots phenolics are analysed in the literature a unspecific extraction methodology is usually used. For a correct characterisation of apricot phenolic composition, which is essential to link a specific polyphenol or group of polyphenols with a particular health effect, an optimised method specific to extract apricots phenolics should be used. To develop a methodology as such, the extraction parameters affecting the extraction of phenolic compounds in apricots were evaluated and optimised.

3.1. Single factor studies:

The LSR, temperature and methanol proportion effect on phenolic extraction were first evaluated individually (Figure 1) to select a relevant variable range for the RSM study. To evaluate these parameters, TPC, TFoC, TEC and TFaC were chosen as dependent variables to give a global view of the extraction of the most representative phenolic families present

in apricots (25,27,28). The extraction time was fixed at 30 min according to previous studies (5,6,12,14).

Choosing an optimal LSR is key to the extraction of phenolics, since working at a low LSR may lead to solvent saturation (8), and working at a high LSR is economically counterproductive. TPC did not increased significantly at values above 20 mL/g, whereas TFoC, TEC and TFaC did not report any changes due to LSR (Figure 1A). Therefore, LSR was fixed at 20 mL/g throughout the rest of the experiment. This LSR is very similar to the optimum in other plant matrixes (12,15,16), including sun-dried apricots (6).

Temperature has been described as a main factors affecting the extraction of phenolics (5,12,34). During extraction, temperature influences compound stability due to chemical and enzymatic degradation (14). Indeed, degradation during phenolic extraction at increased temperatures has been previously reported for several phenolic families (4,9). Nevertheless, in this study, TPC was poorly influenced by an increase of temperature whilst no effect was reported for TFaC extraction (Figure 1B). Consistent with these findings, Gan *et al*. and Cacace *et al*. did not report a significant effect of temperature on the TPC extractability in *Parkia speciose* pods and black currants, respectively (4,15). In our study, TFoC and TEC decreased as temperature increased, and these decreases were higher above 55 °C. This contradicts the findings of the study of Van Der Sulis *et al*. in apple juice that demonstrated epicatechin to be more sensitive to temperature than chlorogenic acid (35). However, factors such as phenolic localisation in fruit tissues and interaction with the plant matrix play an important role in extraction (34), which could explain the observed effect for this specific fruit and phenolic families. Given our results, a range of temperatures between 25 and 55 °C was selected for the RSM study.

Throughout all experiments, methanol was always prepared at a formic acid concentration of 1 %. Low concentrations of organic acids, such as formic acid, promote the degradation of the plant matrix, which enhances the extraction rate of phenolic compounds (36). The effect of methanol proportion on TPC was not clear, which could be due to the wide variety of phenolic families present in apricots (27). However, TFoC and TEC were found to be higher at 90 %. Consistent with this finding, extractions with high proportions (80 and 100 %) of acidified methanol are reported to be capable of extracting tartaric esters and flavonols in apricot fruits, jams and nectars (25,27,28). TFaC was found to achieve the greatest extraction yield at methanol of 50 %, which is in line with the results obtained for flavonoids in *Morinda citrifolia* fruits (37). Given the few quantitative differences reported between 50 and 60 % MetOH in the extraction of flavanols and the important differences in the extraction of flavonols and tartaric esters at higher proportions of methanol, a range of methanol proportions between 60 and 100 % was selected for the RSM study.

3.2. Analysis of response surfaces:

The extraction of apricot phenolics was optimised using an RSM approach. A face-centered central composite design with two factors and three levels was selected, consisting of 11 randomised runs with 3 centre points. The previously optimised LSR (20 mL/g) and extraction time (30 min) were fixed as constant variables during the RSM experiment. The independent variables used in the RSM were methanol proportion (60-100 %, X_j) and temperature (25-55 °C, X_i). The compounds catechin, chlorogenic acid and rutin were analysed individually by the HPLC-DAD method and were included in the RSM as relevant representatives of the phenolic families present in apricots (25,27,28). The obtained experimental extraction of catechin, chlorogenic acid and rutin for all runs are reported in Table 1.

3.2.1. Multiple linear regression and model's adequacy:

The experimental data (Table 1) was used to determine the regression coefficients of equation 1. All compounds analysed generated a significant model, implying that at least one of the extraction variables could explain the variation in the response variables (Table 2). Indeed, the coefficients of determination (R^2) were above 0.9, which means that the model represented the data accurately. In addition, the lack of fit values were not significant ($p > 0.05$), thus further validating the model.

3.2.2. Analysis of regression coefficients and response surface plots:

The regression coefficients of the model for catechin, chlorogenic acid and rutin obtained from the multiple linear regressions are reported in Table 2. Dependent variables (i.e., catechin, chlorogenic acid and rutin) allowed direct interpretation of the effect of the independent variables (i.e., methanol proportion and extraction temperature). The visualisation of the statistical significance of the independent variables on the dependent variables was facilitated by the generated surface contour plots (Figure 2).

Regarding the effect of temperature, chlorogenic acid was not influenced by this factor. However, a significant positive linear effect and a negative quadratic effect were observed for rutin, indicating that rutin extraction increases with temperature to a point after which it begins to decrease (Table 2). This behaviour was previously described for flavanols (12) and total phenolics (5,9) in other plant matrixes. For catechin, a linear negative effect was found to be statistically significant, possibly due to the thermosensitivity of flavanols (12). Indeed, the optimal extraction temperature for catechin according to our optimised results was 32 °C (Figure 2A), in agreement with the optimal extraction temperature of 30 °C reported by Wani *et al*. in sun-dried apricots (6). The optimal extraction temperatures reported for chlorogenic acid and rutin were 53 and 46 °C, respectively (Figure 2B and 2C), which are very similar to those reported

for several phenolics in other plant materials (9,16). Cellular localisation and interaction with cell structural components could explain the difference in the optimal extraction temperatures between the studied compounds (34).

When studying methanol percentage, a negative linear effect of chlorogenic acid and rutin and a negative quadratic effect for catechin, chlorogenic acid and rutin were observed and were statistically significant. This implies that an increase in methanol proportion will result in a reduction of the extraction of rutin and chlorogenic acid, as evidenced in Figure 2. In this sense, Yang *et al.* also reported a negative linear and quadratic effect of the extraction solvent for total phenolics in *Phyllanthus emblica L* (17) and Liyana-Pathirana *et al.* in the antioxidant activity of soft wheat bran (13). In addition, in this study, catechin was reported to have an optimal methanol proportion of 78 % (Figure 2A), which is highly similar to the optimal EtOH proportion (84 %) reported by *Liu et al*. for epicatechin in hawthorn fruits (38). Indeed, methanol proportions of 80 % have been shown to be efficient in extracting several phenolics, including epicatechin and procyanidin B3 (39). In agreement with the optimal reported methanol proportion of 63 % for rutin (Figure 2B), Wijngaard *et al*. found an ethanol concentration of 64 % to be optimal for the extraction of total flavonols (18). In this sense, solvents of approximately 50 and 60 % have been reported to be optimal in the extraction of total phenolics (4,5). The optimal methanol proportion obtained in this study for chlorogenic acid extraction was 60 % (Figure 2C), in agreement with Yilmaz *et al*., who reported an optimal ethanol concentration for neochlorogenic acid at 56 % (5) and Wijngaard *et al*. at 58 % for chlorogenic acid (18). Notably, aqueous mixtures of organic solvents usually yield better extraction rates (37,40). In fact, the addition of water in extraction solvents promotes fruit particle swelling. This increases the contact area between fruit particles and the extraction solvent, which

allows the solvent to penetrate more easily into the food matrix, leading to increased phenolic extraction yields (40).

A negative interaction (crossover effect) between temperature and methanol proportion was found to be statistically significant for chlorogenic acid. Silva *et al*. reported a significant interaction between ethanol and temperature for the total flavonols in *Inga edulis* leaves (12). Similarly, Pompeu *et al*. reported this type of interaction for antioxidant activity in *Euterpe oleracea* fruits (9), and Karacabey *et al*. reported a significant negative interaction between ethanol concentration and temperature in the antioxidant capacity of grape crane extracts (20).

3.2.3. Validation of the model:

The optimised combination of extraction variables at the highest desirability (0.861) were a temperature of 38 °C and methanol at 72 %. To validate the veracity of the model, 3 extractions were performed under the optimised conditions. No significant differences were reported between the experimental and predicted extraction rates for catechin, chlorogenic acid and rutin (Supplementary Table 4). Therefore, the model can accurately predict the behaviour of the response variables within the range of extraction variables studied. Thus, the extraction temperature and methanol proportion were fixed at 38 °C and 72 % throughout the rest of the study. Similar to our results, Wani *et al*. reported a temperature of 30 °C to be optimal in the extraction of total phenolics in sun-dried apricots (6), and Tabaraki *et al*. reported an optimal ethanol concentration between 60 and 70 % for rice brans (16).

3.3. Effect of time on phenolic extraction:

There are many literature examples in which time exerts a significant influence on the extraction of several phenolics (5,10,17,24). It has been postulated that time can promote the solubilisation of polyphenols in a the solvent due to the cell-wall weakening effects of optimised methanol and

temperature conditions (16,17). Therefore, we further evaluate the effect of time on the phenolic extraction of apricots under the optimised conditions of 20 mL/g, 72 % of methanol (1 % formic acid), 38 °C within a range of 0 to 120 minutes (500 rpm) (Figure 3). Variables that encompass multiple representatives of the same phenolic family provide a general view of the effect of a particular extraction parameter. TPC, TFoC, TEC and TFaC represent the major phenolic families found in apricots (25,27,28) and were used to study the effect of extraction time in apricots. However, no significant changes were reported due to time in any of the studied parameters. Therefore, considering practical and economic aspects, centrifugation immediately after vortexing was set as the optimal procedure (i.e., 0 min as extraction time). Consistent with this finding, Thoo *et al.* found no effect of time on the extraction of flavonoids in *Morinda citrifolia* (37). In addition, Yang *et al.* reported a time of 23.16 min and Wani *et al.* a time of 30 min as optimal in *Phyllanthus emblica L*. bark and sun-dried apricots, respectively (6,17), which are relatively short extraction times.

3.4. Multi-step extractions:

To evaluate whether better extraction could be attained with this optimised method, three consecutive extractions were performed under the optimised conditions of 20 mL/g, 38 °C, 72 % of methanol (1 % formic acid). Samples were vortexed with pre-heated extraction solvents and immediately centrifuged. Values above 70 % were observed for TPC, TFoC and TEC in the first extraction step (Figure 4). Values between 89 % and 94 % were reached in the second extraction step. Similarly, Mané *et al*. reported that flavanols, phenolic acids and anthocyanins in grape skins, seeds and pulp were primarily extracted in the first extraction step (8). Chirinos *et al*. also reported a yield of 60 % in a single step for *Tropaeolum tuberosum* phenolic extraction (10). However, since our aim was to extract a maximum content of phenolics for accurate quantification, we set two

extraction steps as the optimal number of extraction steps in the rest of the experiment. Remarkably, in all the parameters studied, no significant differences were reported between the second and third extraction steps, while the first and second steps were significantly different.

3.5. Characterisation of apricot phenolics and the effect of the extraction solvent:

Considering that apricots characterisation has been mainly realised using unspecific phenolic extraction methodologies (30,27,25,31,26,28), we used our specific optimised method to precisely and fully characterize the phenolic profile of Charisma apricots. Moreover, methanolic and ethanolic extractions were performed under the optimised conditions to evaluate the effect of these solvents on the phenolic extraction and characterisation. Our results demonstrate that the methanol and the ethanol-base extractions reported different extraction yields in TFoC (Table 3) and in individual compounds determined by HPLC-ESI-MS/MS (Table 4). In most individual phenolics, methanol achieved higher extractions than ethanol, which is consistent with the literature (10,24). For example, methanol achieved significantly higher extractions protocatechuic acid and rutin. Paradoxically, the TFoC was significantly higher in the ethanol-based extraction. Ethanol also reported some higher extraction rates in individual compounds than methanol. For instance, the ethanol-based extraction achieved significantly higher extractions of chlorogenic acid. Remarkably, chlorogenic acid and related compounds such as caffeic acid have reported important health promoting activities (2). Moreover, there are no differences in the TPC, TEC and TFaC of both extraction solvents. Therefore, considering that ethanol is used in the food industry due to methanol's toxicity (41), our results suggest that the adaptation of this methodology in a larger scale could be used to produce phenolic rich extracts with potential bioactive functions.

Methanol, which generally achieved better extraction rates, was demonstrated to be the optimal solvent to accurately profile the phenolic content of apricots. It is worth considering that, since apricot phenolics vary due to variety, maturity stage and region of cultivation (25–28), comparing the extraction rate of different methods is controversial. Our optimised method achieved 5.33 ± 0.16 mg GAE/g dw, within the range of TPC (8.18 – 4.23 mg GAE/g dw) reported for several apricot varieties (23). However, our method extracted higher TPC than apricots from different varieties in different studies (29). In agreement with the phenolic profile of apricots, we obtained rutin and hydroxycinnamic acids (Table 4) to be the most abundant compounds in apricots (24,26,29). In the case of the most representative phenolic compounds in apricots, higher quantities were extracted with this optimized method than the values reported in the literature. For example, rutin, which was reported to range between 15.50 and 69.52 mg/Kg dw in several apricot varieties, was found at 278.94 ± 9.71 mg/Kg dw in our study (26). Similar concentrations of chlorogenic acid were found in the study of Karabulut *et al*. than the one we found (29). However, our method extracted 178.60 ± 13.11 mg chlorogenic acid/Kg dw, which is greater than the concentrations found in Ordubat (42.41 ± 0.01 mg/Kg dw), Stark early orange (93.86 ± 0.01 mg/Kg dw) and Wilson delicious (147.31 ± 0.00 mg/Kg dw), among other varieties (25). Similarly, lower concentrations of chlorogenic acid were found in the study of Kan *et al*. (26). Karabulut *et al*. reported neochlorogenic acid at concentrations between 103.72 and 285.29 mg/Kg dw (29), and, in this study, the observed neochlorogenic acid's concentration was 565.79 ± 16.82 mg/Kg dw.

All in all, better extraction rates than the reported in the literature using unspecific methods for apricots were achieved for most of the representative phenolic compounds. In addition to the good extraction rates achieved, this method is faster than the one developed for sun-dried apricots (6) and other plant matrixes (5,12). Moreover, the required

temperatures (5,12,14) and LSR (14,19) are lower than other methods for different plant matrixes, making our method more economically feasible.

4. Conclusions:

In this study, we developed a fast and economically feasible method for the specific extraction of apricot phenolics. The optimised conditions were: 20 g/mL, 38 °C and 72 % methanol (1 % formic acid). The phenolic extraction using this method allowed an accurate characterisation of the phenolic profile of apricots, essential in the study of their bioactivity. Additionally, the ethanol-based extraction has potential to be adapted to the food industry for the production of phenolic-rich extracts with potential bioactive effects.

5. Declaration of interest:

All authors declare that they do not have any conflict of interest that could inappropriately influence this manuscript.

6. Acknowledgment:

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Figure legends:

Figure 1: Individual effects of liquid-to-solid ratio (LSR) (A), temperature (T) (B) and methanol (MetOH) percentage (C) on the extraction of apricot total phenolic content (TPC); total flavonols content (TFoC); tartaric ester content (TEC) and total flavanol content (TFaC). The results are expressed as mg of phenolic component per gram of dry weight (mg/g dw) ± SD (n=3). [a, b, c, d] Mean values with different letters were significantly different between extraction conditions (one-way ANOVA with Tukey's *post hoc* test, $p < 0.05$). Abbreviations: caffeic acid (Caff), catechin (Cat), gallic acid equivalents (GAE), and quercetin (Quer).

Figure 2: Response surface plots for catechin (A), rutin (B) and chlorogenic acid (C) in function of extraction temperature and methanol proportion. Extractions were performed at a liquid-to-solid ratio of 20 mL/g under 500 rpm agitation for 30 minutes. Abbreviations: Methanol (MetOH), catechin (Cat), rutin (Rut) and chlorogenic acid (Chl).

Figure 3: Effect of time on the extraction of total phenolic (A), total flavonol (B), tartaric ester (C), and total flavanol (D) contents. Extractions were performed at 20 mL/g, 38 °C, 72 % methanol (1 % formic acid), and agitation at 500 rpm. The results are expressed as mg of phenolic component per gram of dry weight (mg/g dw) ± SD (n=3).

Figure 4: Effect of multi-step extraction on total phenolic (A), total flavonol (B), tartaric ester (C), and total flavanol (D) contents. Extractions were performed at 20 mL/g, 38 °C and 72 % methanol (1 % formic acid). Total quantity of phenolic component extracted after third step is expressed as mg of phenolic component per gram of dry weight (mg/g dw) ± SD (n=3).

Table 1: Face-centred settings of independent variables and experimental results of (+)-catechin, chlorogenic acid and rutin

Run	T (°C)	MetOH (%)	Cat	Chl	Rut
1	25	60	1.21	1.51	0.54
2	55	60	1.08	1.61	0.58
3	25	100	1.14	1.23	0.31
4	55	100	1.10	1.16	0.34
5	25	80	1.27	1.47	0.50
6	55	80	1.24	1.51	0.56
7	40	60	1.18	1.55	0.61
8	40	100	1.17	1.29	0.36
9	40	80	1.29	1.52	0.56
10	40	80	1.24	1.48	0.54
11	40	80	1.33	1.52	0.53

Abbreviations: Temperature (T); methanol (MetOH); (+)-catechin (Cat); chlorogenic acid (Chl); and rutin (Rut). Results are expressed as mg of phenolic component per gram of dry weight (mg/g dw) ± SD (n=3).

Table 2: Analysis of variance and regression coefficients of predicted model for response variables in apricots.

Model parameters	**Regression coefficient**	**Cat**	**Chl**	**Rut**
Intercept	β0	1.29	1.51	$5.49x10^{-1}$
Linear				
T x T	β1	$-3.33x10^{-2}$*	$1.17x10^{-2}$	$2.17x10^{-2}$*
MetOH	β2	$-1.00x10^{-2}$	$-1.65x10^{-1}$*	$-1.18x10^{-1}$*
Interaction				
T x MetOH	β12	$2.25x10^{-2}$	$-4.25x10^{-2}$*	$-2.50x10^{-3}$
Quadratic				
T x T	β11	$-3.73x10^{-2}$	$-3.03x10^{-2}$	$-2.87x10^{-2}$*
MetOH x MetOH	β22	$-1.17x10^{-1}$*	$-1.00x10^{-1}$*	$-7.37x10^{-2}$*
R^2		0.9161	0.9746	0.9884
Adjusted R^2		0.8322	0.9491	0.9767
p-value		0.0101	0.0005	0.0001
F-value		10.92	38.30	84.96
Lack of fit[a]		0.8957	0.2826	0.4910

Abbreviations: Temperature (T); methanol (MetOH); determination coefficient (R^2); (+)-catechin (Cat); Chlorogenic acid (Chl); rutin (Rut).
Differences between groups determined by ANOVA. *$p<0.05$.[a] p-value of lack of fit test.

Table 3: Quantification of apricot (*Prunus armeniaca*) phenolics by HPLC-DAD using methanol or ethanol as extraction solvents.

	MetOH			EtOH			*p*-value
TPC	5.33	±	0.16	5.24	±	0.14	0.232
TFoC	1.21	±	0.10	1.47	±	0.09	0.002
TEC	1.51	±	0.10	1.55	±	0.07	0.451
TFaC	0.25	±	0.02	0.25	±	0.01	0.556

Abbreviations: total phenolic content (TPC); total flavonol content (TFoC); tartaric ester content (TEC); and total flavanol content (TFaC). Extractions were performed twice at optimised conditions: organic solvent 72 % and formic acid 1 %, at 20 mL/g and 38 °C. Results are expressed as mg of phenolic component per gram of dry weight (mg/g dw) ± SD (n=3). Statistics by Student's t-test.

Table 4: Quantification of apricot (*Prunus armeniaca*) phenolics by HPLC-ESI-MS/MS

Compound	MetOH	EtOH	p-Value
Benzoic Acid	5.88 ± 0.30	6.21 ± 0.16	0.16
Hydroxybenzoic acid [a]	2.34 ± 0.05	2.45 ± 0.10	0.17
Protocatechuic acid	185.11 ± 5.10	6.63 ± 0.21	<0.01
Dihydroxybenzoic acid [b]	56.52 ± 1.30	n.q.	
p-Coumaric acid	n.q.	n.q.	
Gallic acid	n.q.	n.q.	
Ferulic acid	n.q.	n.q.	
Resveratrol	0.16 ± 0.00	0.15 ± 0.00	<0.01
Kaempferol	0.05 ± 0.03	0.03 ± 0.00	0.31
Catechin	20.00 ± 0.93	18.45 ± 0.83	0.10
Epicatechin	16.44 ± 0.90	15.31 ± 0.34	0.11
Quercetin	n.q.	n.q.	
Protocatechuic acid glucoside [b]	1.54 ± 0.05	1.59 ± 0.03	0.19
Gallic acid O-glucoside d [c]	0.01 ± 0.00	0.02 ± 0.00	0.03
Gallic acid O-glucoside d2[c]	0.18 ± 0.01	0.20 ± 0.01	0.01
Caffeic acid O-glucoside d1 [d]	2.79 ± 0.04	3.25 ± 0.26	0.04
Caffeic acid O-glucoside d2 [d]	4.77 ± 0.21	5.16 ± 0.25	0.11
Caffeic acid O-glucoside d3 [d]	20.43 ± 0.60	21.69 ± 0.68	0.07
Neochlorogenic acid [e]	565.79 ± 16.82	586.19 ± 13.60	0.18
Chlorogenic acid	178.60 ± 13.11	216.36 ± 4.81	0.01
Cryptogenic acid [e]	41.55 ± 3.50	36.60 ± 2.61	0.12
Feruloylquinnic acid [f]	3.32 ± 0.04	3.45 ± 0.05	0.02
Resveratrol O-glucoside d1 [g]	0.16 ± 0.00	0.15 ± 0.00	0.01
Resveratrol O-glucoside d2 [g]	0.13 ± 0.01	0.15 ± 0.01	0.06
Catechingallate [h]	0.70 ± 0.01	0.71 ± 0.02	0.24
Kaempferol-3-*O*-glucoside	0.47 ± 0.04	0.46 ± 0.01	0.75
Eriodictyol-7-*O*-glucoside	0.32 ± 0.01	0.32 ± 0.00	0.32
Catechin O-glucoside [h]	0.51 ± 0.04	0.46 ± 0.01	0.10
Hyperoside	13.20 ± 0.84	12.87 ± 0.40	0.57
Isorhamnetin-3-*O*-glucoside	0.03 ± 0.00	0.03 ± 0.00	0.72
Procyanidin dimer d1 [i]	14.39 ± 0.73	11.44 ± 0.24	<0.01
Procyanidin dimer d2 [i]	6.63 ± 0.22	5.99 ± 0.34	0.05
Procyanidin dimer B2	6.69 ± 0.21	5.80 ± 0.26	0.01
Procyanidin dimer d3 [i]	1.00 ± 0.08	0.85 ± 0.04	0.04
Procyanidin dimer d4 [i]	2.80 ± 0.20	2.50 ± 0.06	0.07
Procyanidin dimer d5 [i]	0.68 ± 0.01	0.62 ± 0.01	<0.01
Kaempferol-3-*O*-rutinoside	14.51 ± 1.05	14.27 ± 0.21	0.72
Rutin	278.94 ± 9.71	238.33 ± 7.81	<0.01
Procyanidin trimer [i]	0.38 ± 0.01	0.30 ± 0.03	0.01

Abbreviations: n.d., not detected; n.q., not quantified. Extractions were performed twice at optimised conditions: organic solvent 72 % and formic acid 1 %, at 20 mL/g and 38 °C. Results are expressed in mg/Kg dw ± SD (n=3). Statistics by Student's t-test. d1, d2, d3, d4 and d5 indicate different isomeric compounds. [a] Quantified using the calibration curve of benzoic acid. [b] Quantified using the calibration curve of protocatechuic acid. [c] Quantified using the calibration curve of gallic acid. [d] Quantified using the calibration curve of caffeic acid. [e] Quantified using the calibration curve of chlorogenic acid. [f] Quantified using the calibration curve of ferulic acid. [g] Quantified using the calibration curve of resveratrol. [h] Quantified using the calibration curve of catechin. [i] Quantified using the calibration curve of procyanidin dimer B2.

Supplementary Table 1: Retention time (RT), detection wavelength, calibration curves, determination coefficient (R^2), linearity range, method precision, method reproducibility, limit of detection (LOD) and limit of quantification (LOQ) for phenolic compounds detected by the HPLC-DAD method.

						Precision (% RSD, n=3)			Reproducibility (% RSD, n=3)				
Compound	**RT (min)**	**Detection wavelenght (nm)**	**Calibration curve**	**R^2**	**Linearity (µM)**	**100 µg/mL**	**50 µg/mL**	**1 µg/mL**	**100 µg/mL**	**50 µg/mL**	**1 µg/mL**	**LOD (µM)**	**LOQ (µM)**
Chlorogenic acid	9.958	320	*y*=23.295x	0.9996	2.822 - 354.310	0.052	0.064	2.151	1.307	1.143	1.390	0.773	2.577
(+)-Catechin	10.662	280	*y*=11.438x	0.9995	3.445 - 290.280	0.368	0.356	2.091	0.912	0.504	9.275	0.165	0.548
Rutin	15.138	340	*y*=25.972x	0.9992	1.638 - 610.520	1.199	0.392	0.153	0.707	0.931	10.099	0.392	1.306

Supplementary Table 2: Molecular weight and optimised MRM (multiple reaction monitoring) conditions of the identified compounds in apricots by HPLC-ESI-MS/MS.

Compound	MW	[M-H]$^{-a}$	Quantification		Confirmation	
			MS/MS	CE (V)	MS/MS	CE (V)
Benzoic acid	122.12	121.0304	121>77	8	121>59	4
Hydroxybenzoic acid	138.12	137.0256	137>93	40	137>65	36
Protocatechuic acid	154.12	153.0217	153>109	16	153>62	40
Dihydroxybenzoic acid	154.12	153.0215	153>109	16	153>62	40
p-Coumaric acid	164.16	163.0774	163>119	16	163>93	36
Gallic Acid	170.12	169.0137	169>125	12	169>79	24
Caffeic acid	180.16	179.0344	179>135	16	179>107	24
Ferulic acid	194.18	193.0520	193>134	12	193>178	12
Resveratrol	228.24	227.0678	227>143	28	227>185	20
Kaempferol	286.24	285.0403	285>239	28	285>117	56
Eriodictyol	288.25	287.0793	287>151	12	271>135	28
Catechin	290.27	289.0739	289>245	12	289>203	10
Epicatechin	290.27	289.0742	289>245	12	289>203	10
Quercetin	302.24	301.0100	301>151	20	301>179	20
Protocatechuic acid glucoside	316.26	315.0737	315>153	10	315>109	20
Gallic acid O-glucoside	332.26	331.0697	331>169	12	331>125	12
Caffeic acid O-glucoside	342.30	341.0896	341>179	20	341>323	10
Chlorogenic acid	354.31	353.0905	353>191	16	353>85	16
Feruloylquinnic acid	368.34	367.1062	367>193	40	367>161	40
Resveratrol O-glucoside	390.38	389.0675	389>227	20	389>185	20
Catechingallate	442.37	441.0806	441>169	20	441>331	0
Kaempferol-3-*O*-glucoside	448.38	447.0974	447>284	28	447>255	40
Eriodictyol-7-*O*-glucoside	450.39	449.1084	449>287	12	449>151	36
Catechin O-glucose	452.41	451.1298	451>289	20	451>245	20
EGCG	458.37	457.0700	457>169	16	457>305	20
Hyperoside	464.38	463.0917	463>299.9	32	463>271	48
Isorhamnetin-3-*O*-glucoside	478.40	477.1014	477>314	32	477>285	40
Procyanidin dimer B2	578.52	577.1389	577>425	12	577>407	28
Kaempferol-3-*O*-rutinoside	594.52	593.1560	593>285	32	593>255	60
Rutin	610.52	609.1508	609>299.9	40	609>271	60
Procyanidin trimer	866.78	865.2041	865>289	20	865>713	20

Abbreviations: MW, molecular weight; CE, collision energy; [a] Mass detected by qTOF.

Supplementary Table 3: HPLC-ESI-MS/MS method quality parameters for the studied phenolic compounds in apricots.

Compound	RT (min)	Calibration curve	R^2	Linearity (µM)	LOQ (nM)	LOD (nM)
Benzoic acid	9.77	y=304.74x	0.993	0.016-40.943	7.18	23.94
Protocatechuic acid	3.90	y=11.11x	0.996	0.013-32.442	18.20	60.68
p-Coumaric acid	7.68	y=3567.30x	0.999	0.012-30.458	0.87	2.89
Gallic Acid	3.02	y=11453.00x	0.996	0.012-29.391	0.10	0.33
Caffeic acid	5.85	y=88.55x	0.999	0.011-27.754	4.73	15.76
Ferulic acid	8.32	y=1019.40x	0.996	0.010-25.749	0.43	1.43
Resveratrol	11.71	y=529,84x	0.998	0.009-21.906	0.08	0.28
Kaempferol	13.03	y=1489.30x	0.997	0.007-17.468	0.16	0.49
Eriodictyol	12.84	y=1556.40x	0.995	0.007-17.346	0.05	0.15
Catechin	5.13	y=369,62x	0.991	0.007-17.225	0.69	2.29
Epicatechin	6.17	y=500,25x	0.995	0.007-17.225	0.66	2.20
Quercetin	13.25	y=1628.00x	0.996	0.007-16.543	0.05	0.17
Chlorogenic acid	4.80	y=1032.10x	0.999	0.006-14.112	0.32	1.07
Kaempferol-3-*O*-glucoside	9.55	y=1073.20x	0.993	0.004-11.151	0.06	0.19
Eriodictyol-7-*O*-glucoside	8.44	y=1968.60x	0.995	0.004-11.101	0.04	0.15
EGCG	6.40	y=1699,3x	0.999	0.004-10.908	0.05	0.16
Hyperoside	8.37	y=1527.80x	0.994	0.004-10.767	0.04	0.14
Isorhamnetin-3-*O*-glucoside	9.71	y=1058.90x	0.990	0.004-10.451	0.04	0.15
Procyanidin dimer B2	5.63	y=192,39x	0.999	0.003-8.634	0.29	0.96
Kaempferol-3-*O*-rutinoside	9.04	y=976.41x	0.998	0.003-8.410	0.03	0.09
Rutin	8.07	y=825.00x	0.998	0.003-8.190	0.06	0.20

Abbreviations: RT, retention time; R^2, determination coefficient; LOD, limit of detection; LOQ, limit of quantification.

Supplementary Table 4: Overall optimal extraction parameters for phenolics in apricots.

Extraction variables			Response variable	Predicted	Experimental
T (°C)	MetOH (%)	Desirability			
38	72	0.861	Cat	1.28	1.32 ± 0.03
			Chl	1.56	1.56 ± 0.14
			Rut	0.59	0.61 ± 0.01

Abbreviations: Temperature (T); methanol (MetOH); (+)-catechin (Cat); chlorogenic acid (Chl); rutin (Rut). Results are expressed as mg of phenolic components per gram of dry weight (mg/g dw) ± SD (n=3).

Figure 1.

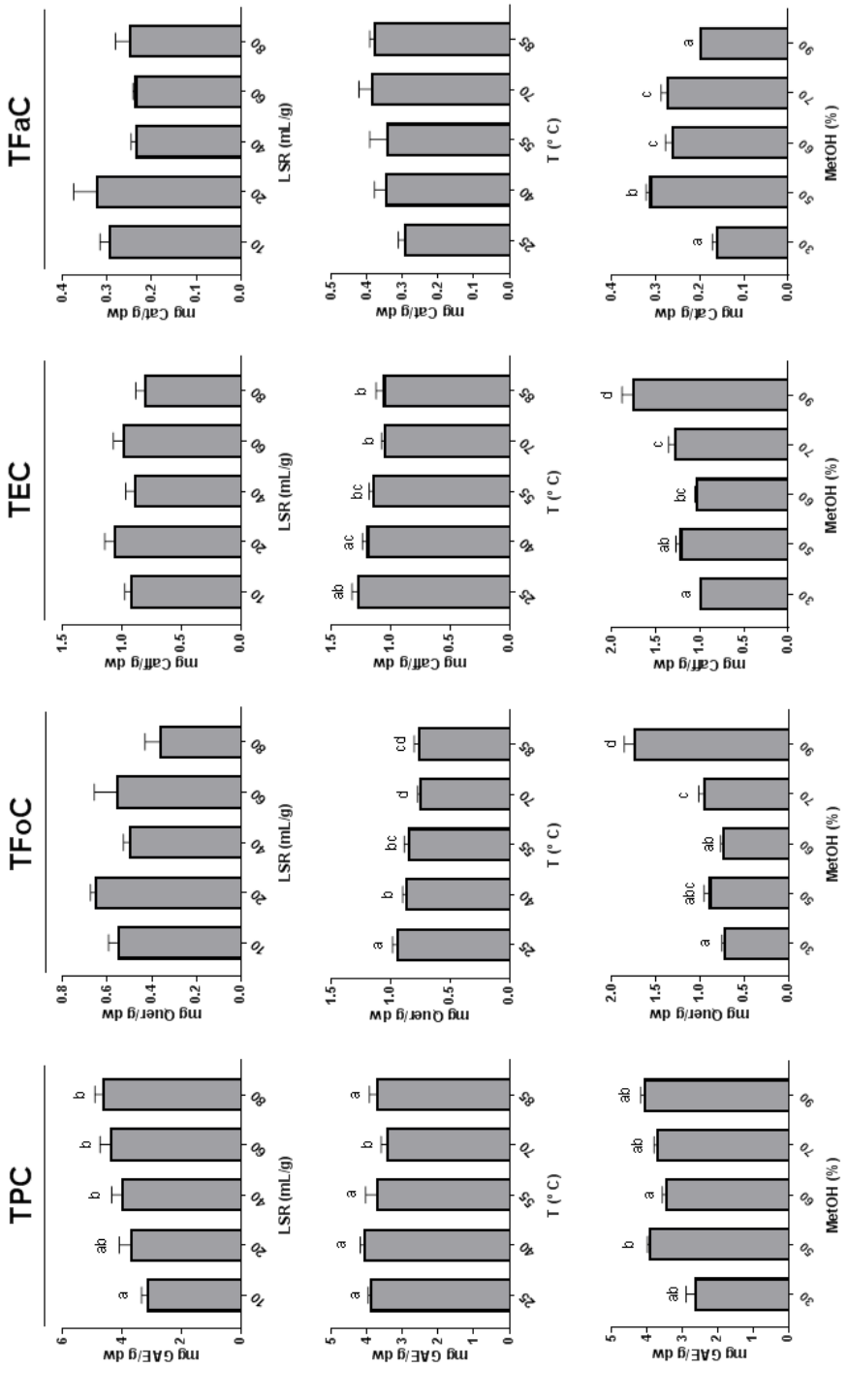

TPC
TFoC
TEC
TFaC
A
B
C
mg GAE/g dw
mg Quer/g dw
mg Cat/g dw
mg Caff/g dw
LSR (mL/g)
T (° C)
MetOH (%)

Figure 2.

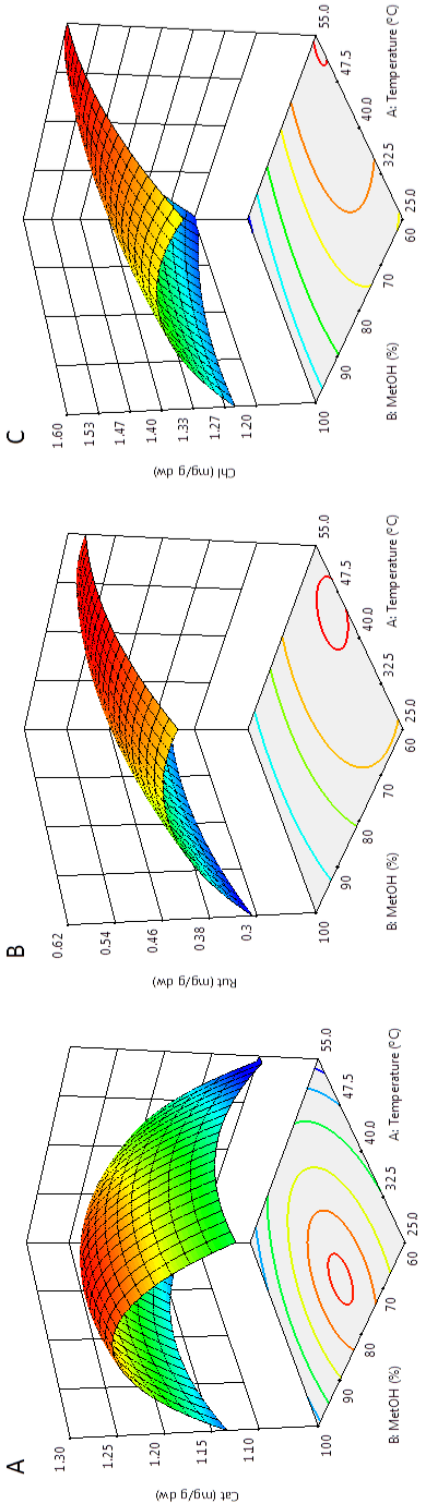

A
Cat (mg/g dw)
1.30
1.25
1.20
1.15
1.10
B
Rut (mg/g dw)
0.62
0.54
0.46
0.38
0.3
C
Chl (mg/g dw)
1.60
1.53
1.47
1.40
1.33
1.27
1.20
B: MetOH (%)
100
90
80
70
60
A: Temperature (°C)
55.0
47.5
40.0
32.5
25.0

Figure 3.

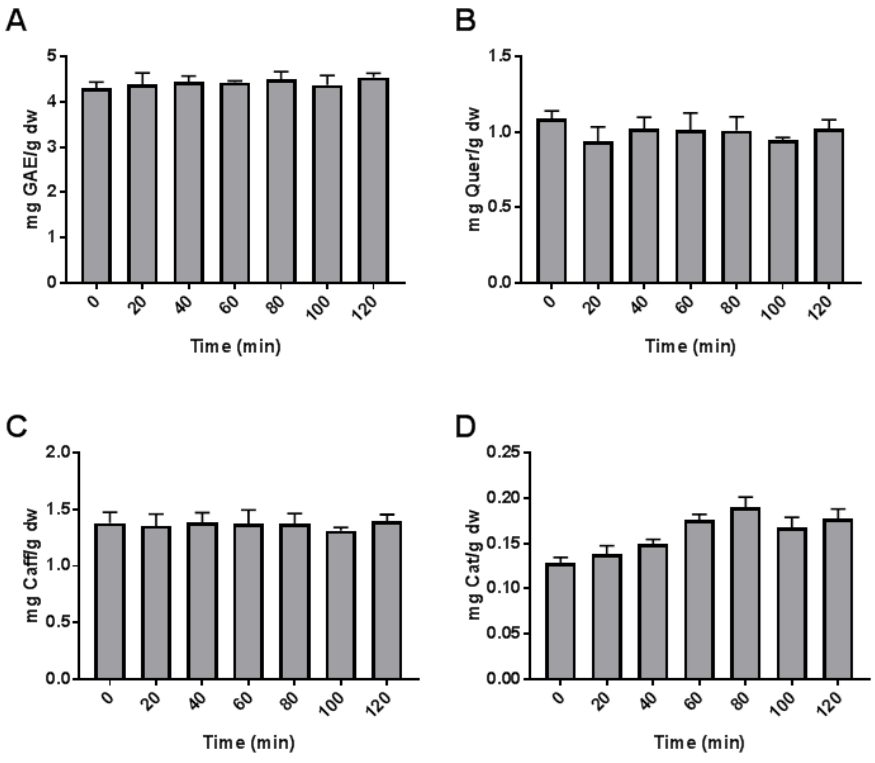
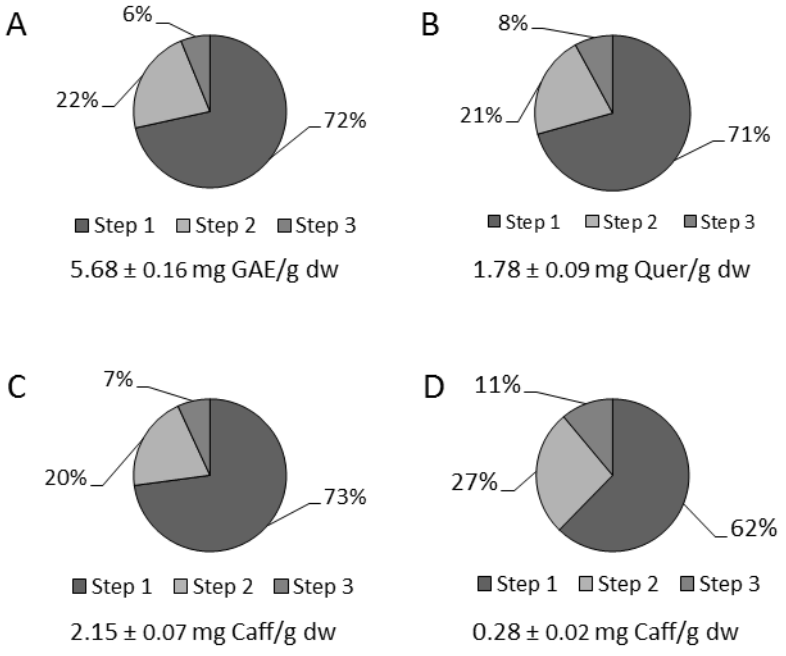

A
mg GAE/g dw
0
1
2
3
4
5
0
20
40
60
80
100
120
Time (min)
B
mg Quer/g dw
0.0
0.5
1.0
1.5
0
20
40
60
80
100
120
Time (min)
C
mg Caff/g dw
0.0
0.5
1.0
1.5
2.0
0
20
40
60
80
100
120
Time (min)
D
mg Cat/g dw
0.00
0.05
0.10
0.15
0.20
0.25
0
20
40
60
80
100
120
Time (min)

Figure 4.


A
6%
22%
72%
Step 1 Step 2 Step 3
5.68 ± 0.16 mg GAE/g dw
B
8%
21%
71%
Step 1 Step 2 Step 3
1.78 ± 0.09 mg Quer/g dw
C
7%
20%
73%
Step 1 Step 2 Step 3
2.15 ± 0.07 mg Caff/g dw
D
11%
27%
62%
Step 1 Step 2 Step 3
0.28 ± 0.02 mg Caff/g dw

MANUSCRIPT 2:

Specific extraction optimization by response surface methodology and full characterization of Royal Dawn sweet cherry (*Prunus avium*) phenolics reveal that this variety is rich in rutin

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MODERN CHANGES IN FRUIT INTAKE PATTERNS MODULATE THE BIOAVAILABILITY AND METABOLISM OF FRUIT PHENOLIC COMPOUNDS IN RATS
Lisard Iglesias Carres

Specific extraction optimization by response surface methodology and full characterization of Royal Dawn sweet cherry (*Prunus avium*) phenolics reveal that this variety is rich in rutin

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Abstract:

To correlate the beneficial effects of cherry consumption with their phenolic composition, a full and precise characterization is required. However, there is not a specific method to fully extract all phenolic compounds from sweet cherries. Thus, this study aimed to optimize the extraction of sweet cherry phenolics by response surface methodology and fully characterize the phenolic profile of Royal Dawn sweet cherries by HPLC-ESI-MS/MS. Extraction conditions were evaluated and optimized at 55 °C, MetOH 72 %, 12 mL/g in two extraction steps. Royal Dawn sweet cherries presented rutin as the predominant phenolic compound, unlike most varieties. Additionally, ethanol was evaluated as replacement solvent, obtaining lower extraction rates, especially for anthocyanins. However, in terms of total amounts, non-anthocyanin compounds were similarly extracted. The developed methodology was fast and can be routinely used in the evaluation of the phenolic profile of sweet cherries and to produce phenolic-rich extracts for the food industry.

Keywords:

Anthocyanins; cherries; flavonoids; polyphenols; response surface methodology.

Abbreviations:

Cy3R, cyanidin-3-*O*-rutinoside; dw, dry weigh; EtOH, ethanol; FO, flavonols; GAE, gallic acid equivalents; HCA, hydroxycinnamic acids; LSR, liquid-to-solid ratio; MetOH, methanol; T, temperature; TAC, total anthocyanin content; and TPC, total polyphenol content.

1. Introduction:

Cherries are one of the most popular fruits (Cao et al., 2015). There exist several species of cherries (Tavaud, Zanetto, David, Laigret, & Dirlewanger, 2004). However, the most relevant ones are *Prunus avium* and *Prunus cerasus* (Cao et al., 2015; Tavaud et al., 2004). *Prunus avium* include sweet cherries and wild cherry trees, which are respectively cultivated for human consumption and their wood. *Prunus cerasus* is cultivated for sour cherry production (Tavaud et al., 2004). Generally, sweet cherry varieties are mainly consumed fresh, whilst sour cherries are mostly used to produce jam, juice or other processed products (Cao et al., 2015; Tavaud et al., 2004).

Cherries are known for their rich nutritional content and their wide range of bioactive compounds, and these include phenolic compounds (Cao et al., 2015). The phenolic profile of sweet cherries (Chockchaisawasdee, Golding, Vuong, Papoutsis, & Stathopoulos, 2016; Di Matteo, Russo, Graziani, Ritieni, & Di Vaio, 2017; Martini, Conte, & Tagliazucchi, 2017; Wang, Jiang, Wang, Jiang, & Feng, 2017) has been widely studied. Sweet cherries are rich in anthocyanins, hydroxycinnamic acids, flavonols and flavan-3-ols (Chockchaisawasdee et al., 2016; Di Matteo et al., 2017; Martini et al., 2017; Wang et al., 2017). Anthocyanins occur mostly as cyanidin glycosyl derivates (Chaovanalikit & Wrolstad, 2004). Among them, cyanidin-3-*O*-rutinoside is widely reported as the anthocyanin with the highest concentration (Martini et al., 2017; Usenik, Fabčič, & Štampar, 2008). Additionally, other anthocyanidins such as peonidin, malvidin and pelargonidin glycosyl derivates occur in sweet cherries at lower concentrations (Kelebek & Selli, 2011; Mozetič, Trebše, Simčič, & Hribar, 2004). Hydroxycinnamic acids are also abundant in sweet cherries (Redondo, Arias, Oria, & Venturini, 2017), and some representatives of this family can reach higher concentrations than that of cyanidin-3-*O*-rutinoside in some varieties (Chockchaisawasdee et al., 2016). Chlorogenic acid, neochlorogenic acid and other cinnamoylquinic acid derivatives have

been described as relevant phenolic compounds in sweet cherries (Chockchaisawasdee et al., 2016; Redondo et al., 2017; Usenik et al., 2008). Flavonols occur mainly as quercetin and kaempferol glycosyl derivates in sweet cherries (Crupi, Genghi, & Antonacci, 2014; Martini et al., 2017), and rutin is the most abundant flavonol (Di Matteo et al., 2017). Monomeric flavan-3-ols usually occur in sweet cherries (Chockchaisawasdee et al., 2016), and epicatechin is found at higher concentrations than those of catechin (Gonçalves et al., 2007; Martini et al., 2017; Wang et al., 2017). Additionally, dimeric procyanidins can be found at relevant concentrations (Crupi et al., 2014; Martini et al., 2017).

Importantly, sweet cherry consumption has been associated with several beneficial effects (Kelley, Rasooly, Jacob, Kader, & Mackey, 2006; Prior et al., 2007; Zhang et al., 2012). To correlate the consumption of cherry phenolic compounds with health effects, proper characterization of the phenolic profile is required. To do so, specific methodologies to fully extract phenolic compounds are necessary. In this sense, extraction factors such as temperature, liquid-to-solid ratio (LSR), solvent, and time influence the extraction of phenolic compounds from anthocyanin-rich (Cacace & Mazza, 2003; Yılmaz, Karaaslan, & Vardin, 2014) and stone fruits (Wani et al., 2017; Yılmaz et al., 2014). In the specific case of sweet cherries, several extraction parameters vary widely between studies. For example, the LSR in the extraction of cherry phenolic compounds has been set at 5 mL/g (Kelebek & Selli, 2011; Usenik et al., 2008), though lower (Chaovanalikit & Wrolstad, 2004) and higher (Gonçalves et al., 2007) ratios have also been used. The wide variability of extraction methods (Chaovanalikit & Wrolstad, 2004; Di Matteo et al., 2017; Gonçalves et al., 2007; Martini et al., 2017; Usenik et al., 2008; Wang et al., 2017) makes it controversial to compare the phenolic profile of sweet cherries among studies.

Considering the chemical complexity and variety of phenolic compounds present in fruits and vegetables (Crozier, Jaganath, & Clifford, 2009; Del Rio et al., 2013), as well as the factors that potentially can affect the

extraction process (Ignat, Volf, & Popa, 2011), it becomes difficult to develop a universal extraction method for all food matrices (Ignat et al., 2011). Hence, the optimization of the extraction of phenolic compounds in different food matrices is essential. In this sense, response surface methodology (RSM) has been effective to optimize polyphenol extraction from different plant materials (Tabaraki & Nateghi, 2011; Yang et al., 2009), including anthocyanin-rich (Borges, Vieira, Copetti, Gonzaga, & Fett, 2011; Pompeu, Silva, & Rogez, 2009; Yılmaz et al., 2014) and stone fruits (Wani et al., 2017; Yılmaz et al., 2014). So far, extraction methods have been developed for the extraction of phenolic compounds from sour cherry pomace (Elez Garofulić, Dragović-Uzelac, Režek Jambrak, & Jukić, 2013; Yılmaz et al., 2014). Although the phenolic profile of sour cherries is similar to the one reported for sweet cherries, relevant differences exist (Kim, Ho, Young, Hyun, & Lee, 2005). In this sense, the most abundant anthocyanin in sour cherries is cyanidin-3-*O*-glucosyl-rutinoside instead of cyanidin-3-*O*-rutinoside (Chaovanalikit & Wrolstad, 2004; Kim et al., 2005; Wojdyło, Nowicka, Laskowski, & Oszmiański, 2014). Additionally, kaempferol-3-*O*-rutinoside instead of rutin is the most relevant flavonol in sour cherries (Wojdyło et al., 2014). Moreover, sour cherries have reported a higher total phenolic content (TPC) than that of sweet cherries (Kim et al., 2005; McCune, Kubota, Stendell-Hollis, & Thomson, 2011). Additionally, relevant components of the food matrix, such as sugar and protein content, are known to differ between sweet and sour cherries (McCune et al., 2011). This evidence suggests that the optimal conditions for the extraction of sweet and sour cherry phenolic compounds might differ.

Recently, Blackhall *at al.* developed a method to extract anthocyanins from Lapins sweet cherries (Blackhall, Berry, Davies, & Walls, 2018). However, to date, no specific methods that aim to fully extract all phenolic compounds from sweet cherries have been developed. Therefore, the aim of this work was to apply RSM to develop an extraction method for all sweet cherry phenolics, which could be routinely used to fully and

precisely characterize sweet cherry phenolics. Moreover, we also aim to characterize the phenolic profile of Royal Dawn sweet cherry by HPLC-ESI-MS/MS, which, to our knowledge, has not been previously characterized.

2. Materials and Methods:

2.1. Plant material:

Royal Dawn sweet cherries (*Prunus avium*) were purchased from Mercabarna (Barcelona, Spain) and were originally from Mendoza (Argentina). Cherry stones were manually removed and flesh was frozen in liquid nitrogen and ground. Next, homogenates were lyophilized for a week in a Telstar LyoQuest lyophilizer (Thermo Fisher Scientific, Madrid, Spain) at -55 °C and ground to a fine powder. The cherry powder was kept dry and protected from humidity and light exposure until extraction.

2.2. Chemicals and reagents:

Acetonitrile, methanol, ethanol (HPLC analytical grade) and glacial acetic acid were purchased from Panreac (Barcelona, Spain). Formic acid was purchased from Scharlab (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q Advantage A10 system (Madrid, Spain). Folin-Ciocalteu reagent was purchased from Fluka/ Sigma-Aldrich (Madrid, Spain). Apigenin, chlorogenic acid, eriodictyol, eriodyctiol-7-*O*-glucoside, hyperoside (quercetin-3-*O*-glucoside), isorhamnetin, isorhamnetin-3-*O*-glucoside, kaempferol, kaempferol-3-*O*-glucoside, and kaempferol-3-*O*-rutinoside were purchased from Extrasynthese (Lyon, France). Benzoic acid, caffeic acid, (+)-catechin, epigallocatechin gallate (EGCG), p-coumaric acid, (-)-epicatechin, ferulic acid, gallic acid, phloroglucinol, procyanidin dimer B2, protocatechuic acid and quercetin were purchased from Fluka/Sigma-Aldrich (Barcelona, Spain). Cyanidin-3-*O*-rutinoside, malvidin-3-*O*-glucoside and peonidin-3-*O*-rutinoside were purchased from PhytoLab (Vestenbergsgreuth, Germany). Resveratrol was purchased from

Quimivita (Barcelona, Spain), and rutin was kindly provided by Nutrafur (Murcia, Spain).

Standard compounds were individually dissolved in methanol (MetOH) at 2000 mg/L, with the exception of isorhamnetin-3-*O*-glucoside (1000 mg/L) and hyperoside (500 mg/L). Additionally, cyanidin-3-*O*-rutinoside, malvidin-3-*O*-glucoside and peonidin-3-*O*-rutinoside were individually dissolved in MetOH (0.01 % HCl) at 500 mg/L. All standard stock solutions were newly prepared every 3 months and stored in amber glass flasks at -20 °C. Mixed standard stock solutions of the standard compounds were prepared in mobile phase A (see sections 2.8.3 and 2.8.4) to obtain the concentration necessary to construct the calibration curves.

2.3. Extraction procedure.:

Cherry powder was weighed to obtain the desired LSR and mixed with 1.5 mL of pre-heated extraction solvent (methanol:water, v:v). Different extraction MetOH proportions, extraction temperatures, times and extraction steps were used throughout the experiment. MetOH was prepared in all cases including 1 % formic acid. Extractions were performed at 500 rpm agitation under protection from light exposure. Once the extraction was completed, samples were centrifuged at 9,500 xg for 10 min at 4 °C, and supernatants were stored at -20 °C until further analyses.

2.4. Response surface design:

The extraction of sweet cherry phenolics was optimized using an experimental design by RSM (Yılmaz et al., 2014). A rotatable central composite design with three factors and five levels was selected. The design consisted of 17 randomized runs with three center point replicates. The independent variables used in the RSM were temperature (T, X_1; 15-65 °C), methanol concentration (MetOH, X_2; methanol:water, 0-100 %) and

LSR (X_3; 4-14 mL/g). Extraction time (30 min) was fixed as a constant during the RSM experiment. Experimental data were fitted to a second polynomial response surface, which follows the equation (Eq 1)

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{\substack{i=1 \\ i<j}}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j$$

where Y is the dependent variable, β_0 the constant coefficient, and β_i, β_{ii} and β_{ij} are the linear, quadratic and interaction regression coefficients, respectively. X_i, X_{ii} and X_{ji} represent the independent variables. Independent variables (see section 2.7) included generic determinations (total phenolic content and total anthocyanin content) and individual compounds detected by HPLC-DAD (cyanidin-3-O-rutinoside, hydroxycinnamic acids and rutin). The results of the RSM design were analyzed with Design-expert 9.0.6 software (Trial version, Stat-Ease Inc., Minneapolis, MN, USA).

2.5. Kinetic study:

A kinetic study was performed to evaluate the effect of time on the polyphenol extraction yield in sweet cherries. Seven extraction times, ranging from 0 to 120 min, were selected. Extraction time of 0 min was established for samples that were mixed with the extraction solvent and immediately centrifuged (9,500 xg, 10 min, 4 °C). The LSR was fixed at 12 mL/g, MetOH percentage at 72 % and temperature at 55 °C. The TPC, TAC and anthocyanins, hydroxycinnamic acids and flavonols quantified by HPLC-DAD (see section 2.7) were determined for all extracts and used to evaluate the effect of time on polyphenol extractability.

2.6. Re-extraction study:

Four consecutive extractions were performed in order to evaluate the influence of multiple extractions on polyphenol extraction yield in sweet cherries. Samples were mixed with the pre-heated (55 °C) extraction

solvent (MetOH of 72 %) in a LSR of 12 mL/g and immediately centrifuged (9,500 xg, 10 min, 4 °C). The TPC, TAC and anthocyanins, hydroxycinnamic acids and flavonols were quantified by HPLC-DAD (see section 2.8), determined for all extracts, and used to evaluate the effect of sequential extractions on the polyphenol extraction yield.

2.7. Phenolic characterization of sweet cherries:

Sweet cherry phenolic profile was accurately quantified in methanol- and an ethanol-based (EtOH) extractions. Briefly, samples were mixed with the pre-heated (55 °C) extraction solvent (MetOH or EtOH of 72 % including 1 % formic acid) in a LSR of 12 mL/g and immediately centrifuged (9,500 xg, 10 min, 4 °C). The characterization of sweet cherries was performed by the developed HPLC-ESI-MS/MS methodology (see section 2.8.4).

2.8. Analysis of response variables:

2.8.1. Total polyphenol content:

The total phenolic content (TPC) of cherry extracts was determined by the Folin-Ciocalteu method adapted from Nenadis *et al.* (Nenadis, Boyle, Bakalbassis, & Tsimidou, 2003). Briefly, 10 µL of the extract and 50 µL of Folin-Ciocalteu reagent were successively added to an Eppendorf tube containing 500 µL of Milli-Q water and mixed. Next, the samples were stored in the dark for 3 min and received 100 µL of Na_2CO_3 (25 %). The samples were brought to a final volume of 1 mL with Milli-Q water and maintained in the dark for 1 hour. The absorbance was read at 725 nm using an Eon BioTek spectrophotometer (Izasa, Barcelona, Spain) against a water sample that underwent equivalent treatment. Gallic acid was used to construct the calibration curve between 40 mg/L and 400 mg/L. The results were expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g dw).

2.8.2. Total anthocyanin content:

The total anthocyanin content (TAC) of cherry extracts was analyzed by the pH differential method (Wrolstad, 1993). Extracts were diluted with sodium acetate buffer (0.4 M, pH 4.5) and potassium chloride buffer (0.025 M, pH 1.0) to relevant spectrophotometric ranges (0.4 – 0.6). Next, absorbance was read at 515 and 700 nm using an Eon BioTek spectrophotometer. TAC was expressed as milligrams of cyanidin-3-*O*-rutinoside equivalents per gram of dry weight (mg Cy3R Eq/g dw). The molar absorbance of Cy3R (595.2 g/mol) used was 28800 L/mol x cm.

2.8.3. HPLC-DAD quantification of phenolic compounds:

Detection and quantification of sweet cherry phenolics for the RSM study was performed by HPLC-DAD. Polyphenol separation was achieved using a ZORBAX Eclipse XDB-C18 (150 mm x 2.1 mm i.d., 5 µm particle size) as the chromatographic column equipped with a Narrow-Bore guard column (2.1 mm x 12.5 mm, 5 µm particle size) (Agilent Technologies, Palo Alto, CA, USA). The mobile phase was (A) water:acetic acid (95:5, v:v) and (B) acetonitrile:acetic acid (95:5, v:v). The HPLC gradient consisted of the following steps: initial conditions 0 % B; 0-30 % B, 0-18 min; 30-100 % B, 18-19 min; 100 % B isocratic, 19-20 min; and 100-0 % B, 20-21 min. A post-run of 6 min was required for column re-equilibration. The flow rate was set at 0.5 mL/min, and the injection volume was 10 µL for all runs. Identification and quantification of the phenolic compounds of interest was achieved with a UV/Vis photodiode array detector (1260 Infinity, Agilent Technologies, Palo Alto, CA, USA) and chromatograms were recorded from 200 to 600 nm. Detected and quantified compounds included the following: hydroxycinnamic acids (HCAs), detected at 320 nm and quantified as chlorogenic acid equivalents; cyanidin-*3-O*-rutinoside (Cy3R), detected at 520 nm; and the flavonol rutin (FO), detected at 340 nm. The results were expressed as milligrams of equivalents per gram of dry weigh. The method quality parameters can be found in Table S1.

2.8.4. HPLC-ESI-MS/MS quantification of phenolic compounds:

The extracts were directly analyzed using a 1200 LC Series coupled to a 6410 MS/MS (Agilent Technologies, Palo Alto, CA, USA). Of note, two different HPLC-ESI-MS/MS systems were used to separate, detect and quantify non-anthocyanin and anthocyanin phenolic compounds. In the case of non-anthocyanin compounds, the column and mobile phases used were the same as those in the HPLC-DAD method (see section 2.8.3). The gradient mode was the following: initial conditions, 0 % B; 0-0.5 min, 0 % B; 0.5-2 min, 0-10 % B; 2-12 min, 10-30 % B; 12-16 min, 30-60 % B; 16-17 min, 60-100 % B; 17-20 min, 100 % B; and 20-21 min, 100-0 % B. A post-run of 6 min was required for column re-equilibration. The flow rate was set at 0.4 mL/min, and the injection volume was 2.5 µL for all runs. Electrospray ionization (ESI) was conducted at 200 °C and 14 l/min with 20 psi of nebulizer gas pressure and 3000 V of capillary voltage. The mass spectrometer was operated in the negative mode, and MS/MS data were acquired in dynamic mode. Separation of anthocyanins was achieved using an Acquity BHE C18 column (50 mm x 2.1 mm, 1.7 5 µm particle size) (Waters, Milford, MA, USA). Mobile phases consisted of water: formic acid (9:1, v:v) (A) and acetonitrile (B). The gradient mode was the following: initial conditions, 0 % B; 0-1 min, 0 % B; 1-5 min, 0-9 % B; 5-10 min, 9-15 % B; 10-15 min, 15-45 % B; 15-16 min, 45-100 % B; 16-17 min, 100 % B; and 17-18 min, 100-0 % B. A post-run of 6 min was required for column re-equilibration. Flow rate was set at 0.4 mL/min, and the injection volume was 2.5 µL for all runs. ESI was conducted as previously descrived. The mass spectrometer was operated in the positive mode, and MS/MS data were acquired in the dynamic mode. Optimized conditions for the analysis of non-anthocyanin and anthocyanin phenolic compounds are summarized in Table S2. In both methodologies, data acquisition was carried out using MassHunter Software (Agilent Technologies, Palo Alto, CA, USA). The calibration curves, coefficient of determination, linearity and

detection and quantification limits for non-anthocyanin and anthocyanin phenolic compounds can be found in Table S3.

2.9. Statistical analysis:

The results of the RSM design were analyzed using Design-expert 9.0.6 software (Trial version, Stat-Ease Inc., Minneapolis, MN, USA). SPSS 19 software (SPSS Inc., Chicago, IL, USA) was used for all other statistical analyses. All experiments were performed in triplicate; the statistics' significance was evaluated using a one-way ANOVA or Student's t-test, and $p<0.05$ was considered to be statistically significant.

3. Results and Discussion:

Sweet cherries are a rich source of anthocyanins, hydroxycinnamic acids, flavonols and flavan-3-ols (Martini et al., 2017). Additionally, sweet cherry phenolic compounds have demonstrated relevant biological activities (Ferretti, Bacchetti, Belleggia, & Neri, 2010). Specific methods that fully extract phenolic compounds for each food matrix are required to completely characterize these compounds and to link food consumption with a health benefit. Methods have been developed for the extraction of anthocyanins in Lapins sweet cherries (Blackhall et al., 2018) and anthocyanin-rich fruits (Blackhall et al., 2018; Grigoras, Destandau, Zubrzycki, & Elfakir, 2012), and phenolic compounds from sour cherry pomace (Elez Garofulić et al., 2013; Yılmaz et al., 2014). However, to our knowledge, no methods that aim to fully extract the most representative phenolic families of sweet cherries exist. Therefore, in this study, we investigated the factors affecting sweet cherry phenolic extraction and optimized them by RSM. Moreover, the extraction method was used to completely characterize by HPLC-ESI-MS/MS the phenolic profile of Royal Dawn sweet cherries for the first time.

3.1. Response surface methodology:

The extraction of sweet cherry phenolics was optimized using the RSM approach previously used by Yılmaz et al. in sour cherries (Yılmaz et al., 2014). Extraction time (30 min) was fixed during the RSM experiment (Blackhall et al., 2018). The TPC, TAC and anthocyanins, hydroxycinnamic acids and flavonols quantified by HPLC-DAD were included in the RSM as relevant representatives of the phenolic families present in sweet cherries (Chockchaisawasdee et al., 2016; Martini et al., 2017). The experimental results for all runs were included in the model (Table 1).

3.1.1. Fitting the model:

The experimental data (Table 1) were used to determine the regression coefficients of equation 1. All the selected compounds generated a significant model, confirming that at least one of the extraction variables could explain the variation of the response variable in comparison with its mean. The coefficients of determination (R^2) and p-values for the lack of fit test can be found in Table 2.

3.1.2. Analysis of regression coefficients:

The effect of MetOH proportion on the extraction of phenolic compounds from sweet cherries was evaluated from 0 to 100 %. A significant ($p<0.05$) positive linear effect of MetOH was found for TAC, Cy3R and FO, while a tendency ($p<0.1$) was observed for TPC. No quadratic effects were reported for HCA or FO, which indicates that an increase in MetOH increases the extraction of those compounds. Linear models have also been reported in the extraction of flavan-3-ols in different plant matrices (Borges et al., 2011). A tendency ($p<0.1$) towards negative quadratic MetOH effects was observed for the TAC. This finding implies that the phenolic extraction increases up to an optimal MetOH percentage, after which it starts to decrease (Figure 1). Positive linear and negative

quadratic effects of the extraction solvent are found for the extraction of TAC in sour cherries (Yılmaz et al., 2014) and other phenolic compounds in different plant materials (Assefa, Saini, & Keum, 2017).

The effect of extraction temperature on the extraction of phenolic compounds from sweet cherries was evaluated from 15 to 65 °C. In line with other studies, we maintained extraction temperature below 65 °C to avoid phenolic degradation (Wani et al., 2017; Yang et al., 2009). No significant linear or quadratic effects were observed for TAC, Cy3R, HCA or FO, which is in disagreement with different studies in different fruit parts (Assefa et al., 2017), stone fruits (Yılmaz et al., 2014) and anthocyanin-rich fruits (Pompeu et al., 2009; Yılmaz et al., 2014). In agreement with our results, Ku *et al*. (Ku & Mun, 2008) did not report a significant effect of temperature on the extraction of anthocyanins from *Rubus coreanus* marc. Temperature was only found to produce a positive quadratic effect on the extraction of TPC. This result suggests that the effect of temperature on the extraction of sweet cherry phenolics is not very relevant (Figure 1). Optimization of the extraction of TPC, tartaric esters and flavonols from black currants was not influenced by the extraction temperature evaluated in a very similar range to our study (Cacace & Mazza, 2003). Indeed, degradation of phenolic compounds has been reported at temperatures higher than 65 °C in RSM designs (Yılmaz et al., 2014). Therefore, the use of lower temperatures in this study could explain the lack of significance of this factor in the extraction of sweet cherry phenolic compounds.

The effect of LSR on the extraction of sweet cherry phenolics was evaluated from 4 to 14 mL/g. TPC, HCA and FO presented a significant positive linear effect of LSR and TAC a significant positive quadratic effect. This result implies that higher LSR will result in a higher extraction of phenolic compounds from sweet cherries (Figure 1). Our results are in agreement with the extraction of different phenolic compounds from sour cherries (Yılmaz et al., 2014), black currants (Cacace & Mazza, 2003) and other plant matrices (Borges et al., 2011; Ku & Mun, 2008) in which the LSR significantly influenced the extraction of phenolic compounds. A

significant interaction (crossover) effect between MetOH and LSR was observed for the extraction of TAC and Cy3R, which was negative in both cases. This effect implies that, depending on the MetOH proportion, the LSR has a different effect. Although crossover effects are not common in the literature, several studies have reported them (Assefa et al., 2017; Borges et al., 2011; Ku & Mun, 2008).

3.1.3. Validation of the model:

To optimize the extraction method for sweet cherry phenolics, the studied variables were maximized. The combination of extraction variables at the highest desirability (0.801) was selected. Specifically, this corresponded to temperature of 55 °C; MetOH of 72 % and LSR of 12 mL/g. Three extractions were performed under those conditions to confirm the model's prediction (Table 3). No differences were obtained between the predicted and experimental values of TAC, Cy3R, HCA and FO, which confirmed the model's accuracy. However, the TPC values were outside the range predicted by the model. Our model could accurately predict all responses except TPC; however, obtaining a higher TPC than that predicted does not represent a serious drawback, as our goal was to extract the maximum phenolic compounds. Therefore, extraction temperature, MetOH proportion and LSR were fixed at 55 °C, 72 % and 12 mL/g throughout the rest of the study. Surprisingly, the optimized extraction LSR was the same as that reported for sour cherry phenolics extraction (Yılmaz et al., 2014) and was very similar to the one reported in the extraction of anthocyanins from Lapins sweet cherries (Blackhall et al., 2018). Despite the same LSR, the solvent/water proportion, optimized at 51 % and 100 %, and extraction temperature, optimized at 75 ° C and 37 °C, were significantly different for the sour and Lapins cherries, respectively (Blackhall et al., 2018; Yılmaz et al., 2014).

3.2. Effect of time on phenolic extraction:

It has been hypothesized that extraction time has a cell-wall weakening effect, promoting the solubilization of phenolic compounds in the solvent (Tabaraki & Nateghi, 2011; Yang et al., 2009). Additionally, the selection of an appropriated extraction time is relevant when considering efficiency, labor and energy costs (Yılmaz et al., 2014). Changes in the response variables due to the effect of time are shown in Supplementary Table 4. Although different studies report a significant effect of time in cherries (Blackhall et al., 2018; Yılmaz et al., 2014) and other anthocyanin-rich fruits (Borges et al., 2011; Cacace & Mazza, 2003; Pompeu et al., 2009), in our study, no significant differences were reported due to the effect of extraction time. The fact that phenolic compounds are rapidly transferred into the extraction solvent makes our method more economically feasible than the methods developed by Yilamez *et al.* (Yılmaz et al., 2014) for sour cherry phenolics and Blackhall *et al.* (Blackhall et al., 2018; Yılmaz et al., 2014) for sweet cherry anthocyanins, which required 100 and 90 min to reach the maximum extraction of phenolic compounds, respectively. However, our results suggest that the solvent is saturated right after the sample is mixed and vortexed with the extraction solvent, indicating that time cannot produce an increase in the polyphenol extraction yield. Consequently, our results open the door to the study of successive sample extractions.

3.3. Effect of multiple-step extractions on phenolic extraction:

Multi-step extractions are a useful strategy to increase the extraction yield of phenolic compounds in food matrices (Yang et al., 2009). To test this strategy in sweet cherries, four sequential extractions were performed. The results show a considerable increase in the extraction of phenolic compounds between the first and second extraction steps (Figure 2). However, after the second extraction step, no significant increases were found in the extraction of sweet cherry phenolics, indicating that the

extraction is mostly completed at the second extraction step, and this result is in agreement with the extraction of phenolic compounds from grapes (Mané et al., 2007). Therefore, two sequential steps were defined as optimal and used throughout the rest of the experiment.

3.4. Phenolic profile of Royal Dawn sweet cherries by HPLC-ESI-MS/MS:

The phenolic profile of Royal Dawn sweet cherries by HPLC-ESI-MS/MS (Table 4) is in agreement with the major phenolic families occurring in other sweet cherries (Chockchaisawasdee et al., 2016; Di Matteo et al., 2017; Martini et al., 2017; Wang et al., 2017). Cyanidin-3-*O*-rutinoside was the anthocyanin with the highest concentration, which is consistent with the phenolic profile of several sweet cherry varieties (Kelebek & Selli, 2011; Martini et al., 2017). In accordance with the literature, cyanidin-based anthocyanins were found to be predominant (Chaovanalikit & Wrolstad, 2004; Chockchaisawasdee et al., 2016). Other anthocyanins, such as delphinidin- and peonidin-based anthocyanins, were found in this study and others (Chockchaisawasdee et al., 2016; Di Matteo et al., 2017; Kelebek & Selli, 2011; Martini et al., 2017). Several hydroxycinnamic acids were found in high concentrations in this study, which is consistent with the fact that stone fruits are rich in these type of phenolic compounds (Redondo et al., 2017). Indeed, caffeoylquinic acid derivatives are widely reported among the phenolic compounds with the highest concentration in sweet cherries (Di Matteo et al., 2017; Usenik et al., 2008). In our study, cyanidin-3-*O*-rutinoside was found at a higher concentration than that of chlorogenic and neochlorogenic acids. This trend is also found in different sweet cherry varieties (Chockchaisawasdee et al., 2016; Martini et al., 2017). Rutin, however, was found at a higher concentration than that of any other compound in our study. Although rutin is reported as the main flavonol in sweet cherries (Di Matteo et al., 2017; Martini et al., 2017), only few varieties report rutin as the predominant phenolic compound

(Kelebek & Selli, 2011; Usenik et al., 2008). Other flavonols described in different sweet cherry varieties, including kaempferol-3-*O*-rutinoside and quercetin-3-*O*-glucoside, were also found in this study (Martini et al., 2017). The main flavan-3-ol representative in Royal Dawn sweet cherries was epicatechin, which had a 3-fold higher concentration than that of catechin. Indeed, epicatechin is widely reported as the main flavan-3-ol in several sweet cherry varieties (Di Matteo et al., 2017; Martini et al., 2017). In agreement with Martini *et al*. (Martini et al., 2017) several procyanidin dimeric compounds were found in Royal Dawn sweet cherries. Precisely, procyanidin dimer B2 was the procyanidin with the highest concentration and reached higher concentrations than those of catechin. Although not common, this trend has been observed in other varieties such as Beritello sweet cherries (Di Matteo et al., 2017). With the exception of flavan-3-ols, non-glycosylated flavonoids were found in very low concentration. Indeed, flavonoids occur mostly as glycosides in plants (Crozier et al., 2009; Del Rio et al., 2013), including sweet cherries (Di Matteo et al., 2017). Free phenolic acids were also found at low concentrations, a trend that has been reported in other sweet cherry varieties (Wang et al., 2017).

3.5. Investigation of solvent replacement:

The methanolic and ethanolic extractions of sweet cherries showed that, in general, phenolic compounds were better extracted in MetOH than they were in EtOH (Table 4), which is consistent with the literature (Borges et al., 2011; Ignat et al., 2011; Lapornik, Prošek, & Golc Wondra, 2005; Silva, Costa, Calhau, Morais, & Pintado, 2017). In the specific case of anthocyanins, methanolic extraction achieved significantly higher extractions, which were also relevant in terms of total amounts. Only a few anthocyanins (i.e., delphinidin *O*-coumaroylglucose d1) were extracted at higher amounts in the ethanol-based extraction. Consistent with our results, methanol was a better extraction solvent for anthocyanins in blueberries (Silva et al., 2017). Additionally, Lapornik *et al*. (Lapornik et al.,

2005) reported 70 % methanol to be a better extraction solvent than that of 70 % ethanol in several anthocyanin-rich fruit by-products. For the non-anthocyanin compounds, methanol based-extraction only achieved statistically significant and relevant higher extraction rates (>20 %) of ferulic acid, quercetin *O*-glucoside, isorhamnetin-3-*O*-glucoside, procyanidin dimer d3 and procyanidin trimer. For the ethanol-based extraction, only protocatechuic acid and quercetin, which were significantly extracted in higher amounts with EtOH, reached a relevant increase (>20 %) of their concentration. Our results are in agreement with other studies that evaluate the extraction of non-anthocyanin phenolic compounds in different vegetal matrices (Tabaraki & Nateghi, 2011; Yılmaz et al., 2014). With the exception of anthocyanins, relevant sweet cherry phenolics with potential bioactivities (Del Rio et al., 2013; Kelebek & Selli, 2011), such as rutin or procyanidin dimer B2, were similarly extracted in both extraction solvents. Consequently, the adaptation of ethanol-based extraction to the food industry could still be useful to produce phenolic extracts with potential health bioactive effects. Additionally, the use of methanol-based methodology can be used to routinely characterize phenolic profiles from sweet cherries.

4. Conclusions:

We optimized by RSM a specific method to rapidly extract all phenolic compounds from sweet cherries. Additionally, we used the optimized method to fully extract and correctly profile by HPLC-ESI-MS/MS the phenolic composition of Royal Dawn sweet cherries and demonstrated that, unlike most sweet cherry varieties, rutin is the predominant phenolic compound. This methodology could be routinely used to extract phenolics from sweet cherries for their full characterization. This characterization is essential to link cherry fruit consumption health-promoting effects with their phenolic profile. Moreover, this method could be applied to produce phenolic-rich extracts for the food industry.

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Figure legends:

Figure 1: Response surface plots for total polyphenol content (TPC; A), total anthocyanin content (TAC; B and C), cyanidin-3-O-rutinoside (Cy3R; D), hydroxycinnamic acids (HCA; E) and flavonols (FO; F) of sweet cherries as a function of extraction temperature, methanol proportion and liquid-to-solid ratio (LSR). A at MetOH=50 %, B at LSR=6 mL/g; and C, D, E and F at T= 40 °C.

Figure 2: Effect of sequential extraction on the extraction of total polyphenol content (TPC; A), total anthocyanin content (TAC; B), cyanidin-3-O-rutinoside (Cy3R; C), hydroxycinnamic acids (HCA; D) and flavonols (FO; E) from sweet cherries. The results are expressed as milligrams of phenolic equivalent per gram of dry weigh ± SD (n=3) and percentage. Different letters (one-way ANOVA) indicate significant differences between extraction steps.

Table 1: Rotatable central settings of independent variables and experimental results of total polyphenol content (TPC), total anthocyanin content (TAC), Cyanidin-3-*O*-rutinoside (Cy3R), hydroxycinnamic acids (HCA) and flavonols (FO).

Run order	T (°C)	MetOH (%)	LSR (mL/g)	TPC	TAC	Cy3R	HCA	FO
1	40	100	9	5.944	1.268	3.288	8.990	0.160
2	55	80	6	6.446	1.768	3.439	9.110	0.166
3	40	0	9	5.158	0.657	1.095	7.865	0.131
4	25	80	6	5.981	1.652	3.546	9.344	0.168
5	40	50	4	5.167	1.920	3.515	8.995	0.173
6	65	50	9	7.414	1.562	3.777	7.855	0.186
7	55	20	6	5.379	0.911	2.020	7.793	0.144
8	15	50	9	7.162	1.596	3.671	9.917	0.176
9	55	80	12	8.461	1.823	4.127	11.806	0.205
10	40	50	9	5.949	1.399	3.378	9.252	0.165
11	40	50	9	6.127	1.306	2.984	8.167	0.151
12	55	20	12	7.556	1.480	3.961	11.676	0.166
13	25	20	12	7.687	1.630	4.217	12.342	0.183
14	40	50	14	6.820	1.540	3.739	11.546	0.181
15	25	20	6	5.038	0.889	1.054	6.123	0.124
16	25	80	12	7.013	1.525	3.383	11.251	0.171
17	40	50	9	6.643	1.265	2.896	7.884	0.145

Abbreviations: temperature (T), methanol (MetOH), liquid-to-solid ratio (LSR). Results are expressed as mg of phenolic components per gram of dry weight (mg/g dw).

Table 2: Analysis of variance and regression coefficients of predicted model for response variables in sweet cherries.

Model parameters	Regression coefficient	TPC	TAC	Cy3R	HCA	FO
Intercept	β_0	6.271	1.969	-0.044	4.957	0.161
Linear						
Temperature	β_1	-1.922×10^{-1}	-4.167×10^{-2}	-6.635×10^{-2}	-1.039×10^{-2}	3.811×10^{-3}
MetOH	β_2	$2.893\times10^{-2\#}$	3.204×10^{-2}*	1.100×10^{-1}*	1.341×10^{-2}	1.043×10^{-2}*
LSR	β_3	3.135×10^{-1}*	-1.960×10^{-1}	1.0342×10^{-2}	4.661×10^{-1}*	1.341×10^{-2}*
Interaction						
Temperature x MetOH	β_{12}	4.731×10^{-4}	1.506×10^{-4}	-2.000×10^{-5}	-	-
Temperature x LSR	β_{13}	1.419×10^{-3}	2.778×10^{-5}	-1.031×10^{-3}	-	-
MetOH x LSR	β_{23}	-2.471×10^{-3}	-1.919×10^{-3}*	-6.360×10^{-3}*	-	-
Quadratic						
Temperature x Temperature	β_{11}	2.104×10^{-3}*	4.377×10^{-4}	1.052×10^{-3}	-	-
MetOH x MetOH	β_{22}	-1.689×10^{-4}	$-1.372\times10^{-4\#}$	-3.500×10^{-4}	-	-
LSR x LSR	β_{33}	8.127×10^{-4}	1.698×10^{-2}*	2.241×10^{-2}	-	-
R^2		0.877	0.853	0.841	0.578	0.451
Adjusted R^2		0.719	0.663	0.635	0.480	0.324
p-value		0.017	0.030	0.038	0.009	0.045
F-value		5.558	4.504	4.100	5.930	3.558
Lack of fit [a]		0.299	0.082	0.140	0.242	0.275

Abbreviations: methanol (MetOH), liquid-to-solid ratio (LSR), total polyphenol content (TPC), total anthocyanin content (TAC), Cy3R (cyanidin-3-*O*-rutinoside), hydroxycinnamic acids (HCA) and flavonols (FO). [#] p *p<0.05. [a] p-value of lack of fit test.

Table 3: Overall optimal extraction parameters for phenolic compounds in sweet cherries.

Extraction variables						
T (°C)	MetOH (%)	LSR (mL/g)	Desirability	Parameters	Predicted	Experimental
55	72	12	0.801	TPC	7.825	10.969 ± 0.543
				TAC	1.647	1.688 ± 0.074
				Cy3R	3.808	2.953 ± 0.134
				HCA	10.944	11.979 ± 0.974
				FO	0.186	0.213 ± 0.014

Abbreviations: Temperature (T), methanol (MetOH), liquid-to-solid ratio (LSR), total polyphenol content (TPC), total anthocyanin content (TAC), Cy3R (cyanidin-3-o-rutinoside), hydroxycinnamic acids (HCA) and flavonols (FO). Results are expressed as mg of phenolic components per gram of dry weight (mg/g dw) ± SD (n=3).

Table 4: Phenolic compounds of sweet cherry extracted using methanol (MetOH) or ethanol (EtOH) as extraction solvents.

Compound	MetOH	EtOH	p-Value
Benzoic acid	2.39 ± 0.17	2.23 ± 0.04	0.20
Phloroglucinol	n.d.	n.d.	
Hydroxybenzoic acid [a]	0.75 ± 0.02	0.81 ± 0.00	0.01
Dihydroxybenzoic acid [b]	0.32 ± 0.00	0.33 ± 0.02	0.71
Protocatechuic acid	1.94 ± 0.04	2.90 ± 0.21	<0.01
p-Coumaric acid	0.12 ± 0.00	0.11 ± 0.00	<0.01
Gallic acid	0.02 ± 0.01	0.02 ± 0.01	0.98
Caffeic acid	0.40 ± 0.01	0.38 ± 0.00	0.07
Ferulic acid	0.36 ± 0.01	0.29 ± 0.01	<0.01
Resveratrol	0.30 ± 0.13	0.11 ± 0.05	0.08
Apigenin	0.04 ± 0.00	0.04 ± 0.01	0.92
Kempferol	0.02 ± 0.00	n.q.	
Eriodictyol	0.06 ± 0.02	0.05 ± 0.02	0.26
Catechin	16.36 ± 0.46	18.49 ± 2.77	0.26
Epicatechin	54.77 ± 0.57	46.46 ± 1.51	<0.01
Quercetin	1.55 ± 0.10	4.03 ± 0.19	<0.01
Caffeoyltartaric acid [c]	2.32 ± 0.11	2.75 ± 0.05	<0.01
Isorhamnetin	3.72 ± 0.08	3.80 ± 0.07	0.26
p-coumaric acid *O*-glucoside d1 [d]	0.91 ± 0.03	0.99 ± 0.02	0.01
p-coumaric acid *O*-glucoside d2 [d]	0.23 ± 0.00	0.22 ± 0.02	0.41
p-coumaric acid *O*-glucoside d3 [d]	0.48 ± 0.01	0.46 ± 0.04	0.68
p-coumaric acid *O*-glucoside d4 [d]	5.01 ± 0.15	5.76 ± 0.09	<0.01
p-coumaric acid *O*-glucoside d5 [d]	0.54 ± 0.01	0.52 ± 0.03	0.31
Gallic acid *O*-glucoside d1 [e]	0.03 ± 0.00	0.03 ± 0.00	0.65
Gallic acid *O*-glucoside d2 [e]	0.11 ± 0.00	0.11 ± 0.00	0.20
Caffeic acid *O*-glucoside [c]	241.95 ± 4.15	276.90 ± 7.12	<0.01
Neochlorogenic acid [f]	263.42 ± 32.21	235.01 ± 43.60	0.42
Chlorogenic acid	111.84 ± 5.94	89.87 ± 28.31	0.26
Cryptogenic acid [f]	34.81 ± 0.19	32.86 ± 1.77	0.13
Feruloylquinic acid [g]	1.66 ± 0.05	1.79 ± 0.04	0.02
Resveratrol O-glucoside d1 [h]	0.37 ± 0.02	0.32 ± 0.01	0.01
Resveratrol O-glucoside d2 [h]	0.52 ± 0.12	0.40 ± 0.01	0.15
Kaempferol-3-*O*-glucoside	2.55 ± 0.11	2.14 ± 0.04	<0.01
Eriodictyol-7-*O*-glucoside	0.40 ± 0.05	0.38 ± 0.16	0.85
Catechin O-glucose [i]	0.18 ± 0.01	0.21 ± 0.02	0.07
EGCG	0.04 ± 0.00	0.05 ± 0.00	<0.01
Quercetin *O*-glucoside [j]	13.11 ± 0.17	10.39 ± 0.25	<0.01
Hyperoside	n.q.	n.q.	
Isorhamnetin-3-*O*-glucoside	0.16 ± 0.03	0.11 ± 0.01	0.03
Procyanidin dimer d1 [k]	6.25 ± 0.10	7.27 ± 1.35	0.26
Procyanidin dimer B2	44.15 ± 0.43	39.34 ± 1.73	0.01
Procyanidin dimer d2 [k]	2.80 ± 0.28	2.54 ± 0.36	0.38
Procyanidin dimer d3 [k]	6.07 ± 0.16	4.74 ± 0.21	<0.01
Kaempferol-3-*O*-rutinoside	46.22 ± 0.50	39.45 ± 0.61	<0.01
Rutin	2141.34 ± 125.08	2194.54 ± 7.54	0.41
Procyanidin trimer [k]	1.63 ± 0.02	1.34 ± 0.07	<0.01
Cyanidin *O*-arabinoside [l]	13.93 ± 0.60	2.09 ± 0.23	<0.01
Cyanidin *O*-caffeoylglucose d1 [l]	0.37 ± 0.03	0.12 ± 0.01	<0.01
Cyanidin *O*-caffeoylglucose d2 [l]	7.78 ± 0.36	1.09 ± 0.04	<0.01

Table 4 (Continued).

Cyanidin *O*-glucose d1 [l]	213.83 ± 41.40	22.21 ± 2.31	<0.01
Cyanidin *O*-glucose d2 [l]	3.13 ± 0.09	0.31 ± 0.03	<0.01
Cyanidin-3-*O*-rutinoside	942.91 ± 170.29	29.21 ± 3.41	<0.01
Delphinidin 3-*O*-rutinoside [l]	0.14 ± 0.01	n.q.	
Delphinidin *O*-coumaroylglucose d1 [l]	0.96 ± 0.07	9.30 ± 0.26	<0.01
Delphinidin *O*-coumaroylglucose d2 [l]	9.91 ± 0.16	5.03 ± 0.08	<0.01
Delphinidin *O*-coumaroylglucose d3 [l]	97.61 ± 20.18	46.37 ± 0.82	0.02
Malvidin *O*-coumaroylglucose [m]	n.q.	0.04 ± 0.01	
Malvidin-3-*O*-glucoside	0.36 ± 0.12	0.50 ± 0.25	0.51
Pelargonidin *O*-glucose d1 [l]	7.81 ± 0.11	0.41 ± 0.04	<0.01
Pelargonidin O-glucose d2 [l]	n.q.	0.37 ± 0.04	
Peonidin-3-*O*-rutinoside	32.97 ± 1.48	5.26 ± 0.20	<0.01

Abbreviations: n.d., not detected, n.q., not quantified. Results are expressed in mg/kg dw ± SD (n=3). Statistics by Student's t-test. d1, d2, d3, d4 and d5 indicate different isomeric compounds.[a] Quantified using the calibration curve of benzoic acid. [b] Quantified using the calibration curve of protocatechuic acid. [c] Quantified using the calibration curve of caffeic acid. [d] Quantified using the calibration curve of p-coumaric acid. [e] Quantified using the calibration curve of gallic acid. [f] Quantified using the calibration curve of chlorogenic acid. [g] Quantified using the calibration curve of ferulic acid. [h] Quantified using the calibration curve of resveratrol. [i] Quantified using the calibration curve of catechin. [j] Quantified using the calibration curve of hyperoside. [k] Quantified using the calibration curve of procyanidin dimer B2. [l] Quantified using the calibration curve of kaempferol-3-*O*- -rutinoside. [l] Compounds quantified using the calibration curve of cyanidin-3-*O*-rutinoside. [m] Compounds quantified using the calibration curve of malvidin-3-*O*-glucoside.

Supplementary Table 1: Retention time (RT), detection wavelength, calibration curves, determination coefficient (R^2), linearity range, method precision, method reproducibility, limit of detection (LOD) and limit of quantification (LOQ) for phenolic compounds detected by the HPLC-DAD method.

Compound	RT (min)	Detection Wavelength (nm)	Calibration Curve	R^2	Linearity (µM)	Precision (% RSD, n=3)			Reproducibility (% RSD, n=3)			LOD (µM)	LOQ (µM)
						100 µg/mL	50 µg/mL	1 µg/mL	100 µg/mL	50 µg/mL	1 µg/mL		
Chlorogenic acid	7.758	320	y=26.295x	0.9995	2.822 - 141.119	0.128	0.018	0.193	0.47	0.19	0.31	0.16	0.54
Cyanidin-3-*O*-rutinoside	14.35	520	y=47.880x	0.9990	1.679 - 83.959	0.092	0.176	0.269	0.51	0.26	0.47	0.01	0.02
Rutin	19.74	340	y=29.296x	0.9992	0.164 - 81.897	0.095	0.026	0.206	0.53	0.19	0.36	0.02	0.06

Supplementary Table 2: Molecular weight, exact mass identified by qTOF and quantification conditions for the quantification of anthocyanins in sweet cherries by HPLC-ESI-MS/MS.

Compound[a]	MW	$[M-H]^-$	Quantification		Confirmation	
		or $[M]^{+b}$	MS/MS	CE (V)	MS/MS	CE (V)
Benzoic acid	122.12	121.0304	121>77	8	121>59	4
Phloroglucinol	126.11	125.0252	125>57	20	125>125	0
Hydroxybenzoic acid	138.12	137.0256	137>93	40	137>65	36
Protocatechuic acid	154.12	153.0217	153>109	16	153>62	40
Dihydroxybenzoic acid	154.12	153.0215	153>109	16	153>62	40
p-Coumaric acid	164.16	163.0774	163>119	16	163>93	36
Gallic Acid	170.12	169.0137	169>125	12	169>79	24
Caffeic acid	180.16	179.0344	179>135	16	179>107	24
Ferulic acid	194.18	193.0520	193>134	12	193>178	12
Resveratrol	228.24	227.0678	227>143	28	227>185	20
Apigenin	270.24	269.0452	269>117	44	269>151	28
Kaempferol	286.24	285.0403	285>239	28	285>117	56
Eriodictyol	288.25	287.0793	287>151	12	271>135	28
Catechin	290.27	289.0739	289>245	12	289>203	10
Epicatechin	290.27	289.0742	289>245	12	289>203	10
Quercetin	302.24	301.0100	301>151	20	301>179	20
p-Coumaric acid *O*-glucoside	326.30	325.0932	325>163	10	325>145	10
Gallic acid *O*-glucoside	332.26	331.0697	331>169	12	331>125	12
Caffeic acid *O*-glucoside	342.30	341.0896	341>179	20	341>323	10
Chlorogenic acid	354.31	353.0905	353>191	16	353>85	16
Feruloylquinnic acid	368.34	367.1062	367>193	40	367>161	40
Resveratrol O-glucoside	390.38	389.0675	389>227	20	389>185	20
Kaempferol-3-*O*-glucoside	448.38	447.0974	447>284	28	447>255	40
Eriodictyol-7-*O*-glucoside	450.39	449.1084	449>287	12	449>151	36
Catechin *O*-glucose	452.41	451.1298	451>289	20	451>245	20
EGCG	458.37	457.0700	457>169	16	457>305	20
Quercetin *O*-glucoside	464.38	463.0858	463>299.9	16	463>271	20
Hyperoside	464.38	463.0917	463>299.9	32	463>271	48
Isorhamnetin-3-*O*-glucoside	478.40	477.1014	477>314	32	477>285	40
Procyanidin dimer B2	578.52	577.1389	577>425	12	577>407	28
Kaempferol-3-*O*-rutinoside	594.52	593.1560	593>285	32	593>255	60
Rutin	610.52	609.1508	609>299.9	40	609>271	60
Procyanidin trimer	866.78	865.2041	865>289	20	865>713	20
Cyanidin *O*-arabinoside	419.36	419.0963	419>287	25		
Cyanidin *O*-caffeoylglucose	611.53	611.1601	611>287	25		
Cyanidin *O*-glucose	449.39	449.1076	449>287	25		
Cyanidin-3-*O*-rutinoside	595.53	595.1656	595>287	40		
Delphinidin 3-*O*-rutinoside	611.53	611.1603	611>303	25		
Delphinidin *O*-coumaroylglucose	611.53	611.1391	611>303	25		
Malvidin *O*-coumaroylglucose	639.58	639.1709	639>331	25		
Malvidin-3-O-glucoside	493.44	493.1337	493>331	25		
Pelargonidin *O*-glucose	433.39	433.1125	433>271	30		
Peonidin-3-*O*-rutinoside	609.56	609.1813	609>301	30		

Abbreviations: MW, molecular weight; CE, collision energy. [a] Anthocyanins were analysed in the positive ion mode. [b] Molecular weight detected by qTOF.

Supplementary Table 3: HPLC-ESI-MS/MS method quality parameters for the studied phenolic compounds in sweet cherries.

Compound[a]	RT (min)	Calibration curve	R^2	Linearity (µM)	LOQ (nM)	LOD (nM)
Benzoic acid	9.77	y=304.74x	0.993	0.016-40.943	7.18	23.94
Phloroglucinol	2.80	y=203.80x	0.992	0.016-39.648	0.40	1.33
Protocatechuic acid	3.90	y=11.11x	0.996	0.013-32.442	18.20	60.68
p-Coumaric acid	7.68	y=3567.30x	0.999	0.012-30.458	0.87	2.89
Gallic Acid	3.02	y=11453.00x	0.996	0.012-29.391	0.10	0.33
Caffeic acid	5.85	y=88.55x	0.999	0.011-27.754	4.73	15.76
Ferulic acid	8.32	y=1019.40x	0.996	0.010-25.749	0.43	1.43
Resveratrol	11.71	y=529,84x	0.998	0.009-21.906	0.08	0.28
Apigenin	14.57	y=1950.50x	0.992	0.007-18.502	0.09	0.32
Kaempferol	13.03	y=1489.30x	0.997	0.007-17.468	0.16	0.49
Eriodictyol	12.84	y=1556.40x	0.995	0.007-17.346	0.05	0.15
Catechin	5.13	y=369,62x	0.991	0.007-17.225	0.69	2.29
Epicatechin	6.17	y=500,25x	0.995	0.007-17.225	0.66	2.20
Quercetin	13.25	y=1628.00x	0.996	0.007-16.543	0.05	0.17
Chlorogenic acid	4.80	y=1032.10x	0.999	0.006-14.112	0.32	1.07
Kaempferol-3-*O*-glucoside	9.55	y=1073.20x	0.993	0.004-11.151	0.06	0.19
Eriodictyol-7-*O*-glucoside	8.44	y=1968.60x	0.995	0.004-11.101	0.04	0.15
EGCG	6.40	y=1699,3x	0.999	0.004-10.908	0.05	0.16
Hyperoside	8.37	y=1527.80x	0.994	0.004-10.767	0.04	0.14
Isorhamnetin-3-*O*-glucoside	9.71	y=1058.90x	0.990	0.004-10.451	0.04	0.15
Procyanidin dimer B2	5.63	y=192,39x	0.999	0.003-8.634	0.29	0.96
Kaempferol-3-*O*-rutinoside	9.04	y=976.41x	0.998	0.003-8.410	0.03	0.09
Rutin	8.07	y=825.00x	0.998	0.003-8.190	0.06	0.20
Cyanidin-3-*O*-rutinoside	8.21	y=19.55x	0.998	0.003-3.358	0.88	2.92
Malvidin-3-*O*-glucoside	10.99	y=258.29x	0.999	0.004-4.053	1.13	3.77
Peonidin-3-*O*-rutinoside	10.59	y=118.74x	0.999	0.003-3.281	0.78	2.61

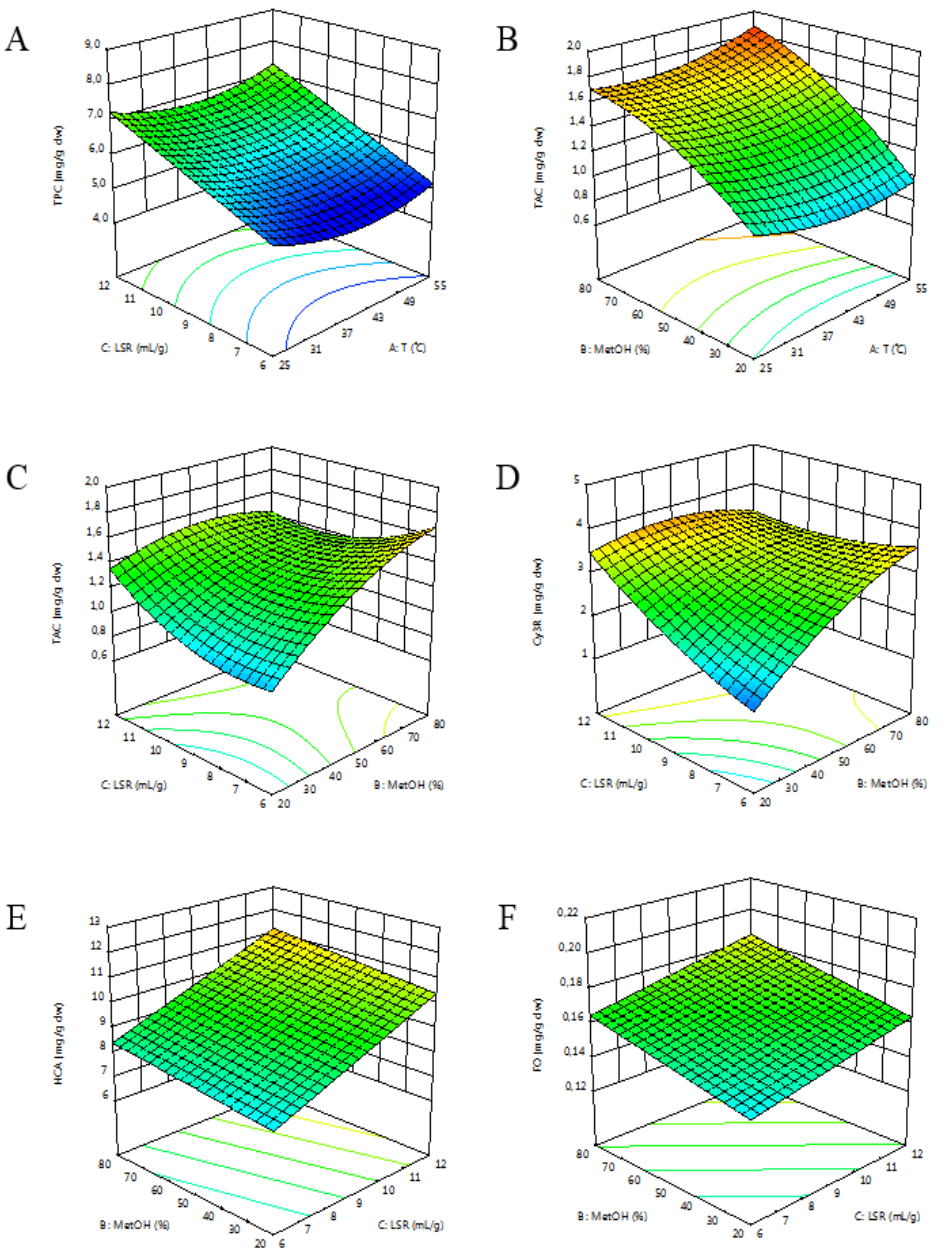
Abbreviations: RT, retention time; R2, determination coefficient; LOD, limit of detection; LOQ, limit of quantification. [a] Anthocyanins were analysed in the positive ion mode.

Supplementary Table 4: Effect of time on the extraction of sweet cherry phenolic compounds.

Time (min) [a]	TPC	TAC	Cy3R	HCA	FO
0	9.64 ± 1.24	1.42 ± 0.10	2.15 ± 0.23	11.67 ± 0.90	0.23 ± 0.03
20	8.98 ± 0.66	1.51 ± 0.12	2.28 ± 0.17	11.85 ± 0.76	0.22 ± 0.01
40	10.41 ± 0.45	1.43 ± 0.02	2.27 ± 0.04	12.23 ± 0.37	0.24 ± 0.02
60	9.99 ± 0.23	1.43 ± 0.05	2.35 ± 0.19	12.72 ± 0.50	0.24 ± 0.02
80	10.42 ± 0.16	1.42 ± 0.08	2.31 ± 0.13	12.25 ± 0.33	0.25 ± 0.01
100	9.41 ± 0.72	1.37 ± 0.13	2.27 ± 0.03	12.60 ± 0.31	0.25 ± 0.04
120	9.23 ± 1.54	1.37 ± 0.11	2.20 ± 0.26	12.19 ± 1.81	0.23 ± 0.05

Abbreviations: total polyphenol content (TPC), total anthocyanin content (TAC), Cy3R (cyanidin-3-o-rutinoside), hydroxycinnamic acids (HCA) and flavonols (FO). Results are expressed as mg of phenolic components per gram of dry weight (mg/g dw) ± SD (n=3). p-values for all parameters were higher than 0.05 by a one-way ANOVA.

Figure 1.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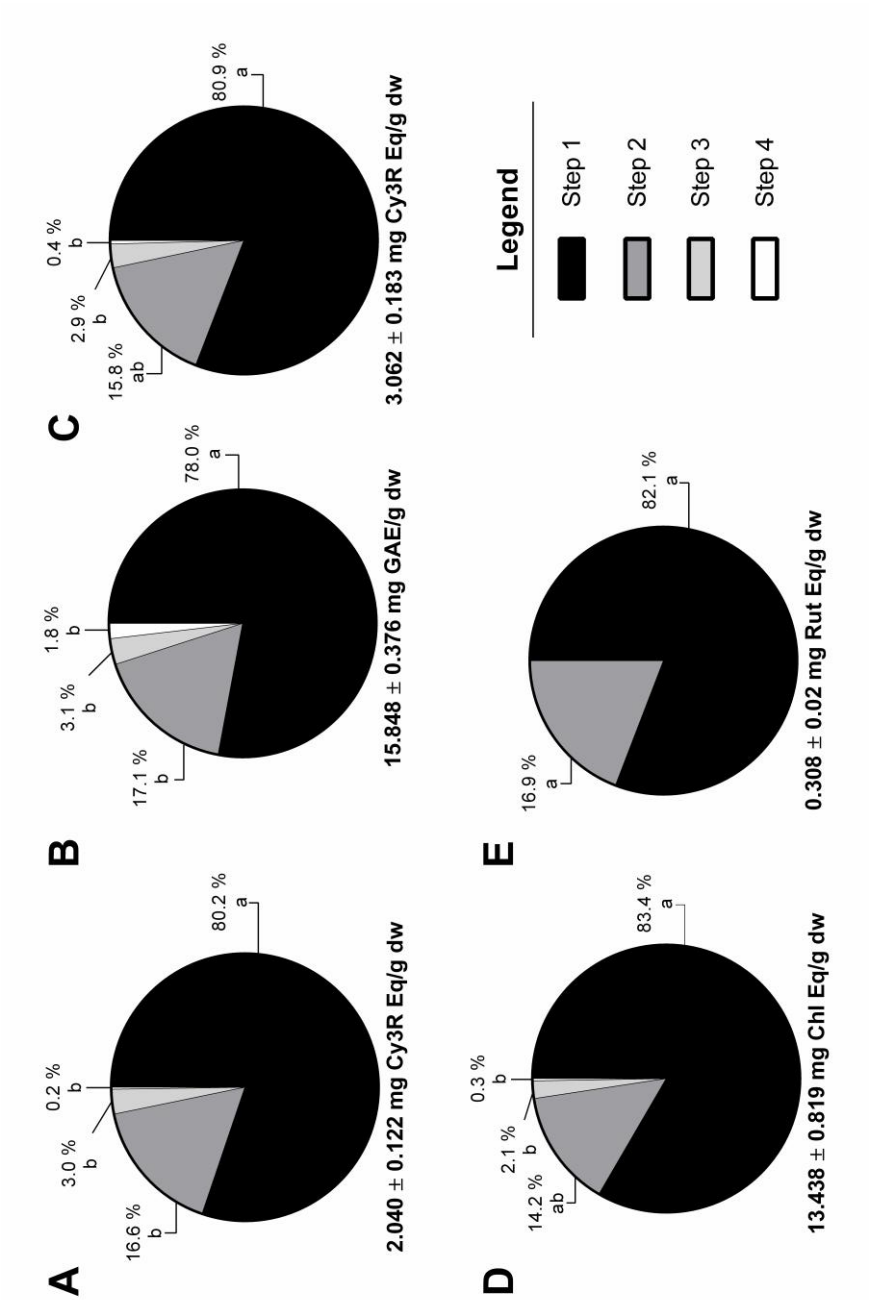
TPC (mg/g dw)
C: LSR (mL/g)
A: T (°C)
B
TAC (mg/g dw)
B: MetOH (%)
A: T (°C)
C
TAC (mg/g dw)
C: LSR (mL/g)
B: MetOH (%)
D
Cy3R (mg/g dw)
C: LSR (mL/g)
B: MetOH (%)
E
HCA (mg/g dw)
B: MetOH (%)
C: LSR (mL/g)
F
FO (mg/g dw)
B: MetOH (%)
C: LSR (mL/g)

Figure 2.


A
80.2 % a
0.2 % b
3.0 % b
16.6 % b
2.040 ± 0.122 mg Cy3R Eq/g dw
B
78.0 % a
1.8 % b
3.1 % b
17.1 % b
15.848 ± 0.376 mg GAE/g dw
C
80.9 % a
0.4 % b
2.9 % b
15.8 % ab
3.062 ± 0.183 mg Cy3R Eq/g dw
D
83.4 % a
0.3 % b
2.1 % b
14.2 % ab
13.438 ± 0.819 mg Chl Eq/g dw
E
82.1 % a
16.9 % a
0.308 ± 0.02 mg Rut Eq/g dw
Legend
Step 1
Step 2
Step 3
Step 4

MANUSCRIPT 3:

Optimized extraction by response surface methodology, characterization and quantification of phenolic compounds in whole red grapes (*Vitis vinifera*).

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UNIVERSITAT ROVIRA I VIRGILI
MODERN CHANGES IN FRUIT INTAKE PATTERNS MODULATE THE BIOAVAILABILITY AND METABOLISM OF FRUIT PHENOLIC COMPOUNDS IN RATS
Lisard Iglesias Carres

Optimized extraction by response surface methodology, characterization and quantification of phenolic compounds in whole red grapes (*Vitis vinifera*).

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Abstract:

Scientific research has focused on the characterization of bioactive polyphenols from grape seeds and skins, and the pulp has often been overlooked. However, since the beneficial properties of grapes are associated with the consumption of whole fruit, a full extraction and posterior characterization of the phenolic compounds in whole grapes is required to identify the involved bioactive compounds. However, such methodologies are not currently available for the whole edible parts of red grapes. Thus, this study aimed to determine the best polyphenol extraction conditions of whole red grapes and to apply the method to characterize and quantify the polyphenol composition of three different grapes. The optimized conditions were 80 mL/g, 65 % methanol (1 % formic acid),72 °C and 100 min. The results of this work suggest a higher quantification of phenolic compounds when red grapes are analysed whole, including the seeds, pulp and skin.

Keywords:

Anthocyanin; flavanols; flavonoids, flavonols; proanthocyanidins; RSM

Chemical compounds studied in this article:

Catechin (PubChem CID: 9064), Chlorogenic acid (PubChem CID: 1794427), Cyanidin-3-*O*-rutinoside (PubChem CID: 441674), Epicatechin (PubChem CID: 72276), Epigallocatechin gallate (PubChem CID: 65064), Malvidin-3-*O*-glucoside (PubChem CID: 443652), p-Coumaric acid (PubChem CID: 637542), Resveratrol (PubChem CID: 445154), and Rutin (PubChem CID: 5280805).

Abbreviations used:

B2, procyanidin dimer B2; Cat, catechin; CG, conventional Grenache grapes; Chl, chlorogenic acid; Cy3R, cyanidin-3-*O*-rutinoside; dw, dry

weight; Ecat, epicatechin; EGCG, (-)-epigallocatechin gallate; GA, gallic acid; GAE, gallic acid equivalents; HCA, hydroxycinnamic acids; LSR, liquid-to-solid ratio; MetOH, methanol; Mv3G, malvidin-3-*O*-glucoside; OG, organic Grenache grapes; pCou, p-coumaric acid; PG, Peruvian Red Globe grapes; RSM, response surface methodology; Rut, rutin; Rvt, resveratrol; TAC, total anthocyanidin content; TFC, total flavanol content; TPC, total phenolic content.

1. Introduction:

Polyphenols are plant secondary metabolites found in high concentrations in a wide variety of foods. More than 8.000 phenol structures have been described, and several are found only in a limited number of species. Due to their beneficial health properties, polyphenols have raised great interest in recent years [1]. Among foods, grapes are one of the fruits with the highest polyphenol content and have been investigated by many authors [2–5]. Phenolic compounds are differentially distributed in the skins, pulp and seeds of grapes [6]. The beneficial health effects of these compounds have been reported from the consumption of phenolic compounds from whole grapes [7], grape juice [8,9] and grape seeds [10].

Red grapes are rich in both flavonoid and non-flavonoid compounds [2,3]. Anthocyanins are the main group of polyphenols found in red grapes. Malvidin-3-*O*-glucoside is the predominant anthocyanin in red grapes, but cyanidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside, petunidin-3-*O*-glucoside and peonidin-3-*O*-glucoside also occur in considerable amounts [3,11]. Flavanols are also present in considerable quantities, and (+)-catechin and (-)-epicatechin the most abundant forms. Several derivatives, such as epicatechin gallate, epigallocatechin gallate, epigallocatechin, and procyanidin dimer B1 and B2, have also been reported [2,3,5]. In red grapes, flavonols are less distributed than anthocyanins. Despite this, quercetin, kaempferol and isorhamnetin glucosides can occur in different cultivars [2,3]. The most abundant phenolic acids in grapes occur as hydroxycinnamic acids, especially as tartaric conjugates [2,5]. In addition, several hydroxybenzoic acid derivates have been described in red grapes, of which gallic acid is the most abundant compound [3]. Resveratrol and its glucoside form trans-polydatin have been identified as the most common components of the stilbene group in red grapes. However, their levels are low and only occur in skins [3,5].

Phenolic compounds are plant stress metabolites [12,13], and several factors have an impact on the phenolic profile of fruits [2], including grapes [3,14,15]. Specifically, the cultivation method can greatly modulate the phenolic content of grapes [16]. For example, organic cultivation is known to modulate the phenolic profile of grapes [15]. Additionally, grape varieties can present markedly different phenolic profiles [4,14,17]. In the specific case of grapes, different phenolic profiles have been demonstrated to give rise to different biological effects [8,9]. For example, the consumption of two different wines with noteworthy phenolic profiles led to different LDL-oxidation outcomes [9]. Therefore, it is essential to characterize the food matrix to correlate it with certain beneficial health effects. To do so, specific extraction methods must be developed, as a multitude of factors can affect the phenolic extraction from food matrixes [18]. In this sense, the extraction temperature, extraction solvent, liquid-to-solid ratio (LSR) and extraction time have been reported to have an impact on the extraction of phenolics from grape cranes, seeds, skins and other grape by-products [19–24]. Traditional optimization studies take into account only one factor at time, while other factors are kept constant. Consequently, this approach is expensive, laborious and time-consuming [25]. Its main disadvantage is that interactions between variables cannot be evaluated and are overlooked [25,26]. In this sense, Response Surface Methodology (RSM) is a useful strategy [18]. RSM enables the evaluation of the effects of different independent variable interactions between themselves and between dependent variables [25,26]. Indeed, RSM has been widely used in the optimization of phenolic compound extraction from several vegetal sources [19,25,26]. However, the one-factor-at-a-time approach is useful in the selection of the experimental ranges used in RSM designs [27].

Notably, phenolic compounds are abundant in grape seeds and skins, and many studies only focus on the characterization of these grape tissues [4,17,28,29]. However, grape pulp also contains phenolic compounds [5,6,30], with the main family being the hydroxycinnamic acids [31]. Although some

authors have evaluated the phenolic content of whole grapes [32,33], the total polyphenol content in red grapes appears to be underestimated. A full profile of the edible parts of grapes, which includes the pulp, is essential to link the health benefits associated with their consumption to their phenolic profile. In this sense, hydroxycinnamic acids have been reported to have diverse health functions [34,35], which makes their contribution to the beneficial bioactivity of whole grape consumption plausible.

There are several studies that have optimized the methods to extract phenolics from grape seeds, skins, cranes, stalks and other grape by-products [19,20,24,28,36,37]. However, studies optimising the extraction of the major phenolic families in red grapes as a whole (including skins, pulp and seeds) are lacking. Given that grapes are typically consumed whole, the study of the whole matrix is of key importance when linking grape consumption and beneficial health effects. Therefore, this study aimed to develop an easy-to-perform extraction method capable of extracting the most representative phenolics from whole red grape varieties. Additionally, this method was used to profile the phenolic content of two whole red Grenache grape cultivars, one produced organically (organic grapes, OG) and the other non-organically (conventional grapes, CG), and a Peruvian Red Globe grape (Peruvian grapes, PG).

2. Materials and methods:

2.1. Plant material:

OG and CG (Grenache, *Vitis vinifera*) were harvested at maturity in the region of Rasquera (Tarragona, Spain). To assure that the only agronomic variable influencing the phenolic profile of the red Grenache grapes was the cultivation system, both OG and CG were harvested on the same day (26 September 2015) from contiguous vineyards. PG (Red Globes, *Vitis vinifera*) were purchased from Mercabarna (Barcelona, Spain). Pedicels were manually removed and whole grapes, which included skins, seeds

and pulp, were frozen in liquid nitrogen and later ground to homogeneity. Next, the homogenates were lyophilized for one week in a Telstar LyoQuest lyophilizer (Thermo Fisher Scientific, Madrid, Spain) at -85 °C. The lyophilized homogenates were ground to a fine powder. The grape powder was kept dry and protected from humidity and light exposure until extraction. OG was used in the optimization studies, while CG and PG were used to provide insights into the different phenolic compositions in different fruit varieties and cultivation methods.

2.2. Chemicals and reagents:

Acetonitrile, methanol, ethanol (HPLC analytical grade) and glacial acetic acid were purchased from Panreac (Barcelona, Spain). Formic acid was purchased from Scharlab (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q Advantage A10 system (Madrid, Spain). The Folin-Ciocalteu and p-dimethylaminocinnamaldehyde (DMACA) reagents were purchased from Fluka/Sigma-Aldrich (Madrid, Spain). Gallic acid (GA), (-)-epicatechin (Ecat), p-coumaric acid (pCou), and (+)-catechin (Cat) were purchased from Fluka/Sigma-Aldrich, chlorogenic acid (Chl), malvidin-3-*O*-glucoside (Mv3G), (-)-epigallocatechin gallate (EGCG) and procyanidin dimer B2 (B2) were purchased from Extrasynthése (Lyon, France); cyanidin-3-*O*-rutinoside (Cy3R) was purchased from PhytoLab (Vestenbergsgreuth, Germany); resveratrol (Rvt) was purchased from Carl Roth (Karlsruhe, Germany) and rutin (Rut) was kindly provided by Nutrafur S.A. (Murcia, Spain).

Ecat, pCou, Cat, Chl, EGCG, B2, Rvt and Rut were individually dissolved in methanol (MetOH) at 2000 mg/L, while Mv3G and Cy3R were dissolved in MetOH (0.01 % HCl) at 500 mg/L. All standard stock solutions were freshly prepared every 3 months and stored in amber glass flasks at -20 °C. Mixed standard stock solutions of Ecat, p-Cou, Cat, Chl, Mv3G, EGCG, B2,

Cy3R, Rvt and Rut were prepared with water:acetic acid (95:5 v/v) to obtain the concentration needed to construct the calibration curves.

2.3. Polyphenol extraction:

Grape powder was weighed to obtain the desired LSR and was mixed with 1 mL of preheated extraction solvent (methanol:water, v:v). Different extraction MetOH proportions (1 % formic acid), extraction temperatures, times and extraction steps were used throughout the experiment. In all cases, the methanol solution included 1 % formic acid. Extractions were performed at 500 rpm agitation with protection from light exposure. Once the extraction was completed, the samples were centrifuged at 9,500 x g for 10 min at 4 °C, and the supernatants were stored at -20 °C until further use.

2.4. Single-factor studies:

To select the working ranges for the RSM independent variables, the effect of LSR, methanol concentration and temperature on the extraction of grape phenolics were evaluated based on total phenolic content (TPC), total anthocyanin content (TAC) and total flavanol content (TFC) extracted from OG, as these variables represent the major phenolic families found in grapes [2,3]. The LSR was evaluated at the ratios of 10, 20, 40 and 80 mL/g; temperatures of 25, 40, 55, 70, and 85 °C; and methanol proportions of 30, 50, 60, 70 and 90 %. All extractions lasted for 30 min, and the extraction variables were kept constant at 80 mL/g, 55 °C, and 50 % when not evaluated.

2.5. Response surface design:

The extraction was optimized with OG using an RSM experimental design. A face-centred central composite design with two factors was selected. It consisted of 11 randomized runs, with three centre point replicates. The

independent variables used in the RSM were MetOH proportion (40-80 %, X_i) and temperature (40-85 °C, X_j). The LSR (80 mL/g) and extraction time (30 min) were fixed as constant variables during the RSM experiment. The experimental data were fitted to a second polynomial response surface, which follows Eq 1:

$$Y = \beta_0 + \sum_{i=1}^{2} \beta_i X_i + \sum_{i=1}^{2} \beta_{ii} X^2{}_{ii} + \sum_{i=1} \sum_{j=i+1} \beta_{ij} X_{ii} X_{ji}$$

where Y is the dependent variable, β_0 the constant coefficient, and $\beta_{i,}$ β_{ii} and β_{ij} are the linear, quadratic and interaction regression coefficients, respectively. $X_{i,}$ X_{ii} and X_{ji} represent the independent variables.

Individual phenolic compounds were quantified by the HPLC-DAD method and used in the RSM optimization study. The results of the RSM design were analysed with Design-expert 9.0.6 software (Trial version, Stat-Ease Inc., Minneapolis, MN, USA). Single parameters that were not influenced by the extraction factors were omitted from the model.

2.6. Kinetic study:

A kinetic study was performed to evaluate the effect of time on the polyphenol extraction yield of OG. Seven extraction times, ranging from 0 to 120 min, were selected. The LSR was fixed at 80 mL/g, the MetOH percentage at 65 % and the temperature at 72 °C. TPC, TAC and TFC were determined for all extracts and used to evaluate the effect of time on the polyphenol extractability.

2.7. Sequential extractions:

Three consecutive extractions were performed to evaluate the influence of multiple extractions on the polyphenol extraction yield in OG. The extractions were carried out under the following conditions: LSR of 80

mL/g, MetOH proportion of 65 %, temperature of 72 °C and extraction time of 100 min. The TPC, TAC and TFC were determined for all extracts and were used to evaluate the effect of sequential extractions on the polyphenol extraction yield.

2.8. Application of the method:

The specific and optimized extraction methodology was used to characterize the phenolic profiles of OG, CG and PG. In brief, the extraction conditions were as follows: LSR of 80 mL/g, MetOH or ethanol (EtOH) proportion of 65 % (1 % formic acid), temperature of 72 °C and extraction time of 100 min.

2.9. Analysis of response variables:

2.9.1. Total Phenolic Content:

The TPC of the extracts was determined by the Folin-Ciocalteu method adapted from Nenadis *et al.* [35]. Briefly, 10 µL of the extract and 50 µL of the Folin-Ciocalteu reagent were successively added to an Eppendorf tube containing 500 µL of Milli-Q water and mixed. The samples were kept in the dark for 3 minutes and 100 µL of Na_2CO_3 (25 %) was added to them. The samples were brought to a final volume of 1 mL with Milli-Q water and were maintained in the dark for 1 hour. The absorbance was read at 725 nm using an Eon BioTek spectrophotometer (Izasa, Barcelona, Spain) against a water sample (blank) that underwent identical treatment. GA was used to construct the calibration curve between 40 mg/L and 400 mg/L. The results were expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g dw).

2.9.2. Total Anthocyanin Content:

The TAC of the extracts was analysed by the pH differential method [11]. The extracts were diluted with sodium acetate buffer (0.4 M, pH 4.5) and

potassium chloride buffer (0.025 M, pH 1.0) to relevant spectrophotometric ranges (0.4-0.6). Next, the absorbance was read at 515 and 700 nm using an Eon BioTek spectrophotometer (Izasa, Barcelona, Spain). The TAC was expressed as milligrams of malvidin 3-O-glucoside equivalents per gram of dry weight (mg Mv3G Eq/g dw). The molar absorbance of Mv3G (493.44 g/mol) used was 28000 L/mol x cm.

2.9.3. Total Flavanol Content:

The TFC of extracts was estimated by the DMACA method [38]. Briefly, the samples (0.1 mL) were mixed with 0.5 mL of DMACA solution (0.1 % 1 N HCl in MetOH) and allowed to react at room temperature for 10 min under protection from light exposure. The absorbance was then read at 640 nm using an Eon BioTek spectrophotometer (Izasa, Barcelona, Spain). Different Cat concentrations between 5 mg/L and 100 mg/L were used to construct the calibration curve. TFC was expressed as milligrams of (+)-Cat equivalents per gram of dry weight (mg Cat Eq/g dw).

2.9.4. HPLC-DAD analysis of phenolic compounds:

Polyphenol separation was achieved using a ZORBAX Eclipse XDB-C18 (150 mm x 2.1 mm i.d., 5 µm particle size) as the chromatographic column (Agilent Technologies, Palo Alto, CA, USA) equipped with a Narrow-Bore guard column (2.1 mm x 12.5 mm, 5 µm particle size). The mobile phase was water:acetic acid (95:5, v:v) (A) and acetonitrile:acetic acid (95:5, v:v) (B) in gradient mode as follows: initial conditions 0 % B; 0-30 % B, 0-18 min; 30-100 % B, 18-19 min; 100 % B isocratic, 19-20 min; 100-0 % B, 20-21 min. A post-run of 6 min was required for column re-equilibration. The flow rate was set at 0.5 mL/min, and the injection volume was 10 µL for all runs. Before the injection, all samples were diluted 1:1 in mobile phase A.

Identification and quantification of the phenolic compounds of interest was achieved with a UV/Vis photodiode array detector (1260 Infinity,

Agilent Technologies, Palo Alto, CA, USA). Chromatograms were recorded from 200 to 600 nm. Flavanols were detected at 280 nm [3,14], p-coumaric acid at 290 nm, hydroxycinnamic acids and stilbenes at 320 nm [3,14], flavonols at 340 nm and anthocyanins at 520 nm [3,20]. When standard compounds were not available, flavanols were quantified as Cat equivalents, hydroxycinnamic acids as Chl equivalents, flavonols as Rut equivalents and anthocyanins as Mv3G equivalents. The results were expressed as milligrams of equivalents per kilogram of dry weight (mg Eq/kg dw).

2.9.5. HPLC-DAD method validation:

Calibration curves, linearity, intraday variability (precision), interday variability (reproducibility), detection and quantification limits were calculated in mobile phase A spiked with polyphenol standards (Supplementary table 1). The peak areas of various concentrations of standards were used to construct the calibration curves. The method's precision was calculated as the relative standard deviation (% RSD) of the concentration in a triplicate analysis of three different spiked samples (50, 25 and 1 μg/mL). Method reproducibility was calculated as the relative standard deviation (% RSD) of three different standard compound concentrations (50, 25 and 1 μg/mL) analysed in triplicate over three consecutive days. Sensitivity was evaluated by determining the limits of detection (LOD) and quantification (LOQ), respectively, which were defined as the concentrations corresponding to 3-fold and 10-fold of the signal-to-noise ratio.

2.10. Statistics:

The results of the RSM design were analysed using Design-expert 9.0.6 software (Trial version, Stat-Ease Inc., Minneapolis, MN, USA). SPSS 19 software (SPSS Inc., Chicago, IL, USA) was used for all other statistical analysis. All experiments were performed in triplicate; the statistical

significance was evaluated using one-way ANOVA or Student's t-test, and *p*-values less than $p<0.05$ were considered to be statistically significant.

3. Results and Discussion:

To quantify and accurately characterize the polyphenol content of foods, it is necessary to optimize the solid-liquid extraction process to obtain the highest polyphenol yield. Importantly, only those compounds that are extracted will be quantified. In this sense, the analysis of grape pulp is of key relevance because this part of the fruit not only contains bioactive compounds such as hydroxycinnamic acids but is also the predominate edible part of grapes [5,6,30,31]. However, to date, no specific methods for the extraction of whole red grape phenolics exist. Some factors, such as LSR, temperature, MetOH proportion, type of solvent and time of extraction, can greatly affect the polyphenol extraction yield from fruit matrixes [25,27]. Thus, it is essential to study the effect of these factors, both independently and combined, on the polyphenol extraction process. In this study, a polyphenol extraction method was optimized using the RSM for OG from *Vitis vinifera*. OG, CG and PG, were analysed whole (including seeds, skins and pulp). The selection of OG was due to its higher phenolic content.

3.1. Single-factor studies:

TPC, TAC and TFC were chosen to evaluate the effect of LSR, extraction temperature and methanol proportion on the extractability of grape phenolic compounds (Figure 1), as they give a global view of the extractability of the major phenolic families found in grapes [2,3].

3.1.1. Effect of liquid-to-solid ratio:

Four LSRs between 10 and 80 mL/g were chosen to evaluate the effect of the LSR on the polyphenol extraction yield and to fix the optimal LSR. No higher LSRs were evaluated, as an increase in this variable increases

production costs and might lead to the lack of detection of phenolic compounds present in low concentrations in grapes. The LSR showed a positive effect on the extraction of TPC, TAC and TFC (Figure 1A), reaching higher yields when the LSR increased. Despite this, only the LSR of 80 mL/g showed a significant difference with other LSRs. Our results are in concordance with those from grape seed [39], black currants [40] and *Inga edulis* leaves [25] where the LSR effect was evaluated and higher yields were obtained with a higher LSR. Moreover, our results are in agreement with the fact that working at a low LSR can cause saturation problems [41]. Thus, a ratio of 80 mL/g was selected for the rest of the study.

3.1.2. Effect of temperature:

Five different temperatures, ranging between 25 and 85 °C, were selected to study the effect of temperature on the polyphenol extraction yield (Figure 1B). The TAC was not statistically influenced by the extraction temperature, although temperatures between 40 and 70 °C appeared to show the highest anthocyanin yield. TAC values at 85 °C decreased slightly in comparison with the other extraction temperatures. This result seems plausible given the thermo-sensitivity of anthocyanins. Indeed, anthocyanin degradation at similar and even lower temperatures was reported for black currants [40]. The TPC and TFC responded in a significant positive manner to temperature. Some studies have shown temperatures similar to 85 °C to be optimal for extracting polyphenols in grape cranes [19,20] and seeds [39]. However, other studies have reported polyphenol degradation when temperatures higher than 50 °C were used [26,27]. Temperatures lower than 40 °C have been reported to have a negative effect on polyphenol extraction [20,27,42]. The observed effect of the temperature can be explained because an increasing temperature modifies solvent properties, weakens polyphenol interactions with cell compounds and compromies cell wall integrity, leading to an increase of polyphenol

transference to the solvent [27,42]. Therefore, 40, 65 and 85 °C were selected as the low, medium and high temperature values for the RSM.

3.1.3. Effect of methanol:

Five different MetOH proportions, ranging between 30 and 90 %, were chosen to study the effect of the percentage of solvent on the polyphenol extraction yield (Figure 1C). The TPC presented a higher extraction yield between 50 and 70 % of MetOH, showing the highest extraction at 50 % MetOH. Other studies have reported similar concentrations of solvent to be optimal for the extraction of TPC, as well as a decreasing TPC tendency when solvent concentrations increased [19,27,40]. The addition of water in organic extraction solvents promotes fruit particle swelling, increasing the contact area between the solvent and the fruit particle. This facilitates solvent penetration into the fruit particles, which enhances the extraction rate of phenolic compounds [43,44]. The TAC values showed significant differences between different percentages of MetOH and were higher at 70 % MetOH. Indeed, anthocyanins are extracted at high organic solvent concentrations in *Euterpe oleracea* [18] and *Euterpe edulis* [45] fruits. The TFC showed a similar tendency as TPC, and concentrations between 50 and 60 % MetOH resulted in the highest TFC. The extraction of apple polyphenols, which includes high quantities of flavanols, reported optimal percentage of MetOH at approximately 60 % [46]. The lowest TAC, TPC and TFC values were obtained at 30 % MetOH, clearly indicating that low MetOH proportions did not extract all of the polyphenols present in OG. Additionally, 90 % MetOH produced a decrease in the extraction of TPC and TFC compared to 50 % MetOH. Consistent with these findings, low MetOH percentages, as well as percentages of approximately 100 %, are inefficient for the extraction of polyphenols [47]. Therefore, 40, 60 and 80 % were selected as the low, medium and high values of MetOH in the RSM study.

3.2. Surface response results:

RSM was used to optimize the polyphenol extraction procedure. MetOH proportions ranging from 40 to 80 % (Xi) and temperatures ranging from 40 to 85 °C (Xj) were selected based on the single factor study results. The previously optimized LSR of 80 mL/g and extraction time of 30 min were fixed throughout the RSM procedure. The selection of the extraction time was selected according to other studies in the literature [25,26,47]. The face-centred design setting of the independent variables using RSM and the experimental values of hydroxycinnamic acids (HCA), flavanols, anthocyanins, stilbenes and flavonols quantified by HPLC-DAD are shown in Table 1. Individual compounds quantified by the HPLC-DAD method were used as dependent variables in the RSM design. The individual responses of anthocyanins, flavanols, flavonols and HCA, were demonstrated to depend on the extraction variables, whereas no Rvt, Chl and p-Cou were detected in any of the extractions. Compounds not responding to the independent variables were omitted from the model.

3.2.1. Fitting the model:

The experimental data in Table 1 were used to determine the coefficients of the second-order polynomial equation (Eq 1). Several compounds generated a significant extraction model, implying that at least one of the extraction variables can explain the variation in the response variables (Table 2). Furthermore, most R^2 values of the parameters were greater than 0.80, meaning that the model accurately represented the experimental data. All of the models generated were highly significant, as the *p*-values ranged between 0.018 and 0.001. In addition, the lack of fit was not significant ($p>0.05$), which indicated that the model could adequately fit the data, thus further validating the model. On the other hand, when Cy3R, Mv3G, malvidin-3-O-glucoside equivalent 1 (Mv3G Eq1), malvidin-3-O-glucoside equivalent 2 (Mv3G Eq2), malvidin-3-O-glucoside

equivalent 4 (Mv3G Eq4) and chlorogenic acid equivalent 1 (Chl Eq1) were analysed, the predicted R^2 was negative (data not shown), implying that the model was not a correct predictor for the response, and the responses were not influenced by either the percentage of MetOH or the temperatures. Therefore, these compounds were omitted in the optimization process. Additionally, the results showed that Cat and Ecat responses had a better fit to a linear model, which is in agreement with the studies of Pinelo M *et al.*, who also reported linear behaviours in phenolic extractions through RSM [21].

When RSM designs are applied, it is important to understand that the effect of a factor in the mass transfer process is not always clear. The chemical characteristics of the solvent and the diversity of structures and composition of natural products allow each combination of material and solvent system to show different behaviours that cannot be predicted [21].

3.2.2. Combined effect of temperature and methanol:

The regression coefficients of the model (Eq 1) for the studied compounds obtained by the multiple linear regression are presented in Table 2. The dependent variables (individual responses of the specific polyphenols studied) allowed a direct interpretation of the effect of the independent variables (MetOH proportion and extraction temperature). The visualization of the statistical significance of the independent variables on the dependent variables was facilitated by generating surface contour plots (Figure 2).

Individual phenolic compounds were affected differently by the extraction temperature and methanol concentration (Table 2). The temperature produced a positive linear effect on B2, Cat, Ecat and Rut Eq2 extraction, and MetOH had a positive linear effect on Mv3G Eq3, Rut, Rut Eq1 and Rut Eq2. Positive linear effects of temperature were also reported for several phenolics in cherry, grape seeds and *Inga edulis* leaves [25,30,47]. A negative quadratic effect of temperature was described for Rut Eq1, EGCG and

Mv3G Eq3, while a negative quadratic effect of MetOH was obtained for EGGG, Mv3G Eq3, Rut and Rut Eq2 (Figure 2). This means that the yields of these compounds increase when the temperature and/or MetOH proportion increases up to a certain point, after which they begin to decrease. Indeed, negative quadratic effects of both solvent concentration and temperature are very common in RSM studies and have been reported in the extraction of polyphenols from vegetal matrixes such as sour cherries [47], *Inga edulis* leaves [25] and *Euterpe oleracea* fruits [18]. A negative linear effect of methanol was found for B2, whereas no linear or quadratic significant effect was reported for Cat and Ecat, suggesting that in the range of MetOH concentrations evaluated, the compounds were not differently extracted. These results are in disagreement with studies were Cat and total flavanols showed positive linear and negative quadratic effects in sour cherries [47] and *Inga edulis* leaves [25]. This may be due to a more restrictive MetOH range and to the different plant matrix. Nevertheless, there are examples of linear behaviours in the literature [21]. Moreover, a positive quadratic effect of methanol was observed for B2, meaning that B2 extraction increase with an increase in MetOH, theoretically up to 100 %. Some studies have reported linear and quadratic effects with total anthocyanins, but the MetOH range used in those studies was less selective than that used in this study [18,47].

In our study, none of the analysed parameters presented an interaction between MetOH and temperature. Although some studies do report these types of interactions [20,45], most of the studies do not [18,25,47].

3.2.3. Method validation:

The combination extraction variables at the highest desirability (0.686) were a temperature of 72 °C and 65 % MetOH (Table 3). To validate the model, 3 extractions were performed under those predicted conditions. HCAs, flavanols, anthocyanins and flavonols were quantified by HPLC-DAD and compared with the model-predicted values. The results showed no

significant differences between the predicted and the experimental values (Table 3). Therefore, the extraction temperature and methanol proportion were fixed at 72 °C and 65 %, respectively, throughout the rest of the study. The optimized extraction temperature of 72 °C is very similar to that reported for sour cherries [47]. However, higher optimal extraction temperatures were reported for the extraction of antioxidants [20] and TPC [19] from grape cranes. Additionally, lower temperatures were also found to be optimal in the extraction of antioxidants from grape stalks [37]. In agreement with our optimized methanol proportion, Karacabey *et al.* found similar ethanol percentages to be optimal in the extraction of phenolics from grape cranes [19]. Furthermore, other plant matrixes show the optimal extraction of phenolic compounds at similar organic solvent percentages [26,27]. Importantly, the differences between the optimal extraction conditions found in this study and the optimal conditions reported for other grape parts [19,20] reflect the need for optimization studies for each food matrix. In agreement with this, Karvela *et al*. demonstrated that the optimized conditions for the extraction of grape seeds from varied among different grape varieties [36].

3.3. Effect of time on polyphenol extraction:

To further optimize the polyphenol extraction method, a kinetic study was performed. Seven extraction times ranging from 0 to 120 min were selected to perform the study. The LSR was fixed at 80 mL/g, the temperature at 72 °C and MetOH at 65 %. The effect of time was evaluated by analysing the extraction rate of TPC, TAC and TFC, which gives a general view of the effect of time on the extraction of the major phenolic families present in grapes [2,3].

TPC and TFC (Figure 3A and 3C) displayed a significant sensitivity to the effect of time during the extraction process, indicating that higher extraction times allow for increased TPC and TFC yields. Indeed, longer extraction times usually result in an increase of polyphenol yields [27]. The

highest TAC (Figure 3B) reported for the anthocyanin yield was obtained at an extraction time of 0 min. This can be explained because anthocyanins are thermo-sensitive and high temperatures can cause anthocyanin degradation over time [40]. Despite this, TAC did not show significant differences with higher extraction times (80-120 min). Therefore, the extraction time was fixed at 100 min throughout the rest of the experiment. Yilmaz *et al.* reported an extraction time of 80–100 min to obtain the maximum TAC and TPC values from sour cherry [47]. Armendola *et al.* reported an extraction time of 120 min to obtain the highest yield of polyphenols from grape marc [22]. The need for such a high extraction time may be a consequence of the high polyphenol content found in grapes or the food matrix itself. In fact, it is known that polyphenols are bonded to some components of the food matrix [23]. Exposure to high temperatures and optimal MetOH concentrations over certain periods of time allows for the solubilisation of these polyphenols due to weakening of the cell wall and polyphenol-component interactions [27,42]. This leads to polyphenol transference into the solvent, thereby increasing the polyphenol yield in the extracts [27].

3.4. Multi-step extractions:

To evaluate the effect that multi-step extractions have on polyphenol extraction yields, three consecutive extractions were performed under the optimized conditions (LSR 80 mL/g, temperature 72 °C, 65 % MetOH, and 100 min extraction time) (Figure 4). The statistical analysis shows that each extraction step statistically increases the TPC (Figure 4A). Specifically, the TPC increased from 25.33 ± 1.77 in the first extraction to 28.93 ± 1.74 mg GAE/g dw in the third extraction. However, no significant increases were achieved for TAC or TFC (Figure 4B and 4C, respectively) between the extraction steps. Similarly, Mané *et al.* reported that flavanols, phenolic acids and anthocyanins in grape skins, seeds and pulp were predominantly extracted in the first extraction step [30]. From an economical

and practical point of view, it was decided that only one extraction step was the optimal condition for grape polyphenol extraction, as TPC only increased by 3 mg GAE/g dw in the third extraction step compared to the first.

3.5. Methanol-ethanol comparison:

Due to the potential application of ethanol in the food industry to obtain extracts for commercialisation, a methanol- and an ethanol-based extraction were carried out to evaluate whether both solvents achieved the same results. Extractions were performed under the optimized conditions of LSR of 80 mL/g, MetOH or EtOH concentrations of 65 % (1 % formic acid), a temperature of 72 °C and an extraction time of 100 min. TPC and TFC did not present significant differences between the methanol and ethanol extractions, although methanol extraction achieved slightly higher values of TPC and TFC (Figure 5A and 5C). These results are in agreement with those obtained by Tabaraki *et al.*, who found only small differences between solvents during the extraction process in grape seeds [42]. However, TAC was reported to be statistically higher in MetOH (1.31 ± 0.08 mg Mv3G Eq/g dw) than in EtOH (0.97 ± 0.08 mg Mv3G Eq/g dw) extraction (Figure 5B). In fact, the anthocyanin yield decreased nearly 26 % in the ethanol extraction. This result is in accordance with the study by Metivier *et al.*, where the grape anthocyanin content was 20 % higher in the methanol extraction [48]. Indeed, methanol extractions are usually more efficient, obtaining higher polyphenol yields than ethanol extractions [24,49]. Even though some differences are reported in the TAC, global differences between the two solvents are minor. Therefore, this method could be used in the food industry. Nevertheless, more studies are needed to adapt it for large-scale use.

3.6. Full characterization of whole red grapes:

The profile and content of phenolic compounds in grapes is known to be affected by many factors [3,14,15], including cultivation conditions [15] and grape variety [4,14,17]. OG, CG, and PG underwent extraction at the optimized conditions of 80 mL/g, 65 % MetOH, and 72 °C for 100 min in one single extraction step to assess the effect of grape variety and culture system on the phenolic profile of whole red grapes. The cultivation methodology (organic or conventional) effect was evaluated by comparing OG and CG, while grape variety was evaluated by comparing PG with CG. When individual polyphenol responses were analysed by the HPLC-DAD method (Table 4), OG, CG and PG presented Mv3G and Cat as the main anthocyanin and flavanols, respectively. Several *Vitis vinefera* varieties reported Cat as the most concentrated flavanol found in both seeds and skins [3,4,17] and Mv3G as the main anthocyanin found in skins [3,29]. Indeed, Mv3G was the most abundant phenolic in all grapes, followed by Chl Eq1 and Cat in OG and CG, and by Cat and Ecat in PG. Grapes have reportedly high concentrations of HCA in skins and also in the pulp [5], which could explain the abundance of Chl EQ1. In all three grape varieties, flavonols were detected in lower quantities than anthocyanins and flavanols, which has been previously described in several *Vitis vinifera* varieties [4,29]. Studies with red grape skins have reported resveratrol at concentrations of approximately 0.6-25 mg/g skins [17]. Given the low concentration of resveratrol in skins and the use of whole red grapes in this study, the concentration of resveratrol in the extracts might be extremely low, thus leading to its lack of detection.

OG showed the highest values of TPC, TAC and TFC, whilst PG showed the lowest. The individual polyphenol responses were in agreement with the previously determined total phenolic and total anthocyanin values (Table 4). Importantly, OG presented higher concentrations of some individual anthocyanins and lower concentrations for some flavonols than did CG.

These results are also in agreement with those found by Mulero *et a*l. in organic and conventional Mourvèdre grapes [15]. Indeed, studies investigating differences between organic and conventional foods have reported higher contents of polyphenols, although not always statistically significant, in organic foods [15,50]. This is in agreement with the fact that phenolic compounds are stress metabolites synthetized under adverse conditions [12]. The PG polyphenolic profile presented fewer polyphenol compounds, in particular anthocyanins, as well as a reduction in their levels when compared to Grenache CG. Indeed, large differences in skin anthocyanin contents between different grape varieties have been described by Kammerer *et al.* and Cantos *et al.* [3,14].

The number of studies evaluating the phenolic profile in whole red grapes is currently limited [32,33]. By applying our method, higher concentrations of relevant phenolic constituents (i.e., catechin, epicatechin and malvidin-3-*O*-glucoside) were found in our grape varieties than in the study by Lingua *et al.*, in which whole red grapes were analysed [33]. Grape pulp contains phenolic compounds, especially hydroxycinnamic acids [5,6,30]. In this sense, Mulero *et al*. reported total hydroxycinnamic acid at concentrations as high as 22.81 ± 0.24 mg/kg of whole fresh weight, when only skins were analysed [15]. In our study, which also includes the pulp, the extraction of CHL EQ1 resulted in clearly superior quantities in the Grenache varieties (OG and CG). In summary, this study suggests that the extraction of phenolic compounds from whole red grapes provides a higher extraction of phenolic compounds, and this is essential to be able to correlate the health effects associated with the consumption of grapes and the phenolic compounds responsible for these effects.

Our study reported the optimal conditions for the extraction of the most representative phenolics from whole red grapes using a MetOH proportion of 65 % (1 % formic acid), a temperature of 72 °C, an LSR of 80 mL/g and an extraction time of 100 min in a one-step extraction. These conditions differed from previous methodologies for specific grape parts (i.e., skins or

seeds), which demonstrated the need for a specific extraction methodology when using whole red grapes. To our knowledge, no similar methodologies have been developed for the extraction of whole red grapes. This work suggests that the analysis of whole grape phenolics, including pulp, will result in a higher quantification of phenolic compounds. Moreover, if adapted to a larger scale, ethanol could be used to produce phenolic-rich extracts for commercialization. This study demonstrates that organic cultivation systems affect the phenolic compound profile of red Grenache grapes, promoting a higher content of phenolic compounds, especially anthocyanins.

3.7. Acknowledgements:

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Figure legends:

Figure 1. Effect of different liquid-to-solid ratios (A), temperatures (B) and methanol proportions (C) on total phenolic (TPC), total anthocyanin (TAC) and total flavanol (TFC) content in organic whole red Grenache grapes. The results are expressed as mg of phenolic equivalents per gram of dry weight (mg Eq/g dw) ± SD (n=3). Letters represent significant differences (One-way ANOVA; $p<0.05$). Abbreviations: liquid-to-solid ratio (LSR), methanol (MetOH), gallic acid equivalents (GAE), malvidin-3-*O*-rutinoside (Mv3R) and catechin (Cat).

Figure 2. Response surface plots for procyanidin dimer B2 (A), catechin (B), epicatechin (C), EGCG (D), malvidin-3-*O*-glucoside equivalent 3 (E), rutin (F), rutin equivalent 1 (G) and rutin equivalent 2 (H) in function of methanol proportion and temperature. Abbreviations: Cat, catechin; Ecat, epicatechin; EGCG, (-)-epigallocatechin gallate; EQ, equivalent; Rut, rutin; Mv3G, malvidin-3-*O*-glucoside; and MetOH, methanol.

Figure 3. Effect of time on total phenolic content (TPC) (A), total anthocyanin content (TAC) (B) and total flavanol content (TFC) (C) of organic whole red Grenache grapes. The results are expressed as mg of phenolic equivalents per gram of dry weight (mg Eq/g dw) ± SD (n=3). Letters represent significant differences (One-way ANOVA; $p<0.05$). Abbreviations: gallic acid equivalents (GAE), malvidin-3-*O*-rutinoside (Mv3R) and catechin (Cat).

Figure 4. Effect of different extraction steps on total phenolic (TPC) (A), total anthocyanin (TAC) (B) and total flavanol (TFC) (C) content of organic whole red Grenache grapes. The total amount after three consecutive extractions is expressed as mg of phenolic equivalents per gram of dry weight (mg Eq/g dw) ± SD (n=3). Letters represent significant differences

(one-way ANOVA; $p<0.05$). Abbreviations: gallic acid equivalents (GAE), malvidin-3-*O*-rutinoside (Mv3R) and catechin (Cat).

Figure 5. Effect of methanol (MetOH) and ethanol (EtOH) on total phenolic content (TPC) (A), total anthocyanin content (TAC) (B), and total flavanol content (TFC) (C) on organic whole red Grenache grapes. Results are expressed as mg of phenolic equivalents per gram of dry weight (mg Eq/g dw) ± SD (n=3). * $p<0.05$. Abbreviations: gallic acid equivalents (GAE), malvidin-3-*O*-rutinoside (Mv3R) and catechin (Cat).

Table 1. Face-centred design setting of the independent variables and experimental results of flavanols, flavonols, hydroxycinnamic acids, stilbenes and anthocyanins quantified by HPLC-DAD of organic whole red Grenache grapes.

Run[a]	MetOH (%)	T (°C)	B2	Cat	Ecat	EGCG	Rut	Rut EQ1	Rut EQ2	Chl	Chl EQ1	pCou	Rvt	Cy3R	Mv3G	Mv3G EQ1	Mv3G EQ2	Mv3G EQ3	Mv3G EQ4
1	40	40	60.66	136.93	74.27	63.95	81.08	133.00	56.47	n.d.	825.48	n.d.	n.d.	135.47	1159.52	104.39	37.57	20.26	181.29
2	80	40	79.63	177.73	100.23	62.89	75.20	146.23	65.66	n.d.	796.71	n.d.	n.d.	151.31	1253.41	119.17	43.96	21.19	216.30
3	40	85	79.82	230.51	124.36	63.03	74.90	135.20	67.32	n.d.	760.54	n.d.	n.d.	144.71	1194.49	111.79	33.17	22.99	188.68
4	80	85	90.47	363.22	180.12	60.74	73.44	146.81	73.75	n.d.	925.75	n.d.	n.d.	139.57	1209.93	113.83	36.78	22.74	201.51
5	40	65	72.29	168.54	128.96	73.44	78.05	143.62	63.99	n.d.	853.2	n.d.	n.d.	142.10	1284.35	133.39	32.91	25.37	210.41
6	80	65	83.00	251.54	124.24	69.12	73.76	153.93	70.50	n.d.	838.44	n.d.	n.d.	133.90	1163.91	113.00	36.41	24.52	200.34
7	60	40	68.95	158.76	78.99	69.09	81.25	147.06	63.77	n.d.	860.99	n.d.	n.d.	145.19	1176.84	119.36	36.24	22.90	198.69
8	60	85	78.02	350.76	146.46	67.14	80.61	138.68	80.25	n.d.	826.5	n.d.	n.d.	141.28	1276.78	117.86	42.36	23.53	214.59
9	60	65	70.79	208.79	128.10	72.67	81.34	150.34	75.91	n.d.	743.96	n.d.	n.d.	134.92	1175.77	120.11	38.09	26.86	191.47
10	60	65	74.60	191.36	122.34	73.67	82.88	151.35	76.61	n.d.	758.96	n.d.	n.d.	140.03	1244.41	136.07	40.21	28.83	205.79
11	60	65	72.44	221.15	129.73	76.89	78.79	156.21	77.25	n.d.	799.85	n.d.	n.d.	132.07	1271.07	117.57	34.31	26.69	216.11

Abbreviations: Methanol (MetOH); temperature (T); not detected (n.d.); procyanidin dimer B2 (B2); (+)-catechin (Cat); (-)-epicatechin (Ecat); (-)-epigallocatechin gallate (EGCG); rutin (Rut); rutin equivalent 1 (Rut Eq1); rutin equivalent 2 (Rut Eq 2); chlorogenic acid (Chl); chlorogenic acid equivalent 1(Chl Eq1); p-coumaric acid (pCou); resveratrol (Rvt); cyanidin-3-rutinoside (Cy3R); malvidin-3-*O*-glucoside (Mv3G); malvidin-3-*O*-glucoside equivalent 1 (Mv3G Eq1); malvidin-3-*O*-glucoside equivalent 2 (Mv3G Eq2); malvidin-3-*O*-glucoside equivalent 3 (Mv3G Eq3) and malvidin-3-*O*-glucoside equivalent 4 (Mv3G Eq4). Results are expressed as milligrams of phenolic compound per kilogram of dry weight (mg /kg dw). [a] All extractions were carried out for 30 min, with 500 rpm agitation.

Table 2. Analysis of the variance and regression coefficients of the predicted model for the response variables of organic whole red Grenache grapes.

Model parameters	Regression coefficient	B2	Cat	Ecat	EGCG	Mv3G Eq3	Rut	Rut Eq1	Rut Eq2
Intercept	β0	66.132	-98.331	-10.753	-20.457	-23.042	55.243	33.532	-45.193
Linear									
MetOH	β1	-0.742*	1.912	0.642	1.293	0.624#	1.228*	1.307*	2.593*
T	β2	0.285*	3.217*	1.475*	1.914	0.973	-0.193#	2.314	0.926*
Interaction									
MetOH x T	β12	-4.775×10^{-3}	-	-	-7.870×10^{-4}	-6.990×10^{-4}	2.422×10^{-3}	-9.810×10^{-4}	-1.579×10^{-3}
Quadratic									
MetOH x MetOH	β11	1.151×10^{-2}*	-	-	-1.089×10^{-2}*	-4.842×10^{-3}*	-1.232×10^{-2}*	-7.934×10^{-3}	-1.924×10^{-2}*
T x T	B22	2.3285×10^{-3}	-	-	-1.523×10^{-2}*	-7.156×10^{-3}*	-1.250×10^{-4}	-1.837×10^{-2}*	-4.548×10^{-3}
R^2		0.961	0.847	0.874	0.9456	0.9283	0.8935	0.9037	0.99553
Adjusted R^2		0.922	0.809	0.843	0.8912	0.8567	0.7871	0.8074	0.9105
p-value		0.002	<0.001	<0.001	0.0035	0.007	0.018	0.014	0.002
F-value		24.63	22.12	27.82	17.38	12.95	8.39	9.38	21.36
Lack of fit		0.442	0.173	0.080	0.776	0.742	0.821	0.495	0.056

Abbreviations: Methanol (MetOH); temperature (T); determination coefficient (R^2); procyanidin dimer B2 (B2); (+)-catechin (Cat); (-)-epicatechin (Ecat); (-)-epigallocatechin gallate (EGCG); Malvidin-3-*O*-glucoside equivalent 3 (MV3G Eq3); rutin (Rut); rutin equivalent 1 (Rut Eq1) and rutin equivalent 2 (Rut Eq2). Differences between groups determined by *ANOVA *p<0.05, # p<0.01*

Table 3. Overall optimal values of extraction parameters for flavanols, anthocyanins, flavonols and phenolic acids of organic whole red Grenache grapes.

Extraction variables [a]			Parameter	Predicted	Experimental		
T (°C)	MetOH (%)	Desirability					
72	65	0.686	B2	76.70	77.73	±	1.17
			Cat	257.57	264.44	±	17.64
			Ecat	137.16	153.03	±	17.03
			EGCG	72.74	73.71	±	3.25
			Cy3R	136.56	144.59	±	1.73
			Mv3G	1233.23	1363.78	±	7.49
			Mv3G Eq1	118.77	131.95	±	8.61
			Mv3G Eq2	37.64	38.23	±	1.45
			Mv3G Eq3	26.72	26.33	±	1.98
			Mv3G Eq4	202.23	219.43	±	6.19
			Rut	79.80	82.75	±	1.05
			Rut Eq1	151.80	151.53	±	1.47
			Rut Eq2	77.80	76.14	±	3.37
			Chl Eq1	828.07	888.50	±	4.73

Abbreviations: Temperature (T); methanol (MetOH); procyanidin dimer B2 (B2); (+)-catechin (Cat); (-)-epicatechin (Ecat); (-)-epigallocatechin gallate (EGCG); cyanidin-3-rutinoside (Cy3R); malvidin-3-O-glucoside (Mv3G); malvidin-3-O-glucoside equivalent (Mv3G Eq); rutin (Rut); rutin equivalent (Rut Eq) and chlorogenic acid equivalent (Chl Eq). Results are expressed as mg of phenolic component per kilogram of dry weight (mg Eq/g dw) ± SD (n=3). No significant differences were determined (Student's t-test; $p<0.05$). [a] All extractions were carried out for 30 min, with 500 rpm agitation.

Table 4. Generic polyphenol variables and individual polyphenol contents analysed by HPLC-DAD of conventional whole Grenache grapes (CG), red organic whole red Grenache grapes (OG) and whole red Peruvian Red Globe grapes (PG).

	CG	**OG**	**PG**
TPC [a]	23.98 ± 2.60	26.37 ± 3.6	17.18 ± 1.87*
TAC [a]	0.98 ± 0.11	1.31 ± 0.08*	0.71 ± 0.05*
TFC [a]	4.42 ± 0.92	4.93 ± 0.58	3.68 ± 0.20
Cat	519.51 ± 179.55	382.30 ± 90.06	255.82 ± 68.72
B2	139.29 ± 43.04	95.88 ± 10.09	39.15 ± 4.14*
EGCG	N.D.	99.47 ± 6.45	N.D.
Ecat	276.45 ± 98.22	162.42 ± 46.17	241.48 ± 14.81
Chl EQ1	797.05 ± 49.07	853.16 ± 47.44	176.31 ± 6.69*
Chl	N.D.	N.D.	N.D.
Rvt	N.D.	N.D.	N.D.
pCou	N.D.	N.D.	N.D.
Mv3G EQ1	34.38 ± 11.23	133.81 ± 10.29*	N.D.
Cy3R	79.57 ± 8.81	159.83 ± 6.20*	161.31 ± 11.97*
Mv3G	1116.80 ± 67.21	1534.89 ± 125.26*	887.14 ± 57.14*
Mv3G EQ2	36.95 ± 10.36	53.36 ± 15.01	N.D.
Mv3G EQ3	69.58 ± 1.50	79.52 ± 10.42	N.D.
Mv3G EQ4	198.09 ± 28.08	199.30 ± 31.98	42.39 ± 5.22*
Rut	157.72± 20.31	117.13 ± 13.26*	60.81 ± 11.36*
Rut EQ1	318.48 ± 63.43	172.48 ± 13.57*	116.17 ± 16.30*
Rut EQ2	111.03 ± 21.27	82.49 ± 4.01	N.D.

Abbreviations: Total polyphenol content (TPC); total anthocyanin content (TAC); total flavanol content (TFC); not detected (n.d.); (+)-catechin (Cat); procyanidin dimer B2 (B2); (-)-epigallocatechin gallate (EGCG); (-)-epicatechin (Ecat); chlorogenic acid equivalent 1 (Chl Eq1); chlorogenic acid (Chl); resveratrol (Rvt); p-coumaric acid (pCou); malvidin-3-O-glucoside equivalent 1 (Mv3G Eq1); cyanidin-3-rutinoside (Cy3R); malvidin-3-O-glucoside (Mv3G); malvidin-3-O-glucoside equivalent 2 (Mv3G Eq2); malvidin-3-O-glucoside equivalent 3 (Mv3G Eq3); malvidin-3-O-glucoside equivalent 4 (Mv3G Eq4); rutin (Rut); rutin equivalent 1 (Rut Eq1) and rutin equivalent 2 (Rut Eq2). Results are expressed as mg of phenolic component per kilogram of dry weight ± SD (n=3). [a] Generic polyphenol variables. The results are expressed as mg of phenolic component per gram of dry weight ± SD (n=3). * represents significant differences with CG by Students t-test (p<0.05).

Supplementary Table 1: Retention time (Rt), detection wavelength, calibration curves, determination coefficient (R^2), linearity range, method precision, method reproducibility, limit of detection (LOD) and limit of quantification (LOQ) for phenolic compound quantification in spiked Milli-Q water:acetic acid (95:5 v:v) solution using HPLC-DAD.

Compound	Rt (min)	Detection wavelength (nm)	Calibration Curve	R^2	Linearity (nM)	Precision (% RSD, n=3)			Reproducibility (% RSD, n=3)			LOD (µM)	LOQ (µM)
						50 µg/mL	25 µg/mL	1 µg/mL	50 µg/mL	25 µg/mL	1 µg/mL		
Cat	6.12	280	y=12.512x	0.997	3.44 - 172.25	3.77	4.70	1.59	6.79	2.31	0.62	0.342	1.140
Chl	7.16	320	y=25.385x	0.997	1.41 - 141.12	5.18	3.37	2.00	4.22	1.47	0.09	0.040	0.135
B2	7.40	280	y=37.814x	0.991	0.17 - 86.43	9.88	5.86	2.03	2.92	5.43	1.45	0.005	0.015
EGCG	8.46	280	y=27.051x	0.998	1.09 - 109.08	4.84	1.81	0.79	3.78	1.67	1.99	0.034	0.113
Ecat	9.27	280	y=13.466x	0.99	1.72 - 172.25	9.49	5.38	9.20	5.14	0.76	1.08	0.089	0.296
Cy3R	10.35	520	y=51.985x	0.995	0.17 - 83.96	5.73	6.10	5.11	4.58	0.22	4.16	0.003	0.010
pCou	10.69	290	y=150.890x	0.996	0.61 - 304.58	5.87	3.35	1.06	4.06	1.01	3.85	0.003	0.010
Mv3R	11.71	520	y=46.252x	0.994	0.20 - 101.33	5.88	6.85	5.84	4.21	0.31	5.56	0.006	0.021
Rut	13.51	340	y=27.135x	0.997	0.16 - 81.90	5.98	3.42	2.41	4.49	1.33	0.19	0.003	0.010
Rvt	16.14	320	y=130.550x	0.997	0.22 - 219.07	5.91	3.34	0.84	4.40	1.42	0.19	0.002	0.006

Abbreviations: (+)-catechin (Cat); chlorogenic acid (Chl); procyanidin dimer B2 (B2); (-)-epigallocatechin gallate (EGCG); (-)-epicatechin (Ecat); cyanidin-3-O-rutinoside (Cy3R); p-coumaric acid (pCou); malvidin-3-O-glucoside (MV3G); rutin (Rut) and resveratrol (Rvt).

Figure 1.

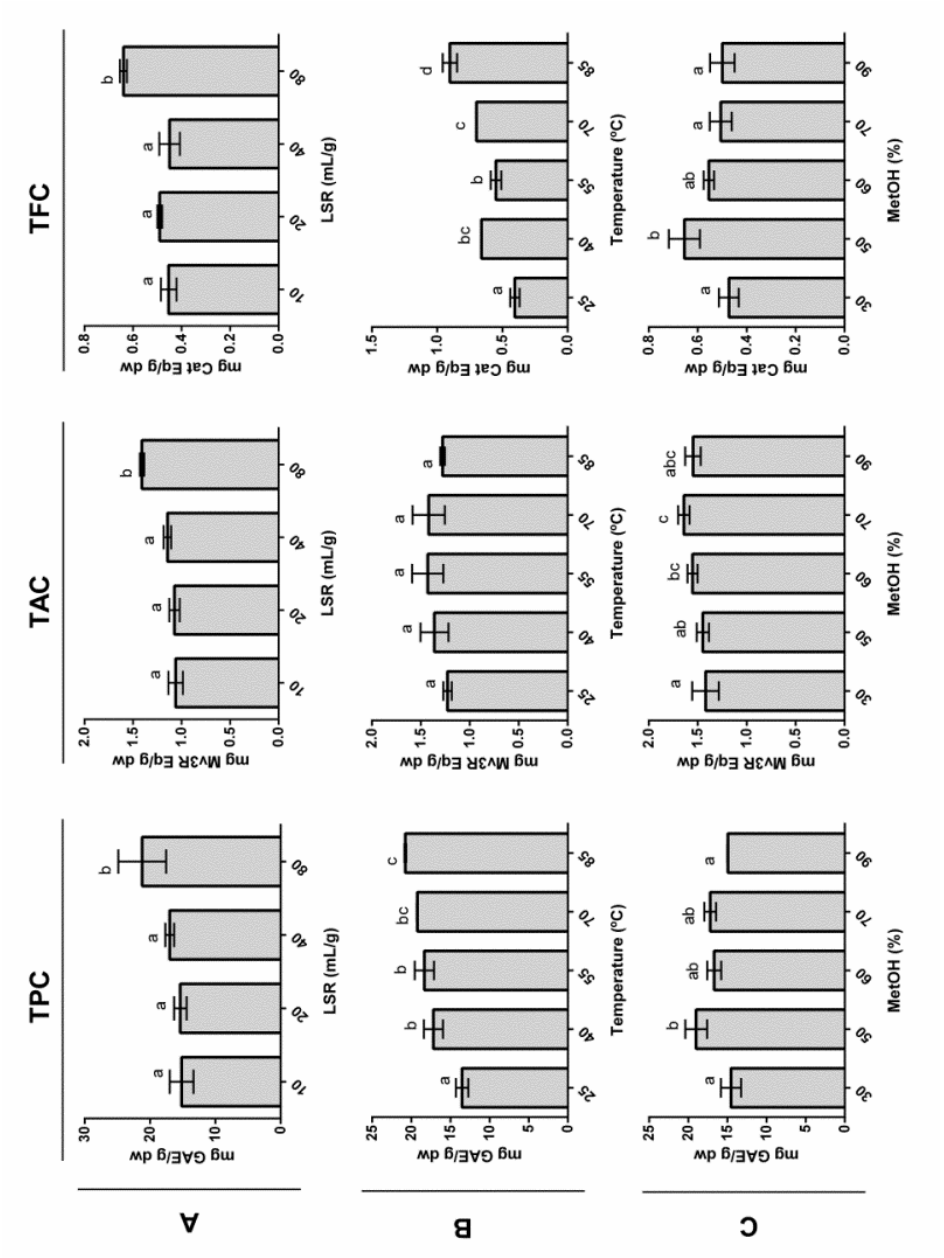

TPC
TAC
TFC
A
B
C
mg GAE/g dw
mg MV3R Eq/g dw
mg Cat Eq/g dw
LSR (mL/g)
Temperature (ºC)
MetOH (%)

Figure 2.

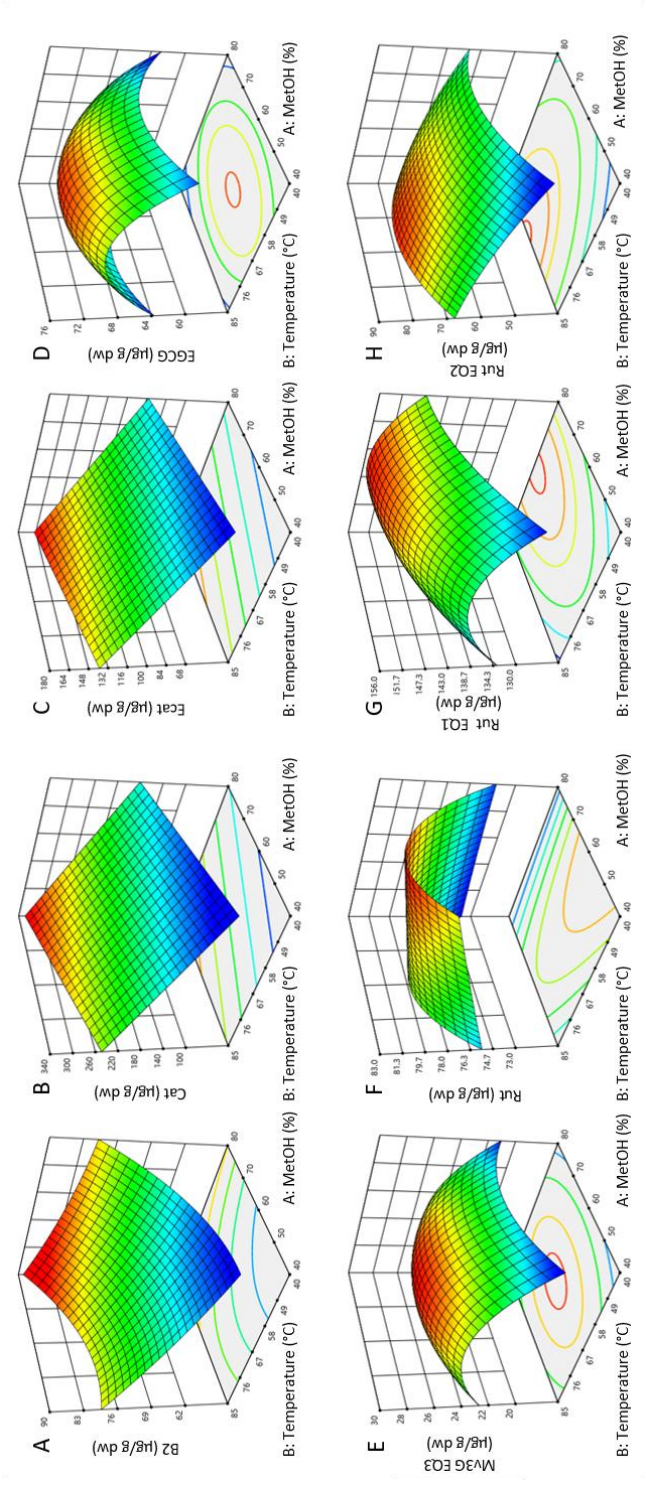
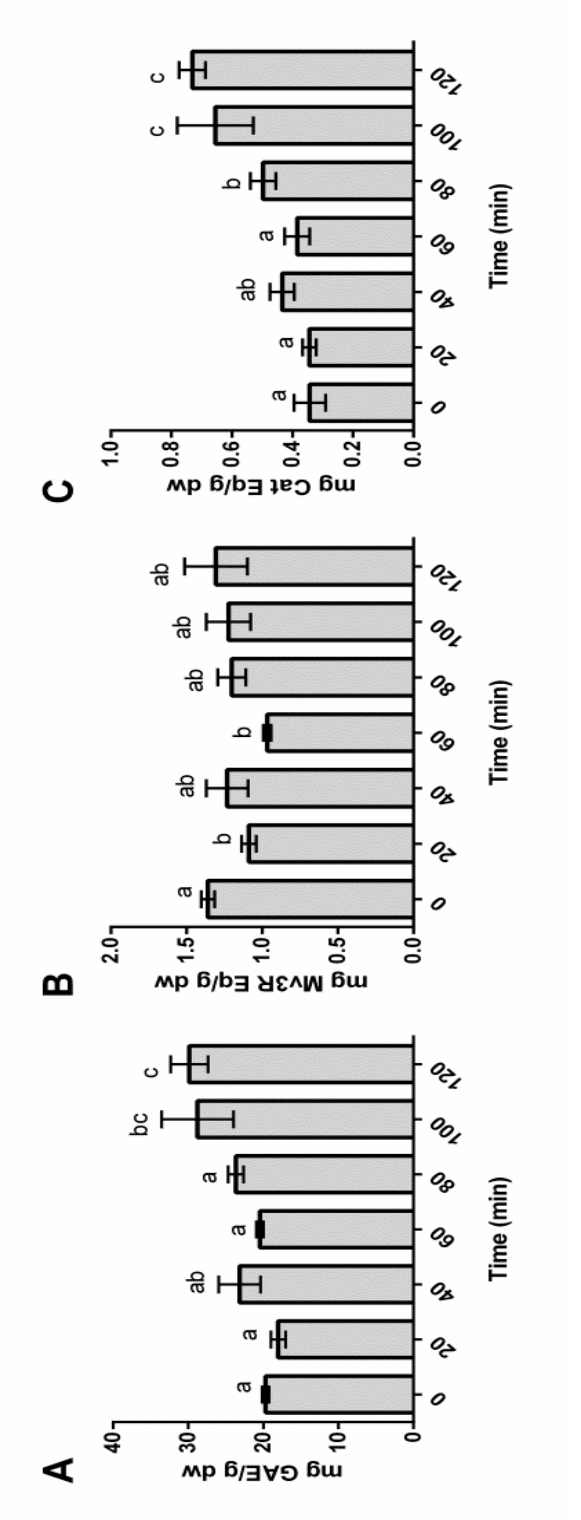

A
B2 (µg/g dw)
B: Temperature (°C)
A: MetOH (%)
B
Cat (µg/g dw)
C
Ecat (µg/g dw)
D
EGCG (µg/g dw)
E
MV3G EQ3 (µg/g dw)
F
Rut (µg/g dw)
G
Rut EQ1 (µg/g dw)
H
Rut EQ2 (µg/g dw)

Figure 3.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0
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20
30
40
a
a
ab
a
a
bc
c
0
20
40
60
80
100
120
Time (min)
B
mg Mv3R Eq/g dw
0.0
0.5
1.0
1.5
2.0
a
b
ab
ab
b
ab
ab
ab
0
20
40
60
80
100
120
Time (min)
C
mg Cat Eq/g dw
0.0
0.2
0.4
0.6
0.8
1.0
a
a
ab
a
b
c
c
0
20
40
60
80
100
120
Time (min)

Figure 4.

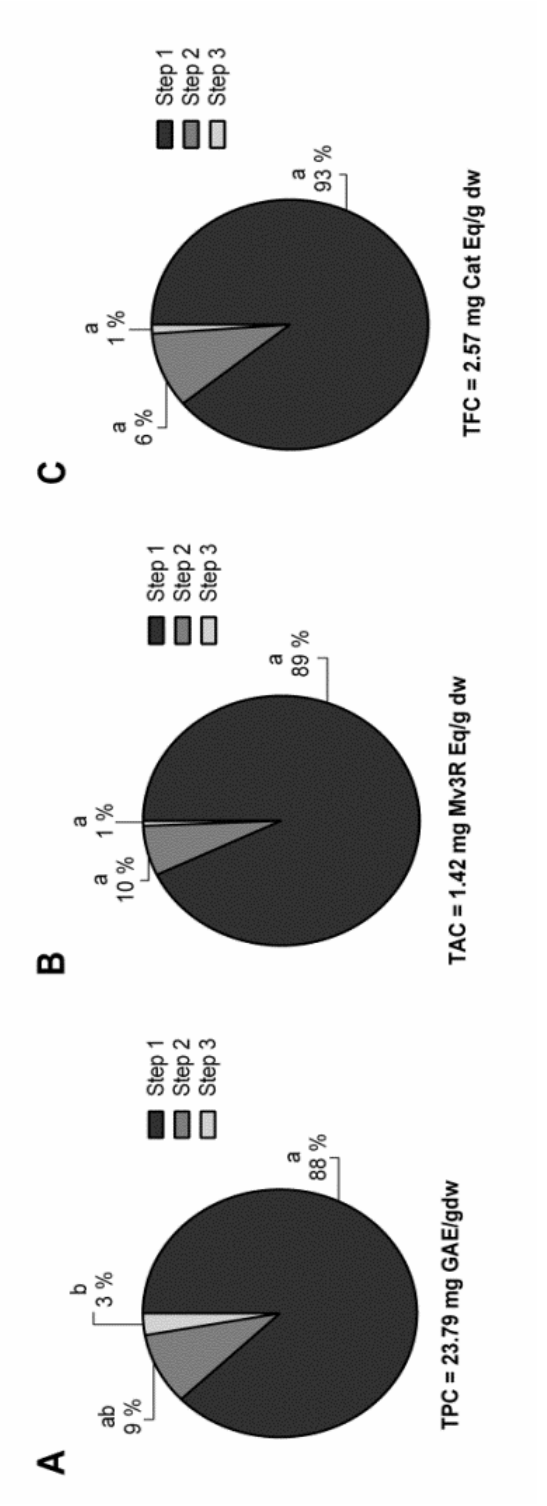
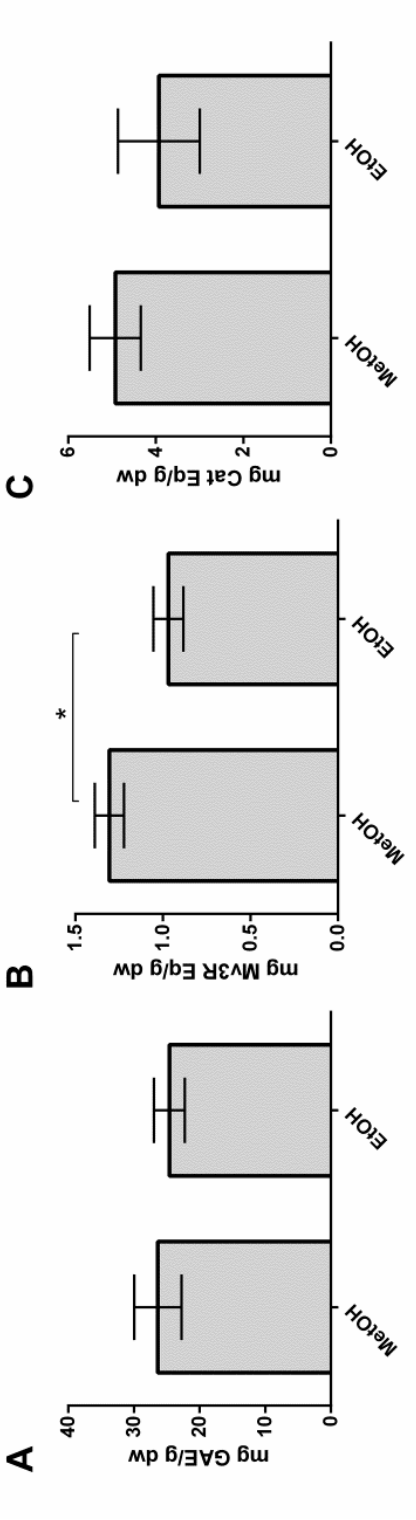

A
Step 1
Step 2
Step 3
b
3 %
ab
9 %
a
88 %
TPC = 23.79 mg GAE/gdw
B
Step 1
Step 2
Step 3
a
1 %
a
10 %
a
89 %
TAC = 1.42 mg Mv3R Eq/g dw
C
Step 1
Step 2
Step 3
a
1 %
a
6 %
a
93 %
TFC = 2.57 mg Cat Eq/g dw

Figure 5.


A
mg GAE/g dw
0
10
20
30
40
MetOH
EtOH
B
mg MV3R Eq/g dw
0.0
0.5
1.0
1.5
*
MetOH
EtOH
C
mg Cat Eq/g dw
0
2
4
6
MetOH
EtOH

UNIVERSITAT ROVIRA I VIRGILI
MODERN CHANGES IN FRUIT INTAKE PATTERNS MODULATE THE BIOAVAILABILITY AND METABOLISM OF FRUIT PHENOLIC COMPOUNDS IN RATS
Lisard Iglesias Carres

MANUSCRIPT 4:

Optimization of a polyphenol extraction method for sweet orange pulp (*Citrus sinensis L.*) to identify phenolic compounds consumed from sweet oranges.

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Abstract:

The consumption of sweet oranges has been linked to several health benefits, many of which are attributed to hesperidin, a flavanone that is present in high amounts in these fruits. However, other phenolic compounds can contribute to the bioactivity of sweet oranges. To link those effects to their phenolic profile, the complete characterization of the phenolic profile is mandatory. Although many studies have profiled the phenolic composition of orange juices, their pulps, which retain phenolic compounds, are overlooked. This fact is particularly relevant because dietary guidelines recommend the consumption of whole fruits. Therefore, this study aimed to develop a specific method for the optimal extraction of phenolics from orange pulp and to use this method to characterize these fruits grown at different locations by HPLC-ESI-MS/MS. The extraction conditions that reported the highest total polyphenol content (TPC) and hesperidin contents were 20 mL/g, 55 °C, and 90 % methanol. The extraction time and number of sequential steps were further evaluated and optimized as 20 min and two extraction steps, respectively. Although lower extraction rates were achieved when using ethanol as the extraction solvent, high TPC and hesperidin yields were obtained, suggesting the potential use of this methodology to produce phenolic-rich extracts for the food industry. By applying the optimized methodology and analyzing the extracts by HPLC-ESI-MS/MS, geographic cultivation regions were demonstrated to affect the phenolic profiles of oranges. In short, we developed a quick, easy-to-perform methodology that can be used to extract orange phenolics from pulp for their identification and quantification and to evaluate the factors that affect the phenolic profile in sweet orange pulps.

Keywords: Flavanones; Flavonoids; Hesperidin; Response Surface Methodology

Chemical compound studied in this article:

Didymin (PubChem CID: 16760075), hesperidin (PubChem CID: 10621), kaempferol-3-*O*-rutinoside (PubChem CID: 5318767), narirutin (PubChem CID: 442431), neoeriocitrin (PubChem CID: 114627) and rutin (PubChem CID: 5280805).

Abbreviations:

GAE, gallic acid equivalents; LSR, liquid-t-solid ratio; MetOH, methanol; RSM, response surface methodology; and TPC, total polyphenol content.

1. Introduction:

Phenolic compounds are secondary plant metabolites that can enter the human diet though the consumption of vegetal products. Importantly, a wide range of health-related activities have been reported for phenolic compounds. In this sense, the phenolic compounds present in citrus fruits have received attention due to their anti-oxidant, anti-inflammatory and cardioprotective activities [1]. Sweet oranges are a rich source of flavanones, and the effects of their consumption are related to cardioprotective effects, among others [2,3]. Remarkably, hesperidin, the most abundant flavanone in sweet oranges [1,4,5], is believed to be the responsible for many of the biological effects linked to sweet orange consumption [1–3]. Other relevant compounds in sweet oranges that can contribute to their health-promoting activities include narirutin, phenolic acids and flavonols [4,6,7].

To link the consumption of sweet orange phenolic compounds to a health effect, characterization of their phenolic profile is required. For flavanones specifically, their extraction and analysis remain challenging due to their chemical complexity and vast distribution in the plant kingdom [1]. Additionally, factors such as the solvent type, temperature, extraction time and liquid-solid ratio (LSR) influence the polyphenol extraction yield [8–11]. Consequently, it becomes difficult to develop a universal extraction method for all the phenolic compounds in all food matrixes [8]. Hence, the extraction method must be optimized for each food matrix. In this sense, response surface methodology (RSM) has been used to optimize the extraction of polyphenols in several plant products, including fruits [9,11–19]. This methodology allows the evaluation of interactions between different independent variables and their effects on the response variables [9].

There are multiple optimized methods for extracting phenolics from citrus peels [10,17,19–21], and many studies have analyzed orange juice without

any extraction methods [6,22,23]. Indeed, the phenolic content of orange juices has been widely profiled [6,7,22,23], and the factors that modulate it, which include orange variety, maturity stage, post-harvest processing and storage conditions, have been studied [5,24,25]. However, orange pulps, which are the edible parts of sweet oranges, are usually overlooked. Although some attempts have been performed to characterize the whole edible parts of oranges, no specific or optimized extraction methodologies have been used. Additionally, only flavanone aglycones and total phenolics were quantified, which is insufficient to link the consumption of a fruit to a specific health benefit [26–28]. Importantly, orange pulp by-products are known to contain phenolic compounds [29,30]. Therefore, only profiling citrus juices can lead to a lower quantification of the real phenolic content in this type of fruit. Additionally, the phenolic profiles of citrus peels, pulps and juices differ. More specifically, the phenolic profile of citrus peels differs from citrus pulps and juices in terms of the type of phenolic compounds, but the differences in the phenolic profiles of citrus juices and pulps are mainly quantitative [3,4,31]. Further, dietary guidelines recommend the consumption of whole fruits instead of juices [32,33]; in the specific case of sweet oranges, this includes their pulps.

Given the chemical diversity of phenolic compounds [1], the different food matrixes [5,34] and the influence of the extraction variables [8–10], specific extraction methods must be developed. However, to our knowledge, there is no optimized extraction method for isolating orange pulp phenolics. Therefore, the aim of this study was to determine the best extraction conditions for polyphenols from sweet orange pulp by RSM. Additionally, the phenolic profile of two different Navelina sweet oranges cultivars was characterized by HPLC-ESI-MS/MS to evaluate whether the geographical growing location could modulate the phenolic profiles of sweet oranges.

2. Materials and Methods:

2.1. Plant Material:

Navelina oranges (*Citrus sinensis L.*), grown in the southern (southern orange, SO) or northern (northern orange, NO) hemisphere, were purchased form Mercabarna (Barcelona, Spain). Fresh oranges were peeled, and the pulp was frozen in liquid nitrogen. Oranges were ground until reaching homogeneity, and the homogenates were lyophilized for one week in a Telstar LyoQuest lyophilizer at - 55 °C, after which the samples were further ground to obtain a fine powder. Orange powder was kept dry and protected from light exposure until extraction. NOs were used to develop the extraction method, and NOs and SOs were used to validate the extraction method and provide insights into the factors affecting the phenolic profile in oranges.

2.2. Chemicals and reagents:

Acetonitrile (HPLC analytical grade) and glacial acetic acid were purchased from Panreac (Barcelona, Spain). Methanol and ethanol (HPLC analytical grade) were purchased from Sigma-Aldrich (Madrid, Spain). Formic acid was purchased from Scharlab (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q Advantage A10 system (Madrid, Spain). The Folin-Ciocalteu reagent was purchased from Fluka/Sigma-Aldrich (Madrid, Spain). Apigenin, chlorogenic acid, diosmetin, eriodictyol, eriodyctiol-7-*O*-glucoside, quercetin-3-*O*-galactoside (hyperoside), isorhamnetin-3-*O*-glucoside, kaempferol, kaempferol-3-*O*-glucoside, and kaempferol-3-*O*-rutinoside were purchased from Exrtasynthese (Genay, France). Benzoic acid, caffeic acid, p-coumaric acid, ferulic acid, gallic acid, naringenin, phloroglucinol, protocatechuic acid and quercetin were purchased from Fluka/Sigma-Aldrich (Madrid, Spain). Hesperidin was purchased from Quimigranel (Barcelona, Spain) and hesperetin, naringin and rutin were kindly provided by Nutrafur (Murcia, Spain).

Standard compounds were individually dissolved in methanol at 2000 mg/L, with the exception of naringenin and isorhamnetin-3-*O*-glucoside, which were dissolved at 1000 mg/L, and hesperidin and hyperoside, which were dissolved at 500 mg/L. All standard stock solutions were newly prepared every 3 months and stored in amber-glass flasks at -20 °C. Mixed standard stock solutions were diluted with Milli-Q water to obtain the desired concentration needed to construct the calibration curves.

2.3. Extraction procedure:

NO powder was weighed to obtain the desired LSR and mixed with 1 mL of pre-heated extraction solvent. Different methanol proportions (methanol:water, v:v), temperatures, times and extraction steps were used throughout the experiment. Additionally, in all cases, methanol was prepared with 1 % formic acid. All extractions were performed with 500 rpm agitation under protection from light exposure. Samples were centrifuged at 8,500 rpm for 10 min at 4 °C, and the supernatant fractions were collected and stored at -20 °C until analysis.

2.4. Analyses of the response variables:

2.4.1. Determination of the total phenolic content (TPC):

The TPC of extracts was determined by the Folin-Ciocalteu method [35]. Briefly, 50 µL of the extract and 50 µL of the Folin-Ciocalteu reagent were consecutively added to an Eppendorf tube containing 500 µL of water. After 3 min, 100 µL of Na_2CO_3 (25 %) was added to the mixture. A final volume of 1 mL was reached by the addition of Milli-Q water. 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. Gallic acid at different concentrations was used as a standard compound to construct the

calibration curves. The results were expressed as milligram gallic acid equivalents per gram of dry weight (GAE/g dw).

2.4.2. HPLC-DAD analysis of phenolic compounds:

An HPLC-DAD method was developed to quantify hesperidin in NO powder. Polyphenol separation was achieved using a ZORBAX Eclipse XDB-C18 (150 mm x 2.1 mm i.d., 5 µm particle size) as the chromatographic column (Agilent Technologies, Palo Alto, CA, USA) equipped with a Narrow-Bore guard column (2.1 mm x 12.5 mm 5 µm particle size, Agilent Technologies, Palo Alto, CA, USA). The mobile phase was 0.25 % acetic acid in water (A) and acetonitrile (B) in a gradient mode as follows: initial conditions, 0 % B; 0-1 min, 0-20 % B; 1-8 min, 20-30 % B; 8-9 min, 30-100 % B; 9-10 min, isocratic at 100 % B; and 10-11 min, 100-0 % B. A 5-min post-run was applied for column equilibration. The flow rate was set at 0.5 mL/min, and the injection volume was 10 µL for all runs.

Identification and quantification of hesperidin was achieved with a UV/Vis photodiode array detector (1260 Infinity, Agilent Technologies, Palo Alto, CA, USA). Chromatograms were recorded from 200 to 400 nm. Hesperidin was detected and quantified at 280 nm [7]. The results were expressed as mg hesperidin/g dw and were used in the RSM design to evaluate the effect of multiple extractions and solvent replacement on the extraction of orange phenolic compounds.

To validate the quantitative HPLC-DAD method, the calibration curves, linearity, intraday variability (precision), interday variability (reproducibility), and detection and quantification limits were calculated using Milli-Q water spiked with hesperidin. The peak areas of the various hesperidin concentrations were used to construct calibration curves. The method's precision was determined as the relative standard deviation (% RSD) of the concentration in a triplicate analysis from three different

spiked samples (10, 200 and 500 μg/mL). Method reproducibility was determined as the relative standard deviation (% RSD) of the concentrations from three different hesperidin concentrations (10, 200 and 500 μg/mL) analyzed in triplicate over three consecutive days. Sensitivity was evaluated by determining the limits of detection (LOD) and quantification (LOQ), which are defined as the concentration corresponding to 3-fold and 10-fold of the signal-to-noise ratio, respectively. The quality parameters are summarized in S1 Table.

2.4.3. HPLC-ESI-MS/MS:

The extracts were directly analyzed using a 1200 LC Series coupled to a 6410 MS/MS (Agilent Technologies, Palo Alto, CA, USA). The column and mobile phases used were the same as in the HPLC-DAD method (see section 2.4.2). The gradient mode was used with the following conditions: initial condition, 0 % B; 0-0.5 min, 0 % B; 0.5-2 min, 0-10 % B; 2-12 min, 10-30 % B; 12-16 min, 30-60 % B; 16-17 min, 60-100 % B, 17-20 min, 100 % B; and 20-21 min, 100-0 % B. A 6-min post-run was required for column re-equilibration. The flow rate was set at 0.4 mL/min, and the injection volume was 2.5 μL for all runs. Electrospray ionization (ESI) was conducted at 200 °C and 14 L/min with 20 psi of nebulizer gas pressure and a capillary voltage of 3000 V. The mass spectrometer was operated in the negative mode and the MS/MS data were acquired in dynamic mode. The optimized conditions for the analysis of the phenolic compounds studied using HPLC-ESI-MS/MS are summarized in S2 Table. Data acquisition was performed using the MassHunter Software (Agilent Technologies, Palo Alto, CA, USA). The calibration curves, coefficient of determination, linearity and detection and quantification limits for the HPLC-ESI-MS/MS method can be found in S3 Table and were evaluated following the same principles as in the HPLC-DAD method (see section 2.4.2).

2.5. Experimental design:

2.5.1. Range selection:

Prior to RSM, the extraction temperature, methanol concentration and LSR effects were individually evaluated on the extraction of the TPC from NO to select the RSM working ranges. The temperature was evaluated at 25, 40, 55, 70 and 85 °C; methanol at 30, 50, 60, 70, and 90 %; and LSR at 5, 10, 20, 30 and 40 mL/g. When not evaluated, the extraction parameters were kept constant at 55 °C, 50 % and 20 mL/g, respectively. The extractions at this stage of the optimization were performed for 30 min (500 rpm).

2.5.2. Response surface methodology:

The extraction was optimized using an experimental design by RSM. A face-centered central composite design with two factors and three levels consisting of 11 randomized runs with 3 center-point replicates was selected. The independent variables were the extraction temperature (25 – 55 °C; X_1) and methanol concentration (50 – 90 % methanol:water, 1 % formic acid, X_2). LSR and the extraction time were fixed at 20 mL/g and 30 min, respectively. The experimental data were fit to a second polynomial response surface using the equation

$$Y = \beta_0 + \sum_{i=1}^{2} \beta_i X_i + \sum_{i=1}^{2} \beta_{ii} X^2{}_{ii} + \sum_{i=1} \sum_{j=i+1} \beta_{ij} X_{ii} X_{ji} \quad (1)$$

where Y is the dependent variable, β0 the constant coefficient, and βi, βii and βij are the linear, quadratic and interaction regression coefficients, respectively. Xi, Xii and Xji represent the independent variables.

TPC and hesperidin levels were used as response (dependent) variables in the RSM optimization step. The results from the RSM design were analyzed with the Design-expert 9.0.6 software (Trial version, Stat-Ease Inc., Minneapolis, MN, USA).

2.5.3. Extraction time:

The contribution of the extraction time to the NO TPC was evaluated by performing phenolic extractions at 55 °C using 90 % methanol (1 % formic acid) with an LSR of 20 mL/g within a range from 0 to 120 min (500 rpm). An extraction time of 0 min was established for samples that were mixed with the extraction solvent and immediately centrifuged (8,500 rpm for 10 min at 4 °C).

2.5.4. Multi-step extractions:

To evaluate the effect of multiple extraction steps on the recovery of the TPC and hesperidin from NO, three sequential extractions were performed on orange powder under the following conditions: 55 °C, 90 % methanol (1 % formic acid), and 20 min (20 mL/g, 500 rpm).

2.5.5. Ethanol-methanol comparison:

To evaluate the efficiency of ethanol on TPC and hesperidin extraction, NO powder was extracted twice under the following conditions: 55 °C, 90 % methanol or ethanol (1 % formic acid), and 20 min (20 mL/g, 500 rpm).

2.6. Statistical analysis:

The experimental results for the RSM design were analyzed using the Design-Expert 9 software (Trial version, Stat-Ease INC., Minneapolis, MN, USA). The SPSS 19 software (SPSS Inc., Chicago, IL, USA) was used for any other statistical analysis. All experiments were performed in triplicate and the statistical significance was evaluated using a one-way ANOVA or Student's t-test. A p-value less than 0.05 was considered to be statistically significant.

3. Results and Discussion:

Citrus polyphenols such as hesperidin are known to exert beneficial health effects when consumed [1–3]. The proper quantification of a food matrix is essential to correlate its consumption and an observed health effect. The diversity of phenolic compounds found among fruits [1], fruit varieties [5] and even fruit parts [31] makes it difficult to develop a universal extraction method. Therefore, specific extraction methods must be developed to accurately profile the phenolic composition of a plant matrix. Moreover, no optimized extraction methods have been developed for orange pulps: they not only contain substantial quantities of phenolic compounds [29,30], but their consumption is also recommended over that of their juices [32,33]. To determine the optimal extraction conditions for orange pulp, RSM was applied to optimize the TPC andas the main compound occurring in sweet oranges, hesperidin [5]. Throughout the optimization process, NO were used due to their higher phenolic content.

3.1. Range selection:

Prior to RSM, the LSR, temperature and methanol proportion effects were individually evaluated for the extraction of the TPC from NO (Fig 1). This allowed the selection of an optimized LSR and the selection of RSM working ranges for methanol and temperature. The extraction time was fixed at 30 min according to other studies [9,12].

The selection of an appropriate LSR is of significant relevance when optimizing an extraction method, as low LSRs lead to solvent saturation and high LSRs are economically inefficient [36]. Therefore, the LSR was evaluated at 5, 10, 20, 30 and 40 mL/g. As the solvent proportion increased, the TPC extraction increased, up to 20 mL/g (Fig 1A). In this sense, Inoue *et al*. reported changes in the hesperidin and narirutin extraction yields due to LSR variations in kiyomi (*Citrus unshiu*) peels [10], which are the most abundant phenolics in oranges. However, no significant

differences were observed at values higher than 20 mL/g. Therefore, the LSR was fixed at 20 mL/g for the rest of the experiments. Similar LSR values were also reported to be optimal for the extraction of phenolics from sun-dried apricots [12] and dried mandarin (*Citrus reticulate*) peels [20]. Additionally, Silva *et al*. reported a similar behavior for TPC extraction from *Inga edulis* leaves [9] and Pompeu *et al*. from *Euterpe oleracea* fruits [13].

Throughout these experiments, methanol was always prepared with formic acid at a concentration of 1 %. Low concentrations of organic acids enhance the extraction of phenolics by promoting plant matrix degradation [15]. The solvent type and percentage can greatly influence the extraction of phenolics in citrus species [37,38] and other plant materials [14,15]. The effect of the methanol proportion (Fig 1B) observed in this study is consistent with the fact that phenolic extractability increases with increasing solvent proportions to a certain point, after which it starts to decrease [11,16], as reported for other citrus species [10,17]. The maximum phenolic extraction was reached at 70 % methanol. Therefore, 50, 70 and 90 % methanol were chosen as the low, medium and high methanol percentages, respectively, for the RSM.

Temperature usually affects the extraction of phenolics by enhancing the solubility of phenolic compounds [39]. However, an increase beyond a certain point can promote the degradation of phenolics due to thermal and enzymatic degradation [40]. Nevertheless, no significant effects of temperature were observed for the extraction of the TPC from oranges within the range studied (Fig 1C); this has also been previously described by S. Chang *et al*. in the extraction of the TPC from kaffir lime (*Citrus hystrix*) peels [17]. Therefore, relatively mild temperatures of 25, 40 and 55 °C were selected to be applied as the low, medium and high levels, respectively, in the RSM. Additionally, working at mild temperatures is recommended in such studies as high temperatures promote extraction

solvent loss due to vaporization, which increases the extraction cost from the food industry's point of view [17].

3.2. Response surface methodology:

The phenolic extraction from orange pulp was further optimized by RSM. Throughout the experimental RSM step optimization, the LSR was fixed at 20 mL/g, as previously determined, and the extraction time was set at 30 min according to other studies [9,12]. A face-centered central composite design with two factors and three levels consisting of 11 randomized runs with 3 center points was selected. The independent variables used in the RSM were temperature (25-55 °C, X_i) and the methanol proportion (50-90 %, X_j). TPC and hesperidin contents were included in the model. Selection of these variables was based on the evaluation of all the phenolic compounds present in sweet orange pulps based on the TPC and the importance of hesperidin in sweet oranges as the main phenolic compound [5] for health promoting activities [1–3].

3.2.1. Multiple linear regression and model adequacy:

The TPC and hesperidin results from all the runs are shown to be dependent on the extraction variables in Table 1. These experimental data were used to determine the regression coefficients in Eq.1. While hesperidin extraction fit a quadratic model, TPC fit a linear one (Table 2). Good fit was achieved and the responses' variability was explained by the model. Additionally, the determination coefficients (R^2) for TPC and hesperidin were higher than 0.9. ANOVA analysis was statistically significant, suggesting that at least one of the model parameters could explain the variation in the responses in relation to the average response. Furthermore, statistical insignificance ($p > 0.05$) in the lack of fit test showed that the model properly fit the experimental data, thus further validating the model.

3.2.2. Analysis of the regression coefficients and response surface plots:

The regression coefficients for the TPC and hesperidin models, reported in Table 2, allow for the interpretation of the effects of the independent variables on their extraction. Surface plots (Fig 2) facilitated the visualization of the significance of the extraction variables on the extraction of TPC and hesperidin.

Regarding the extraction temperature, the linear positive and negative effects were found to be statistically significant for TPC and hesperidin, respectively. For TPC, this indicates that increasing the temperature will result in greater TPC extraction (Fig 2B). Temperature usually produces positive linear and negative quadratic effects in RSM [9,13,17], including designs evaluating flavanones [19]. However, a significant positive quadratic effect was reported for hesperidin. This behavior has been previously reported for flavonols in *Inga edulis* leaves [9] and suggests that hesperidin requires more energetic conditions to be released into the solvent.

For the methanol proportion, positive linear effects were reported to be statistically significant for TPC and hesperidin. Additionally, a significant negative quadratic effect was found for hesperidin. This indicates that there is a maximum methanol proportion in the hesperidin extraction, after which its extraction starts to decrease; this effect can be observed in Fig 2A. Solvent positive linear and negative quadratic effects are widely reported in the literature [9,13]. In this sense, hesperidin and naringin extraction from citrus peels have been reported to present negative quadratic effects [19].

The combination of extraction variables at the highest desirability (0.971) was used to determine the optimal extraction conditions. In our study, the optimal extraction temperature was 55 °C. Similarly, orange peels have

been reported to have an optimal extraction temperature of 60 °C for the extraction of total flavonoids, total phenolic compounds and antioxidant activity [18]. Additionally, TPC was reported to show an optimal extraction temperature at 55.56 °C in yuzu (*Citrus junos*) [19] and 48.3 °C in kaffir lime (*Citrus hystrix*) [17] peels. Further, 90 % was found to be the optimal methanol proportion, possibly due to the low solubility of hesperidin in water [41]. Consistent with this result, high ethanol proportions have been reported as optimal for the extraction of phenolics from different citrus species [19,21]. For example, Silva *et al*. found 86.8 % ethanol to be optimal for the extraction of *Inga edulis* phenolics at very similar conditions of 58.2 °C, 24 mL/g and 30 min [9]. Additionally, pure methanol is more efficient than methanol:water (50:50, v:v) for the extraction of lemon phenolics [38]. However, water ethanol mixtures of approximately 50 % are optimal for the extraction of phenolics from bitter orange (*Citrus aurantium*) flowers [42] and kaffir lime (*Citrus hystrix*) peels [17].

3.2.3. Model validation:

To validate the veracity of the model, 3 extractions were performed using the optimized conditions. No differences were obtained between the predicted and experimental values (Table 3). Therefore, the model accurately predicted the behavior of the response variables within the studied range for the extraction variables. Consequently, the extraction temperature and methanol proportion were fixed at 55 °C and 90 %, respectively, throughout the rest of the study. Remarkably, the optimized solvent percentage significantly differed from other optimized methods for citrus peels [17,19]. This result demonstrates that each fruit matrix requires specific extraction conditions and studies such as ours for each specific fruit.

3.3. Effect of time on TPC:

Theoretically, working at the optimal methanol proportion and temperature for a certain period of time allows the solubilization of phenolic compounds due to weakened cell wall interactions with phenolic compounds, leading to a greater transference of phenolics into the extraction solvent [11,14]. To test this in our orange sample, a range of extractions between 0 and 120 min was performed under the optimized conditions of 55 °C, 90 % of methanol and 20 mL/g. Our results show that TPC increases at 20 min of extraction time, after which additional time does not produce any significant increases or decreases on the TPC (Fig 3). In this sense, increases in the extraction of different phenolic families due to time can reach a plateau after which they do not become more concentrated [9,39]. Importantly, the extraction of polyphenols from citrus peels requires extraction times approximately 120 min [17,19], further proving that the fruit matrix plays an important role in the optimal extraction conditions for citrus phenolics. Considering practical and economic issues, 20 min was fixed throughout the rest of the study.

3.4. Effect of sequential steps on the TPC and hesperidin content:

To test whether our optimized method was able to extract most of the TPC and hesperidin from oranges in a single step, orange powder was subjected to three consecutive extractions under the optimized conditions (55 °C, 90 %, 20 mL/g, and 20 min). Most of the hesperidin (93 %) and TPC (87 %) were extracted in the two first extraction steps (Fig 4). Because only 1.84 ± 0.12 mg hesperidin/g dw and 2.30 ± 0.34 mg GAE/g dw remained to be extracted in the third extraction, 2 sequential steps was selected as the optimized number of extractions. In agreement with this result, Mané *et al*. reported few increases in flavanol extraction with two, three and four consecutive extractions for grape skins, seeds and pulp [43]. Additionally, Yang *et al*. reported the same tendency for *Phyllanthus*

emblica L. barks [11]. Remarkably, this method obtained 24.77 ± 1.28 mg hesperidin/g dw with two consecutive extractions.

3.5. Investigation of methanol replacement for ethanol:

Orange pulps are food industry by-products and could potentially be revalorized to obtain phenolic-rich extracts [29]. Ethanol is used in the food industry due to methanol's toxicity [8]. To evaluate whether ethanol could replace methanol as the extraction solvent, NO powder was subjected to two consecutive extractions under the optimized conditions of 55 °C, 90 % organic solvent, 20 mL/g, and 20 min agitation. Hesperidin and TPC were found to be statistically higher in methanolic extractions than in ethanolic extractions (Fig 5), in agreement with the fact that methanol usually achieves higher extraction rates than ethanol [8,15]. Indeed, Maguaza *et al*. reported a higher extraction of hesperidin from mandarin (*Citrus reticulata*) rinds when methanol was used as an extraction solvent [37]. Additionally, methanol has previously been used to extract phenolics from citrus species [10,17]. The fact that a fair amount of the total phenolics and hesperidin was extracted (17.93 ± 0.70 mg hesperidin/g dw and 12.95 ± 0.51 mg GAE/g dw) using ethanol as an extraction solvent and the important health activities of hesperidin [1–3] opens the door to possible adaptations of this method to produce phenolic-rich extracts with potential bioactive functions.

3.6. Detailed characterization of sweet oranges:

Once the extraction method was optimized (two sequential extractions at 55 °C, 90 % MetOH, 20 mL/g and 20 min agitation), it was used to study the differences in the phenolic profiles generated by different geographical growing regions. To complete this objective, the phenolic profile of NO and SO pulps were compared. Characterization of the phenolic profiles of both orange cultivars was performed using an HPLC-ESI-MS/MS methodology. The phenolic profiles of the pulps from both oranges (Table 4) is in

agreement with the phenolic profiles of juices from different sweet orange varieties [5,22,34]. In both studied sweet oranges, hesperidin dominated narirutin, which is in accordance with the phenolic profiles of orange juices [5]. Compounds such as didymin, kaempferol-3-*O*-rutinoside, protocatechuic acid *O*-glucoside, neoeriocitrin and rutin were found in lower but still relevant concentrations compared to hesperidin and narirutin. This trend has previously been found for different phenolics in several sweet orange varieties [22,34]. Minor phenolic compounds such as eriocitrin, *p*-coumaric acid, chlorogenic acid and ferulic acid were also found in orange pulp, in agreement with different studies in orange juices [5,6]. Previous studies have evaluated the phenolic profiles of orange pulps. However, only the total phenolic content and the content of flavanone aglycones after hydrolysis were evaluated [26–28]. Hence, this study provides an accurate and complete characterization of the phenolic contents found in sweet orange pulps.

Phenolic compounds are plant-stress metabolites. Environmental conditions, which include temperature, water accessibility and light exposure, are known to modulate the phenolic profile of different fruits [44]. Therefore, changes in the phenolic profiles of oranges could occur depending on their geographical localization. However, in terms of the phenolic representatives, NO and SO were very similar, though the amounts of phenolic compounds reported in each cultivar were different. In general, most phenolic compounds reported significantly higher concentrations in NO than in SO. In agreement with our results, differences in the individual phenolic compound contents were found in pummelo (*Citrus grandis*) [45], bergamot (*Citrus bergamia*) [46] and lemons (*Citrus limon*) [47] grown in different regions. Surprisingly, no significant differences were reported for the content of the major phenolic, hesperidin, in sweet oranges (p=0.06). Our results indicate that the region of cultivation has an impact on the concentrations of individual phenolic compounds found in sweet orange pulps.

4. Conclusions:

In this study, we developed an optimized extraction method that is capable of extracting *Citrus sinensis* phenolics from its pulp. This method consists of two relatively fast sequential extractions (20 min each) using a relatively mild temperature (55 °C) and a high methanol concentration (90 %). Importantly, our method is able to extract high quantities of phenolic compounds, which is essential for determining the accurate phenolic profile present in the fruit. Given that no previous extraction methods for orange pulps are reported in the literature, we propose this method as a routine methodology to study the phenolic profile of the edible parts of sweet oranges. Additionally, this method could be useful for producing phenolic-rich extracts with potential bioactive functions. This study also indicates that the geographical region affects the phenolic profile of orange pulps.

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Figure legends:

Figure 1. Individual effect of LSR (A), methanol proportion (B) and temperature (C) on the extraction of total phenolic compounds from *Citrus sinensis* pulp. The results are expressed as mg of gallic acid equivalents per g of dry weigh ± SD (mg GAE/g dw). [a, b, c] Mean values (n=3, each) with different letters denote significant differences by one-way ANOVA with Tukey's post-hoc test ($p<0.05$).

Figure 2. Response surface plots for hesperidin (A) and the total phenolic content (B) from *Citrus sinensis* pulp at a functional methanol proportion (MetOH) and temperature.

Figure 3. Effect of extraction time on the extraction of total phenolic compounds from *Citrus sinensis* pulp. The results (n=3, each) are expressed as mg of gallic acid equivalents per g of dry weigh ± SD (mg GAE/g dw). Statistical analysis was performed by one-way ANOVA with Tukey's post-hoc test (significant at $p<0.05$).

Figure 4. Effect of sequential extractions on the extraction of hesperidin (A) and the total phenolic compounds (B) from *Citrus sinensis* pulp. The results (n=3, each) are expressed as mg of hesperidin or gallic acid equivalents per g of dry weigh ± SD (mg GAE or hesperidin/g dw).

Figure 5. Comparison between the extraction efficiency of methanol (MetOH) and ethanol (EtOH) on the extraction of hesperidin (A) and the total phenolic compounds (B) from *Citrus sinensis* pulp**.** The results (n=3, each) are expressed as mg of hesperidin or gallic acid equivalents per g of dry weigh ± SD (mg GAE or hesperidin/g dw). Statistical analysis was performed using Student's t-test (significant at $p<0.05$).

Table 1: Face-centered settings for the independent variables and experimental results for hesperidin and the total phenolic content (TPC) in *Citrus sinensis* pulp.

Run	T^a (ºC)	MetOH (%)	Hesperidin (mg/g dw)	TPC (mg GAE/g dw)
1	25	50	8.60	8.85
2	55	50	11.08	9.59
3	25	90	18.20	10.75
4	55	90	20.17	11.55
5	25	70	16.68	10.37
6	55	70	18.14	10.46
7	40	50	8.19	9.35
8	40	90	18.86	10.78
9	40	70	16.07	9.93
10	40	70	15.86	10.30
11	40	70	16.13	10.18

Abbreviations: T^a, temperature; MetOH, methanol; and GAE, gallic acid equivalents.

Table 2: Analysis of the variance and regression coefficients for the predicted model for the response variables in *Citrus sinensis* pulp.

Model parameters	Regression coefficients	Hesperidin	TPC
Intercept	β_0	-29.62	6.38
Lineal			
T^a	β_1	-0.32**	$1.82x10^{-2}$*
MetOH	β_2	1.21**	$4.41x10^{-2}$**
Interaction	β_{12}	$-4.25x10^{-4}$	-
Quadratic			
$T^a x T^a$	β_{11}	$5.24x10^{-3}$**	-
MetOH x MetOH	β_{22}	$-6.77x10^{-3}$**	-
p-value		<0.0001	<0.0001
R^2		0.9946	0.9191
Adjusted R^2		0.9892	0.8989
F-value		184.3	45.44
Lack of fit [a]		0.0636	0.4094

Abbreviations: T^a, Temperature; MetOH, methanol; and R^2, determination coefficient. Differences between the groups were determined by ANOVA. [a] p-value of the lack of fit test.* $p<0.05$; ** $p<0.01$.

Table 3: Overall optimal values for the extraction parameters for hesperidin and the total phenolic content in *Citrus sinensis* pulp.

Model parameters			Response	Predicted value	Experimental value
Tª (ºC)	**MetOH (%)**	**Desirability**			
55	90	0.971	Hesperidin	20.34	19.55 ± 0.52
			TPC	11.35	11.23 ± 0.29

Abbreviations: Tª, Temperature; MetOH, methanol; and TPC, total polyphenol content. The results are expressed as mg of hesperidin or gallic acid equivalents per g of dry weight ± SD (n=3).

Table 4: The phenolic contents from Navelina sweet orange pulps from the northern (NO) and southern (SO) hemispheres by HPLC-ESI-MS/MS expressed as mg/kg dw ± SD (n=3, each).

Phenolic compound	NO	SO	p-value
Benzoic Acid	n.q.	n.d.	
Phloroglucinol	2.34 ± 0.13	n.d.	<0.01
Hydorxybenzoic acid [a]	n.q.	n.q.	
Protocatechuic acid	3.89 ± 0.10	n.q.	
p-Coumaric acid	0.28 ± 0.00	0.09 ± 0.01	<0.01
Gallic acid	n.d.	n.q.	
Caffeic acid	n.q.	n.d.	
Ferulic acid	1.48 ± 0.02	0.84 ± 0.03	<0.01
Apigenin	n.q.	n.q.	
Naringenin	0.90 ± 0.08	0.25 ± 0.03	<0.01
Kaempferol	n.d.	n.d.	
Eriodictyol	n.q.	0.10 ± 0.00	
Diosmetin	n.q.	n.q.	
Quercetin	n.d.	n.d.	
Hesperetin	2.13 ± 0.12	1.85 ± 0.61	0.48
Protocatechuic acid *O*-glucoside [b]	315.59 ± 20.95	254.88 ± 20.49	0.02
p-Coumaric acid *O*-glucoside [c]	3.30 ± 0.09	2.22 ± 0.10	<0.01
Gallic acid *O*-glucoside d1 [d]	0.01 ± 0.00	0.01 ± 0.00	<0.01
Caffeic acid *O*-glucoside d2 [e]	10.25 ± 0.40	8.19 ± 0.56	0.01
Caffeic acid *O*-glucoside d3 [e]	23.73 ± 0.65	10.89 ± 0.42	<0.01
Caffeic acid *O*-glucoside d4 [e]	90.39 ± 3.18	36.14 ± 1.24	<0.01
Chlorogenic acid d1 [f]	2.64 ± 0.12	0.48 ± 0.22	<0.01
Chlorogenic acid d2 [f]	0.93 ± 0.02	n.q.	<0.01
Chlorogenic acid	7.70 ± 0.29	0.69 ± 0.03	<0.01
Feruloylquinnic acid d1 [g]	0.14 ± 0.00	0.13 ± 0.00	0.02
Feruloylquinnic acid d2 [g]	0.23 ± 0.01	n.d.	
Phloridzin [h]	n.d.	0.45 ± 0.02	
Kaempferol-3-*O*-glucoside	n.q.	0.07 ± 0.01	
Eriodictyol-7-*O*-glucoside	n.d.	0.50 ± 0.03	
Isorhamnetin-3-*O*-glucoside	0.07 ± 0.00	0.06 ± 0.00	<0.01
Myricetin-*O*-glucoside [i]	0.20 ± 0.00	0.11 ± 0.00	<0.01
Narirutin [j]	$8.07x10^3 \pm 0.62x10^3$	$3.54x10^3 \pm 0.57x10^3$	<0.01
Naringin	n.d.	n.d.	
Kaempferol-3-*O*-rutinoside	516.20 ± 46.00	457.77 ± 16.52	0.11
Didymin [k]	545.70 ± 52.62	444.57 ± 85.08	0.15
Eriocitrin [l]	1.39 ± 0.07	0.73 ± 0.05	<0.01

Table 4 (Continued).

Neoeriocitrin[l]	127.13 ± 7.14	44.44 ± 6.49	<0.01
Rutin	91.23 ± 6.51	16.56 ± 0.79	<0.01
Hesperidin	$38.28x10^3 \pm 3.91x10^3$	$25.83x10^3 \pm 5.87x10^3$	0.06

d1, d2, d3 and d4 indicate different isomeric compounds[a] Quantified using the calibration curve of benzoic acid. [b] Quantified using the calibration curve of protocatechuic acid. [c] Quantified using the calibration curve of p-coumaric acid. [d] Quantified using the calibration curve of gallic acid. [e] Quantified using the calibration curve of caffeic acid. [f] Quantified using the calibration curve of chlorogenic acid. [g] Quantified using the calibration curve of ferulic acid. [h] Quantified using the calibration curve of phloroglucinol. [i] Quantified using the calibration curve of hyperoside. [j] Quantified using the calibration curve of naringin. [k] Quantified using the calibration curve of hesperidin. [l] Quantified using the calibration curve of eriodictyol-7-*O* -glucose. Abbreviations: n.d., not detected; n.q., not quantified. Statistical analysis was performed using Student's t-test.

S1 Table: HPLC-DAD method parameters for hesperidin.

Compound	Detection wavelength (nm)	Calibration curve	R^2	Linearity[a]	Precision (% RSD, n=3)[b]			Reproducibility (% RSD, n=3)[b]			LOD[a]	LOQ[a]
					1	20	50	1	20	50		
Hesperidin	280	Y= 8.24X	>0.99	3.31-165.41	0.70	0.78	1.96	2.44	0.53	2.06	0.42	1.40

[a] μM. [b] μg/mL. Abbreviations: R^2, determination coefficient; LOD, limit of detection; and LOQ, limit of quantification.

S2 Table: Molecular weight, detected ion mass by qTOF and optimized MRM (multiple reaction monitoring) conditions for the identified polyphenol compounds in Navelina sweet orange pulps by HPLC-ESI-MS/MS.

Compound	MW	$[M-H]^-$	Quantification		Confirmation	
			MS/MS	CE (V)	MS/MS	CE (V)
Benzoic acid	122.12	121.0304	121>77	8	121>59	4
Phloroglucinol	126.11	125.0265	125>57	20	125>125	0
Hydroxybenzoic acid	138.12	137.0256	137>93	40	137>119	40
Protocatechuic acid	154.12	153.0215	153>109	16	153>62	40
p-Coumaric acid	164.16	163.0774	163>119	16	163>93	36
Gallic Acid	170.12	169.0137	169>125	12	169>79	24
Caffeic acid	180.16	179.0344	179>135	16	179>107	24
Ferulic acid	194.18	193.0520	193>134	12	193>178	12
Apigenin	270.24	269.0452	269>117	44	269>151	28
Naringenin	272.25	271.0619	271>151	20	271>119	32
Kaempferol	286.24	285.0403	285>239	28	285>117	56
Eriodictyol	288.25	287.0793	287>151	12	271>135	28
Disometin	300.26	299.0565	299>284	20	299>256	36
Quercetin	302.24	301.0100	301>151	20	301>179	20
Hesperetin	302.28	301.0525	301>164	28	301>286	16
Protocatechuic acid glucoside	316.26	315.0737	315>153	10	315>109	20
Gallic acid O-glucoside	332.26	341.0896	331>169	12	331>125	12
Caffeic acid O-glucoside	342.30	341.0896	341>179	20	341>323	10
Chlorogenic acid	354.31	353.0905	353>191	16	353>85	16
Feruloylquinnic acid	368.34	367.1062	367>193	40	367>161	40
Phloridzin	436.41	435.1331	435>273	10	435>167	40
Kaempferol-3-*O*-glucoside	448.38	447.0974	447>284	28	447>255	40
Eriodictyol-7-*O*-glucoside	450.39	449.1084	449>287	12	449>151	36
Hyperoside	464.38	463.0917	463>300	32	463>271	48
Isorhamnetin-3-*O*-glucoside	478.40	477.1014	477>314	32	477>285	40
Myricetin-3-*O*-glucoside	480.38	479.0841	479>317	20	479>461	10
Narirutin	580.53	579.1762	579>271	40	579>151	48
Naringin	580.53	579.1762	579>271	40	579>151	48
Kaempferol-3-*O*-rutinoside	594.52	593.1560	593>353	32	593>255	60
Didymin	594.56	593.1963	593>285	20		
Eriocitrin	596.53	595.1705	595>287	20		
Neoeriocitrin	596.53	595.1711	595>287	20		
Rutin	610.52	609.1508	609>300	40	609>271	60
Hesperidin	610.56	609.1869	609>301	20	609>286	52

Abbreviations: MW, molecular weight; CE, collision energy; Ms>Ms, mass-mass transitions.

S3 Table: HPLC-ESI-MS/MS method quality parameters for the study of polyphenol compounds in Navelina sweet orange pulps.

Compound	RT (min)	Calibration curve	R^2	Linearity (µM)	LOD (nM)	LOQ (nM)
Benzoic acid	9.77	y=304.74x	0.993	0.016-40.943	7.18	23.94
Phloroglucinol	2.80	y=203.80x	0.992	0.016-39.648	0.40	1.33
Protocatechuic acid	3.90	y=11.11x	0.996	0.013-32.442	18.20	60.68
p-Coumaric acid	7.68	y=3567.30x	0.999	0.012-30.458	0.87	2.89
Gallic Acid	3.02	y=11453.00x	0.996	0.012-29.391	0.10	0.33
Caffeic acid	5.85	y=88.55x	0.999	0.011-27.754	4.73	15.76
Ferulic acid	8.32	y=1019.40x	0.996	0.010-25.749	0.43	1.43
Apigenin	14.57	y=1950.50x	0.992	0.007-18.502	0.10	0.32
Naringenin	14.62	y=864.67x	0.999	0.007-18.365	0.18	0.61
Kaempferol	13.03	y=1489.30x	0.997	0.007-17.468	0.16	0.49
Eriodictyol	12.84	y=1556.40x	0.995	0.007-17.346	0.05	0.15
Disometin	14.81	y=2782.00x	0.997	0.007-16.543	0.14	0.47
Quercetin	13.25	y=1628.00x	0.996	0.007-16.543	0.05	0.17
Hesperetin	14.99	y=520.95x	0.995	0.007-16.541	0.08	0.25
Chlorogenic acid	4.80	y=1032.10x	0.999	0.006-14.112	0.32	1.07
Kaempferol-3-*O*-glucoside	9.55	y=1073.20x	0.993	0.004-11.151	0.06	0.19
Eriodictyol-7-*O*-glucoside	8.44	y=1968.60x	0.995	0.004-11.101	0.04	0.15
Hyperoside	8.37	y=1527.80x	0.994	0.004-10.767	0.04	0.14
Isorhamnetin-3-*O*-glucoside	9.71	y=1058.90x	0.990	0.004-10.451	0.04	0.15
Naringin	9.57	y=591.45x	0.997	0.003-8.613	0.14	0.48
Kaempferol-3-*O*-rutinoside	9.04	y=976.41x	0.998	0.003-8.410	0.03	0.09
Rutin	8.07	y=825.00x	0.998	0.003-8.190	0.06	0.20
Hesperidin	9.02	y=938.04x	0.994	0.003-8.189	0.52	1.74

Figure 1.

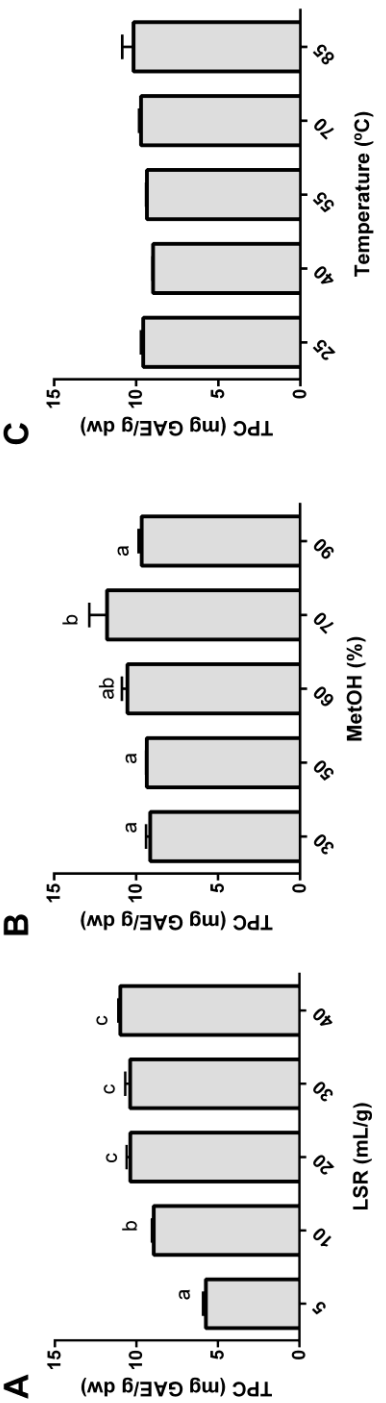


Figure 2.

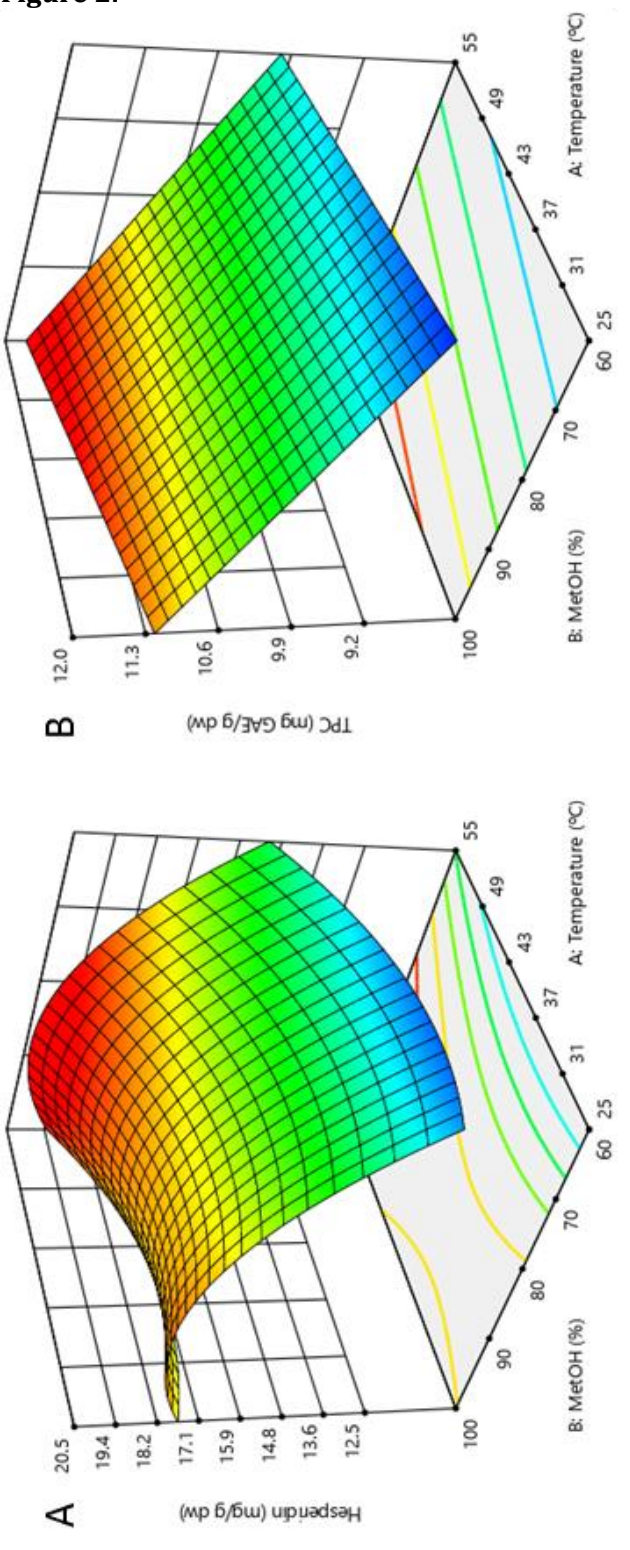

B
TPC (mg GAE/g dw)
12.0
11.3
10.6
9.9
9.2
A: Temperature (°C)
55
49
43
37
31
25
B: MetOH (%)
60
70
80
90
100
A
Hesperidin (mg/g dw)
20.5
19.4
18.2
17.1
15.9
14.8
13.6
12.5
A: Temperature (°C)
55
49
43
37
31
25
B: MetOH (%)
60
70
80
90
100

Figure 3.

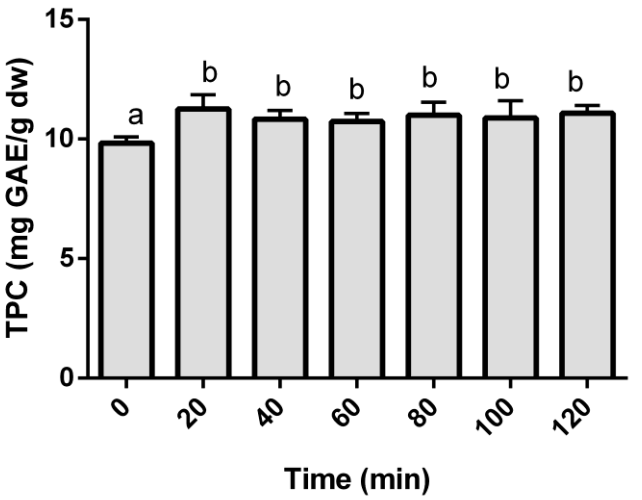


Figure 4.

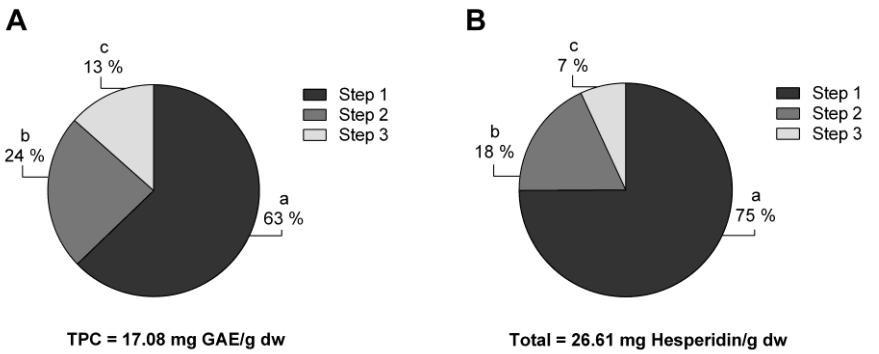
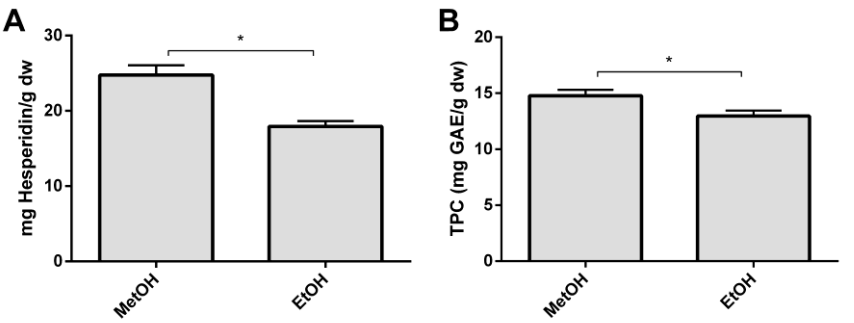

A
c
13 %
b
24 %
a
63 %
Step 1
Step 2
Step 3
TPC = 17.08 mg GAE/g dw
B
c
7 %
b
18 %
a
75 %
Step 1
Step 2
Step 3
Total = 26.61 mg Hesperidin/g dw

Figure 5.


A
mg Hesperidin/g dw
30
20
10
0
*
MetOH
EtOH
B
TPC (mg GAE/g dw)
20
15
10
5
0
*
MetOH
EtOH

UNIVERSITAT ROVIRA I VIRGILI
MODERN CHANGES IN FRUIT INTAKE PATTERNS MODULATE THE BIOAVAILABILITY AND METABOLISM OF FRUIT PHENOLIC COMPOUNDS IN RATS
Lisard Iglesias Carres

CHAPTER 2: EVALUATION OF THE EFFECT OF MODERN LIFESTYLE PATTERNS ON THE BIOAVAILABILITY AND METABOLISM OF FRUIT PHENOLIC COMPOUNDS.

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MANUSCRIPT 5:

Organic red grapes have a particular phenolic bioavailability in rats.

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Abstract:

Fruit consumption is known to promote health, and this is mainly attributed to their polyphenol content. However, the health-promoting functions of phenolic compounds are attributed to their metabolic products. Importantly, several factors can modulate their bioavailability and metabolism. Nowadays, the organic cultivation of fruits is becoming popular. In this sense, this study aimed to evaluate whether the differences in red Grenache grapes that derive from organic culture practice conditions the bioavailability and metabolism of their phenolic compounds in rats. For this, organic (organic grapes, OG) and non-organic (conventional grapes, CG) red Grenache grapes were characterised and administered to Wistar rats at a dose of 65 mg GAE/Kg bw. Serum samples were recollected before and 2, 4, 7, 24 and 48 h after OG or CG administration, and phenolic metabolites were quantified by HPLC-ESI-MS/MS. Results showed that organic cultivation has an effect of the phenolic profile of red Grenache grapes and their dietary components, showing an increase of oligomeric proanthocyanidins and anthocyanidins, and a decrease of free flavanols and dietary fibre. The serum profile of OG-administered rats was different from the CG-administered rats. OG-administered rats showed a higher metabolites´ concentration at the 2 h, suggesting higher small intestine metabolism, and a reduced 24 h metabolites´ concentration, indicating reduced colonic biotransformation of phenolic compounds compared with CG-administered rats. This study demonstrates that OG metabolites presented a particular serum kinetic behaviour compared with CG, which might have an effect on the bioactivity of their phenolic compounds.

Abbreviations:

Non organic conventional grapes (CG); conventional grape-administered rats (CGR); organic grapes (OG); organic grape-administered rats (OGR).

Keywords:

Anthocyanins; flavanols; Flavonoids; Phase-II metabolism; Phenolic compounds; Microbial metabolism.

Compounds studied in this article:

Benzoic acid (PubChem CID: 243), Catechin (PubChem CID: 9064), Chlorogenic acid (PubChem CID: 1794427), Cyanidin-3-O-rutinoside (PubChem CID: 441674), Epicatechin (PubChem CID: 72276), Epigallocatechin gallate (PubChem CID: 65064), Hippuric acid (PubChem CID: 464), Homovanillic acid (PubChem CID: 1738), Malvidin-3-O-glucoside (PubChem CID: 443652), p-Coumaric acid (PubChem CID: 637542), Phenylpropionic acid (PubChem CID: 107), Resveratrol (PubChem CID: 445154), and Rutin (PubChem CID: 5280805).

1. Introduction:

Fruit consumption is known to be healthy and this is mainly attributed to their phenolic content [1,2]. In this sense, red grapes have been reported as one of the richest dietary sources of phenolic compounds [3]. The main phenolic families found in red grapes are flavan-3-ols, anthocyanins, flavonols, hydroxycinnamic acids and stilbenes [4,5]. Flavan-3-ols in grapes occur in monomeric as well as oligomeric and polymeric forms. Several anthocyanidins, including cyanidin, petunidin, malvidin and delphinidin glycosides can occur in grapes [4,6]. Flavonols occur in grapes mainly as quercetin and kaempferol glycosyl derivates [4,7] and resveratrol is mainly found in grape skins [5]. The synthesis of phenolic compounds in plants can be modulated by environmental conditions such as water availability and light exposure [7]. Particularly, the phenolic profile of fruits is known to vary depending on culture practises [7]. Nowadays the grape organic cultivation system is increasing [8], and its production is subjected to strict rules that regulate type and amount of chemicals used for their production [9]. Additionally, organic (organic grapes, OG) and non-organic (conventional grapes, CG) grapes have been shown to produce different health effects in animal models of obesity and epilepsy [10,11].

The health beneficial effects of phenolic compounds have been attributed to their metabolic products rather than the naturally-occurring forms [12,13]. In this sense, phenolic compounds must be bioavailable to exert a systemic function, which entails their digestion, absorption and metabolism [14,15]. Buccal, pancreatic and intestinal enzymes can cleavage the glycosyl moiety of phenolic compounds, generating aglycone phenolics [14,16]. Nevertheless, glycosylated phenolic compounds can be absorbed by the host and found in plasma [17]. Importantly, anthocyanidins can be absorbed at the stomach [18] and reach the systemic circulation as glycosides [17]. Phenolic compounds, including

anthocyanins, can be absorbed at the small intestine [18,19]. There, these compounds undergo phase-II detoxification, which include glucuronidation and methylation by uridine 5´-diphosphate glucuronosyltransferases (UGTs) and catechol-*O*-methyltransferase (COMT), respectively. Before reaching the plasma, these compounds can undergo further phase-II metabolism in the liver, which additionally includes sulphuration by cytosolic sulfotransferases (SULTs) [19–21]. Non-absorbed phenolic compounds are known to reach the colon where they can be subjected to microbial metabolism [19,22]. For example, high molecular weight proanthocyanidins are unlikely to be absorbed in the small intestine and reach the colon [19,23,24]. When these non-absorbed phenolics reach the gut, microbiota can hydrolyse them [23–26]. Metabolic products are known to reach the systemic circulation and reach different organs and tissues [20,27] to finally reach the kidneys, where they will be eliminated via the urine [14,21].

Importantly, different factors are known to modulate the bioavailability and metabolism of phenolic compounds [27–31]. Some of these factors include administration dose, content of dietary components in the food matrix (i.e. dietary fibre and fat) and metabolic state of the host [13,27,28,32]. Therefore, this study aimed to evaluate whether OG have a particular bioavailability and metabolism of their phenolic compounds in rats when compared to CG.

2. Materials and methods:

2.1. Chemical and reagents:

Acetone, acetonitrile, methanol (all HPLC analytical grade) and phosphoric acid were purchased from Sigma-Aldrich (Barcelona, Spain). Glacial acetic acid was purchased from Panreac (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q Advantage A10 system (Madrid, Spain). Eriodyctiol-7-O-glucoside, quercetin-3-*O*-galactoside (hyperoside), isorhamnetin-3-O-glucoside, kaempferol-3-O-glucoside, and kaempferol-3-

O-rutinoside were purchased from Exrtasynthese (Lyon, France). Benzoic acid, caffeic acid, (+)-catechin, epigallocatechin gallate (EGCG), p-coumaric acid, (-)-epicatechin, ferulic acid, gallic acid, hippuric acid, 3-hydroxybenzoic acid, 3-(4-hydroxy)phenylpropionic acid, phloroglucinol, proanthocyanidin dimer B2, protocatechuic acid, pyrocatechol (Internal Standard, IS), quercetin and vanillic acid were purchased from Fluka/Sigma-Aldrich (Madrid, Spain). Cyanidin-3-O-rutinoside, malvidin-3-*O*-glucoside and peonidin-3-O-rutinoside were purchased from PhytoLab (Vestenbergsgreuth, Germany). Resveratrol was purchased from Quimivita (Barcelona, Spain), 5-(3',4'-dihydroxyphenyl)-γ-valerolactone was purchased from MicroCombiChem e.K. (Wiesbaden, Germany) and rutin was kindly provided by Nutrafur (Murcia, Spain).

Standard compounds were individually dissolved in methanol (MetOH) at 2000 mg/L, with the exception of isorhamnetin-3-*O*-glucoside, dissolved at 1000 mg/L, and hyperoside, dissolved at 500 mg/L. Also, cyanidin-3-*O*-rutinoside, malvidin-3-*O*-glucoside and peonidin-3-*O*-rutinoside were individually dissolved in MetOH (0.01% HCl) at 500 mg/L. All standard stock solutions were newly prepared every 3 months and stored in amber-glass flasks at -20 °C. Mixed standard stock solutions of the standard compounds were prepared in acetone/water/acetic acid (70/29.5/0.5; v/v/v) or methanol to construct the calibration curves for phenolic metabolites or grape phenolic quantification, respectively.

2.2. Plant material:

OG and CG from the red variety Grenache were harvested at maturity on the same day in the same geographical region of Rasquera (Tarragona, Spain). Grape pedicles were manually removed and grapes, including seeds, skins and pomace, were frozen in liquid nitrogen. Frozen grapes were grounded to homogeneity. Homogenates were then freeze-dried for one week at -85 °C using a Telstar LyoQuest lyophiliser (Thermo Fisher Scientific, Madrid, Spain). Liophilised grapes were further grounded to

obtain a fine powder, which was kept at room temperature and protected from light exposure and humidity until further use.

2.3. Characterisation of dietary components of grapes:

Characterisation of the dietary components of OG and CG was performed according to the official AOAC methods [33]. Briefly, water content was determined by weigh difference between samples before and after dehydration (98 °C, 24 h). Ash content was determined weight difference between samples before and after complete incineration of organic matter (500 °C, 24 h). Protein was quantified by the Kjeldahl method (conversion factor 6.25), and lipid content was determined by continuous extraction with n-hexane in a Soxhlet extractor. Total Dietary fibre (TDF) content was determined by treatment of grapes with heat-stable α-amylase, protease from *Bacilus licheniformis* and amyloglucosidase from *Aspargilus* niger (Sigma-Aldrich, Madrid, Spain) and sequential weight of the dry residue. Carbohydrate content was mathematically calculated by subtracting the water, ash, lipid, protein and TDF of OG and CG. All methodologies were applied to freeze-dried OG and CG in triplicates.

2.4. Extraction, detection and quantification of phenolic compounds from grapes:

Phenolic compounds from OG and CG were extracted according to nthe methodology described by Iglesias-Carres *et al*. [34]. The extracts were directly analysed using a 1200 LC Series coupled to a 6410 MS/MS (Agilent Technologies, Palo Alto, CA, USA). Two different HPLC-ESI-MS/MS systems were used to separate, detect and quantify non-anthocyanin and anthocyanin phenolic compounds. In the case of non-anthocyanin compounds, a ZORBAX Eclipse XDB-C18 (150 mm x 2.1 mm i.d., 5 μm particle size), equipped with a Narrow-Bore guard column (2.1 mm x 12.5 mm, 5 μm particle size) (Agilent Technologies, Palo Alto, CA, USA), was used as the chromatographic column. The mobile phase was (A)

water:acetic acid (95:5, v:v) and (B) acetonitrile:acetic acid (95:5, v:v). The gradient mode was the following: initial conditions, 0 % B; 0-0.5 min, 0 % B; 0.5-2 min, 0-10 % B; 2-12 min, 10-30 % B; 12-16 min, 30-60 % B; 16-17 min, 60-100 % B; 17-20 min, 100 % B; and 20-21 min, 100-0 % B. A post-run of 6 min was required for column re-equilibration. The flow rate was set at 0.4 mL/min, and the injection volume was 2.5 µL for all runs. Electrospray ionization (ESI) was conducted at 200 °C and 14 L/min with 20 psi of nebulizer gas pressure and 3000 V of capillary voltage. The mass spectrometer was operated in the negative mode, and MS/MS data were acquired in dynamic mode. Separation of anthocyanins was achieved using an Acquity BHE C18 column (50 mm x 2.1 mm, 1.7 5 µm particle size) (Waters, Milford, MA, USA). Mobile phases consisted of water: formic acid (9:1, v:v) (A) and acetonitrile (B). The gradient mode was the following: initial conditions, 0 % B; 0-1 min, 0 % B; 1-5 min, 0-9 % B; 5-10 min, 9-15 % B; 10-15 min, 15-45 % B; 15-16 min, 45-100 % B; 16-17 min, 100 % B; and 17-18 min, 100-0 % B. A post-run of 6 min was required for column re-equilibration. Flow rate was set at 0.4 mL/min, and the injection volume was 2.5 µL for all runs. ESI was conducted as previously described. The mass spectrometer was operated in the positive mode, and MS/MS data were acquired in dynamic mode. Method quality parameters can be found in Supplementary Table 1.

2.5. Experimental procedure in rats and serum collection:

Male Wistar rats (640 ± 43 g) of 30 weeks of age were housed at 22 °C with a light/dark cycle of 12 h (lights on at 9:00 am), and consumed tap water and a standard chow diet (AO4, Panlab, Barcelona, Spain) *ab libitum* during the experiment. Rats were randomly distributed in two groups (n=6, each): the OG-administered and the CG-administered rat (OGR and CGR, respectively) group. In both groups, rats were orally administered a dose of 65 mg GAE/Kg bw, which corresponds to 2.45 mg dw OG/Kg bw and 2.71 mg dw CG/Kg bw. Oral administration was performed to 8-hours-

starved rats by intragastric intubation between 9 and 10 am. Blood samples were obtained via saphenous vein extraction using non-heparinised vials (Sarstedt, Barcelona, Spain) before (0 h) and 2, 4, 7, 24 and 48 h after grape administration (Figure 1). Blood samples remained at room temperature for 1 hour and were then centrifuged (2000 x g, 15 min, 4 °C) to obtain serum. Serum samples were stored at – 80 °C until use. Samples were pooled (n=6) to obtain sufficient volumes for the analysis. This study was performed in accordance with institutional guidelines for the care and use of laboratory animals. Also, the experimental procedure was performed was approved by the Ethical Committee for Animal Experimentation of the Universitat Rovira i Virgili (reference number 4249).

2.6. Extraction of phenolic metabolites from serum:

Prior to serum phenolic metabolites quantification, samples were pre-treated with the previously developed methodology based on micro solid-phase extraction (μ-SPE). Serum samples were cleaned and concentrated by μ-SPE using 30 μm OASIS HLB μ-Elution Plates (Waters, Barcelona, Spain) as previously described by Margalef *et al.* [30].

2.7. Serum phenolic metabolites quantification:

Grape serum metabolites were analysed with two different chromatographic systems depending on their structure. Chromatographic separation of non-anthocyanin metabolites was achieved with a Kinetex EVO C18 (2.6 μm, 150 x 2.1mm) column. Mobile phases consisted on water/acetic acid (99.8/0.2; v/v) (mobile phase A) and acetonitrile (mobile phase B) with the following gradient: initial conditions, 0 % B; 0 – 0.5 min, 0 % B; 0.5 – 15 min, 0 – 40 % B; 15 – 15.5 min, 40 – 100 % B; 15.5 – 19 min, 100 % B; 19 – 20 min, – 100 –0 % B. A post run of 3 min was required for column re-equilibration. Flow rate was set constant at 0.4 mL/min. Quantification was achieved by coupling the above system with a

6490 MS/MS system (Agilent Technologies, Palo Alto, CA, USA). Electrospray ionization (ESI) was conducted at 200 °C, 14 L/min and with 20 psi of nebulizer gas pressure and 3000 V of capillary voltage. The mass spectrometer was operated in the negative mode and data was acquired using the Dynamic mode. Optimized fragmentation conditions for the analysis of phenolic metabolites are summarized in supplementary Supplementary Table 2. The analysis of anthocyanin metabolites was conducted as described in Nagy *et al*. [35]. ESI was conducted as previously described, and the mass spectrometer was operated in the positive mode. For quantification of phenolic metabolites, serum obtained from the 0 h time-point was spiked with 8 different standard concentrations to construct calibration curves. Any compound present in the 0 h time-point was subtracted from the serum concentration in all the other time-points. Samples were quantified by interpolating the analyte/IS peak abundance ratio in the standard curves. Data acquisition was performed by using MassHunter Software (Agilent Technologies, Palo Alto, CA, USA). Method quality parameters can be found in Supplementary Table 3.

3. Results:

This study aimed to elucidate whether organic cultivation system conditions the bioavailability and metabolism of red Grenache grape polyphenols. Thus, the characterisation of the food matrix, which included the phenolic and non-phenolic dietary constituents of both grape varieties, was required. OG and CG were harvested from contiguous vineyards and in the same day to assure that the only agronomic difference between them was the cultivation system. Before and 2, 4, 7, 24 and 48 h after the administration of 65 mg GAE/Kg bw of OC or CG serum samples were obtained to study the bioavailability and metabolism of OG and CG polyphenols.

3.1. Non-phenolic constituents of organic and conventional red Grenache grapes:

Water, ash, protein, lipid, fibre and carbohydrate content of freeze-dried OG and CG were determined (Table 1). The quantities of food constituents were very similar in both grape cultivars, with the exception of total dietary fibre, which was 1.7 times higher in conventional grapes. Specifically, OG and CG were dominated by carbohydrates (proportions higher than 70 %). Also, a small proportion of approximately 12 % water still remained after the freeze-drying procedure. Lipids, protein and ashes represented a small proportion of the grape dietary components.

3.2. Phenolic profile of organic and conventional red Grenache grapes:

OG and CG showed a different phenolic profile (Table 2). OG had higher quantities of anthocyanidins than CG. In these sense, OG showed a 1.6 times more content of malvidin-based anthocyanidins than CG. Regarding flavan-3-ols, OG had higher concentrations of proanthocyanidin dimers and trimers and of the monomeric glycosylated and gallate flavan-3-ols, although free catechin and epicatechin were found in higher concentrations in CG. Moreover, CG presented higher quantities of the predominant flavonol quercetin-3-*O*-glucoside, and also of kaempferol *O*-galactose and isorhamnetin-3-*O*-glucoside. Also, caffeoyltartaric acid, the second most abundant phenolic acid in both grape varieties, was more abundant in CG. As for the stilbene family, CG reported higher concentrations of this family of compounds than OG.

3.3. Serum kinetic behaviour of organic and conventional red Grenache grapes metabolites:

After an oral administration of whole OG and CG at the same phenolic dose of 65 mg GAE/Kg bw to rats, which corresponded to an administration

dose of 2.45 mg dw OG/Kg bw and 2.71 mg dw CG/Kg bw, the kinetic profile of the total sum of all detected grape phenolic metabolites in rat serum presented a similar behaviour between groups (Figure 2 A and Table 3), and this included two serum peaks, one at 2 h and the other at 24 h, while very few metabolites were detected at 7 and 48 h. However, important differences appeared. In this sense, OGR presented a total metabolite concentration of 4289.75 nM 2 h after grape administration, while CGR presented a 1.8-fold lower total concentration (2390.11 nM). Contrarily, the total metabolite concentration at 24 h in CGR was of 2495.41 nM, which represents a 1.4-fold increase of total metabolite concentration when compared to OGR (1822.21 nM).

3.3.1. Flavonoid metabolites:

Flavonoid glycosides (i.e. rutin) and non-conjugated flavan-3-ols (i.e. catechin or epicatechin) were not detected in the serum of these animals. However, flavonoid phase-II metabolites were detected, but those were only flavan-3-ol phase-II metabolites. The kinetic plasma behaviour of this group of metabolites followed a similar behaviour as the total phenolic metabolite concentration, and also showed two serum peaks, one at 2 h and the other at 24 h with concentrations that ranged between 1500 and 50 nM (Figure 2B and Table 3). Precisely, OGR presented higher concentrations of total flavan-3-ol phase-II metabolites than CGR at all the time points studied. Maximum concentrations of these compounds were found 2 h after grape administration, with the exception of methylcatechin, which was found at 7 h. Methylcatechin glucuronide was the flavonoid phase II metabolite that reached the highest serum concentration in both OGR and CGR.

3.3.2. Phenolic acid metabolites:

The serum kinetic profile of phenolic acids also presented the two serum peaks, one at 2 and one at 24 h, and at 2 h total concentrations were higher

in OGR (2031.82 nM) than in CGR (791.69 nM) (Figure 2C and Table 3). In this sense, methylgallic, homovanillic, hippuric and phenylpropionic acids were the most abundant compounds at 2 h in OGR, but the last two were found at significantly lower concentrations in CGR at the same time point. While the 2 h serum peak was the maximum in OGR, the 24 h peak reached higher concentrations than the 2 h peak in CGR. In this sense, at 24 h CGR reported a higher total phenolic acid concentration (2110.21 nM) than OGR (1191.27 nM). Indeed, higher total concentrations at 24 h in CGR were mainly due to a much higher increase of hippuric acid concentrations in CGR (1395.63 nM in CGR and 570.86 nM in OGR).

3.3.3. Cinnamic acids metabolites:

The cinnamic acids detected in this study were p-coumaric, caffeic and ferulic acids. The total serum kinetic profile of cinnamic acid derivates was very similar when OG or CG was administered to rats, and both showed like the other types of metabolites two serum peaks, one at 2 and the other at 24 h after grape administration as well as a lack of detection of these metabolites at 7 and 48 h (Figure 2D and Table 3). However, the kinetic profile of this metabolic family in serum was mainly due to p-coumaric acid concentrations. In this sense, p-coumaric acid appeared at very similar concentrations in serum after OG or CG administration.

3.3.4. Valerolactone and valeric acid metabolites:

In CGR, 4-hydroxy-(3',4'-dihydroxyphenyl)valeric acid first appeared in serum at 4 h (5.99 nM) and increased at 7 h (17.13 nM) maintaining a similar levels of concentration in serum until 48 h. The behaviour of this compound in serum was different in ORG. In this sense, it first appeared at 2 h (12.79 nM) and reach maximum concentrations by 7 h (19.59 nM). The concentrations between 24 and 48 h, although reduced when compared to the 7 h, were maintained (11.87 and 11.48 nM, respectively). As for 5-(3,4-dihydroxyphenyl)-γ-valerolactone glucuronide, both rat groups reported a

maximum concentration peak 24 h after grape administration (Table 3), which was higher in OGR (76.98 nM) than in CGR (13.65 nM). However, a less concentrated serum peak also appeared at 4 h in OGR (19.92 nM) and CGR (7.75 nM).

4. Discussion:

The consumption of fruits is known to produce health effects [1], and these are partially attributed to their phenolic content [2]. However, recent studies suggest metabolic products after phenolic ingestion to be the real effectors of those biological functions [12,13,36]. Thus, the study of the factors that modulate polyphenol bioavailability and metabolism is essential to understand their bioactivity. In this sense, many factors can modulate polyphenol bioavailability and metabolism, including dose and food matrix [13,32,37]. Currently, the production of grapes grown under organic cultivation systems is increasing [8], and growing conditions are known to modulate the phenolic profile of fruits [38]. Thus, this change in the production of grapes could condition the bioavailability and metabolism of grape polyphenols. Therefore, this study aimed to elucidate whether organic cultivation of grapes can condition grape polyphenol bioavailability and metabolism in rats. To assure that the only independent variable was the cultivation system, OG and CG were harvested at maturity the same day from contiguous vineyards.

The complete characterisation of the food administered is of mandatory requirement to understand the bioavailability and metabolism of phenolic compounds. To provide relevant information of the food matrixes administered to rats, the phenolic profile of OG and CG was determined by HPLC-ESI-MS/MS, as well as their content in relevant dietary components. The profile of non-phenolic constituents was very similar between cultivars, but OG had a 1.6-fold lower dietary fibre content than CG. Similarly, Dani *et al.* [6] reported a reduced fibre content in Niagara grape juices produced organically when compared to their conventional

counterparts. As for the phenolic constituents, the profile in both variety agrees with the major phenolic families in several grape varieties [4,5]. OG presented a higher content in phenolic compounds, and this agrees with the fact that phenolic compounds are synthetized under stressful conditions [3,7,39]. Thus, the limited amount of chemical treatment allowed in organic cultivation systems might have promoted a higher stress to grapes that lead to the modulation of their phenolic profile. Agreeing with this, OG presented a higher anthocyanin content than CG, and this flavonoid family is a hallmark of plant stress [39]. Moreover, organic cultivation systems have previously been demonstrated to modulate anthocyanin [6,11], free flavan-3-ol [40], oligomeric flavan-3-ol [6] and flavonol [4] content of grapes and grape-derived products in the same tendency found in this study. Paradoxically, stilbenes, which are considered phytoalexins produced by plants under stress, injury or disease [3], were found in greater amounts in CG.

The bioavailability of grape phenolics form OG and CG was studied in rat serum at different times after an acute administration. Polyphenol dose administration is a well-known factor affecting the bioavailability and metabolism of phenolic compounds [13,32,41]. To avoid that any difference in the phenolics bioavailability could arise from a different phenolic compound dose administration, rats were administered a total of 65 mg GAE/Kg bw, which in the case of OGR consisted on 2.45 g OG/Kg bw and on 2.71 g CG/Kg bw in CGR. This total polyphenol dose was selected considering the total polyphenol content in a dose of 125 mg/Kg bw of a grape seed flavan-3-ol extract in which flavanol metabolites were correctly detected in plasma [13]. The selection of the time points of blood recollection was chosen taking into account that the early extraction times (i.e. 2 – 4 h) give information of small intestinal absorption of phenolic compounds, while later time points (i.e. 7 – 48 h) give information about colonic metabolism of phenolic compounds [30]. Remarkably, different studies evaluate the bioavailability and metabolism of grape polyphenols

in rats [13,23,28,30,41–43] and humans [14,16,44,45], but only few last longer than 7 h and evaluate the contribution of microbial-derived metabolites into the serum and/or plasma metabolic profile [23,28,44]. Importantly, the study of the microbial metabolism of polyphenols is of relevance as these compounds also hold the key of the bioactive effects associated with polyphenol consumption [22,36].

Generally, no flavonol, anthocyanin or stilbene phase-II metabolites were detected in this study. Indeed, only flavonoid phase-II metabolites detected in this study were flavan-3-ol metabolites, and those were mainly glucuronide metabolites, trend that has been observed for grape, cocoa and hazelnut flavan-3-ols [27,28,46–48]. Although some authors quantify phase-II metabolites of flavonols and stilbens in serum, plasma or other tissues after the consumption of foods or extracts rich in those compounds, the administration doses used were much higher [17,18,20,21,25,43]. For example, in rats administered a grape pomace polyphenol extract, Rodriguez Lanzi *et al.* [43] found quercetin methyl-glucuronide in plasma when quercetin was administered at a dose of 0.84 mg/Kg bw, but not at a dose of 0.42 mg/Kg bw. In the case of anthocyanin phase-II metabolites, the kinetics of these compounds are fast as they can appear as early as 6 minutes after oral administration [18] and be almost removed from the plasma by the 4th hour after administration [17]. Also, their bioavailability seems to be low, as reported by Kuntz *et al.* [49]. In this sense, in humans, the administration of 0.33 L of a smoothie rich in anthocyanins (total anthocyanin concentration of 983 ± 38 mg/L) reported a maximum plasma concentration of glycosylated compounds of 2.79 nM (maximum concentration around 55 – 60 min after smoothie intake) and a maximum plasma concentration of glucuronides of 2.8 nM (maximum concentration around 110 min after smoothie intake) [49]. In addition, anthocyanidins, and specially the acylated anthocyanidins, are very unstable under intestinal conditions [16,50]. In this sense, Kuntz *et al.* [49] attributed the low anthocyanin bioavailability in human plasma due to their degradation

under physiological pH. Another group of metabolites that reported a wide lack of detection in OGR and CGR were phenylacetic acid derivates. Precisely, in our study, the only phenylacetic representative found was homovanillic acid. Similarly, Castello *et al*. [44] did not report phenylacetic acid derivates after administration of a grape pomace extract in humans.

Important differences were found in the phenolics bioavailability and metabolism of OG and CG. In the first serum peak (2 h), which would reflect the small intestinal absorption of grape polyphenols, the total phase-II flavan-3-ol metabolite concentration was higher in OGR than in CGR. However, CG presented a higher content in monomeric flavan-3-ols than OG, which are more bioavailable than their oligomeric counterparts [30]. Therefore, the food matrix seems to influence the bioavailability and metabolism of grape phenolic compounds [32,37]. Indeed, polyphenol bioavailability has been reported to vary depending on food matrix characteristics. In this sense, the plasma kinetic of different phenolic compounds present in wine depended on their administration as wine, wine juice or vegetable juice in humans in the study of Goldberg *et al*. [51]. Specifically, dietary components of the fruit matrix, such as fibre or lipid content, can modulate the bioavailability and metabolism of phenolic compounds [37]. In this sense, CG presented a higher content in dietary fibre, which is known to bound phenolic compounds under gastrointestinal conditions, thus impeding their small intestinal absorption and promoting a higher arrival of these compounds to the colon [15,37]. This would also be in agreement with the higher total phenolic acid concentration at 24 h and the short side-chain phenolic acids, such as benzoic acid, at 48 h in CGR than ORG. Flavan-3-ols phase-II metabolites re-peaked in serum 24 h after grape administration in both treatment groups, and OGR reached higher concentrations than CGR. The higher dimeric and trimeric content of flavan-3-ols in OG could contribute to the higher concentration of flavan-3-ol phase-II metabolites 24 h after grape administration. In this sense, high molecular weight of flavan-3-ols are

known to be hydrolysed by gut microbiota to form monomeric flavan-3-ols, and these structures can be absorbed *in situ* and undergo phase-II metabolism [19,23]. Thus, the phenolic profile of red grapes seems not to be the only factor modulating the bioavailability of flavan-3-ols in this study but rather other fruit-related factors, such as dietary fibre, seem to significantly contribute to the differences between the serum metabolite profile of OGR and CGR. Grapes are known to contain non-extractable proanthocyanidins (NEPAs), which are proanthocyanidins linked to food matrix constituents (i.e. fibre or proteins), which cannot be extracted by either digestive enzymes or acidic methanol-water solvents [52]. Thus, these proanthocyanidins, which may not be extracted and quantified by our grape extraction method, could also contribute to the differences reported in OGR and CGR in the formation of the 24-h flavan-3-ol phase-II metabolites peak since these compounds are not absorbed in the small intestine [52]. The higher content in oligomeric flavan-3-ols in OG had also an impact on the profile of valerolactones, which are compounds mainly formed by the microbial metabolism of dimeric flavan-3-ols [23], and that were found at higher concentrations at 24 h in ORG than in CGR.

At 2 h, the serum of both treatment groups also showed relevant concentrations of phenolic acids which, in the case of OGR, where even higher than the ones reported at 24 h. Phenolic acids such as hippuric or homovanillic acids, are abundant microbial derived metabolites from flavan-3-ols, flavonols and anthocyanins [23,24,26,53]. In our study, the main phenolic acids found at 2 h in both administration groups were hippuric, homovanillic, phenylpropionic and methylgallic acids. Notoriously, in grape-seed flavan-3-ol bioavailability studies in rats, phenolic acids such as phenylpropionic and homovanillic acid also peaked at 2 h after administration, and their concentrations were also higher at 2 h than at 24 h [23]. Moreover, when whole grapes are consumed, under small intestinal conditions, anthocyanins can be degraded to form phenolic acids [54], and, thus, the higher anthocyanin content in OG could also

explain the higher serum concentration of phenolic acids at 2 h in OGR. Phenolic acids found at 24 h are compounds that arise from the microbial metabolism of flavan-3-ols, flavonols and anthocyanins [23,24,26,53]. For example, hippuric acid, which was the most concentrated phenolic metabolite at 24 h in both administration groups, is formed due to the microbial metabolism of flavan-3-ols [23].

The highest serum concentration of cinnamic acid derivates was found at 2 h after grape administration, which was similar for OGR and CGR. In both cases, p-coumaric acid accounted for > 95 % of the cinnamic acids found in serum at the 2 h despite their low concentration in both OG and CG. However, anthocyanins acetylated with p-coumaric acid were abundant in both grape cultivars. These compounds are unstable under gastrointestinal conditions and can be hydrolysed, thus releasing the acylated compounds such as p-coumaric acid that can thereafter be absorbed [16,50]. However, and despite the higher content in acylated anthocyanins with p-coumaric acid in OG, OGR did not reach much higher concentrations of p-coumaric acid than CGR. Thus, these results suggest another metabolic rout for the formation of p-coumaric acid in CGR at 2 h. The only cinnamic acid derivate present at 24 h was p-coumaric acid. Although anthocyanins can also give rise to p-coumaric acid when metabolised by the gut microbiota [55], other metabolic routs could lead to the p-coumaric acid at 24h. For example, caffeic acid derivates such as caffeoyltartaric acid, found at higher concentrations in CG than in OG, could undergo hydrolysis and dehydroxylation to form p-coumaric acid, and this would agree with the higher p-comaric concentration at 24 h of CGR than OGR. Similarly, gallic acid dehydroxylation has been proposed as a possible metabolic rout for the generation of protocatechuic acid in humans [21].

All in all, it seems that the metabolism and serum bioavailability of whole red grape polyphenols is conditioned not only by phenolic profile changes that occur due to organic cultivation system but also by the modulation of

the dietary components (i.e. fibre content) by this cultivation system. Importantly, the bioavailability and metabolism of phenolic compounds are the main limiting factors for their bioactivity [37]. Thus, the differences reported in this study could involve important change in the biological effects associated with the consumption of OG and CG. In agreement with this, Cardozo *et al.* [10] reported chronic intake of organic grape juice for 12 weeks to normalise the nitric oxide levels in the cerebral cortex and hippocampus of rats fed a high fat diet which was not found when rats consumed conventional grape juice. Similarly, Rodrigues *et al.* [11] reported different effects on the activity of antioxidant enzymes in the cerebellum of rats that consumed organic or conventional juices in a chemical rat model of epilepsy. Moreover, Dani *et al.* [56] reported an increase of the liver lipid peroxidation in rats when organic grape juice was consumed for 30 day which was not found when grape juice was produced conventionally. However, functional studies should be performed to elucidate whether the differences in the bioavailability and metabolism triggered by OG cultivation affect the bioactivity associated with red Grenache grape consumption.

5. Conclusions:

The differences in the phenolic and non-phenolic composition of red Grenache grapes submitted to an organic cultivation system resulted into different grape phenolic serum metabolites kinetic profiles than CG. The serum metabolite concentration in OGR were found at higher concentrations at the 2 h time point than in CGR, suggesting higher small intestinal metabolism of OG polyphenols. Contrarily, OGR showed lower concentrations at 24 h, which suggests a lower metabolism of OG phenolic compounds by the gut microbiota. As a result of this different bioavailability and metabolism of OG phenolic compounds, the potential health effects associated with their consumption could differ from conventional grapes.

Supplementary Materials: Table S1: HPLC-ESI-MS/MS method quality parameters for the studies phenolic compounds in red Grenache grapes. Table S2: Optimised conditions for the analysis of grape phenolic metabolites by HPLC-ESI-MS/MS. Table S3: The calibration curve, determination coefficient (R2), linearity, LOQ, LOD, MDL and MQL for standard phenolic compounds spiked in serum samples using HPLC-ESI-MS/MS.

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Figure legends:

Figure 1: Graphical representation of the experimental design used in this study.

Figure 2: Kinetic profile of organic (organic grapes, OG) and non-organic (conventional grapes, CG) red Grenaceh grapes in rat serum: (**a**) total serum metabolites; (**b**) total flavan-3-ol metabolites; (**c**) total phenolic acid metabolites; (**d**) total cinnamic acid metabolites; (**e**) total other metabolites. Concentrations (nM) were quantified using the HPLC-ESI-MS/MS method in rat pooled serum (n=6, each) 2, 4, 7, 24 and 28 h after the ingestion of red Grenache grapes at a dose of 65 mg GAE/Kg bw, corresponding to 2.45 mg dw OG/Kg bw and 2.71 mg dw CG/Kg bw.

Table 1: Non-phenolic constituents in organic (organic grapes, OG) and non-organic (conventional grapes, CG) red Grenache grapes freeze-dried powders

	OG	CG
Water	12.44 ± 0.07	11.51 ± 0.04
Ashes	1.46 ± 0.04	1.88 ± 0.09
Protein	2.29 ± 0.07	3.44 ± 0.03
Lipids	2.02 ± 0.16	1.91 ± 0.05
Total fiber	4.51 ± 0.31	7.71 ± 0.04
Carbohydrates	77.29 ± 0.64	73.55 ± 0.25

Results are expressed as g/100 g dw ± SD (n=3).

Table 2: Phenolic compounds in organic (organic grapes, OG) and non-organic (conventional grapes, CG) red Grenache grapes quantified by HPLC-ESI-MS/MS.

Compound	OG	CG
Flavanols		
Catechin	609.62 ± 155.92	1365.37 ± 43.86
Epicatechin	280.81 ± 75.60	429.92 ± 4.28
Gallocatechin [a]	n.d.	0.06 ± 0.01
Epigallocatechin [a]	0.11 ± 0.01	n.q.
Catechin gallate [a]	85.25 ± 24.64	43.69 ± 3.85
(Epi)catechin *O*-glucoside d1 [b]	12.29 ± 2.74	11.14 ± 1.07
(Epi)catechin *O*-glucoside d2 [b]	6.40 ± 1.74	5.95 ± 1.29
(Epi)catechin *O*-glucoside d3 [b]	6.94 ± 1.49	6.43 ± 1.33
(Epi)catechin *O*-glucoside d4 [b]	46.52 ± 8.24	35.35 ± 3.95
Gallocatechin gallate [a]	0.23 ± 0.04	0.11 ± 0.02
Epigallocatechin gallate	0.86 ± 0.08	0.78 ± 0.07
Procyanidin dimer d1 [c]	339.90 ± 91.28	239.90 ± 12.42
Procyanidin dimer d2 [c]	115.20 ± 28.92	103.03 ± 3.97
Procyanidin dimer B2	319.50 ± 70.84	189.61 ± 12.28
Procyanidin dimer d3 [c]	9.98 ± 2.51	7.84 ± 0.83
Procyanidin dimer d4 [c]	34.40 ± 4.81	25.30 ± 2.09
Procyanidin dimer d5 [c]	11.20 ± 2.42	6.42 ± 0.12
Procyanidin trimer d1 [c]	3.95 ± 0.80	3.27 ± 0.20
Procyanidin trimer d2 [c]	2.98 ± 0.72	1.94 ± 0.05
Anthocyanins		
Pelargonidin *O*- glucoside [d]	1.80 ± 0.15	5.30 ± 0.11
Cyanidin *O*-glucoside [d]	48.44 ± 6.39	31.72 ± 1.57
Delphinidin *O*-glucoside [d]	106.59 ± 10.26	199.42 ± 26.66
Petunidin *O*-glucoside [d]	122.59 ± 16.59	311.93 ± 21.00
Malvidin-3-*O*-glucoside	564.55 ± 24.67	854.56 ± 50.43
Malvidin *O*-acetylglucoside [e]	7.41 ± 1.27	7.93 ± 0.29
Peonidin *O*-coumaroylglucoside d1 [f]	35.11 ± 5.41	22.14 ± 1.83
Peonidin *O*-coumaroylglucoside d2 [f]	2.04 ± 0.26	1.44 ± 0.26
Peonidin 3-*O*-rutinoside	n.d.	n.d.
Delphinidin *O*-coumaroylglucoside d1 [d]	n.q.	15.29 ± 2.70
Delphinidin *O*-coumaroylglucoside d2 [d]	3.13 ± 0.30	6.27 ± 0.60
Delphinidin *O*-coumaroylglucoside d3 [d]	2.02 ± 0.34	1.17 ± 0.03
Petunidin *O*-acetylglucoside d1 [d]	1.08 ± 0.15	1.37 ± 0.08
Petunidin *O*-acetylglucoside d2 [d]	1.91 ± 0.33	n.q.
Petunidin *O*-acetylglucoside d3 [d]	1.71 ± 0.16	3.46 ± 0.23
Petunidin *O*-acetylglucoside d4 [d]	32.67 ± 4.05	57.43 ± 3.35
Malvidin *O*-coumaroylglucoside d1 [e]	13.61 ± 1.47	1.27 ± 0.10
Malvidin *O*-coumaroylglucoside d2 [e]	50.60 ± 6.19	5.50 ± 0.23
Malvidin *O*-coumaroylglucoside d3 [e]	830.52 ± 111.56	82.99 ± 9.16

Table 2 (Continued).

Flavonols		
Quercetin	1.39 ± 0.19	1.46 ± 0.10
Kaempferol *O*-galactose [g]	8.61 ± 2.00	14.08 ± 1.19
Kaempferol-3-*O*-galucose	33.45 ± 7.33	56.81 ± 4.09
Hyperoside	n.d.	n.d.
Quercetin-3-*O*-glucoside [h]	187.43 ± 60.16	470.51 ± 96.61
Isorhamnetin-3-*O*-glucoside	23.40 ± 2.77	75.62 ± 3.26
Kaempferol-3-*O*-rutinoside	1.84 ± 0.40	2.72 ± 0.31
Rutin	20.46 ± 3.47	24.82 ± 1.86
Stilbenes		
Resveratrol	4.46 ± 1.59	7.78 ± 0.39
Resveratrol *O*-glucoside d1 [i]	7.49 ± 1.12	60.96 ± 7.38
Resveratrol *O*-glucoside d2 [i]	17.47 ± 6.72	151.04 ± 17.37
Phenolic acids		
Benzoic Acid	9.55 ± 0.79	7.46 ± 0.41
3-Hydroxybenzoic acid [j]	n.q.	n.q.
Dihydroxybenzoic acid d1 [k]	4.78 ± 0.72	2.06 ± 0.16
Protocatechuic acid	15.76 ± 5.42	11.08 ± 1.50
p-Coumaric acid	0.90 ± 0.14	0.44 ± 0.05
Gallic acid	3.51 ± 1.25	2.54 ± 0.39
Caffeic acid	n.q.	0.63 ± 0.08
Ferulic acid	n.q.	n.q.
Caffeoyltartaric acid [l]	3433.78 ± 275.71	5863.78 ± 361.82
Protocatechuic acid *O*-glucoside [k]	4381.98 ± 360.54	3656.60 ± 40.89
Coumaric acid *O*-glucoside [m]	5.32 ± 1.44	1.23 ± 0.02
Gallic acid *O*-glucoside d1 [n]	0.70 ± 0.17	0.79 ± 0.07
Gallic acid *O*-glucoside d2 [n]	13.54 ± 4.56	35.23 ± 0.34
Caffeic acid *O*-glucoside d1 [l]	n.d.	13.46 ± 1.02
Caffeic acid *O*-glucoside d2 [l]	n.d.	7.74 ± 0.44
Caffeic acid *O*-glucoside d3 [l]	65.57 ± 2.12	67.68 ± 3.92
Flavanones		
Eriodictyol	n.q.	n.q.
Eriodictyol-7-*O*-gucoside	0.44 ± 0.04	0.40 ± 0.00

Results are expressed as mg/Kg dw ± SD (n=3). d1, d2, d3, d4 and d5 indicate different isomeric compounds. Abbreviations: n.d., not detected; n.q., not quantified. [a] Quantified using the calibration curve of epigallocatechin gallate. [b] Quantified using the calibration curve of catechin. [c] Quantified using the calibration curve of procyanidin dimer B2. [d] Quantified using the calibration curve of cyanidin-3-*O*-rutinoside. . [e] Quantified using the calibration curve of malvidin-3-*O*-glucoside. [f] Quantified using the calibration curve of peonidin-3-*O*-rutinoside. [g] Quantified using the calibration curve of kaempferol-3-*O*-glucoside. [h] Quantified using the calibration curve of hyperoside. [i] Quantified using the calibration curve of resveratrol. [j] Quantified using the calibration curve of benzoic acid. [k] Quantified using the calibration curve of protocatechuic acid. [l] Quantified using the calibration curve of caffeic acid. [m] Quantified using the calibration curve of p-coumaric acid. [n] Quantified using the calibration curve of gallic acid.

Table 3: Grape phenolic metabolites quantified in pooled (n=6, each) rat serum at 2, 4, 7, 24 and 48 h after the administration of organic (organic grapes, OG) or non-organic (conventional grapes, CG) red Grenache grapes at a dose of 65 mg GAE/Kg bw, corresponding to 2.45 mg dw OG/Kg bw and 2.71 mg dw CG/Kg bw.

	Metabolite	Serum concentration (nM)									
		OG					CG				
		2 h	4 h	7 h	24 h	48 h	2 h	4 h	7 h	24 h	48 h
FM	Catechin glucuronide [a]	210.92	124.64	n.d.	31.46	n.d.	226.05	108.46	n.d.	31.14	n.d.
	Epicatechin glucuronide [b]	110.07	70.83	n.d.	30.72	n.d.	80.48	44.18	n.d.	15.06	n.d.
	Methylcatechin glucuronide [a]	1431.23	912.64	3.80	358.25	35.55	998.24	566.59	0.00	201.36	25.99
	Methylepicatechin glucuronide [b]	373.68	279.15	2.60	93.26	3.54	199.44	126.00	2.54	47.21	15.00
	Methylcatechin [a]	n.d.	14.11	52.30	11.30	19.83	n.d.	n.d.	47.67	39.56	34.59
PAM	3-(4-hydroxyphenyl)propionic acid	n.q.	23.65	5.79	31.71	4.17	n.d.	15.66	n.d.	48.82	n.d.
	3-(3-hydroxyphenyl)propionic acid [c]	11.55	11.04	n.d.	2.15	n.d.	3.02	6.46	n.d.	7.81	n.d.
	3-(3,4-Dihydroxyphenyl)propionic acid [c]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Phenylpropionic acid [c]	407.96	333.39	n.d.	464.55	n.d.	8.25	136.10	n.d.	352.11	n.d.
	Homovanillic [d]	495.95	152.58	n.d.	28.27	n.d.	430.47	112.50	88.00	133.17	3.48
	Hippuric acid	570.86	599.20	n.d.	610.93	n.d.	n.d.	178.12	n.d.	1395.63	n.d.
	Methylgallic acid [e]	384.04	193.99	n.d.	37.77	2.08	288.94	103.64	n.d.	25.57	1.55
	Dihydrocaffeic acid glucuronide [f]	8.61	n.q.	n.d.	n.d.	n.d.	4.44	3.98	1.86	n.q.	n.q.
	Dihydroferulic acid glucuronide [g]	14.64	n.d.	n.d.	n.d.	n.d.	12.52	n.d.	n.d.	n.d.	n.d.
	Vanillic Acid	18.56	n.d.	n.d.	5.88	n.d.	6.22	n.q.	7.03	9.39	7.17
	Benzoic acid	92.13	12.50	n.d.	10.02	n.d.	37.83	21.45	27.93	132.59	83.70
	3-Hydroxybenzoic	27.54	n.d.	n.d.	n.q.	n.d.	n.q.	n.d.	2.05	5.13	14.43
CAM	p-Coumaric acid	110.26	31.19	n.d.	17.09	n.d.	90.68	29.44	n.d.	25.15	1.50
	Caffeic	n.q.	15.85	n.d	n.d	n.d	n.q.	5.71	n.d	n.d.	n.d.
	Ferulic acid	5.16	n.d.	n.d.	n.q.	n.d.	1.68	n.q.	n.d.	n.d.	n.d.
OM	4-Hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid [h]	12.79	8.79	19.56	11.87	11.48	n.d.	5.99	17.13	12.07	19.36
	5-(3,4-Dihydroxyphenyl)-γ-valerolactone glucuronide [h]	3.81	19.92	n.d.	76.98	n.d.	1.85	7.75	n.d.	13.65	n.d.
Total metabolites		**4289.75**	**2803.48**	**84.05**	**1822.21**	**76.65**	**2390.11**	**1472.02**	**194.21**	**2495.41**	**206.78**

Abbreviations: FM, flavan-3-ol phase-II metabolites; PAM, phenolic acid metabolites, CAD, Cinnamic acid metabolites; OM, other metaboilites. [a] Quantified using the calibration curve of catechin. [b] Quantified using the calibration curve of epicatechin. [c] Quantified using the calibration curve of 3-(4-hydroxyphenyl)propionic acid. [d] Quantified using the calibration curve of vanillic acid. [e] Quantified using the calibration curve of gallic acid. [f] Quantified using the calibration curve of caffeic acid. [g] Quantified using the calibration curve of ferulic acid. [h] Quantified using the calibration curve of 5-(3,4-Dihydroxyphenyl)-γ-valerolactone glucuronide.

Table S1: HPLC-ESI-MS/MS method quality parameters for the studied phenolic compounds in red Grenache grapes.

Compound[a]	RT (min)	Calibration curve	R^2	Linearity (µM)	LOQ (nM)	LOD (nM)
Benzoic acid	9.77	y=304.74x	0.993	0.016-40.943	7.18	23.94
Protocatechuic acid	3.90	y=11.11x	0.996	0.013-32.442	18.20	60.68
p-Coumaric acid	7.68	y=3567.30x	0.999	0.012-30.458	0.87	2.89
Gallic Acid	3.02	y=11453.00x	0.996	0.012-29.391	0.10	0.33
Caffeic acid	5.85	y=88.55x	0.999	0.011-27.754	4.73	15.76
Ferulic acid	8.32	y=1019.40x	0.996	0.010-25.749	0.43	1.43
Resveratrol	11.71	y=529.84x	0.998	0.009-21.906	0.08	0.28
Catechin	5.13	y=369.62x	0.991	0.007-17.225	0.69	2.29
Epicatechin	6.17	y=500.25x	0.995	0.007-17.225	0.66	2.20
Quercetin	13.25	y=1628.00x	0.996	0.007-16.543	0.05	0.17
Kaempferol-3-*O*-glucoside	9.55	y=1073.20x	0.993	0.004-11.151	0.06	0.19
Eriodictyol-7-*O*-glucoside	8.44	y=1968.60x	0.995	0.004-11.101	0.04	0.15
EGCG	6.40	y=1699.3x	0.999	0.004-10.908	0.05	0.16
Hyperoside	8.37	y=1527.80x	0.994	0.004-10.767	0.04	0.14
Isorhamnetin-3-*O*-glucoside	9.71	y=1058.90x	0.990	0.004-10.451	0.04	0.15
Procyanidin dimer B2	5.63	y=192.39x	0.999	0.003-8.634	0.29	0.96
Kaempferol-3-*O*-rutinoside	9.04	y=976.41x	0.998	0.003-8.410	0.03	0.09
Rutin	8.07	y=825.00x	0.998	0.003-8.190	0.06	0.20
Cyanidin-3-*O*-rutinoside	8.21	y=19.55x	0.998	0.003-3.358	0.88	2.92
Malvidin-3-*O*-glucoside	10.99	y=258.29x	0.999	0.004-4.053	1.13	3.77
Peonidin-3-*O*-rutinoside	10.59	y=118.74x	0.999	0.003-3.281	0.78	2.61

[a] Anthocyanins were analysed in the positive ion mode. Abbreviations: RT, retention time; R^2, determination coefficient; LOD, limit of detection; LOQ, limit of quantification.

Table S2: Optimised conditions for the analysis of grape phenolic metabolites by HPLC-ESI-MS/MS.

Compound	MW	$[M-H]^-$ by qTOF	RT(min)	Quantification		Confirmation	
				MS>MS	CE(V)	MS>MS	CE(V)
Benzoic acid	122.00	121.0493	8.27	121>77	8	121>59	4
Phloroglucinol	126.11	125.0280	2.30	125>57	10	125>125	0
3-Hydroxybenzoic	138.12	137.0243	5.33	137>93	40	137>65	36
Phenylpropionic acid	150.17	149.0256	10.75	149>105	10		
Protocatechuic acid	154.12	153.0599	4.29	153>109	16	153>62	40
p-Coumaric acid	164.05	163.0439	8.00	163>119	16	163>93	36
3-(4-hydroxyphenyl)propionic acid	166.17	165.0580	6.36	165>121	10	165>59	0
3-(3-hydroxyphenyl)propionic acid	166.17	165.0580	6.86	165>121	10	165>59	0
Vanillic Acid	168.19	167.0401	6.15	167>108	10	167>123	5
Gallic acid	170.12	169.0193	4.00	169>125	12	167>79	24
Hippuric acid	179.17	178.0565	5.71	178>134	5	178>77	10
Caffeic	180.16	179.0401	6.49	179>135	16	179>107	24
Homovanillic	182.17	181.0547	4.67	181>163	10	181>134	5
3-(3,4-Dihydroxyphenyl)propionic acid	182.17	181.0547	12.92	181>137	10		
Methylgallic acid	184.15	183.0302	5.08	183>168	10	183>124	10
Ferulic acid	194.18	193.0561	8.56	193>134	12	193>178	12
5-(3,4-Dihydroxyphenyl)-γ-valerolactone	208.21	207.0727	6.62	207>85	10	207>121	10
4-Hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid	226.23	225.0699	11.42	225>163	10	225>181	10
Resveratrol	228.23	227.2080	11.36	227>143	28	227>185	20
Kaempferol	285.23	285.0481	14.14	285>239	28	285>117	56
Catechin	289.26	289.0794	6.11	289>203	10	285>245	12
Epicatechin	289.26	289.0794	7.23	289>203	10	285>245	12
Quercetin	302.24	301.0797	12.52	301>151	20	301>179	20
Methylcatechin	304.29	303.0244	6.73	303>137	20	303>285	10
Dihydrocaffeic acid glucuronide	358.30	357.0901	4.95	357>181	20	357>137	20
Dihydroferulic acid glucuronide	372.32	371.1063	6.21	371>195	20	371>136	20
5-(3,4-Dihydroxyphenyl)-γ-valerolactone glucuronide	384.33	383.0158	6.64	383>207	24	383>163	40
Catechin glucuronide	466.39	465.1128	6.19	465>289	20	465>203	40
Epicatechin glucuronide	466.39	465.1128	6.50	465>289	20	465>203	40
Methylcatechin glucuronide	480.42	479.1288	7.61	479<303	20	479>289	20
Methylepicatechin glucuronide	480.42	479.1288	7.79	479<303	20	479>289	20
Procyanidin dimer B2	578.52	577.1476	6.91	557>425	10	577>407	30

Abbreviations: MW, molecular weight; CE, collision energy.

Table S3: The calibration curve, determination coefficient (R^2), linearity, LOQ, LOD, MDL and MQL for standard phenolic compounds spiked in serum samples using HPLC-ESI-MS/MS.

Compound	Calibration curve	R^2	Linearity (µM)	LOD (nM)	LOQ (nM)	MDL (nM)	MQL (nM)
Benzoic acid	y=3.024x	0.9976	0.008-8.197	0.42	1.40	0.17	0.56
Phloroglucinol	y=30.107x	0.9979	0.008-3.965	21.63	72.09	8.65	28.83
3-Hydroxybenzoic acid	y=10.687x	0.9994	0.007-7.240	0.97	3.24	0.39	1.30
Protocatechuic acid	y=5.191x	0.9938	0.013-6.488	3.07	10.24	1.23	4.10
p-Coumaric acid	y=27.827x	0.9962	0.006-6.096	1.08	3.62	0.43	1.45
3-(4-Hydroxyphenyl)propionic acid	y=0.960x	0.9994	0.006-6.018	1.02	3.41	0.41	1.37
Vanillic acid	y=3.4722x	0.9986	0.006-5.946	1.41	4.69	0.56	1.88
Gallic acid	y=0.606x	0.9981	0.006-5.878	0.58	1.93	0.23	0.77
Hippuric acid	y=2.136x	0.9919	0.006-5.581	0.46	1.53	0.18	0.61
Caffeic acid	y=14.335x	0.9902	0.006-5.551	0.87	2.89	0.35	1.16
Ferulic acid	y=7.214x	0.9987	0.010-5.150	0.90	3.01	0.36	1.20
5-(3,4-Dihydroxyphenyl)-γ-valerolactone	y=7.489x	0.9990	0.010-4.803	0.44	1.48	0.18	0.59
Resveratrol	y=0.541x	0.9989	0.044-2.191	3.46	11.53	1.38	4.61
Kaempferol	y=4.111x	0.9936	0.035-7.012	6.19	20.62	2.47	8.25
Catechin	y=3.5780x	0.9976	0.07-3.457	1.30	4.32	0.52	1.73
Epicatechin	y=4.618x	0.9942	0.03-3.457	0.61	2.03	0.24	0.81
Quercetin	y=12.992x	0.9978	0.007-3.309	1.10	3.68	0.44	1.47
Procyanidin dimer B2	y=5.5367x	0.9922	0.002-8.643	0.35	1.15	0.14	0.46

[a] MDL and MQL for the analysis of 250 µL of serum. Abbreviations: LOD, limit of detection; LOQ, limit of quantification; MDL, method detection limit; MQL, method quantification limit.

Figure 1.

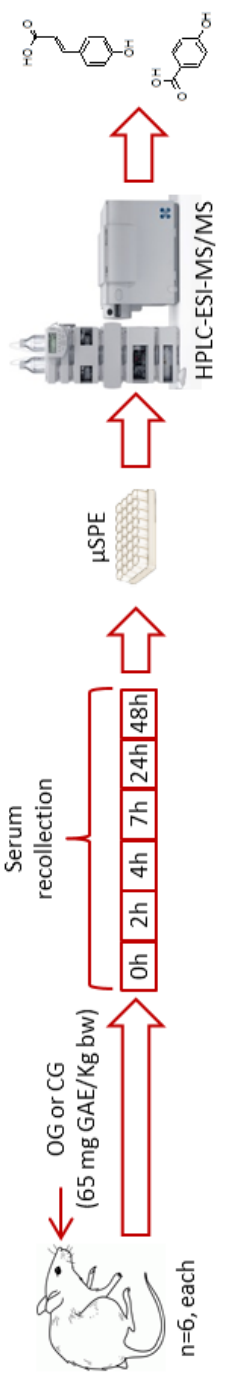
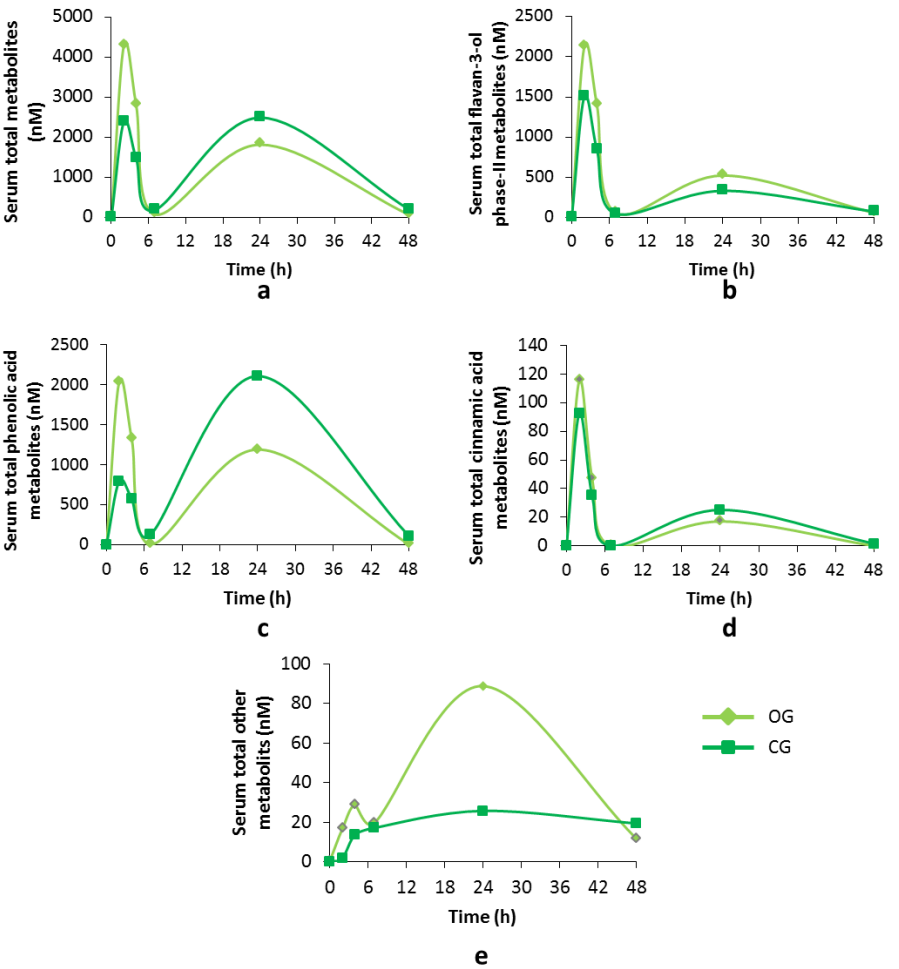

HPLC-ESI-MS/MS
µSPE
Serum recollection
0h
2h
4h
7h
24h
48h
OG or CG (65 mg GAE/Kg bw)
n=6, each

Figure 2.


Serum total metabolites (nM)
Time (h)
a
Serum total flavan-3-ol phase-II metabolites (nM)
Time (h)
b
Serum total phenolic acid metabolites (nM)
Time (h)
c
Serum total cinnamic acid metabolites (nM)
Time (h)
d
Serum total other metabolits (nM)
Time (h)
e
OG
CG

UNIVERSITAT ROVIRA I VIRGILI
MODERN CHANGES IN FRUIT INTAKE PATTERNS MODULATE THE BIOAVAILABILITY AND METABOLISM OF FRUIT PHENOLIC COMPOUNDS IN RATS
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MANUSCRIPT 6:

The bioavailability and metabolism of orange phenolic compounds is conditioned by orange growing region and photoperiod exposure.

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Abstract:

In modern occidental societies orange consumption occurs regardless of photoperiod exposure, and consumers have availability of oranges produced at different geographical regions. These consumption patterns could involve relevant changes in orange phenolic compound bioavailability and metabolism, which seems to be key modulators of the bioactivity associated with phenolic compound consumption. Thus, this study aimed to evaluate whether orange production at different regions and/or consumed under different photoperiod exposures can modulate orange phenolic compound bioavailability and metabolism. To do so, Fischer 344 rats were supplemented with two different sweet orange cultivars, either cultivated in the southern (southern orange, SO) or northern (northern orange, NO) hemispheres, for 10 weeks at a dose of 100 mg dw/Kg bw day under 18, 12 or 6 hours of light per day (L18, L12 and L6 photoperiods, respectively). Serum concentration of relevant metabolites were evaluated by HPLC-ESI-MS/MS. Results indicate that the bioavailability of orange phenolic compound is conditioned by the different phenolic content in the studied orange cultivars and that SO-supplemented rats presented higher quantities of native compounds in their serums than NO-supplemented rats. Additionally, photoperiod was able to influence type and amount of relevant metabolic products, especially in SO-supplemented rats under L6 photoperiod regime. Further studies should be performed to evaluate the relevance of these changes in the bioactivity associated with sweet orange consumption.

Keywords:

Bioavailability, circannual, hesperidin, orange, photoperiod, polyphenols, seasonal variation, xenohormesis.

Compounds studied in this study:

3-(Phenyl)propionic acid (PubChem ID: 107); ferulic acid (PubChem ID: 445858); hesperetin (PubChem ID: 72281); hesperetin 7'-*O*-glucuronide (PubChem ID: 71777476); hesperidin (PubChem ID: 10621); naringenin (PubChem ID: 932); naringenin glucuronide sulphate (PubChem ID: 101371492); and narirutin (PubChem ID: 442431).

1. Introduction:

The consumption of fruits and vegetables has been associated with different health effects [1], and this has been linked with their content in phytochemicals such as phenolic compounds [2]. In this sense, the consumption of hesperidin, a flavanone found at high concentrations in oranges, has reported a wide range of biological effects in *in vitro*, animal and human studies [3–5]. However, several studies suggest that the health beneficial effects of phenolic compound consumption, including hesperidin [6–8], are attributed to their metabolic products [7,9,10]. Precisely, the bioavailability and metabolism of phenolic compounds are key limiting factors for their bioactivity [11]. Thus, the study of the bioavailability and metabolism of phenolic compounds is essential to stablish clear relationships with phenolic compound consumption and a health benefit.

Although absorption and appearance in plasma of fruit-native phenolic compounds occurs [12], these compounds are recognized as xenobiotics and undergo vast detoxification metabolism in the host [13–15]. Flavanone phase-II relevant reactions include sulphuration by cytosolic sulphotransferases (SULTs) and glucuronidation by uridine 5'-diphosphate glucuronosyltransferases (UGTs) [11,16]. However, dietary phenolic compounds that are not absorbed at the small intestine can reach the colon [17,18]. Additionally, food matrix components can also modulate phenolic compounds' absorption, distribution, metabolism and excretion (ADME) [13]. For example, dietary fibre is known to bound phenolic compounds naturally and under gastrointestinal digestion conditions, promoting a lower small intestinal absorption and, consequently, a higher arrival of phenolic compounds to the colon [11,19]. There, gut microbiota is able to catabolise these non-absorbed compounds into smaller molecules, which can be later absorbed *in situ* [20]. Relevant flavanone microbial-derived metabolites include 3-(3'-hydroxy-4'-methoxyphenyl)propionic acid, 3-(3',4'-dihydroxyphenyl)propionic acid, 3-(3'-hydroxyphenyl)propionic acid

and 3-(phenyl)propionic acid [20]. Phase-II and microbial-derived metabolites are known to reach the systemic circulation to be finally eliminated via the urine [13,14,21,22].

Among the factors that can modulate the bioavailability and metabolism of phenolic compounds, dose, food matrix and phenolic compound structure have been studied for sweet orange phenolic compounds [8,13,15,23,24]. Importantly, changes in animal physiology can modulate the bioavailability and metabolism of phenolic compounds as previously evidenced in obese and hypertensive rats [25–27]. Humans, like other mammals, are sensitive to photoperiod changes and their physiology and behaviour change depending on photoperiod exposure [28]. For example, in months with lower light exposure, humans tend to be less active [29] and blood pressure and body fat percentage increase [30]. Moreover, important changes with potential implications for hesperidin and other phenolic compounds bioavailability and metabolism occur during low-light exposure months [31,32]. For example, gut microbiota, responsible for type and quantity of microbial-derived phenolic metabolites [18], is known to change depending on photoperiod exposure in experimental models and humans [33–35]. Moreover, kidney glomerular filtration rate, estradiol plasma levels and metabolic rate also present circannual variations [36–38]. Remarkably, all these circannual changes suggest that the bioavailability of phenolic compounds could significantly differ depending on the season of the year in which they are consumed. However, to date, no studies that evaluate orange phenolic bioavailability depending on photoperiod exist.

The consumption of oranges and other citrus fruits occurs throughout the year, but they are mostly consumed during fall and winter [39]. Also, in the European Union countries (EU-28), oranges are imported from different countries, and this includes countries form the southern hemisphere such as Argentina [40]. This implies relevant changes in orange consumption patterns. Importantly, these are factors that could potentially affect the

bioavailability and metabolism of orange polyphenols, and, thus, the health associated effects of their consumption [27]. Moreover, the xenohormesis theory postulates that chemical cues like phenolic compounds are recognised by mammals, which will adapt their physiology in a pre-emptive fashion to increase survival chances [41]. Thus, the consumption of fruits from different regions and out of their consumption season could involve an erroneous signalling.

Therefore, the aim of this study was to evaluate whether the phenolic compounds' bioavailability and metabolism of two sweet oranges cultivars, one grown in the southern (southern orange, SO) and the other in the northern (northern orange, NO) hemisphere was modulated by photoperiod exposure.

2. Materials and methods:

2.1. Chemicals and reagents:

Acetone, acetonitrile, methanol (all HPLC analytical grade), phosphoric acid and ammonium acetate were purchased from Sigma-Aldrich (Madrid, Spain). Glacial acetic acid was purchased from Panreac (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q Advantage A10 system (Madrid, Spain). Hesperetin, naringenin, narirutin, were purchased from Extrasynthese (Genay, France). Hesperidin-D3, hesperetin 7-*O*-β-D-glucuronide and hesperetin 7-*O*-sulphate were purchased from Carbosynth Ltd (Compton, United Kingdom). Caffeic acid, p-coumaric acid, and ferulic acid were purchased from Fluka/Sigma-Aldrich (Madrid, Spain). Hesperidin was purchased from Quimigranel (Barcelona, Spain).

Standard compounds were individually dissolved at concentrations in between 1 – 2 mg/mL and stored at - 80 °C. A mixed standard stock solution of all the standard compounds, with the exception of Internal Standard (IS), was prepared in methanol (20 µg/mL) and diluted in acetone/Milli-Q

water/acetic acid (70/29.5/0.5; v/v/v) solution to the desired concentration and to construct the calibration curves.

2.2. Fruit material:

Navelina sweet oranges (*Citrus sinensis L.*), either SO or NO, were purchased form Mercabarna (Barcelona, Spain) on the same month (September 2015) to emulate the commercial availability and consumption of oranges produced at distant geographical regions on the same season of the year. SO were originally from Argentina (Mendoza) and NO from Spain (Valencia). Briefly, fresh oranges were peeled and pulp was frozen in liquid nitrogen. Orange pulp was grounded to homogeneity and lyophilized for one week in a Telstar LyoQuest lyophilizer (Thermo Fisher Scientific, Madrid, Spain) at -55 °C. Orange pulps were then further grounded to obtain a fine powder and kept dry and protected from light exposure throughout the supplementation procedure in rats.

Phenolic composition of SO and NO can be found in Table 1. Analysis of non-phenolic relevant food matrix components, which included water, ash, protein, fibre, lipid and carbohydrate content was performed on the freeze-dried SO and NO powders according to the official AOAC methods [42]. Water content was determined by weight difference between samples before and after dehydration (98 °C, 24 h). Ash content was determined weight difference between samples before and after complete incineration of organic matter (500 °C, 24 h). Protein was quantified by the Kjeldahl method, and lipid content was determined by continuous extraction with n-hexane in a Soxhlet extractor. Total dietary fibre content was determined by treatment of oranges with heat-stable α-amylase, protease from amyloglucosidase from *Aspargilus* niger and *Bacilus licheniformis* (Sigma-Aldrich, Madrid, Spain) and sequential weighing of the dry residue. Carbohydrate content was mathematically calculated by subtracting the water, ash, lipid, protein and total dietary fibre of SO and NO.

2.3. Experimental procedure in rats and serum collection:

A total of 54 male Fischer 344 rats of 8 weeks of age were purchased from Charles River Laboratories (Barcelona, Spain). Rats were randomly sub-divided into 3 (n=18, each) light exposure regimes (photoperiod), consisting on 18 h light/day (L18), 12 h light/day (L12) or 6 h light/day (L6). Rats underwent photoperiod adaptation for 4 weeks. Thereafter, a daily supplementation period of 10 weeks with 100 mg SO dw/Kg bw, 100 mg NO dw/Kg bw, or vehicle (VH; glucose 10 mg/Kg bw and fructose 10 mg/Kg bw) followed (n=6, each). Supplementation was performed by voluntary oral administration between 9:00 and 10:00 am (lights on at 9:00 am) to avoid circadian interferences. Throughout the experiment rats consumed a standard chow diet (Panlab A04, Planlab, Barcelona, Spain) and tap water ab libitum. At the end of the experiment, rats were deprived from food after supplementation with SO, NO or VH and put down after 1 hour (between 10:00 am and 11:00 am) (Figure 1). Blood was recollected in non-heparinised tubes and after 1 h at room temperature was centrifuged (2000 x g, 15 min, 4 °C) to obtain serum. Serum was stored at – 80 °C until phenolic metabolites analysis. Rat weights before adaptation to photoperiod, after adaptation of photoperiod and after SO, NO or VH supplementation can be found in Table 2. This study was performed in accordance with institutional guidelines for the care and use of laboratory animals. Also, the experimental procedure performed was approved by the Ethical Committee for Animal Experimentation of the Universitat Rovira i Virgili (reference number 4249).

2.4. Purification and concentration of orange phenolic compound serum metabolites:

Serum samples were pre-treated according to the previously developed methodology based on micro-solid-phase extraction (μ-SPE) by using 30 μm OASIS HLB μ-Elution Plates (Waters, Barcelona, Spain) [43,44]. Briefly, μ-cartridges were loaded with 250 μL of methanol and 250 μL acetic acid 0.2 %. Serum samples (250 μL) were mixed with 300 μL phosphoric acid 4 % and 50 μL of IS (100 ppb) and were then loaded into the μ-cartridges. Then, plates were washed with 200 μL of Milli-Q water and 200 μL acetic acid 0.2 %. Retained phenolic metabolites were eluted with two sequential additions of 50 μL of acetone/water/acetic acid (70/29.5/0.5; v/v/v). A total of 5 μL of the eluted solutions were directly injected into the HPLC-ESI-MS/MS system.

2.5. Chromatographic analysis of orange phenolic compound metabolites:

Chromatographic separation of sweet orange phenolic metabolites was achieved with a Kinetex EVO C18 (2.6 μm, 150x2.1 mm) column (Phenomenex, Torrance, CA, USA) . Mobil phase A consisted on 10 mM of ammonium acetate in water:acetic acid (99.5:0.5; v:v), and mobile phase B was pure acetonitrile. The gradient used to separate sweet orange phenolic metabolites was the following: initial conditions, 0% B; 0 – 0.5 min, 0 % B; 0.5 – 4 min, 0-25 % B; 4 – 9 min, 25 – 50 % B; 9 – 9.3 min, 50 – 100 % B; 9.3 – 10.3 min, 100 % B; 10.3 – 10.7, 100 – 0 % B. A post run of 2 min was required for column re-equilibration. Flow rate was set constant at 0.5 mL/min. Quantification was achieved by coupling the above system with a 6490 MS/MS system (Agilent Technologies, Palo Alto, CA, USA). Electrospray ionization (ESI) was conducted at 240 °C and 12 L/min with 25 psi of nebulizer gas pressure and 3500 V of capillary voltage. The mass spectrometer was operated in the negative mode and data was acquired

using the Dynamic mode. Optimized fragmentation conditions for the analysis of phenolic metabolites can be found Supplementary Table 1.

2.6. Sample quantification:

Serum obtained from the VH groups was pooled and spiked with 10 different standard concentrations to construct calibration curves. Samples were quantified by interpolating the analyte/IS peak abundance ratio in the standard curves. A pool of each VH photoperiod group was also analysed. Any compound present in those pools was subtracted from SO and NO respective counterparts. Data acquisition was performed by using MassHunter Software (Agilent Technologies, Palo Alto, CA, USA). Sensitivity was evaluated by limits of detection (LOD) and quantification (LOQ) for plasma spiked with the standard compounds, which were respectively defined as x 3 and x 10 of the signal-to-noise ratio. Method detection (MDL) and quantification (MQL) limits were calculated for the analysis of 250 µL of serum. Method quality parameters are reported in Supplementary Table 2.

2.7. Statistics:

Two-way ANOVA was used to estimate any statistical difference ($p<0.05$) in the final weight of the animals depending on photoperiod exposure and treatment administration with SPSS 19 software (SPSS Inc., Chicago, IL, USA).

3. Results:

3.1. Food matrix components in SO and NO:

The proportion of non-phenolic dietary components in SO and NO was very similar (Figure 2). Remarkably, carbohydrates represented the highest proportion of all components, which was around 44 % in both orange

cultivars. Then dietary fibre and retained water followed. Protein, lipid and ash content were low in both orange cultivars.

3.2. Serum distribution of phenolic metabolites after chronic supplementation with SO or NO:

The total concentration of sweet orange phenolic metabolites (Table 3), as well as the concentration and proportion (Figure 3) of metabolic families in rat serum after a period of 10 weeks with a supplementation of 100 mg dw/Kg bw day of SO and NO were modulated by orange cultivar supplementation and photoperiod exposure. The total concentration of phenolic metabolites was found at higher concentrations in NO-supplemented rats than in SO-suppemented rats in a given photoperiod. Precisely, the differences between treatment groups were approximately 2-fold higher in NO-supplemented rats in the L18 and L12 photoperiods and of 5.6-fold higher in NO-supplemented rats in the L6. In this sense, total metabolite concentrations for NO ranged in between 25.42 and 30.32 nM, and in rats supplemented with SO in the L18 and the L12 photoperiod groups total serum metabolite concentration were 15.31 and 15.59 nM, respectively. However, total metabolite concentration in SO-supplemented rats in the L6 was of 4.51 nM. Notoriously, in both orange supplementation groups, a L12>L18>L6 trend in total metabolite concentration was conserved.

The principal metabolic family found in this study was phenolic acids, both in proportions and total amounts (Figure 3) in all experimental groups, with the exception of SO-supplemented rats in the L6. In this sense, phenolic acids reported proportions of the total metabolites higher than 85 % in NO-supplemented rats and accounted for 73.84 % and 94.20 % in SO-supplemented rats under the L18 and L12 photoperiods, respectively. In the case of SO-supplemented rats in the L6 photoperiod, phenolic acids only reached a 15.46 % of the total metabolite concentration (Figure 3B). In terms of individual representatives, only 3-(phenyl)propionic acid and

ferulic acid were quantified rat serums (Table 3). The most abundant of these two metabolites was 3-(phenyl)propionic acid, which was found at higher concentrations in NO-supplemented rats (concentrations between 15.60 and 24.41 nM) than in their counterparts in a given photoperiod. Remarkably, 3-(phenyl)propionic acid was not detected in SO-supplemented rats in the L6 photoperiod. Ferulic acid was also found at higher concentrations in NO-supplemented rats, reaching a maximum serum concentration of 9.12 nM in the L18 photoperiod. Remarkably, only SO-supplemented rats in the L6 photoperiod reported this metabolite in serum, and at a low concentration (0.70 nM) (Table 3).

Phase-II metabolites were also quantified in this study, and all of them were glucuronide metabolites (Table 3). Total concentrations of phase-II metabolites were, in general, low. However, this metabolic family was more relevant in NO-supplemented rats than in SO-supplemented rats (Figure 3). In this sense, total concentrations were of 1.49 nM in the L18, 1.87 nM in the L12 and 2.46 nM in the L6 in NO-supplemented rats (Table 3), and their proportion in the total metabolite concentration ranged between 5.03 and 9.69 % (Figure 3B). In SO-supplemented rats, this metabolic family was only present in the L6 photoperiod (Figure 3B) and at a low concentration (2.03 nM), but represented a 45.02 % of the total metabolite concentration in serum (Figure 3B). In terms of individual representatives, hesperidin glucuronide d1 and naringenin glucuronide sulphate were the only phase-II metabolites quantified in plasma. Specifically, hesperidin glucuronide d1 was found in NO-supplemented rats in the L18 photoperiod and in SO- and NO-supplemented rats in the L6 photoperiod. Remarkably, hesperidin glucuronide d1 was the only representative of phase-II metabolites in NO-supplemented rats in the L18 and L6 photoperiods. Naringenin glucuronide sulphate was found in SO-supplemented rats in the L6 photoperiod and in NO-supplemented rats in the L12 photoperiod, and this phase-Ii metabolite was the only representative of this family in NO-supplemented rats in the L12 Specifically

Native compounds, defined as non-metabolised compounds in serum naturally present in fruits, were also detected in this study (Table 3). This metabolic family was found in all SO-supplementation groups, but only found in NO-supplemented rats in the L6 photoperiod (Figure 3A). Particularly, these compounds followed a L18>L6>L12 concentration trend in SO-supplemented rats, reporting concentrations between 0.90 and 4.01 nM. However, the proportions reach in serum were high in the L6 (39.52 %) followed by L18 (26.16 %) (Figure 3B). In terms of individual representatives hesperidin was the only compound present in the serum of rats, being the exception SO-supplemented rats in the L18, that reported both hesperidin (2.77 nM) and narirutin (1.23 nM) (Table 3).

4. Discussion:

Fruit consumption has been associated with a reduced risk to develop chronic diseases, and this has been linked to their content in phenolic compounds [1,2]. Among fruits, oranges stand out due to their content of hesperidin, a potent cardioprotective flavanone [3–5]. Currently, the consumption of oranges, as well as other citrus fruits, takes place throughout the year [39] with the consumption of oranges of several geographic regions. However, the cultivation of fruits under different conditions (i.e. cultivated at distant geographical regions) can potentially modulate the bioavailability of their phenolic compounds [27]. Moreover, since scientific evidence demonstrates changes in relevant physiological parameters for phenolic compound bioavailability and metabolism due to photoperiod exposure [28–38], in this study we hypothesise that orange phenolic compounds bioavailability and metabolism can be influence by the photoperiod at which this fruit is consumed. This is of key relevance as many of the effects reported by phenolic compound consumption are mediated by their metabolic products [6–8]. To evaluate if the bioavailability and metabolism of orange phenolic compound are conditioned by the geographic region at which oranges are cultivated

and/or by the photoperiods at which this fruit is consumed, we administered 100 mg dw/Kg day of sweet orange cultivated in the southern or northern hemispheres for 10 weeks to rats under different photoperiods. A dose 100 mg dw fruit/Kg bw day was selected to reach an achievable orange human consumption. In this sense, although water significantly contributes to orange fresh weight [45], a human of 80 Kg would have to consume around 60 g fresh fruit/day to reach the dose stablished in this study, and this approximates to the consumption of a piece of peeled fresh Navel sweet orange a day. Moreover, to emulate the commercial availability of sweet oranges produced from distant geographical regions on the same season of the year, SO and NO were purchased on the same month (September 2015). Consequently, NO were purchased during their traditional production season, while SO can be considered as out-of-season fruits, as September in the south hemisphere is not within the traditional production season of oranges. Finally, selection of Fischer 334 rats was motivated by their sensitivity to photoperiod exposure [31,32].

Food matrix components are relevant in phenolic compound bioavailability and metabolism [11,27]. For example, in the specific case of orange flavanones, plasma maximum concentrations, time to reach it and elimination half-life, were modified by co-administration of flavanones with yoghurt [13]. Also, dietary fibre can play an important role as it can bound to phenolic compounds under gastrointestinal conditions, thus modifying their bioavailability and metabolism [11,19]. However, the orange cultivars used in this study (SO and NO) were very similar in terms of dietary components such as fibre or carbohydrates. Therefore, differences in the bioavailability of sweet orange phenolic compounds between SO and NO in a given photoperiod must arise from their different phenolic content rather than due to other food matrix components like fibre. Indeed, NO approximately presented a 1.6-fold increase in main phenolic representatives (hesperidin and narirutin) than SO, which can explain why NO-supplemented rats presented an approximated 2-fold increase in total

metabolites concentration, with the exception of L6 rats. In this sense, NO-supplemented rats always presented a higher concentration of the flavanone microbial metabolites 3-(phenyl)propionic acid than SO-rats in a given photoperiod. Moreover, the higher concentrations of caffeic acid derivates in NO than SO could have led to a higher serum concentration of ferulic acid in NO-supplemented rats than their counterparts since ferulic acid has not been described as a microbial-derived metabolite of flavanones, but as a consequence of the host metabolism of caffeic acid derivates present in NO and SO. Indeed, in SO-supplemented rats, ferulic acid was only quantified in the L6, and in low amounts. The 2-fold increase in total metabolites concentration in NO-supplemented rats than in SO are in line with the study of Manach *et al*. in humans, who reported a 2-fold increase in the maximum concentration of hesperetin and naringenin metabolites after the consumption of 1 L of orange juice when compared to humans that consumed 0.5 L of orange juice [22]. Similarly, the consumption of 0.93 mg hesperidin/Kg bw day and 2.92 mg hesperidin/Kg bw in healthy humans produced a plasma maximum concentration of hesperetin of 0.48 ± 0.27 mM and 1.05 ± 0.25 mM, respectively [23]. Interestingly, this 2-fold increase in the total serum concentration found in this study also evidences that the host phase-II enzymes are not saturated in the doses and analysis time of this study [9].

The serum phenolic profile of SO- and NO-supplemented rats was dominated by the metabolic family of phenolic acids, with the exception of SO in the L6 photoperiod. Several phenolic acids can be formed by the action of the gut microbiota on orange phenolic compounds [21]. For example, *in vitro* fermentation studies demonstrated hydroxylated and methylated phenylpropionic acids to be formed at high concentrations in between 1 – 4 hours after exposure of hesperetin and naringenin to gut microbiota [20]. Studies that evaluate the accumulation of phenolic metabolites after long-term supplementation doses have concluded that phenolic acids are not accumulated over time but are rather rapidly removed from animal tissues

[46]. Thus, the lack of detection of relevant flavanone microbial metabolites (i.e. hydroxylated and methylated phenylpropionic acids) of this study could be a direct consequence of the early (1 h) sacrifice of the animals after the last supplementation dose considering the time required for these metabolites to be produced by the gut microbiota, and their lack of accumulation in serum in chronic supplementation studies. Despite that, 3-(phenyl)propionic acid was found in abundant proportions in all rats groups, with the exception of SO-supplemented rats in the L6. Importantly, 3-(phenyl)propionic acid, which is the main end-term metabolic product of flavanone fermentation, is formed at later (8 – 24 h) times than any other relevant flavanone microbial metabolite after the exposure of flavanones to colonic microbiota in *in vitro* fermentation studies [20]. Thus, it seems plausible that the circulating 3-(phenyl)propionic acid in the serum of SO- and NO-supplemented rats could be the residual and last-term microbial metabolite of orange flavanones administered the day before the sacrifice (25 h before the sacrifice). Interestingly, 3-(phenyl)propionic acid was the metabolite that contributed the most in the serum of SO- and NO-supplemented rats, with the exception of SO-supplemented rats in the L6. Thus, the contribution of orange metabolites in the serum profile of SO- and NO-supplemented rats in this study is mainly due to host microbiota contribution. However, it should be noted that the xenobiotic metabolism is dynamic, and the type of metabolites and their concentration highly depend on the time of analysis, as previously reported for orange flavanones [13,24] and grape seed flavanols [26,43]. Thus, the contribution of other microbial metabolites, as well as other type of metabolites, should not be discarded.

When flavanones are absorbed, they are rapidly metabolized by phase-II detoxification enzymes generating glucuronide and sulphate derivatives [13–16]. In our study, SO- and NO-supplemented rats reported a small contribution of flavanone phase-II metabolites. Importantly, different studies using orange-derived products report a maximum concentration of flavanone phase-II derivates 5 – 8 hours after ingestion [13,22,23] and in

our study, serum was obtained after 1 hour of the last supplementation dose. Moreover, the dose used was a physiologically normal administration doses and studies evaluating phenolic compound bioavailability and metabolism usually use higher administration doses [8,22,25], and even doses that can saturate the phase-II detoxification systems [26,43,47]. However, some differences depending on orange cultivar supplementation can be observed.

Interestingly, native compounds (hesperidin and narirutin) were found in the serum of both SO- and NO-supplemented rats. Indeed, compounds such as hesperidin can be found in biological samples such as plasma [12]. In this sense, Kobayashi *et al.* proposed hesperidin to be absorbed by paracellular passive diffusion [48]. Through this rout, hesperidin is not in contact with intestinal phase-II enzymes, which could explain the detection of hesperidin in their native form in our study. Remarkably, the effect of orange cultivar supplementation was evident for native compounds. Precisely, SO-supplemented rats reported hesperidin and/or narirutin in each photoperiod regime, while NO-supplemented rats only presented hesperidin in serum in the L6 photoperiod. These results are surprising as they seem not to agree with the phenolic content in SO and NO because NO presented a higher concentration in hesperidin and narirutin than SO. Thus, our results suggest orange cultivar supplementation to have a differential effect on thigh junctions. In this sense, quercetin has been found to promote the expression of proteins involved in tight junction formation as well as their assembly which are key in regulating the paracellular transport of substances [49]. Precisely, the lower contents of glycosides of quercetin, such as rutin, in SO agrees with the presence of native compounds in SO-supplemented rats. Therefore, and considering that this study was carried out for a period of 10 weeks with SO or NO supplementation, the regulation of tight junction protein expression and assembly is a plausible mechanism to explain the differences in serum concentration of native compounds between rats supplemented with the different orange cultivars.

The effect of photoperiod was evident for the flavanone microbial metabolites 3-(phenyl)propionic acid, and especially in SO-supplemented rats under the L6 photoperiod. Precisely, in the L6, the administration of either of the studied cultivars reported the lowest serum concentration than any other photoperiod, and, in the case of SO-supplemented rats, this metabolite was not detected. Moreover, and regardless of orange cultivar administration, the L12 photoperiod reported the highest 3-(phenyl)propionic acid concentration and the L18 the medium one. Importantly, gut microbiota is responsible for the amount and type of microbial-derived metabolites formed [18], and photoperiod exposure can modulate it in different species [34,35]. Thus, these differences in 3-(phenyl)propionic acid, maintained in both supplementation orange cultivars interventions, would agree with the modulation of microbiota by the photoperiod.

Considering the effect of the photoperiod on phase-II metabolites, phase-II metabolites with a glucuronide acid moiety were more abundant in the L6 groups, especially for SO-supplemented rats, that did not report glucuronides at any other photoperiod regime than the L6. Light signals have an important effect on the expression of different hepatic detoxifying enzymes [50]. For example, in mice, UGT1a1 and SULT1a1 find their hepatic expression at the highest before lights are turned off in 12/12 light/dark cycles [51]. Thus, it seems plausible that different photoperiod exposures modulate the expression of this phase-II enzymes. Also, photoperiod exposure is able to modulate several physiological parameters, such as metabolic rate or estradiol plasma levels, an important regulator of COMT activity [36,38,52]. In this sense, caffeic acid can be methylated by host catechol-*O*-methyl transferase (COMT) to form ferulic acid [53]. Therefore, ferulic acid presence in rat serum would be a direct consequence of the host metabolism of caffeic acid derivates present in NO and SO. The effect of photoperiod on the concentration of ferulic acid in NO-supplemented rats followed a L18>L6>12 tendency. Therefore, the changes reported due to

photoperiod exposure within a given orange cultivar supplementation group could, at least partially, arise from the differential expression or activity of phase-II enzymes regulated by light signals.

Nevertheless, considering that flavanone phase-II metabolites reach their maximum plasma or serum concentration 5 – 8 hours after ingestion [13,22,23], it is plausible that serum recollection at a later time point (i.e. 6 h) would have provided more information on the modulation of photoperiod exposure on the phase-II metabolism of sweet orange phenolic compounds. Thus, a greater contribution of orange cultivar and/or photoperiod exposure on the modulation of the bioavailability and metabolism of sweet orange phenolic compounds should not be ruled out. The significance of the different serum profiles generated due to orange cultivar administration or photoperiod exposure on the bioactivity associated with orange consumption should be evaluated in further functional studies.

Regarding native compounds found in serum, in SO-supplemented rats, photoperiod also played a role on the bioavailability of these phenols. Precisely, serum concentrations of total native compounds followed a L18>L6>L12 tendency, which agrees with the results for ferulic acid and further suggests that the modulation of physiology by photoperiod can modulate the bioavailability of phenolic compounds [36–38].

Importantly, the photoperiod that showed a most marked reduction on sweet orange polyphenol bioavailability was the L6, and this effect was most marked in SO-supplemented rats. These results are interesting as the traditional consumption season of oranges includes the winter, which is emulated by the L6 photoperiod [39]. However, SO were produced outside their traditional production season, and thus, would not naturally be consumed under L6-like conditions. Therefore, this important decrease in the bioavailability of SO polyphenols could be explained by this mismatch between the production season of SO in this study and its consumption in

the L6. Indeed, this agrees with the xenohormesis theory, since the consumption of fruits produced under conditions that consuming animals are not under might involve an erroneous signalling in the host [41]. Moreover, the differences in the bioavailability in SO in the L6 could involve important changes in the bioactivity associated with SO consumption in the winter. In this sense, phenolic compounds must be bioavailable to produce a systemic function [11]. Thus, the systemic biological effects associated with SO consumption during the winter might be scarce.

When the data generated in this study is viewed as a whole, it is clear that both orange cultivar and photoperiod exposure can, either alone or in a synergic effect, modulate the bioavailability and metabolism of sweet orange phenolic compounds. These results are in line with the xenohormesis theory, as the different bioavailability and metabolism of sweet orange phenolic compounds reported in this study could be the chemical cues that pre-emptive physiological adaptations to be more adapted to the environment [41]. Agreeing with this, the consumption of cherries out of their traditional consumption season has recently been shown to change the regulation of key clock genes [54].

5. Conclusions:

This study demonstrates orange growing region and photoperiod exposure to condition sweet orange phenolic compound bioavailability and metabolism. However, functional studies should be performed to assess whether the differences in bioavailability and serum profile of sweet orange phenolics involve relevant implications in health. Also, further studies should be performed to corroborate the serum differences depending on photoperiod exposure in humans.

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Figure legends:

Figure 1: Graphical representation of the experimental design of this study. Abbreviations: southern orange (SO), northern orange (NO), vehicle (VH), light hours a day (L).

Figure 2: Percentage of non-phenolic fruit matrix components of sweet orange pulp cultivated in the southern (A) and northern (B) hemispheres. Abbreviations: SO, southern orange; and NO, northern orange.

Figure 3: Serum concentration (A) and percentage (B) of phenolic acids (PA), phase-II metabolites (PIIM), and native compounds (NC) after the supplementation to Fisher 344 rats with sweet orange pulp grown in the southern (southern oranges, SO) and northern (northern oranges, NO) hemispheres (100 mg dw/Kg bw day) for 10 weeks under different photoperiod exposure (L18, L12, and L6).

Table 1: The phenolic contents from Navelina sweet orange pulps from the northern (northern orange, NO) and southern (sourthern orange, SO) hemispheres by HPLC-ESI-MS/MS expressed as mg/kg fw ± SD (n=3, each).

Phenolic compound	NO	SO
Phloroglucinol	0.30 ± 0.02	n.d.
Protocatechuic acid	0.50 ± 0.01	n.q.
p-Coumaric acid	0.04 ± 0.00	0.01 ± 0.00
Ferulic acid	0.19 ± 0.00	0.11 ± 0.00
Naringenin	0.12 ± 0.00	0.03 ± 0.00
Eriodictyol	n.q.	0.01 ± 0.00
Hesperetin	0.28 ± 0.02	0.24 ± 0.08
Protocatechuic acid O-glucoside [a]	41.03 ± 2.72	33.13 ± 2.66
p-Coumaric acid O-glucoside [b]	0.43 ± 0.01	0.29 ± 0.01
Caffeic acid O-glucoside d1 [c]	1.33 ± 0.05	1.06 ± 0.07
Caffeic acid O-glucoside d2 [c]	3.08 ± 0.08	1.42 ± 0.05
Caffeic acid O-glucoside d3 [c]	11.75 ± 0.41	4.70 ± 0.16
Chlorogenic acid d1 [d]	0.34 ± 0.02	0.06 ± 0.03
Chlorogenic acid d2 [d]	0.12 ± 0.00	n.q.
Chlorogenic acid	1.00 ± 0.04	0.09 ± 0.00
Feruloylquinnic acid d1 [e]	0.02 ± 0.00	0.02 ± 0.00
Feruloylquinnic acid d2 [e]	0.03 ± 0.00	n.d.
Phloridzin [f]	n.d.	0.06 ± 0.00
Kaempferol-3-O-glucoside	n.q.	0.01 ± 0.00
Eriodictyol-7-O-glucoside	n.d.	0.07 ± 0.00
Isorhamnetin-3-O-glucoside	0.00 ± 0.00	0.01 ± 0.00
Myricetin-O-glucoside [g]	0.00 ± 0.00	0.01 ± 0.00
Narirutin [h]	1049.10 ± 80.60	460.20 ± 75.14
Kaempferol-3-O-rutinoside	67.08 ± 5.98	59.51 ± 2.15
Didymin [i]	70.85 ± 6.84	57.79 ± 11.06
Eriocitrin [j]	0.18 ± 0.01	0.09 ± 0.01
Neoeriocitrin [j]	16.53 ± 0.93	5.78 ± 0.84
Rutin	11.86± 0.85	2.15 ± 0.10
Hesperidin	4976.40 ± 508.30	3357.90 ± 763.10

d1, d2 and d3 indicate different isomeric compounds. [a] Quantified using the calibration curve of p-coumaric acid. [b] Quantified using the calibration curve of gallic acid. [c] Quantified using the calibration curve of caffeic acid. [d] Quantified using the calibration curve of chlorogenic acid. [e] Quantified using the calibration curve of ferulic acid. [f] Quantified using the calibration curve of phloroglucinol. [g] Quantified using the calibration curve of hyperoside. [h] Quantified using the calibration curve of naringin. [i] Quantified using the calibration curve of hesperidin. [j] Quantified using the calibration curve of eriodictyol-3-*O*-glucose.

Table 2: Rat weight at the beginning, after photoperiod adaptation and end of the experiment.

		Initial weight (g)	Weight after adaptation (g)	Final weight (g)
L18	**VH**	195.0 ± 10.1	297.3 ± 13.9	386.5 ± 12.7
	SO	199.5 ± 5.1	291.2 ± 8.1	374.3 ± 9.9
	NO	198.0 ± 7.0	295.8 ± 8.3	380.3 ± 11.3
L12	**VH**	201.0 ± 3.6	298.0 ± 5.3	381.0 ± 6.6
	SO	196.8 ± 9.5	290.8 ± 7.9	383.3 ± 7.1
	NO	195.8 ± 3.6	285.7 ± 7.1	365.5 ± 9.1
L6	**VH**	179.8 ± 8.2	286.2 ± 8.3	370.3 ± 11.0
	SO	179.2 ± 6.9	284.3 ± 9.5	378.3 ± 10.9
	NO	180.0 ± 5.7	278.3 ± 7.3	359.8 ± 8.7

Results are expressed as g body weight ± SEM (n=6). No statistical differences were reported by two-way ANOVA (p <0.05) between VH vs SO and VH vs NO in the same photoperiod group. Abbreviations: Vehicle (VH), southern orange (SO), Northern orange (NO).

Table 3: Serum metabolite concentrations (nM) after the supplementation with sweet orange pulp grown in the south (SO) and north (NO) hemispheres (100 mg dw/Kg bw day) for 10 weeks under different photoperiod exposure.

	L18		L12		L6	
	SO	**NO**	**SO**	**NO**	**SO**	**NO**
Hesperidin	2.77	n.q.	0.90	n.q.	1.78	0.85
Narirutin	1.23	n.q.	n.q.	n.q.	n.q.	n.q.
Total native compounds	**4.01**	**0.00**	**0.90**	**0.00**	**1.78**	**0.85**
Hesperetin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Naringenin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total aglycone compounds	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
Hesperetin 7-*O*-β-D-glucuronide	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
Hesperetin glucuronide d1 [a]	n.q.	1.49	n.q.	n.q.	1.05	2.46
Hesperetin 7-*O*-sulphate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Naringenin *O*-glucuronide [a]	n.d.	n.q.	n.q.	n.q.	n.q.	n.q.
Naringenin glucuronide sulphate [b]	n.d.	n.q.	n.d.	1.87	0.98	n.q.
Naringenin sulphate [c]	n.q.	n.q.	n.d.	n.q.	n.d.	n.d.
Total phase-II metabolites	**0.00**	**1.49**	**0.00**	**1.87**	**2.03**	**2.46**
3-(Phenyl)propionic acid [d]	11.30	19.01	14.68	24.41	n.d.	15.60
3-(3'-Hydroxyphenyl)propionic acid [d]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3-(4'-Hydroxyphenyl)propionic acid [d]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3-(3',4'-Dihydroxyphenyl)propionic acid [e]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3-(3'-hydroxy-4'-methoxyphenyl)propionic acid [f]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
p-Coumaric acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Caffeic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ferulic acid	n.q.	9.12	n.q.	4.04	0.70	6.51
Total phenolic acids	**11.30**	**28.13**	**14.68**	**28.45**	**0.70**	**22.11**
Total compounds	**15.31**	**29.61**	**15.59**	**30.32**	**4.51**	**25.42**

[a] Quantified using the calibration curve of hesperetin 7-*O*-glucuronide. [b] Quantified using the calibration curve of hesperetin 7-*O*-sulphate. [c] Quantified using the calibration curve of naringenin. [d] Quantified using the calibration curve of p-coumaric acid. [e] Quantified using the calibration curve of caffeic acid. [f] Quantifiec using the calibration curve of ferulic acid. Abbreviations: Vehicle (VH), southern orange (SO), Northern orange (NO).

Supplementary table 1: Optimised conditions for the quantification of sweet orange phenolic metabolites.

	RT	Quantification		Confirmation	
	(min)	MS>MS	CE (V)	MS>MS	CE (V)
Hesperetin-D3 (IS)	6.47	304.09>286.00	16	304.09>164.10	20
Hesperidin	4.80	609.18>301.10	20	609.18>242.10	48
Narirutin	4.57	579.17>270.90	20	579.17>151.20	40
Hesperetin	6.47	301.07>164.10	20	301.07>136.00	32
Naringenin	6.16	271.06>119.10	24	271.06>151.10	12
Hesperetin 7-*O*-glucuronide	4.78	477.10>301.10	24	477.10>163.80	48
Hesperetin 7-*O*-β-D-glucuronide	4.55	477.10>301.10	24	477.10>163.80	48
Hesperetin glucuronide derivate d1 [a]	4.20	477.10>301.10	24	477.10>163.80	48
Hesperetin 7-*O*-sulphate	5.59	381.03>301.10	16	381.03>163.90	36
Naringenin *O*-glucuronide	4.82	447.09>270.90	20	447.09>119.00	48
Naringenin glucuronide sulfate	4.28	527.20>351.10	24	527.20>351.10	24
Naringenin sulphate	5.32	351.10>271.10	24		
3-(Phenyl)propionic acid	5.12	149.00>105.00	10		
3-(3'-Hydroxyphenyl)propionic acid	3.50	165.00>121.00	10	165.00>59.00	0
3-(4'-Hydroxyphenyl)propionic acid	3.79	165.00>121.00	10	165.00>59.00	0
3-(3',4'-Dihydroxyphenyl)propionic acid	3.22	181.00>137.00	10		
3-(3'-hydroxy-4'-methoxyphenyl)propionic acid	4.14	195.00>136.00	20	195.00>121.00	20
p-Coumaric acid	3.81	163.00>119.00	16	163.00>93.00	36
Caffeic acid	3.21	179.00>135.00	16	179.00>107.00	24
Ferulic acid	4.12	193.00>134.00	12	193.00>178.00	12

[a] Unidentified hesperetin glucuronide derivate. Abbreviations: retention time (RT), collision energy (CE), internal standard (IS).

Supplementary Table 2: Quality parameters for the detection of serum sweet orange phenolic metabolites

Compound	Calibration curve	R^2	Linearity (µM)	LOD (µM)	LOQ (µM)	MDL[a] (nM)	MQL[a] (nM)
Hesperidin	y=7.011x	0.9940	0.002-0.819	0.203	0.675	0.081	0.270
Narirutin	y=2.079x	0.9912	0.004-0.983	0.885	2.950	0.354	1.180
Hesperetin	y=4.914x	0.9913	0.003-1.654	1.341	4.470	0.536	1.788
Naringenin	y=3.936x	0.9979	0.007-0.918	2.029	6.762	0.811	2.705
Hesperetin 7-*O*-β-D-glucuronide	y=4.336x	0.9985	0.004-1.045	0.801	2.668	0.320	1.067
Hesperetin 7-*O*-sulphate	y=43.999x	0.9993	0.005-0.654	0.973	3.245	0.389	1.298
p-Coumaric acid	y=1.046x	0.9961	0.006-3.048	1.793	5.976	0.717	2.390
Caffeic acid	y=7.790x	0.9911	0.006-2.776	1.714	5.714	0.686	2.286
Ferulic acid	y=0.354x	0.9969	0.011-2.575	0.468	1.561	0.187	0.624

[a] MDL and MQL for 250 µL of serum. Abbreviations: Coefficient of determination (R^2), limit of detection (LOD), limit of quantification (LOQ), method detection limit (MDL), and method quantification limit (MQL).

Figure 1.

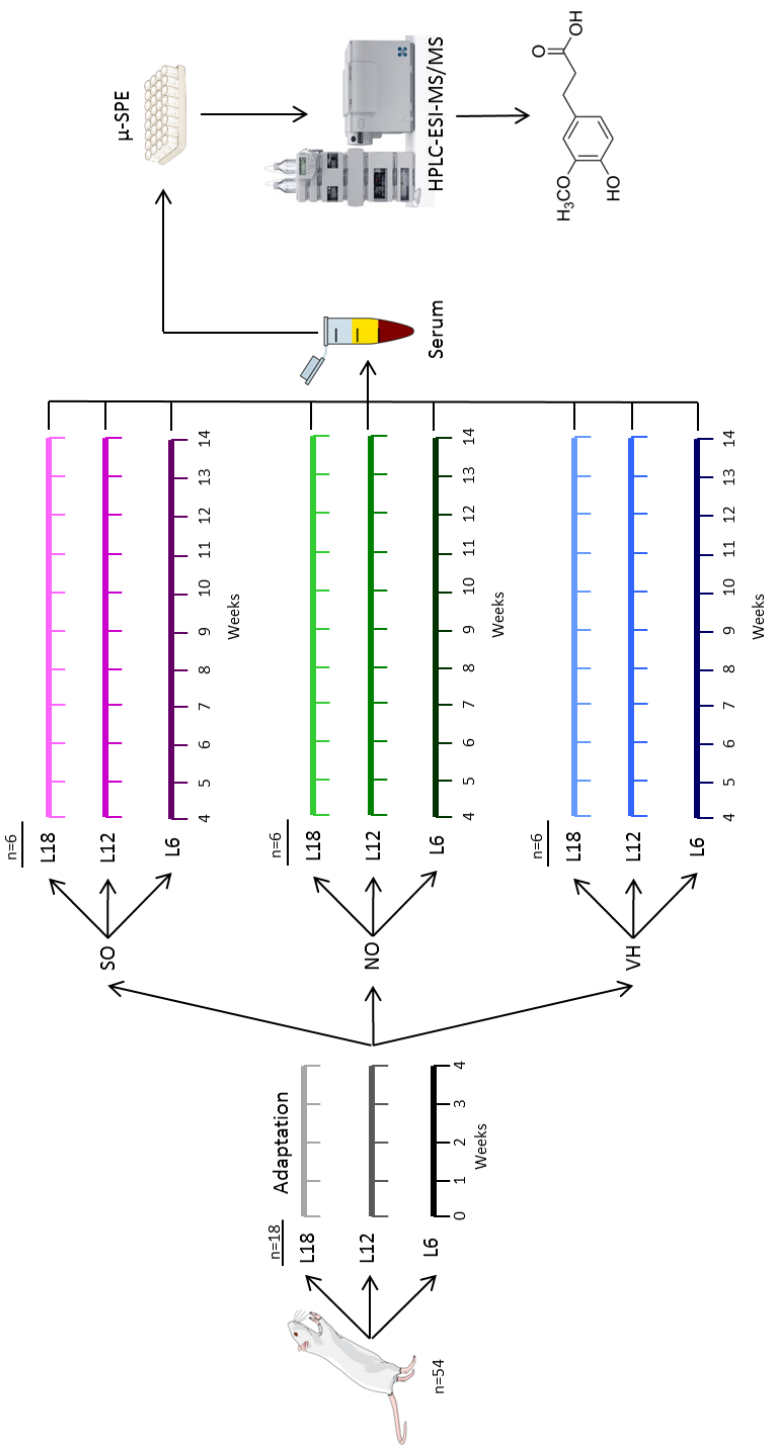


Figure 2.

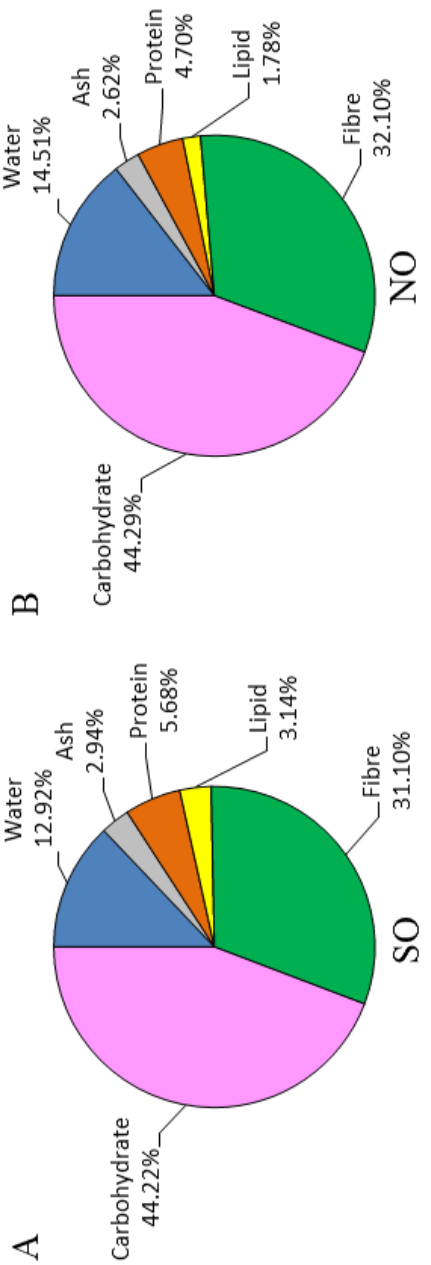
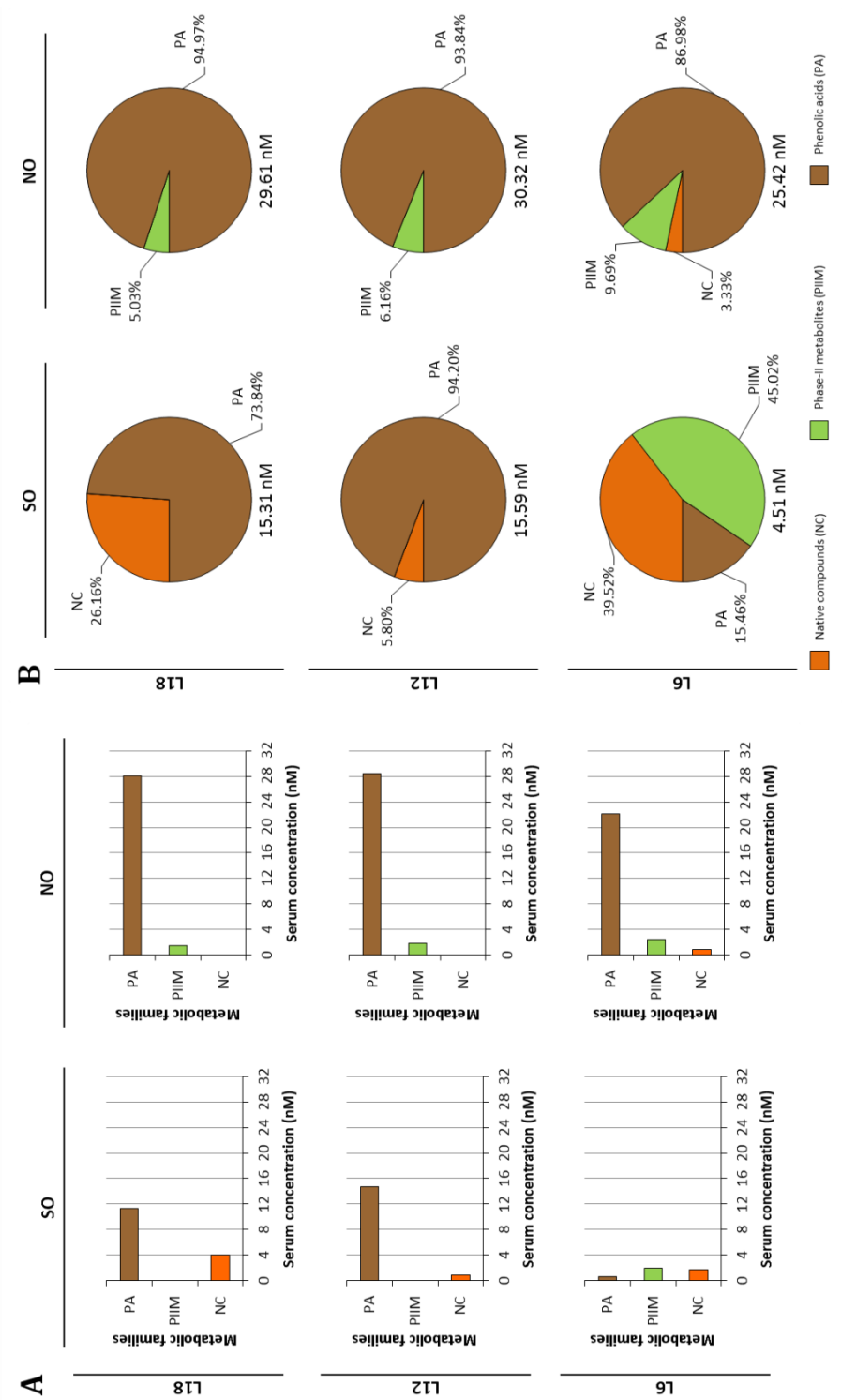

A
Water 12.92%
Ash 2.94%
Protein 5.68%
Lipid 3.14%
Fibre 31.10%
Carbohydrate 44.22%
SO
B
Water 14.51%
Ash 2.62%
Protein 4.70%
Lipid 1.78%
Fibre 32.10%
Carbohydrate 44.29%
NO

Figure 3.


A
B
SO
NO
L18
L12
L6
Metabolic families
PA
PIIM
NC
Serum concentration (nM)
0 4 8 12 16 20 24 28 32
NC 26.16%
PA 73.84%
15.31 nM
PIIM 5.03%
PA 94.97%
29.61 nM
NC 5.80%
PA 94.20%
15.59 nM
PIIM 6.16%
PA 93.84%
30.32 nM
NC 39.52%
PIIM 45.02%
PA 15.46%
4.51 nM
PIIM 9.69%
PA 86.98%
NC 3.33%
25.42 nM
Native compounds (NC)
Phase-II metabolites (PIIM)
Phenolic acids (PA)

UNIVERSITAT ROVIRA I VIRGILI
MODERN CHANGES IN FRUIT INTAKE PATTERNS MODULATE THE BIOAVAILABILITY AND METABOLISM OF FRUIT PHENOLIC COMPOUNDS IN RATS
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MANUSCRIPT 7:

The bioavailability of red grape polyphenols is higher in winter-like conditions.

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Abstract:

Humans are animals sensitive to photoperiod (day length), and, in the winter, physiological adaptations increase the cardiovascular risk (CVR). Winter consumption of whole red grapes could be beneficial to attenuate CVR due to their content in bioactive polyphenols. Notoriously, the phenolic metabolites are considered to be the effectors of these biological functions. Scientific evidence suggests that the bioavailability and metabolism of phenolic compounds could change depending on seasonal time due to seasonal physiological changes. However, to date, no studies that evaluate this exist. Therefore, the aim of this study was to evaluate whether the bioavailability and metabolism of red grape phenolics was modulated by photoperiod exposure and grape cultivar administration. To do so, the serum phenolic metabolites were profiled in a 10-week supplementation study with organic (organic grape, OG) or non-organic (conventional grape, CG) red Grenache grapes. Our results indicated grape cultivar administration and photoperiod exposure to condition grape phenolics' bioavailability and metabolism. Among grape cultivars, CG presented the highest variability depending on photoperiod exposure. Among photoperiod groups, those rats with the lowest light exposure reported a higher bioavailability of grape phenolics. Importantly, microbial-derived metabolites were the most abundant in all groups. The results of this study suggest that the consumption of whole grapes could be beneficial in the management of CVR, especially during winter months.

Keywords:

Cardiovascular risk; Bioavailability; Polyphenols; Seasonal variation.

1. Introduction:

It is known that the behaviour and physiology of humans can change depending on photoperiod (day hours) exposure [1]. For instances, physical activity, energy expenditure and body fat change in the winter at longitudes far from the equator, and those changes make human more susceptible to cardiovascular risk (CVR) [2–4]. This higher CVR is reflected in the higher incidence of cardiovascular deaths in low-light exposure months such as November [4]. Epidemiological studies have shown that a diet rich in fruits and vegetables is able to reduce the development of cardiovascular disease [5]. Importantly, many of the effects of fruit consumption are mediated by plant phytochemicals such as polyphenols [6,7]. Among the foods with the highest polyphenol content, red grapes stand out [8]. Dietary guidelines often recommend the consumption of whole fruits [9], and in the specific case of grapes, this includes the consumption of grape seeds and skins, which are known to be rich in phenolic compounds [10] with relevant biological activities [7,11], including reduction of cardiovascular risk [12]. This dietary recommendation is of key relevance as the consumption of a whole food or isolated compounds might not have the same biological effect [6]. Additionally, food matrix components, such as fat or fibre, are known to modulate the bioavailability and metabolism of phenolic compounds [13–15].

Current knowledge suggests phenolic metabolites to be the responsible molecules for the health benefits associated with polyphenol consumption [7,16–18]. Indeed, phenolic compounds from grapes are known to be rapidly and extensively metabolised [19,20]. Once absorbed, phenolic compounds are recognised as xenobiotics and undergo phase-II detoxification in the small intestine and liver. Typical phase-II reactions include glucuronidation by uridine 5'-diphosphate glucuronosyltransferases (UGTs), methylation by catechol-O-

methyltransferase (COMT), and sulfuration by cytosolic sulfotransferases (SULTs) [14]. However, between 90 – 95 % of dietary phenolic are not absorbed in the small intestine and reach the colon where they are subjected to extensive microbial metabolism [16,21,22]. Both phase-II and microbial-derived metabolites are known to appear in the serum and plasma to reach different organs and tissues [17,23–25]. Finally, these compounds are eliminated via the urine [14].

The metabolic fate of phenolic compounds from grapes and grape phenolic extracts has been widely studied [19–21,23,24,26] as well as their biological functions under different health conditions [11,27–30]. Also, there is scientific evidence that demonstrates different health effects after consumption of grapes and grape-derived products produced organically or conventionally [29,30]. Moreover, several factors can modulate the bioavailability and metabolism of phenolic compounds, which, in turn, might also affect their bioactivity [19,23,24,26]. In this sense, one of the important factors for grape phenolic bioavailability and metabolism is microbiota. In this sense, the quantity and type of microbial-derived metabolites depends largely on the microbial composition of the gut [16]. Factors like diet and dietary components such as fibre and phenolic compounds can modulate host's microbiota composition [16,27,31,32]. Scientific evidence also demonstrates photoperiod to modulate the microbiota composition of different animal models and humans [33–36]. Additionally, important physiological changes occur under exposure to different photoperiods [37–41], and some of them, such as plasma estradiol levels or kidney glomerular filtration rate, might also have an important effect of polyphenol bioavailability and metabolism. Additionally, grape phenolics administration can modulate clock genes, which are also regulated by light and diet and maintain host's homeostasis [42–48]. Importantly, these genes are linked with the control of relevant physiological factors for CVR such as glucose homeostasis [49] and blood pressure [50].

Given the importance of photoperiod exposure in health [2,3], winter consumption of whole grapes could play an important protective role. The scientific evidence previously described suggests that grape phenolics could present different bioavailability depending on photoperiod exposure, and that grape cultivar could further modulate their bioavailability. However, to date, there are no studies that evaluate grape phenolics bioavailability depending on photoperiod exposure. Hence, this study aimed to evaluate whether the bioavailability of grape phenolics and the metabolite serum profile varies depending on photoperiod exposure after a chronic supplementation with grapes. This was evaluated is male Fischer 344 rats using two different red Grenache grape cultivars; one produced organically (organic grapes, OG) and the other non-organically (conventional grapes, CG).

2. Materials and methods:

2.1. Chemical and reagents:

Acetone, acetonitrile, methanol (all HPLC analytical grade) and phosphoric acid were purchased from Sigma-Aldrich (Madrid, Spain). Glacial acetic acid was purchased from Panreac (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q Advantage A10 system (Madrid, Spain). Benzoic acid, caffeic acid, catechin, epicatechin, ferulic acid, gallic acid, hippuric acid, 2-(4-hydroxyphenyl)acetic acid, 2-(3-hydroxyphenyl)propionic acid, kaempferol p-coumaric acid, 3-hydroxybenzoic acid, pyrocatechol (Internal Standard, IS), quercetin and vanillic acid were purchased from Fluka/Sigma-Aldrich (Madrid, Spain). Procyanidin dimer B2 and protocatechuic acid were purchased from Extrasynthese (Genay, France). Resveratrol was purchased from Quimivita (Barcelona, Spain) and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone was purchased from MicroCombiChem e.K. (Wiesbaden, Germany). Standard compounds were individually prepared in methanol (2000 mg/L) every three months and stored in amber-dark glass flasks at -20 °C. A mixed standard stock solution of all

the standard compounds, with the exception of IS, was prepared weekly in methanol (20 μg/mL) and stored under the same conditions. This mixture was diluted daily in acetone/Milli-Q water/acetic acid (70/29.5/0.5; v/v/v) solution to the desired concentration and stored under the same conditions.

2.2. Plant material:

Red Grenache grapes, namely OG and CG, were harvested at maturity in the region of Rasquera (Tarragona, Spain) by contiguous vineyards on the same day. Then, whole grapes (seeds, skins and pomace) were frozen in liquid nitrogen and grounded to homogeneity. Homogenates were then freeze-dried for one week using at -85 °C using a Telstar LyoQuest lyophilizer (Thermo Fisher Scientific, Madrid, Spain) and further grounded to obtain a fine powder. This powder was kept at room temperature and protected from light exposure and humidity until administered to the rats. Administration doses of the phenolic constituents present in OG and CG are summarized in Table 1, which is adapted from Iglesias-Carres *et al*. [51]. Ash, carbohydrate, fibre, lipid, protein and water contents in OG and CG can be found elsewhere [52].

2.3. Experimental design in rats and serum collection:

A total number of 54 male Fisher-334 rats were purchased from (Charles River, Barcelona, Spain). Rats consumed a standard chow diet (Panlab A04, Planlab, Barcelona, Spain) *ab libitum* and had access to tap water was *ab libitum*. Rats were randomly divided into 3 different light/dark cycles (n=18, each): L12 (12 h of light/day), L18 (18 h of light/day) and L6 (6 h of light/day), respectively emulating spring and fall, summer and winter light exposure. Light/dark cycles groups were further divided into 3 different groups (n=6, each): supplementation with OG at a dose of 100 mg/Kg day; supplementation with CG at a dose of 100 mg/Kg day; or supplementation with vehicle (VH), consisting on glucose (10 mg/Kg day) and fructose (10

mg/Kg day) in water. All rats groups underwent light/dark cycle adaptation for 4 weeks before treatment administration. In all cases, lights were turned on at 9.00 am and treatments were orally administered in between 9.00 and 10.00 am for a period of 10 weeks. Animals were kept at 22 °C in animal quarters. On the day of the sacrifice, rats were deprived from food once the treatments (OG, CG or VH) were administered, and were put down after 1 hour. Blood was recollected in non-heparinised flasks and waited an hour at room temperature. Then, blood was centrifuged (2000 x g, 15 min, 4 °C) to obtain serum samples, which were aliquoted and frozen at – 80 °C. Rat weights can be found in Table 2.

This study was performed in accordance with institutional guidelines for the care and use of laboratory animals, and the experimental procedure was approved by the Ethical Committee for Animal Experimentation of the Universitat Rovira i Virgili (reference number 4249).

2.4. Extraction of phenolic metabolites:

Before being subjected to chromatographic separation, serum samples were pre-treated with the developed micro solid-phase extraction (μ-SPE) methodology [24]. Serum samples were cleaned and concentrated by μ-SPE using 30 μm OASIS HLB μ-Elution Plates (Waters, Barcelona, Spain). Briefly, micro-cartridges were sequentially loaded with 250 μL of methanol and 250 μL acetic acid 0.2 %. Then, plates were loaded with a mixture containing 300 μL phosphoric acid 4 %, 250 μL serum samples and 50 μL of IS (200 ppb). Plates were then sequentially washed with 200 μL of Milli-Q water and 200 μL acetic acid 0.2 %. Retained phenolic metabolites were eluded by the addition of 50 μL of acetone/water/acetic acid (70/29.5/0.5; v/v/v) twice. Later, 2.5 μL of the eluted solutions were directly injected into the HPLC-ESI-MS/MS system.

2.5. Chromatographic analysis of phenolic metabolites:

Chromatographic separation was achieved with a Kinetex EVO C18 (2.6 µm, 150x2.1mm) column. Mobile phases consisted on water/acetic acid (99.8/0.2; v/v) (mobile phase A) and acetonitrile (mobile phase B) with the following gradient: initial conditions, 0 % B; 0-0.5 min, 0 % B; 0.5-15 min, 0-40 % B; 15-15.5 min, 40-100 % B; 15.5-19 min, 100 % B; 19-20 min, 100-0 % B. A post run of 3 min was required for column re-equilibration. Flow rate was set constant at 0.4 mL/min. Quantification was achieved by coupling the above system with a 6490 MS/MS system (Agilent Technologies, Palo Alto, CA, USA). Electrospray ionization (ESI) was conducted at 200 °C and 14 L/min with 20 psi of nebulizer gas pressure and 3000 V of capillary voltage. The mass spectrometer was operated in the negative mode and data was acquired using the Dynamic mode. Optimized fragmentation conditions for the analysis of phenolic metabolites can be found in supplementary Table 1.

2.6. Sample quantification:

Serum obtained from the VH groups was spiked with 10 different standard concentrations to construct calibration curves. Samples were quantified by interpolating the analyte/IS peak abundance ratio in the standard curves. Any compound present in the VH groups was substracted from OG and CG groups. Data acquisition was performed by using MassHunter Software (Agilent Technologies, Palo Alto, CA, USA). Method quality parameters are reported in Table 3.

2.7. Statistics:

Two-way ANOVA was used to estimate any statistical difference ($p<0.05$) in the final weight of the animals depending on photoperiod exposure and administration of OG, CG or VH with SPSS 19 software (SPSS Inc., Chicago, IL, USA).

3. Results:

Chronic supplementation with OG or CG was found to give rise to different phenolic metabolite serum profiles, which depended on the photoperiod (L12, L18 or L6) and grape cultivar (OG or CG) supplementation (Table 4). Bioavailability of CG phenolics was found greater in the L6 group (2667.40 nM) than in the L12 (337.57 nM) or L18 (563.07 nM) groups. This increase was mainly produced by a higher serum concentration of hippuric acid (Table 4). As a matter of fact, most of the metabolites were present in higher concentrations in the L6 group. Relevant increases of homovanillic acid, phenylpropionic acid and catechin glucuronide were found in the L6 group. Despite that, metabolites such as methylepicatechin glucuronide and caffeic acid showed a marked higher concentration in the L12 group. These changes in concentration affected the proportion of metabolite families found in rat serum, which variated depending on photoperiod exposure (Figure 2). For example, the high concentrations of hippuric acid in the L6 group were translated into a high proportion of the other metabolites family in the L6 rats. In the L18 group, phenylpropionic acids dominated, while cinnamic acid derivates dominated in the L12 group due to caffeic acid's contribution (185.04 nM). Remarkably, the most abundant metabolic families in each photoperiod were almost negligible in the other photoperiods, with the exception of phenylpropionic acid derivates in the L6 group.

Rats supplemented with OG showed, in each photoperiod, that the total serum metabolite concentration was higher than the one found in CG-supplemented rats. Importantly, a similar pattern of grape phenolics bioavailability depending on photoperiod exposure was found (L6>L18>L12) (Table 4). The increase of serum metabolite concentration in L6-OG was mainly mediated hippuric and phenylpropionic acids, which were also abundant in the L6 photoperiod of CG-supplemented rats. Few metabolites were reduced in the L6 group when compared with the other

two OG-supplemented photoperiod groups, and compounds such as benzoic and 3-(4-hydroxyphenyl)propionic acids were increased in L6 group. Interestingly, homovanillic acid was found at lower concentrations the L6 rats supplemented with OG than their L12 and L18 counterparts. The percentage of phenolic families of OG-supplemented rats differed mainly between the L6 group and the L12 and L18 groups due to the differences in plasma concentrations of individual compounds. Neverthgeless, these differences were not as marked as in the CG-suppplemented group. In all three photoperiods, the metabolite class of other metabolites was the most abundant (62.7 – 31.4 %). However, while phenylpropionic acid and phenylacetic acid derivates presented similar proportions in the L12 and L18 groups, which were in between 19.2 % and 28.3 %, phenylpropionic acid derivates represented a superior proportion (22.9 %) than phenylacetic acid derivates (4.5 %).

4. Discussion:

The CVR events is greater in the winter due to physiological changes associated with photoperiod [1–3], and the consumption of whole red grapes in the winter could help attenuate this risk given the cardiovascular protective effect of grape phenolics [12]. Importantly, these effects are mediated by the metabolic products of phenolic compounds rather than the natural forms [7,16–18]. Although physiological changes relevant in phenolic compound bioavailability and metabolism occur under exposure to different photoperiods [33,35–38,53,54], no studies that evaluate this exist in the literature. Therefore, the aim of this study was to evaluate whether grape polyphenol bioavailability and metabolism is modulated under photoperiods that simulate winter (L6), summer (L18) and spring and fall (L12) in healthy rats. Fischer 344 rats were selected due to their physiological sensibility to photoperiod [37,38]. Selection of CG and OG grape cultivars was motivated due to their similar phenolic profile and different serum bioavailability of phenolic compounds [51], as well as the

increasing trend in organic grape production [55]. Selection of serum pooling was performed due to increase in homogeneity and sensibility, which allows the detection of all potential metabolites [21,24].

It is important to note that the last supplementary dose of CG and OG was administered 1 hour before the sacrifice. Previous studies with grape administration demonstrate two independent serum metabolite peaks, being the first at 2 h and the second at 24 h after grape administration [51]. Therefore, the phenolic metabolite profile found in this study reflects the metabolism of the two last grape supplementary doses administered to rats. Consequently, the results herein presented do not demonstrate serum metabolite accumulation due to long-term grape administration. Indeed, lack of accumulation of grape phenolic metabolites has been previously demonstrated in rats treated chronically with grape seed flavan-3-ols [23].

No non-metabolised compounds were detected in the serum rats supplemented with CG, or in the OG group for that matter (Table 4). This is in agreement with the fact that phenolic compounds undergo rapid metabolism in the host [19,20] and has previously been reported in acute CG and OG administration studies [51]. Although the proportions of flavonoid phase-II metabolites changed between photoperiods in both OG- and CG-supplemented rats (Figure 1), the total amounts of this family were relatively constant in rats supplemented with CG (58.10 – 132.86 nM) and OG (126.93 – 239.96 nM). Notoriously, OG-supplemented rats reached higher serum concentrations of flavan-3-ols phase-II metabolites with regards to their CG-supplemented counterparts. This effect has also been reported in acute OG or CG administration studies and linked to the higher fibre content in CG [51].

It has been estimated that 90 – 95 % of dietary phenolic compounds reach the colon where they are subjected to extensive microbial metabolism [16,21,22]. Later absorption and appearance in relevant organs and tissues of these metabolites has been previously after the consumption of extracts

rich in flavan-3-ols [23,24] and anthocyanins [25]. In this study, the serum profile of rat groups was dominated by microbial derived-metabolites (Table 4), which is in agreement with the better absorption of these compounds than the native phenolic compounds [16]. Additionally, previous studies involving chronic administration of flavan-3-ols also showed microbial-derived metabolites to be the most abundant metabolites in different organs such as the liver, brain and aorta of male Wistar rats [23]. However, important differences were found between grape cultivar administration (CG or OG) and photoperiod exposure (L12, L18 or L6) in the type and proportions of serum microbial-derived metabolites.

Rats supplemented with CG showed a clear different serum metabolite profile in each photoperiod (Figure 1), mediated by changes in the serum concentration of relevant microbial-metabolites belonging to different phenolic metabolite families (Table 1). Importantly, the quantity and type of microbial-derived metabolites depends on the gut microbial composition [16]. Evidence in Sprague-Dawley rats [33], Siberian hamsters [36], broiler roosters [35] and humans [34] shows different microbiota composition of these organisms depending on photoperiod exposure. This microbiota changes associated to photoperiod exposure could be responsible for the important serum metabolite profile changes in CG-supplemented rats. However, although OG-supplemented rats showed a shift in the serum metabolite profile depending on photoperiod (Figure 1), those changes were not as marked as in CG-supplemented rats. Importantly, other factors, such as fibre consumption, are known to significantly modulate the host microbiota [31]. Additionally, dietary fibre can naturally and/or under gastrointestinal conditions bind to phenolic compounds, modulating their bioavailability [13]. Moreover, phenolic compounds can also have a modulatory effect of gut's microbiota diversity and intestinal barrier function [16,27], respectively relevant in polyphenols' metabolism and bioavailability. Therefore, changes in

phenolic composition (Table 1) as well as other food matrix parameters such as dietary fibre, found at 7.71 g/100 g dw in CG and 4.51 g/100 g dw in OG [52], could lead to the different production of microbial-metabolites in CG- and OG-supplemented rats under the same photoperiod. Remarkably, these differences could have important health implications as many of the biological effects of phenolic compounds are mediated by microbial metabolites [7,16,18]. Thus, the consumption of one cultivar over another could involve different bioactivities. In this sense, chronic intake of organic grape juice for 12 weeks, but not conventional juice intake, normalised the nitric oxide levels in the cerebral cortex and hippocampus of rats fed a high fat diet [29]. Also, the consumption of organic or conventional grape juices reported different effects on the activity of antioxidant enzymes in the cerebellum of a rat epilepsy model [30]. Moreover, the consumption of OG or CG in different seasons of the year cold also involve different health effects. Precisely, our group has demonstrated that the consumption of CG in the L6 and L18 to produce different effects on the signalling pathway of leptin in STD Fischer 344 rats [56]. Additionally, the consumption of grapes out of their traditional consumption season also modulates differently several genes involved in the circadian control [57].

Importantly, the total amount of metabolites found in the L6 group, regardless of grape cultivar administration, was higher than in L12 or L18 (Table 4). These results are of special relevance as in low-light exposure months the CVR is higher [4,58]. Therefore, the type and higher bioavailability of phenolic metabolites in the L6 groups could be beneficial to reduce the incidence of cardiovascular mortality. Thus, these results could set the basis of red grape consumption recommendation in the winter, provided results were reproduced in humans. However, functional studies should be performed to confirm the reduction of CVR associated with grape consumption in the winter.

5. Conclusions:

To our knowledge, this is the first study to evaluate the effect of photoperiod exposure on the bioavailability and metabolism of grape phenolics, and phenolics in general for that matter. In this sense, this study demonstrates grape phenolics bioavailability and metabolism to depend on photoperiod exposure. Specifically, relevant changes were found in microbial-derived metabolites, which could involve alterations on the bioactivity of grape phenolics. Further studies should be performed to corroborate microbiota changes triggered by photoperiod exposure suggested by different serum metabolites profiles. Also, functional studies are required to reveal whether these differences in bioavailability and serum metabolite profile involve different health effects relevant for CVR management.

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Conflicts of interest:

No conflicts of interest exist in this study.

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Figure legends:

Figure 1: Graphical representation of the experimental design used in this study.

Figure 2: Distribution of phenolic metabolite families in the serum of rats administered organic (organic grapes, OG) or non-organic (conventional grapes, CG) red Grenache grapes at a dose of 100 mg/KG bw. Abbreviations: L12, 12 h light/day; L18, 18 h light/day; and L6, 6 h light/day.

Table 1: Administration doses of phenolic compounds (μg/Kg bw day) present in organic (organic grapes, OG) and non-organic (conventional grapes, CG) red Grenache grapes.

Compound	OG	CG
Benzoic Acid	0.96 ± 0.08	0.75 ± 0.04
Dihydroxybenzoic acid d1	0.48 ± 0.07	0.21 ± 0.02
Protocatechuic acid	1.58 ± 0.54	1.11 ± 0.15
p-Coumaric acid	0.09 ± 0.01	0.04 ± 0.01
Gallic acid	0.35 ± 0.13	0.25 ± 0.04
Caffeic acid	0.00 ± 0.00	0.06 ± 0.01
Resveratrol	0.45 ± 0.16	0.78 ± 0.04
Apigenin	0.00 ± 0.00	0.01 ± 0.00
Catechin	60.96 ± 15.59	136.54 ± 4.39
Epicatechin	28.08 ± 7.56	42.99 ± 0.43
Quercetin	0.14 ± 0.02	0.15 ± 0.01
Gallocatechin	0.00 ± 0.00	0.01 ± 0.00
Epigallocatechin	0.01 ± 0.00	0.00 ± 0.00
Caffeoyltartaric acid	343.38 ± 27.57	586.38 ± 36.18
ProtocatechuiC acid O-glucoside	438.20 ± 36.05	365.66 ± 4.09
Coumaric acid O-glucoside	0.53 ± 0.14	0.12 ± 0.00
Gallic acid O-glucoside d1	0.07 ± 0.02	0.08 ± 0.01
Gallic acid O-glucoside d2	1.35 ± 0.46	3.52 ± 0.03
Caffeic acid O-glucoside d1	0.00 ± 0.00	1.35 ± 0.10
Caffeic acid O-glucoside d2	0.00 ± 0.00	0.77 ± 0.04
Caffeic acid O-glucoside d3	6.56 ± 0.21	6.77 ± 0.39
Resveratrol O-glucoside d1	0.75 ± 0.11	6.10 ± 0.74
Resveratrol O-glucoside d2	1.75 ± 0.67	15.10 ± 1.74
Catechin 3-O-gallate	8.53 ± 2.46	4.37 ± 0.38
Kaempferol O-galactose	0.86 ± 0.20	1.41 ± 0.12
Kaempferol-3-O-galacucose	3.35 ± 0.73	5.68 ± 0.41
Eriodictyol-7-O-gucoside	0.04 ± 0.00	0.04 ± 0.00
(Epi)catechin O-glucoside d1	1.23 ± 0.27	1.11 ± 0.11
(Epi)catechin O-glucoside d2	0.64 ± 0.17	0.60 ± 0.13
(Epi)catechin O-glucoside d3	0.69 ± 0.15	0.64 ± 0.13
(Epi)catechin O-glucoside d4	4.65 ± 0.82	3.54 ± 0.39
Gallocatechin gallate	0.02 ± 0.00	0.01 ± 0.00
Epigallocatechin gallate	0.09 ± 0.01	0.08 ± 0.01
Quercetin-3-O-glucoside	18.74 ± 6.02	47.05 ± 9.66
Isorhamnetin-3-O-glucoside	2.34 ± 0.28	7.56 ± 0.33

Table 1 (Continued).

Procyanidin dimer d1	33.99 ± 9.13	23.99 ± 1.24
Procyanidin dimer d2	11.52 ± 2.89	10.30 ± 0.40
Procyanidin dimer B2	31.95 ± 7.08	18.96 ± 1.23
Procyanidin dimer d3	1.00 ± 0.25	0.78 ± 0.08
Procyanidin dimer d4	3.44 ± 0.48	2.53 ± 0.21
Procyanidin dimer d5	1.12 ± 0.24	0.64 ± 0.01
Kaempferol-3-o-rutinoside	0.18 ± 0.04	0.27 ± 0.03
Rutin	2.05 ± 0.35	2.48 ± 0.19
Procyanidin trimer d1	0.40 ± 0.08	0.33 ± 0.02
Procyanidin trimer d2	0.30 ± 0.07	0.19 ± 0.01
Pelargonidin O-glucoside	0.18 ± 0.18	0.53 ± 0.01
Cyanidin O-glucoside	4.84 ± 0.64	3.17 ± 0.16
Delphinidin O-glucoside	10.66 ± 1.03	19.94 ± 2.67
Petunidin O-glucoside	12.26 ± 1.66	31.19 ± 2.10
Malvidin-3-O-glucoside	56.46 ± 2.47	85.46 ± 5.04
Malvidin O-acetylglucoside	0.74 ± 0.13	0.79 ± 0.03
Peonidin O-coumaroylglucoside d1	3.51 ± 0.54	2.21 ± 0.18
Peonidin O-coumaroylglucoside d2	0.20 ± 0.03	0.14 ± 0.03
Peonidin-3-O-rutinoside	0.00 ± 0.00	0.00 ± 0.00
Delphinidin O-coumaroylglucoside d1	0.00 ± 0.00	1.53 ± 0.27
Delphinidin O-coumaroylglucoside d2	0.31 ± 0.03	0.63 ± 0.06
Delphinidin O-coumaroylglucoside d3	0.20 ± 0.03	0.12 ± 0.00
Petunidin O-acetylglucoside d1	0.11 ± 0.02	0.14 ± 0.01
Petunidin O-acetylglucoside d2	0.19 ± 0.03	0.00 ± 0.00
Petunidin O-acetylglucoside d3	0.17 ± 0.02	0.35 ± 0.02
Petunidin O-acetylglucoside d4	3.27 ± 0.41	5.74 ± 0.34
Malvidin O-coumaroylglucoside d1	1.36 ± 0.15	0.13 ± 0.01
Malvidin O-coumaroylglucoside d2	5.06 ± 0.62	0.55 ± 0.02
Malvidin O-coumaroylglucoside d3	83.05 ± 11.16	8.30 ± 0.92

Results are expressed as μg/Kg bw day ± SD (n=3). d1, d2, d3, d4 and d5 indicate different isomeric compounds. Adapted from Iglesias-Carres *et al*. (53).

Table 2: Weights of Fischer 344 rats at the beginning (initial weight), after photoperiod adaptation and end (final weight) of dietary intervention.

Light/Dark cycle	Treatment	Initial weigh (g)	Weight after adaptation (g)	Final weight (g)
L18	VH	195 ± 10.1	297.3 ± 13.9	386.5 ± 12.7
	CG	193.3 ± 6.2	289.5 ± 8.6	380.8 ± 6.6
	OG	189.2 ± 4.2	302.2 ± 8.1	395 ± 13.3
L12	VH	201 ± 3.6	298 ± 5.3	381 ± 6.6
	CG	202.5 ± 3.8	294.5 ± 8.8	382.7 ± 8.5
	OG	198.7 ± 3.7	300.5 ± 9.1	382.7 ± 11.8
L6	VH	179.8 ± 8.2	286.2 ± 8.3	370.3 ± 11
	CG	182.8 ± 5.1	280.7 ± 7.7	361.3 ± 7.2
	OG	178.2 ± 3.9	277.2 ± 8.4	363 ± 8.8

Results are expressed as grams of body weight ± SEM (n=6). No statistical differences ($P<0.05$) were detected in the final weight of animals in this study by Two-way ANOVA. Abbreviations: L12, 12 hours of light/day; L18, 18 hours of light/day; L6, 6 hours of light/day; VH, vehicle; OG, organic red Grenache grape; and CG, conventional red Grenache grape.

Table 3: Calibration curve, determination coefficient (R^2), working linearity range, LODs, LOQs, MLDs and MQLs for phenolic compound quantification in spiked serum samples using HPLC-ESI-MS/MS.

Compound	Retention time (min)	Calibration curve	R^2	Linearity (µM)	LOD (nM)	LOQ (nM)	MDL[a] (nM)	MQL[a] (nM)
Benzoic acid	8.13	y=0.1271x	0.991	0.008-16.377	2.602	8.674	1.041	3.470
3-Hydroxybenzoic acid	5.33	y=0.5206x	0.999	0.007-14.480	0.770	2.566	0.308	1.027
2-(4-Hydroxyphenyl)acetic acid	5.81	y=0.0826x	0.993	0.007-13.145	0.190	0.632	0.076	0.253
Protocatechuic acid	3.96	y=0.3361x	0.998	0.006-12.977	0.618	2.060	0.247	0.824
p-Coumaric acid	7.82	y=1.6366x	0.996	0.006-12.191	1.805	6.017	0.722	2.407
3-(4-Hydroxyphenyl)propionic acid	6.76	y=0.0579x	0.996	0.006-12.035	1.389	4.629	0.555	1.852
Vanillic acid	6.03	y=0.1982x	0.998	0.006-11.895	0.834	2.779	0.334	1.112
Hippuric acid	5.91	y=0.1084x	0.990	0.006-11.163	0.235	0.785	0.094	0.314
Caffeic acid	6.39	y=0.9491x	0.992	0.006-11.101	0.447	1.492	0.179	0.597
Ferulic acid	8.41	y=0.5645x	0.999	0.005-10.300	0.519	1.731	0.208	0.692
5-(3,4-Dihydroxyphenyl)-γ-valerolactone	6.52	y=0.4319x	0.992	0.005-9.606	0.335	1.117	0.134	0.447
Resveratrol	11.22	y=0.1928x	0.990	0.004-2.191	1.195	3.983	0.478	1.593
Kaempferol	14.02	y=0.1731x	0.995	0.035-3.494	6.165	20.551	2.466	8.220
Catechin	5.98	y=0.2281x	0.997	0.003-6.890	0.795	2.650	0.318	1.060
Epicatechin	6.96	y=0.1597x	0.998	0.003-6.890	0.886	2.953	0.354	1.181
Quercetin	12.36	y=0.1597x	0.998	0.003-6.617	0.735	2.451	0.294	0.980
Procyanidin dimer B2	6.78	y=0.3912x	0.994	0.002-1.790	0.474	1.580	0.190	0.632

Abbreviations: R^2, determination coefficient; LOD, limit of detection; LOQ, limit of quantification; MDL, method detection limit, and MLD, method quantification limit.[a] MDL and MQL for the analysis of 250 µL of serum.

Table 4: Individual phenolic metabolites in rat serum after chronic supplementation with organic (organic grapes, OG) and non-organic (conventional grapes, CG) Grenache grapes in Fischer 344 rats kept at different photoperiods.

Compound	L18		L12		L6	
	OG	CG	OG	CG	OG	CG
Resveratrol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Kaempferol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Catechin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Epicatechin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Quercetin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Procyanidin dimer B2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total non-metabolised compounds	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
Methylcatechin [a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Catechin glucuronide [a]	38.34	13.26	26.57	12.70	38.27	23.29
Epicatechin glucuronide [b]	43.41	10.67	25.15	11.82	53.50	26.07
Methylcatechin glucuronide [a]	142.73	38.19	75.20	33.59	148.20	83.51
Methylepicatechin glucuronide [b]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total flavonoid phase-II metabolites	**224.48**	**62.12**	**126.93**	**58.10**	**239.96**	**132.86**
p-Coumaric acid	n.d.	n.d.	8.22	n.d.	n.d.	12.05
Caffeic acid	n.d.	n.d.	n.d.	185.04	n.q.	4.45
Ferulic acid	n.d.	n.d.	n.d.	n.d.	n.d.	10.95
Total cinnamic acid derivates	**0.00**	**0.00**	**8.22**	**185.04**	**0.00**	**27.45**
Phenylpropionic acid [c]	417.14	373.13	199.03	n.d.	724.00	591.89
3-(4-Hydroxphenyl)propionic acid	26.91	18.79	n.d.	n.d.	49.81	12.19
3-(3-Hydroxphenyl)propionic acid [c]	n.d.	n.d.	n.d.	n.d.	2.23	1.70
3-(3,4-Dihydroxyphenyl)propionic acid [c]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Dihydrocaffeic acid glucuronide [d]	9.30	n.d.	14.36	4.24	3.94	n.d.
Dihydroferulic acid glucuronide [e]	7.64	n.d.	10.41	2.60	10.24	n.d.
Total phenylpropionic acid derivates	**461.00**	**391.92**	**223.81**	**6.84**	**790.22**	**605.78**
2-(3-Hydroxypheyl)acetic acid [f]	80.18	10.97	22.43	n.d.	64.57	170.06
2-(4-Hydroxypheyl)acetic acid	2.75	3.70	n.d.	n.d.	2.66	1.49
Homovanillic acid [g]	291.30	8.54	201.88	30.79	87.64	387.84
Total phenylacetic acids	**374.23**	**23.20**	**224.31**	**30.79**	**154.87**	**559.39**
Benzoic acid	45.0	67.2	7.2	27.2	85.2	63.4
3-Hydroxybenzoic acid	n.d.	n.d.	n.d.	n.d.	9.8	52.0
4-Hydroxybenzoic acid [h]	n.d.	13.6	n.d.	n.d.	7.8	29.8
Protocatechuic acid	n.q.	n.q.	n.d.	n.d.	n.q.	n.q.
Dihydroxybenzoic acid [i]	n.q.	n.d.	9.2	n.d.	n.d.	n.d.
Vanillic acid	n.d.	n.q.	n.d.	n.q.	n.d.	3.4
Gallic acid	13.3	n.d.	n.d.	n.d.	n.d.	5.8
Methylgallic acid [j]	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.
Total benzoic acid derivates	**58.3**	**80.8**	**16.4**	**27.2**	**102.8**	**154.4**
Hippuric acid	510.07	n.d.	567.50	n.d.	2159.86	1186.29
5-(3,4-Dihydroxyphenyl)-γ-valerolactone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4-Hydroxy-5-(3′,4′-dihydroxyphenyl) valeric acid [k]	0.99	2.63	n.q.	n.q.	3.77	1.22
5-(3,4-Dihydroxyphenyl)-γ-valerolactone glucuronide [k]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total other metabolites	**511.06**	**2.63**	**567.50**	**0.00**	**2163.63**	**1187.51**
Total metabolites	**1629.11**	**560.71**	**1167.15**	**307.93**	**3451.47**	**2667.40**

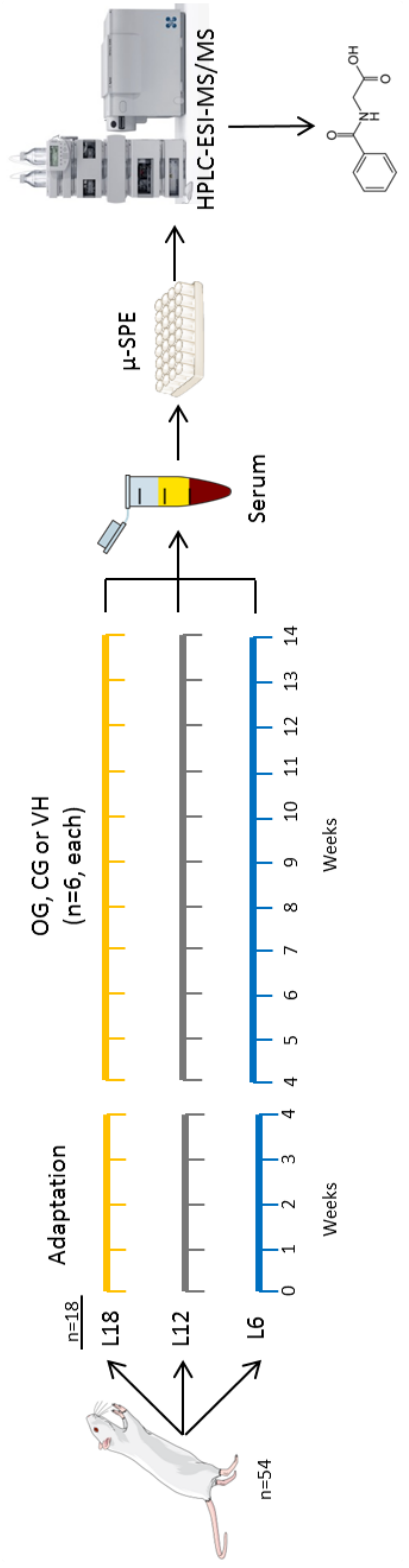
Abbreviations: L12, 12 h light/day; L18, 18 h light/day; and L6, 6 h light/day.[a] Quantified using the calibration curve of catechin. [b] Quantified using the calibration curve of epicatechin. [c] Quantified using the calibration curve of 3-(4-hydroxyphenyl)propionic acid. [d] Quantified using the calibration curve of caffeic acid. [e] Quantified using the calibration curve of ferulic acid. [f] Quantified using the calibration curve of 2-(4-hydroxyphenyl)acetic acid. [g] Quantified using the calibration curve of vanillic acid. [h] Quantified using the calibration curve of 3-hydroxybenzoic acid. [i] Quantified using the calibration curve of protocatechuic acid. [j] Quantified using the calibration curve of gallic acid. [k] Quantified using the calibration curve of 5-(3,4-Dihydroxyphenyl)-γ-valerolactone.

Supplementary Table 1: Optimised conditions for the analysis of grape phenolic metabolites by HPLC-ESI-MS/MS.

Compound	MW	[M-H]$^{-a}$	RT(min)	Quantification		Confirmation	
				MS>MS	CE(V)	MS>MS	CE(V)
Benzoic acid	122.00	121.0493	8.271	121>77	8	121>59	4
3-Hydroxybenzoic	138.12	137.0243	5.326	137>93	40	137>65	36
Phenylpropionic acid	150.17	149.0256	10.752	149>105	10		
2-(3-Hydroxyphenyl)acetic acid	152.15	151.0382	5.318	151>107	5	151>93	20
2-(4-Hydroxyphenyl)acetic acid	152.15	151.0382	5.809	151>107	5	151>65	30
Protocatechuic acid	154.12	153.0599	4.286	153>109	16	153>62	40
p-Coumaric acid	164.05	163.0439	7.996	163>119	16	163>93	36
3-(4-hydroxyphenyl)propionic acid	166.17	165.0580	6.361	165>121	10	165>59	0
3-(3-hydroxyphenyl)propionic acid	166.17	165.0580	6.862	165>121	10	165>59	0
Vanillic Acid	168.19	167.0193	6.152	167>108	10	167>123	5
Gallic acid	170.12	169.0193	3.998	169>125	12	167>79	24
Hippuric acid	179.17	178.0565	5.706	178>134	5	178>77	10
Caffeic	180.16	179.0401	6.491	179>135	16	179>107	24
Homovanillic	182.17	181.0547	4.667	181>163	10	181>134	5
3-(3,4-Dihydroxyphenyl)propionic acid	182.17	181.0547	12.915	181>137	10		
Methylgallic acid	184.15	183.0302	5.075	183>168	10	183>124	10
Ferulic acid	194.18	193.5610	8.558	193>134	12	193>178	12
5-(3,4-Dihydroxyphenyl)-γ-valerolactone	208.21	207.0727	6.623	207>85	10	207>121	10
4-Hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid	226.23	225.0699	11.420	225>163	10	225>181	10
Resveratrol	228.23	227.2080	11.358	227>143	28	227>185	20
Kaempferol	285.23	285.0481	14.140	285>239	28	285>117	56
Catechin	289.26	289.0794	6.109	289>203	10	285>245	12
Epicatechin	289.26	289.0794	7.231	289>203	10	285>245	12
Quercetin	302.24	301.0797	12.523	301>151	20	301>179	20
Methylcatechin	304.29	303.0244	6.725	303>137	20	303>285	10
Dihydrocaffeic acid glucuronide	358.30	357.0901	4.954	357>181	20	357>137	20
Dihydroferulic acid glucuronide	372.32	371.1063	6.214	371>195	20	371>136	20
5-(3,4-Dihydroxyphenyl)-γ-valerolactone glucuronide	384.33	383.0158	6.641	383>207	24	383>163	40
Catechin glucuronide	466.39	465.1128	6.194	465>289	20	465>203	40
Epicatechin glucuronide	466.39	465.1128	6.497	465>289	20	465>203	40
Methylcatechin glucuronide	480.42	479.1288	7.613	479<303	20	479>289	20
Methylepicatechin glucuronide	480.42	479.1288	7.785	479<303	20	479>289	20
Procyanidin dimer B2	578.52	577.1476	6.911	557>425	10	577>407	30

Abbreviations: MW, molecular weight; CE, collision energy. [a] Studied by qTOF.

Figure 1.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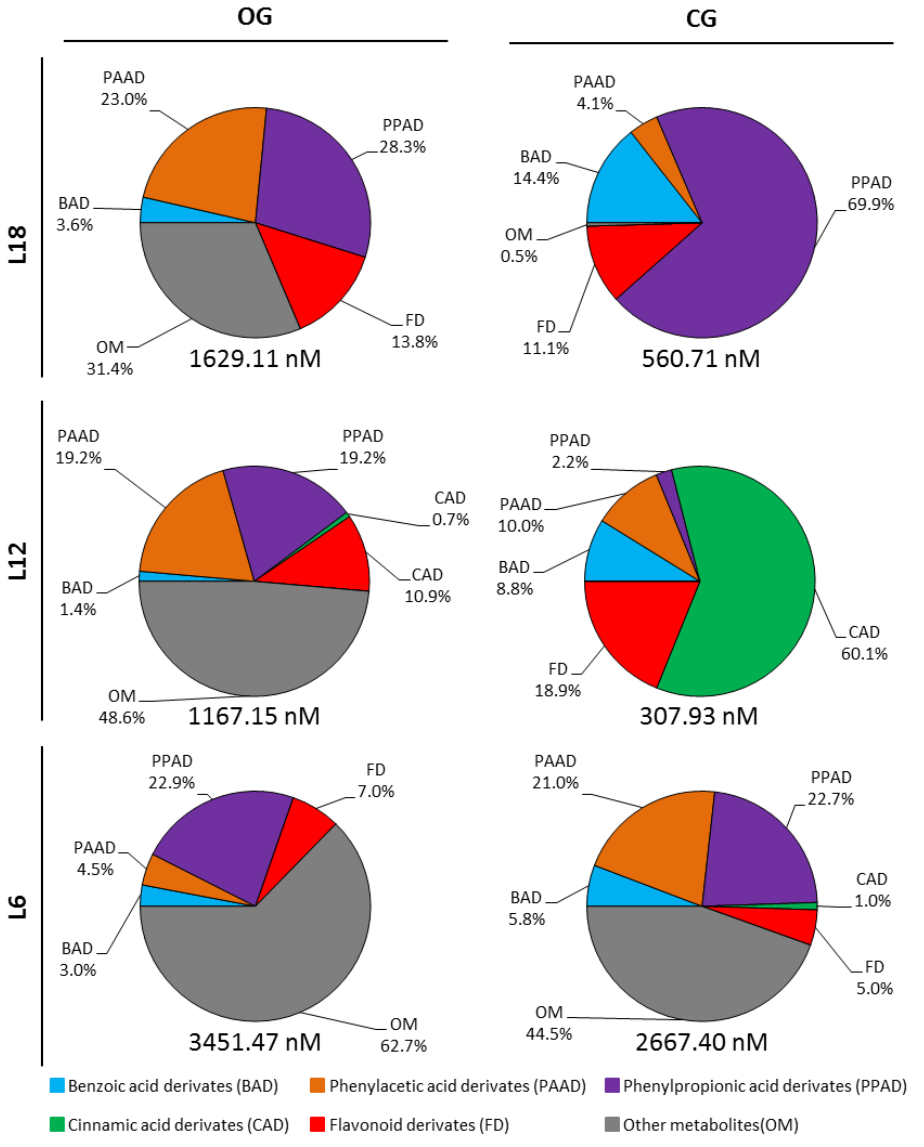
μ-SPE
Serum
OG, CG or VH
(n=6, each)
Adaptation
n=18
L18
L12
L6
n=54
Weeks
Weeks
0 1 2 3 4
4 5 6 7 8 9 10 11 12 13 14

Figure 2.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L12
L6
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BAD 1.4%
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307.93 nM
PPAD 22.9%
FD 7.0%
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BAD 3.0%
OM 62.7%
3451.47 nM
PAAD 21.0%
PPAD 22.7%
CAD 1.0%
BAD 5.8%
FD 5.0%
OM 44.5%
2667.40 nM
Benzoic acid derivates (BAD)
Phenylacetic acid derivates (PAAD)
Phenylpropionic acid derivates (PPAD)
Cinnamic acid derivates (CAD)
Flavonoid derivates (FD)
Other metabolites(OM)

UNIVERSITAT ROVIRA I VIRGILI
MODERN CHANGES IN FRUIT INTAKE PATTERNS MODULATE THE BIOAVAILABILITY AND METABOLISM OF FRUIT PHENOLIC COMPOUNDS IN RATS
Lisard Iglesias Carres

MANUSCRIPT 8:

The bioavailability and metabolism of red grape polyphenols is conditioned by its consumption season metabolically-altered Fischer 344 rats .

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The bioavailability and metabolism of red grape polyphenols is conditioned by its consumption season in metabolically-altered Fischer 344 rats.

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Abstract:

Metabolic syndrome (MetS) is known to increase the cardiovascular risk (CVR), and grape polyphenol consumption has been proposed as a strategy to manage the pathologies associated with MetS. Moreover, CVR is higher in low-light exposure months. Importantly, natural physiological changes triggered by amount of day light exposure (photoperiod) could modulate the bioavailability and metabolism of grape polyphenols. Therefore, the aim of this study was to evaluate whether the bioavailability and metabolism of red grape phenolics was modulated by photoperiod exposure in MetS-altered homeostatic control. To do so, the serum phenolic metabolites were profiled in long-term supplementation with conventional grapes (CG) in rats consuming a cafeteria diet (CAF). Our results in the serum of Fischer 344 rats with CAF diet-induced MetS demonstrate that photoperiod is able to modulate the bioavailability and metabolism of CG polyphenols. Precisely, rats with the lowest light exposure reported a higher bioavailability of grape phenolics. Also, the type of metabolites was different between photoperiod groups. The results of this study suggest that the consumption of whole grapes by in patients with MetS could be beneficial in the management of CVR during winter months.

Keywords:

Cardiovascular risk; Bioavailability; Metabolic syndrome; Polyphenols; Seasonal variation.

1. Introduction:

Metabolic syndrome (MetS) is a cluster of pathologies that disrupt host's homeostasis. Among these pathologies, obesity, cardiovascular disease and type-II diabetes stand out [1]. Importantly, the consumption polyphenols has been associated with a reduction of the risk to develop these pathologies [2–4], and current scientific evidence suggests the supplementation with grapes to manage MetS-associated pathologies [5]. However, recent studies suggest that the compounds that have a real bioactive effect are the polyphenol metabolites rather than the natural-occurring compounds in fruits [3,6–8]. Indeed, phenolic compounds are known to be rapidly and extensively metabolised [9,10]. Once absorbed, phenolic compounds are recognised as xenobiotics and undergo phase-II detoxification in the small intestine and liver [11]. However, between 90 – 95 % of dietary phenolic are not absorbed in the small intestine and reach the colon. There, these compounds are subjected to microbial metabolism, generating small phenolic acid compounds [6,12,13]. Both phase-II and microbial-derived metabolites appear in the serum and plasma and reach different organs and tissues [7,14–16] to be finally eliminated via the urine [11].

Potentially, any factor that modulates host physiology can have important implications for polyphenol bioavailability and metabolism [17]. In the specific case of grape polyphenols, their metabolic fate has been widely studied [9,10,12,14,15,18]. In this sense, cafeteria (CAF) diet consumption, known to promote MetS [19], has been previously reported to modulate grape-seed phenolics' bioavailability and metabolism in Wistar rats with MetS [18]. Moreover, humans and other mammals are known to present changes on their physiology hat fluctuate in cycles of approximately a year, and the amount of light hours a day (photoperiod) is one of the main responsible factors [20]. Specifically, some of these changes might have important implications for polyphenol bioavailability and metabolism [21–

29], while others places humans at a higher cardiovascular risk (CVR) in the winter [30–32].

Thus, given the importance of photoperiod and MetS in CVR [30,31,33], winter consumption of whole grapes could play an important protective role, especially for the management of CVR [5]. Hence, this study aimed to evaluate whether the bioavailability and metabolism of grape polyphenols varies depending on photoperiod exposure in MetS rats after a chronic supplementation with grapes. This was evaluated male Fischer 344 male rats that consumed a cafeteria (CAF) diet, known to promote MetS [19], using grapes produced conventionally (conventional grapes, CG).

2. Materials and methods:

2.1. Chemical and reagents:

Acetone, acetonitrile, methanol (all HPLC analytical grade) and phosphoric acid were purchased from Sigma-Aldrich (Madrid, Spain). Glacial acetic acid was purchased from Panreac (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q Advantage A10 system (Madrid, Spain). Benzoic acid, caffeic acid, catechin, epicatechin, ferulic acid, gallic acid, hippuric acid, 2-(4-hydroxyphenyl)acetic acid, 2-(3-hydroxyphenyl)propionic acid, kaempferol p-coumaric acid, 3-hydroxybenzoic acid, pyrocatechol (Internal Standard, IS), quercetin and vanillic acid were purchased from Fluka/Sigma-Aldrich (Madrid, Spain). Procyanidin dimer B2 and protocatechuic acid were purchased from Extrasynthese (Genay, France). Resveratrol was purchased from Quimivita (Barcelona, Spain) and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone was purchased from MicroCombiChem e.K. (Wiesbaden, Germany). Standard compounds were individually prepared in methanol (2000 mg/L) every three months and stored in amber-dark glass flasks at -20 °C. A mixed standard stock solution of all the standard compounds, with the exception of IS, was prepared weekly in methanol (20 μg/mL) and stored under the same conditions. This mixture

was diluted daily in acetone/Milli-Q water/acetic acid (70/29.5/0.5; v/v/v) solution to the desired concentration and stored under the same conditions.

2.2. Plant material:

CG of the variety Grenache (*Vistis vinifera)* were harvested at maturity in the region of Rasquera (Tarragona, Spain). Whole grapes (seeds, skins and pomace) were frozen in liquid nitrogen and grounded to homogeneity. Homogenates were then freeze-dried for one week using at -85 °C using a Telstar LyoQuest lyophilizer (Thermo Fisher Scientific, Madrid, Spain) and further grounded to obtain a fine powder, which was kept at room temperature and protected from light exposure and humidity until administered to the rats. The phenolic composition of CG can be found in Table 1, which is adapted from Iglesias-Carres *et al*. [34].

2.3. Experimental design in rats and serum collection:

A total number of 36 male Fisher 334 rats were purchased from (Charles River, Barcelona, Spain) and kept at 22 °C in animal quarters. Rats were randomly divided into 3 different light/dark cycles (n=12, each): L12 (12 h of light/day), L18 (18 h of light/day) and L6 (6 h of light/day), respectively emulating spring and fall, summer and winter light exposure. For a period of 4 weeks, rats underwent adaptation to photoperiod and consumed a standard (STD) chow diet (Panlab A04, Planlab, Barcelona, Spain) and tap water *ab libitum*. Then, rats in each photoperiod were divided into two different supplementation groups (n=6, each): CG and vehicle (VH). CG supplementation consisted on 100 mg CG dw/Kg bw in water, and VH on consisted on glucose (10 mg/Kg day) and fructose (10 mg/Kg day) in water. This supplementation period lasted for 7 weeks and included the administration of CAF diet, in addition to the STD diet. CAF diet consists on sausage, bacon, biscuits with paté, cheese, ensaïmada (sweetened pastry), carrots and sweetened milk (20% sucrose w/v). CAF was freshly provided

to the animals daily, and animals could choose what to eat and ate *ad libitum*. The nutritional composition of the CAF diet was 14% protein, 35% fat and 51% carbohydrates [19]. Access to tap water was *ab libitum* in both dietary interventions. Both dietary groups were randomly sub-divided into. In all cases, lights were turned on at 9.00 am and treatments were orally administered in between 9.00 and 10.00. On the day of the sacrifice, rats were deprived from food once the treatments (CG or VH) were administered, and were put down after 1 hour. Blood was recollected in non-heparinised flasks and waited 1 hour at room temperature. Then, blood was centrifuged (2000 x g, 15 min, 4 °C) to obtain serum samples, which were aliquoted and frozen at – 80 °C. Rat weights can be found in Table 2. This study was performed in accordance with institutional guidelines for the care and use of laboratory animals. Moreover, the experimental procedure that was performed was approved by the Ethical Committee for Animal Experimentation of the Universitat Rovira i Virgili (reference number 4249).

2.4. Extraction of phenolic metabolites:

Before chromatographic separation, serum samples were purified and concentrated by micro solid-phase extraction (μ-SPE) methodology [15] with 30 μm OASIS HLB μ-Elution Plates (Waters, Barcelona, Spain). Briefly, micro-cartridges were sequentially loaded with 250 μL of methanol and 250 μL acetic acid 0.2 %. Then, plates were loaded with a mixture containing 300 μL phosphoric acid 4 %, 250 μL serum samples and 50 μL of IS (200 ppb). Plates were then sequentially washed with 200 μL of Milli-Q water and 200 μL acetic acid 0.2 %. Retained phenolic metabolites were eluded with of 50 μL of acetone/water/acetic acid (70/29.5/0.5; v/v/v) twice. Later, 2.5 μL of the eluted solutions were directly injected into the HPLC-ESI-MS/MS system.

2.5. Chromatographic analysis of phenolic metabolites:

Chromatographic separation of CG polyphenol metabolites was achieved with a Kinetex EVO C18 (2.6 μm, 150x2.1mm) column. Mobile phases consisted on water/acetic acid (99.8/0.2; v/v) (mobile phase A) and acetonitrile (mobile phase B) with the following gradient: initial conditions, 0 % B; 0-0.5 min, 0 % B; 0.5-15 min, 0-40 % B; 15-15.5 min, 40-100 % B; 15.5-19 min, 100 % B; 19-20 min, 100-0 % B. A post run of 3 min was required for column re-equilibration, and flow rate was set constant at 0.4 mL/min. Quantification was achieved by coupling the above system with a 6490 MS/MS system (Agilent Technologies, Palo Alto, CA, USA). Electrospray ionization (ESI) was conducted at 200 °C and 14 L/min with 20 psi of nebulizer gas pressure and 3000 V of capillary voltage. The mass spectrometer was operated in the negative mode and data was acquired using the Dynamic mode. Optimized fragmentation conditions for the analysis of phenolic metabolites can be found in Supplementary Table 1.

2.6. Sample quantification:

Serum obtained from the VH groups was spiked with 10 different standard concentrations to construct calibration curves. Samples were quantified by interpolating the analyte/IS peak abundance ratio in the standard curves. Any compound present in the VH groups was substracted from CG groups. Data acquisition was performed by using MassHunter Software (Agilent Technologies, Palo Alto, CA, USA). Method quality parameters are reported in Supplementary Table 2.

2.7. Statistics:

Two-way ANOVA was used to estimate any statistical difference ($p<0.05$) in the final weight of the animals depending on photoperiod exposure and administration CG or VH with SPSS 19 software (SPSS Inc., Chicago, IL, USA).

3. Results:

The serum profile of CG-supplemented rats considerably differed between photoperiod groups in Fischer 344 rats that consumed a CAF diet (Table 3). The L6 group reported the highest total serum concentration (2132.00 nM), followed by the L12 (161.90 nM) and L18 (12.13 nM) groups, showing an increasing tendency on the bioavailability of CG polyphenols as light hours a day decreased. Interestingly, the only compound quantified in the L18 group was gallic acid, which was not an abundant compound in any of the other photoperiods (Table 1). Instead, in the L12 group, phenylpropionic acid derivates were the main representatives (Figure 1), accounting for 98.3 % of all the metabolites. This was due to the contribution of phenylpropionic acid. The L6 presented the most diverse serum profile of the three photoperiods (Figure 1). Like in the L18, benzoic acids dominated in the L6 group (64.2 %), but the dominating metabolite was benzoic acid (Table 3). The proportions of phenylpropionic acid derivates (8.1 %), phenylacetic acid derivates (16.2 %) and other metabolites (11.0 %) were mainly due to the contribution of phenylpropionic acid (132.99 nM), homovanillic acid (279.61 nM) and hippuric acid (226.82 nM), respectively (Table 1).

4. Discussion:

Individuals suffering from MetS usually are hypertensive, and present dyslipidaemia and type II diabetes, and these are relevant CVR factors[1]. Given the cardiovascular protective effect of grape phenolics [4,5], consumption of whole red grapes in the winter could help attenuate the higher CVR during low-light exposure months, and especially in population groups with elevated CVR such as people suffering from MetS [5]. Importantly, these health effects of fruit consumption are mediated by the metabolic products of phenolic compounds rather than the natural forms [3,6–8]. Although physiological changes relevant in phenolic compound bioavailability and metabolism occur under exposure to different

photoperiods [21,22,26,28,29,35,36], no studies that evaluate the effect of photoperiod in animals with MetS exist in the literature. Therefore, the aim of this study was to evaluate whether grape polyphenol bioavailability and metabolism is modulated under photoperiods that simulate winter (L6), summer (L18) and spring and fall (L12) in rats with MetS. Fischer 344 rats were selected due to their physiological sensibility to photoperiod [21,22], and CAF diet due to their known modulation of grape-seed flavan-3-ols bioavailability and metabolism [14,18] as well as its promotion of MetS development [19]. Moreover, MetS in CAF rats also promotes a higher CVR [37,38], which is also observed in humans with MetS [33]. To detect of all potential metabolites generated after CG consumption, serum was pooled to increase in homogeneity and sensibility [12,15].

Grape polyphenols are rapidly absorbed and their metabolites can appear in plasma as early as 1 h after their consumption [39]. However, previous studies with CG demonstrated that serum phenolic metabolites are present at relevant concentrations 24 h after the intake of CG [34]. Thus, the results of this experimental design most probably not only represent the serum metabolites of the CG supplementation dose on the day of the sacrifice but rather a combination of this and the circulating metabolites generated due to the dose administered the day before the sacrifice. Consequently, the results herein presented do not demonstrate serum metabolite accumulation due to long-term grape administration. Indeed, lack of accumulation of grape phenolic metabolites has been previously demonstrated in CAF rats treated chronically with grape seed flavan-3-ols [14].

No non-metabolised compounds were detected in the serum of rats supplemented with CG (Table 3), which is consistent with the fact that phenolic compounds undergo rapid metabolism in the host [9,10]. Indeed, non-metabolised compounds only appear in animal tissues such as serum when phenolic compounds are administered at high doses that saturate

the phase-II detoxification system [9,40]. However, in this study, the only flavonoid phase-II metabolites detected in serum was methylcatechin glucuronide, and it was only detected in the L6 photoperiod at the concentration of 2.71 nM. These results are in clear disagreement with several studies evaluating grape polyphenol bioavailability in serum and plasma, which find flavan-3-ol glucuronides in abundant quantities [9,10,15,39]. Nevertheless, this effect has been previously observed in male Wistar rats with MetS administered with grape seed polyphenols [18]. Precisely,

The phenolic profile of rats with MetS consuming CG was dominated by low molecular weight metabolites, which belonged to different metabolic families (Figure 2), and these metabolites are normally generated by the gut microbiota [12,41]. Indeed, it has been estimated that 90 – 95 % of dietary phenolic compounds reach the colon where they are subjected to microbial metabolism [6,12,13]. These compounds can be absorbed *in situ* and then reach other organs and tissues as evidenced in administration studies with extracts rich in flavan-3-ols [14,15] and anthocyanins [16]. Previous studies involving chronic administration of flavan-3-ols also showed microbial-derived metabolites to be the most abundant metabolites in different organs such as the liver, brain and aorta of male Wistar rats consuming a CAF diet [14]. In this study, the type and the proportions of serum microbial-derived metabolites changed depending on photoperiod exposure. Importantly, the quantity and type of microbial-derived metabolites depends on the gut microbial composition [6], which is known to change in different mammals due to photoperiod exposure [26,27,29]. Therefore, this effect could explain the wide differences in proportions of microbial-derived metabolites in the serum of Fischer 344 rats with MetS.

Although the differences in the serum proportion of metabolic families were relevant, especially when comparing the L12 with the other two

photoperiods, the total serum concentration found in each photoperiod group was most remarkable. In this sense, rats in the L6 photoperiod reach a total serum metabolite concentration of 2132.00 nM, which is far superior than the one found in rats under the L12 (161.90 nM) and in the L18 (12.13 nM). Thus, it seems that photoperiod has a huge effect on the bioavailability of CG polyphenols in Fischer 344 rats with MetS. Precisely, CG polyphenol bioavailability is increased when CG are consumed under the photoperiod conditions (L6) that simulate their traditional consumption season [42].

Importantly, phenolic compounds must be bioavailable to produce a systemic bioactive effect [11]. The total amount of metabolites found in the L6 group was the highest. Also, benzoic acid derivates reached the highest concentrations under those photoperiod conditions, and these are known to possess relevant cardioprotective functions [8,43–45]. Thus, both the differences in type of metabolites and total serum concentrations could have important health implications as many of the biological effects of phenolic compounds are mediated by microbial metabolites [3,6,8,43–45]. Precisely, the consumption of CG by Fischer 344 rats with MetS has already been demonstrated to produce different health effects. In this sense, our group has demonstrated that the consumption of CG out of their traditional consumption season (L6) modulates differently several genes involved in the circadian control in Fischer 344 rats with MetS [46]. Moreover the consumption of CG in the L6 and L18 produce different effects on the signalling pathway of leptin in Fischer 344 rats with MetS [47]. However, no information is available on the effects of CG consumption under different photoperiods on CVR management. Thus, functional studies should be performed to confirm wehther the consumption of CG in the winter is able to reduce CVR associated with MetS.

5. Conclusions:

This study demonstrates grape phenolics bioavailability and metabolism to depend on photoperiod exposure under MetS-disrupted homeostatic control promoted by CAF diet consumption in Fischer 344 rats. The relevant changes found in microbial-derived metabolites could involve alterations on the bioactivity associated with the consumption of CG, which would depend on its consumption season. However, further studies should be performed to evaluate whether or not these change involve different bioactive effects. Most notoriously, the bioavailability of red grape phenolics was at its highest photoperiod conditions that emulate their traditional consumption season, suggesting that the recommendation of grape consumption should be encouraged during the winter.

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Conflicts of interest:

No conflicts of interest exist in this study.

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Figure legends:

Figure 1: Graphical representation of the experimental design used in this study.

Figure 2: Distribution of phenolic metabolite families in the serum rats administered conventional red Grenache grapes (CG) at a dose of 100 mg/KG bw. Abbreviations: L12, 12 h light/day; L18, 18 h light/day; and L6, 6 h light/day.

Table 1: Administration doses of phenolic compounds (μg/Kg bw day) present conventional red Grenache grapes (CG).

Compound	CG
Benzoic Acid	0.75 ± 0.04
Dihydroxybenzoic acid d1	0.21 ± 0.02
Protocatechuic acid	1.11 ± 0.15
p-Coumaric acid	0.04 ± 0.01
Gallic acid	0.25 ± 0.04
Caffeic acid	0.06 ± 0.01
Resveratrol	0.78 ± 0.04
Apigenin	0.01 ± 0.00
Catechin	136.54 ± 4.39
Epicatechin	42.99 ± 0.43
Quercetin	0.15 ± 0.01
Gallocatechin	0.01 ± 0.00
Epigallocatechin	0.00 ± 0.00
Caffeoyltartaric acid	586.38 ± 36.18
ProtocatechuiC acid O-glucoside	365.66 ± 4.09
Coumaric acid O-glucoside	0.12 ± 0.00
Gallic acid O-glucoside d1	0.08 ± 0.01
Gallic acid O-glucoside d2	3.52 ± 0.03
Caffeic acid O-glucoside d1	1.35 ± 0.10
Caffeic acid O-glucoside d2	0.77 ± 0.04
Caffeic acid O-glucoside d3	6.77 ± 0.39
Resveratrol O-glucoside d1	6.10 ± 0.74
Resveratrol O-glucoside d2	15.10 ± 1.74
Catechin 3-O-gallate	4.37 ± 0.38
Kaempferol O-galactose	1.41 ± 0.12
Kaempferol-3-O-galacucose	5.68 ± 0.41
Eriodictyol-7-O-gucoside	0.04 ± 0.00
(Epi)catechin O-glucoside d1	1.11 ± 0.11
(Epi)catechin O-glucoside d2	0.60 ± 0.13
(Epi)catechin O-glucoside d3	0.64 ± 0.13
(Epi)catechin O-glucoside d4	3.54 ± 0.39
Gallocatechin gallate	0.01 ± 0.00
Epigallocatechin gallate	0.08 ± 0.01
Quercetin-3-O-glucoside	47.05 ± 9.66
Isorhamnetin-3-O-glucoside	7.56 ± 0.33

Table 1 (Continued).

Procyanidin dimer d1	23.99 ± 1.24
Procyanidin dimer d2	10.30 ± 0.40
Procyanidin dimer B2	18.96 ± 1.23
Procyanidin dimer d3	0.78 ± 0.08
Procyanidin dimer d4	2.53 ± 0.21
Procyanidin dimer d5	0.64 ± 0.01
Kaempferol-3-o-rutinoside	0.27 ± 0.03
Rutin	2.48 ± 0.19
Procyanidin trimer d1	0.33 ± 0.02
Procyanidin trimer d2	0.19 ± 0.01
Pelargonidin O-glucoside	0.53 ± 0.01
Cyanidin O-glucoside	3.17 ± 0.16
Delphinidin O-glucoside	19.94 ± 2.67
Petunidin O-glucoside	31.19 ± 2.10
Malvidin-3-O-glucoside	85.46 ± 5.04
Malvidin O-acetylglucoside	0.79 ± 0.03
Peonidin O-coumaroylglucoside d1	2.21 ± 0.18
Peonidin O-coumaroylglucoside d2	0.14 ± 0.03
Peonidin-3-O-rutinoside	0.00 ± 0.00
Delphinidin O-coumaroylglucoside d1	1.53 ± 0.27
Delphinidin O-coumaroylglucoside d2	0.63 ± 0.06
Delphinidin O-coumaroylglucoside d3	0.12 ± 0.00
Petunidin O-acetylglucoside d1	0.14 ± 0.01
Petunidin O-acetylglucoside d2	0.00 ± 0.00
Petunidin O-acetylglucoside d3	0.35 ± 0.02
Petunidin O-acetylglucoside d4	5.74 ± 0.34
Malvidin O-coumaroylglucoside d1	0.13 ± 0.01
Malvidin O-coumaroylglucoside d2	0.55 ± 0.02
Malvidin O-coumaroylglucoside d3	8.30 ± 0.92

Results are expressed as μg/Kg bw day ± SD (n=3). d1, d2, d3, d4 and d5 indicate different isomeric compounds. Adapted from Iglesias-Carres *et al*. [34].

Table 4: Individual phenolic metabolites in rat serum after chronic supplementation conventional red Grenache grapes (CG) in rats with Metabolic syndrome kept at different photoperiods.

Compound	L18	L12	L6
Resveratrol	n.d.	n.d.	n.d.
Kaempferol	n.d.	n.d.	n.d.
Catechin	n.d.	n.d.	n.d.
Epicatechin	n.d.	n.d.	n.d.
Quercetin	n.d.	n.d.	n.d.
Procyanidin dimer B2	n.d.	n.d.	n.d.
Total non-metabolised compounds	**0**	**0**	**0**
Methylcatechin [a]	n.d.	n.d.	n.d.
Catechin glucuronide [a]	n.d.	n.d.	n.d.
Epicatechin glucuronide [b]	n.d.	n.d.	n.d.
Methylcatechin glucuronide [a]	n.d.	n.d.	2.71
Methylepicatechin glucuronide [b]	n.d.	n.d.	n.d.
Total flavonoid phase-II metabolites	**0**	**0**	**2.71**
p-Coumaric acid	n.d.	n.d.	3.09
Caffeic acid	n.q.	n.d.	n.q.
Ferulic acid	n.d.	n.d.	4.45
Total cinnamic acid derivates	**0**	**0**	**7.54**
Phenylpropionic acid [c]	n.d.	152.87	132.99
3-(4-Hydroxphenyl)propionic acid	n.d.	6.27	n.q.
3-(3-Hydroxphenyl)propionic acid [c]	n.d.	n.d.	33.83
3-(3,4-Dihydroxyphenyl)propionic acid [c]	n.d.	n.d.	n.d.
Dihydrocaffeic acid glucuronide [d]	n.d.	n.d.	5.2
Dihydroferulic acid glucuronide [e]	n.d.	n.d.	n.d.
Total phenylpropionic acid derivates	**0**	**159.14**	**172.02**
2-(3-Hydroxypheyl)acetic acid	n.d.	n.d.	59.53
2-(4-Hydroxypheyl)acetic acid	n.d.	n.d.	7.06
Homovanillic acid	n.d.	n.d.	279.61
Total phenylacetic acids	**0**	**0**	**346.2**
Benzoic acid	n.d.	n.d.	1338.7
3-Hydroxybenzoic acid [w]	n.d.	n.d.	n.d.
4-Hydroxybenzoic acid [w]	n.d.	n.d.	5.7
Protocatechuic acid	n.d.	n.d.	4.1
Dihydroxybenzoic acid	n.d.	n.d.	8.1
Vanillic acid	n.d.	n.d.	12.7
Gallic acid	12.13	n.q.	n.d.
Methylgallic acid	n.q.	n.q.	n.q.
Total benzoic acid derivates	**12.13**	**0**	**1369.4**
Phloroglucinol	n.d.	n.d.	n.d.
Hippuric acid	n.d.	n.d.	226.82
5-(3,4-Dihydroxyphenyl)-γ-valerolactone	n.d.	n.d.	n.d.
4-Hydroxy-5-(3′,4′-dihydroxyphenyl) valeric acid	n.d.	2.75	7.36
5-(3,4-Dihydroxyphenyl)-γ-valerolactone glucuronide	n.d.	n.d.	n.d.
Total other metabolites	**0**	**2.75**	**234.18**
Total metabolites	**12.13**	**161.9**	**2132**

Abbreviations: L12, 12 h light/day; L18, 18 h light/day; and L6, 6 h light/day.[a] Quantified using the calibration curve of catechin. [b] Quantified using the calibration curve of epicatechin. [c] Quantified using the calibration curve of 3-(4-hydroxyphenyl)propionic acid. [d] Quantified using the calibration curve of caffeic acid. [e] Quantified using the calibration curve of ferulic acid. [f] Quantified using the calibration curve of 2-(4-hydroxyphenyl)acetic acid. [g] Quantified using the calibration curve of vanillic acid. [h] Quantified using the calibration curve of 3-hydroxybenzoic acid. [i] Quantified using the calibration curve of protocatechuic acid. [j] Quantified using the calibration curve of gallic acid. [k] Quantified using the calibration curve of 5-(3,4-Dihydroxyphenyl)-γ-valerolactone.

Table 2: Weights of Fischer 344 rats at the beginning (initial weight), after photoperiod adaptation and end (final weight) of the experiment.

Light/Dark cycle	Treatment	Initial weigh (g)	Weight after adaptation (g)	Final weight (g)
L18	**VH**	239.5 ± 5.5	297.7 ± 4.7	411.0 ± 7.8
	CG	248.8 ± 3.5	305.1 ± 3.9	433.5 ± 7.7
L12	**VH**	251.8 ± 6.1	311.2 ± 6.4	440.5 ± 11.0
	CG	238.2 ± 7.0	303.8 ± 8.2	418.4 ± 8.3
L6	**VH**	235.4 ± 6.8	290.4 ± 7.4	407.1 ± 12.0
	CG	244.1 ± 7.7	292.8 ± 9.3	410.2 ± 11.4

Results are expressed as grams of body weight ± SEM (n=6). No statistical differences ($P<0.05$) were detected in the final weight of animals in this study by Two-way ANOVA. Abbreviations: L12, 12 hours of light/day; L18, 18 hours of light/day; L6, 6 hours of light/day; VH, vehicle; and CG, conventional red Grenache grape.

Supplementary Table 1: Optimised conditions for the analysis of grape phenolic metabolites by HPLC-ESI-MS/MS.

Compound	MW	[M-H]$^{-a}$	RT(min)	Quantification		Confirmation	
				MS>MS	CE(V)	MS>MS	CE(V)
Benzoic acid	122.00	121.0493	8.271	121>77	8	121>59	4
3-Hydroxybenzoic	138.12	137.0243	5.326	137>93	40	137>65	36
Phenylpropionic acid	150.17	149.0256	10.752	149>105	10		
2-(3-Hydroxyphenyl)acetic acid	152.15	151.0382	5.318	151>107	5	151>93	20
2-(4-Hydroxyphenyl)acetic acid	152.15	151.0382	5.809	151>107	5	151>65	30
Protocatechuic acid	154.12	153.0599	4.286	153>109	16	153>62	40
p-Coumaric acid	164.05	163.0439	7.996	163>119	16	163>93	36
3-(4-hydroxyphenyl)propionic acid	166.17	165.0580	6.361	165>121	10	165>59	0
3-(3-hydroxyphenyl)propionic acid	166.17	165.0580	6.862	165>121	10	165>59	0
Vanillic Acid	168.19	167.0193	6.152	167>108	10	167>123	5
Gallic acid	170.12	169.0193	3.998	169>125	12	167>79	24
Hippuric acid	179.17	178.0565	5.706	178>134	5	178>77	10
Caffeic	180.16	179.0401	6.491	179>135	16	179>107	24
Homovanillic	182.17	181.0547	4.667	181>163	10	181>134	5
3-(3,4-Dihydroxyphenyl)propionic acid	182.17	181.0547	12.915	181>137	10		
Methylgallic acid	184.15	183.0302	5.075	183>168	10	183>124	10
Ferulic acid	194.18	193.5610	8.558	193>134	12	193>178	12
5-(3,4-Dihydroxyphenyl)-γ-valerolactone	208.21	207.0727	6.623	207>85	10	207>121	10
4-Hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid	226.23	225.0699	11.420	225>163	10	225>181	10
Resveratrol	228.23	227.2080	11.358	227>143	28	227>185	20
Kaempferol	285.23	285.0481	14.140	285>239	28	285>117	56
Catechin	289.26	289.0794	6.109	289>203	10	285>245	12
Epicatechin	289.26	289.0794	7.231	289>203	10	285>245	12
Quercetin	302.24	301.0797	12.523	301>151	20	301>179	20
Methylcatechin	304.29	303.0244	6.725	303>137	20	303>285	10
Dihydrocaffeic acid glucuronide	358.30	357.0901	4.954	357>181	20	357>137	20
Dihydroferulic acid glucuronide	372.32	371.1063	6.214	371>195	20	371>136	20
5-(3,4-Dihydroxyphenyl)-γ-valerolactone glucuronide	384.33	383.0158	6.641	383>207	24	383>163	40
Catechin glucuronide	466.39	465.1128	6.194	465>289	20	465>203	40
Epicatechin glucuronide	466.39	465.1128	6.497	465>289	20	465>203	40
Methylcatechin glucuronide	480.42	479.1288	7.613	479<303	20	479>289	20
Methylepicatechin glucuronide	480.42	479.1288	7.785	479<303	20	479>289	20
Procyanidin dimer B2	578.52	577.1476	6.911	557>425	10	577>407	30

Abbreviations: MW, molecular weight; CE, collision energy. [a] Studied by qTOF.

Supplementary Table 2: Calibration curve, determination coefficient (R^2), working linearity range, LODs, LOQs, MLDs and MQLs for phenolic compound quantification in spiked serum samples using HPLC-ESI-MS/MS.

Compound	Retention time (min)	Calibration curve	R^2	Linearity (μM)	LOD (nM)	LOQ (nM)	MDL[a] (nM)	MQL[a] (nM)
Benzoic acid	8.13	y=0.1271x	0.991	0.008-16.377	2,602	8,674	1,041	3,470
3-Hydroxybenzoic acid	5.33	y=0.5206x	0.999	0.007-14.480	0.770	2,566	0.308	1,027
2-(4-Hydroxyphenyl)acetic acid	5.81	y=0.0826x	0.993	0.007-13.145	0.190	0.632	0.076	0.253
Protocatechuic acid	3.96	y=0.3361x	0.998	0.006-12.977	0.618	2,060	0.247	0.824
p-Coumaric acid	7.82	y=1.6366x	0.996	0.006-12.191	1,805	6,017	0.722	2,407
3-(4-Hydroxyphenyl)propionic acid	6.76	y=0.0579x	0.996	0.006-12.035	1,389	4,629	0.555	1,852
Vanillic acid	6.03	y=0.1982x	0.998	0.006-11.895	0.834	2,779	0.334	1,112
Hippuric acid	5.91	y=0.1084x	0.990	0.006-11.163	0.235	0.785	0.094	0.314
Caffeic acid	6.39	y=0.9491x	0.992	0.006-11.101	0.447	1,492	0.179	0.597
Ferulic acid	8.41	y=0.5645x	0.999	0.005-10.300	0.519	1,731	0.208	0.692
5-(3,4-Dihydroxyphenyl)-γ-valerolactone	6.52	y=0.4319x	0.992	0.005-9.606	0.335	1,117	0.134	0.447
Resveratrol	11.22	y=0.1928x	0.990	0.004-2.191	1,195	3,983	0.478	1,593
Kaempferol	14.02	y=0.1731x	0.995	0.035-3.494	6,165	20,551	2,466	8,220
Catechin	5.98	y=0.2281x	0.997	0.003-6.890	0.795	2,650	0.318	1,060
Epicatechin	6.96	y=0.1597x	0.998	0.003-6.890	0.886	2,953	0.354	1,181
Quercetin	12.36	y=0.1597x	0.998	0.003-6.617	0.735	2,451	0.294	0.980
Procyanidin dimer B2	6.78	y=0.3912x	0.994	0.002-1.790	0.474	1,580	0.190	0.632

Abbreviations: R^2, determination coefficient; LOD, limit of detection; LOQ, limit of quantification; MDL, method detection limit, and MLD, method quantification limit.[a] MDL and MQL for the analysis of 250 μL of serum.

Figure 1.

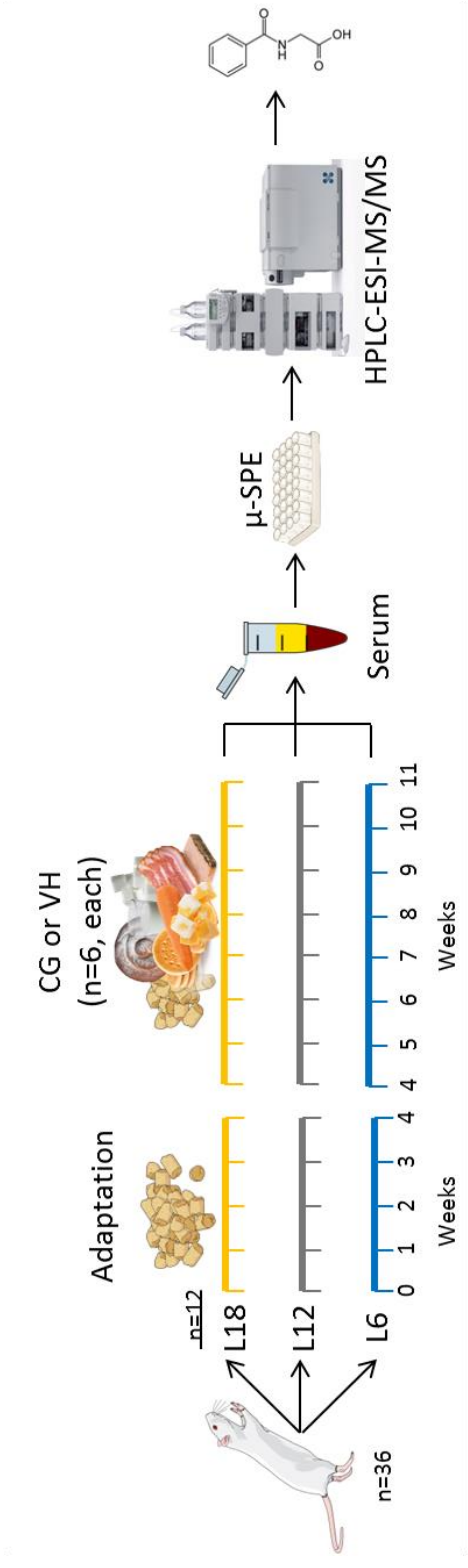
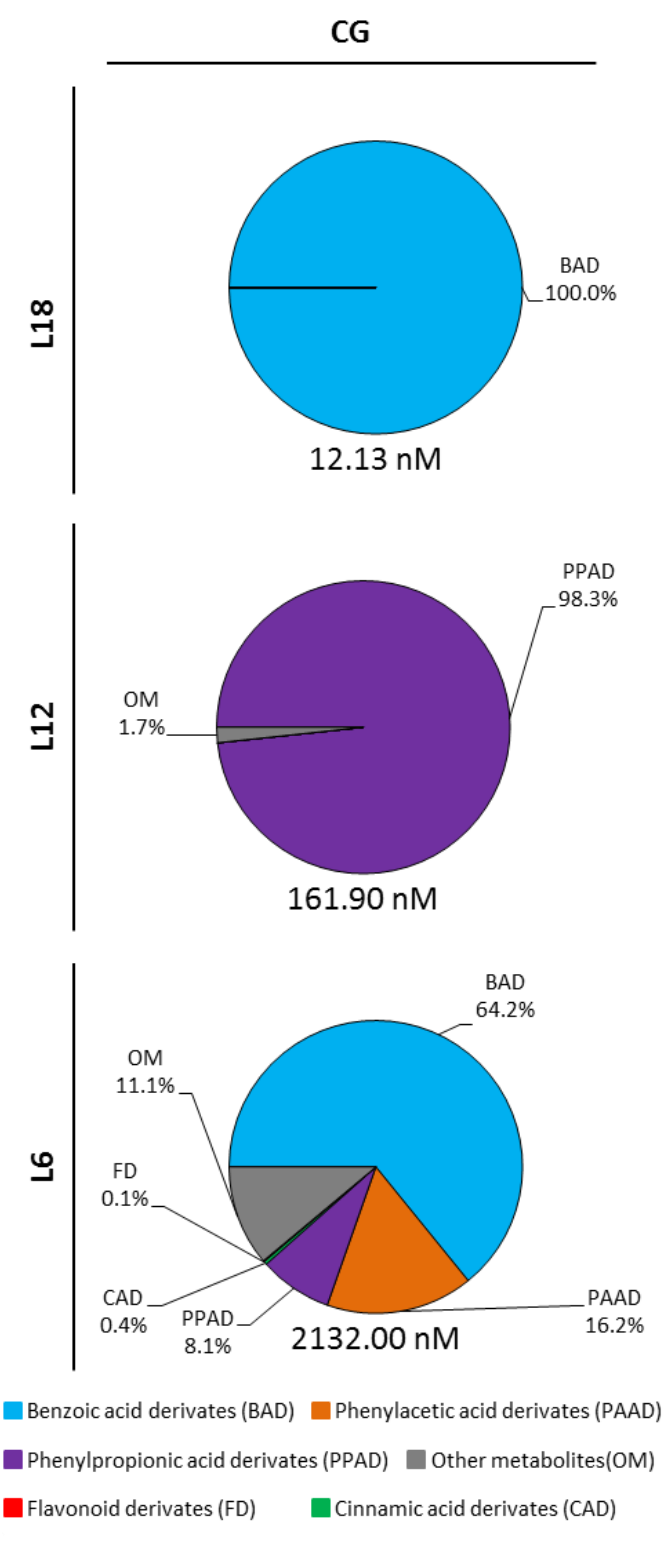

OH
HPLC-ESI-MS/MS
µ-SPE
Serum
CG or VH
(n=6, each)
Adaptation
n=12
L18
L12
L6
n=36
Weeks
0 1 2 3 4
4 5 6 7 8 9 10 11
Weeks

Figure 2.


CG
L18
BAD
100.0%
12.13 nM
L12
PPAD
98.3%
OM
1.7%
161.90 nM
L6
BAD
64.2%
OM
11.1%
FD
0.1%
CAD
0.4%
PPAD
8.1%
PAAD
16.2%
2132.00 nM
Benzoic acid derivates (BAD)
Phenylacetic acid derivates (PAAD)
Phenylpropionic acid derivates (PPAD)
Other metabolites(OM)
Flavonoid derivates (FD)
Cinnamic acid derivates (CAD)

GENERAL DISCUSSION:

UNIVERSITAT ROVIRA I VIRGILI
MODERN CHANGES IN FRUIT INTAKE PATTERNS MODULATE THE BIOAVAILABILITY AND METABOLISM OF FRUIT PHENOLIC COMPOUNDS IN RATS
Lisard Iglesias Carres

GENERAL DISCUSSION

Modern occidental societies face the problem of obesity and metabolic diseases such as the metabolic syndrome (MetS) and diabetes. This higher prevalence of metabolic diseases has been linked with changes in lifestyle, and dietary changes play an important role on that tendency (1–5). In this sense, modern occidental societies consume high-fat high-sugar diets that promote the development of these diseases (3,6). Moreover, fruit intake patterns have also changed. In this sense, there is now commercial availability of fruits at any time of the year and from different geographical origins (7,8). Also, the consumption of non-ecological (conventional) fruits, chemically treated with pesticides, is generalised (9–11). Traditionally, fruits were produced, and hence, consumed, only during their natural harvest season, proceeded from nearby regions and were cultivated without any chemical treatment (organically) (7,12,13). The consumption of fruits has been linked with several health-promoting benefits (14–21), and this has been partially attributed to their phenolic content (18,22). Remarkably, many of these effects reduce the risk to develop chronic metabolic diseases such as MetS, obesity and associated pathologies (14–21). Importantly, the bioavailability and metabolism of fruit phenolic compounds are the main limiting factors for their bioactivity. In this sense, changes that affect the food matrix (i.e. fibre or polyphenol content of fruits) or host's physiology can involve changes in the bioavailability and metabolism of polyphenols (23). Therefore, the modern occidental changes in lifestyle can potentially condition the bioavailability and metabolism of fruit polyphenols. Thus, the study of the factors related with modern lifestyle patterns with a potential effect on the bioavailability and metabolism of fruit polyphenols is of relevance.

Given that the biological effects of fruit consumption have been partially attributed to their phenolic content (18,22), the full characterisation of the phenolics of fruits are required to stablish clear relationships between

fruit consumption and the associated health effects. Among the most freshly consumed fruits, apricots, sweet cherries, red grapes and sweet oranges stand out (24). These fruits present a traditional seasonality on their commercial availability and, hence, on their consumption. In this sense, apricots and cherries are traditionally produced during high-light exposure months, and grapes and oranges during low-light exposure months (12,25). Moreover the consumption of these fruits has been associated with several health effects (26–29). Some of these include the prevention, management and reduction of the risk to develop chronic diseases such as hypertension, diabetes and other pathologies associated with obesity (18,30–36). The phenolic profile of apricots, cherries, grapes and oranges has been widely studied (37–50). However, for a proper characterisation of the phenolic profile occurring in fruits, as well as other foods for that matter, specific and optimised extraction methodologies are required. The effect of extraction conditions on the extraction of phenolic compounds has been widely studied in different fruit and vegetal sources (51–68). In this sense, LSR, extraction solvent, extraction temperature, extraction time and number of extractions are among the factors with the greatest contribution on polyphenol extraction yield. Until now, different optimised extractions methodologies are available for the extraction of different fruit and other vegetal sources (51–57). Dietary guidelines recommend the consumption of whole fruits over their juices (21,69), and apricots, sweet cherries, red grapes and sweet oranges are among the most consumed fresh fruits (70). Despite that, most optimisation studies are intended for the revalorisation of the industrial wastes of these fruits (58–68). All in all, this evidences the need for specific and optimised extraction methods for the whole edible parts of these fruits.

Thus, we evaluated and optimised the extraction parameters that influence the extraction of phenolic compounds from the whole edible parts of apricots **[Manuscript 1]**, sweet cherries **[Manuscript 2]**, red grapes **[Manuscript 3]**, and sweet oranges **[Manuscript 4]**. The different optimal

extraction conditions significantly differed between seasonal fruits, confirming that the extraction of phenolic compounds in each fruit matrix requires specific conditions. For example, while the extraction of phenolic compounds required a high methanol proportion in the case of sweet orange polyphenols, middle proportions were required for the other studied fruits. The development of these methods allowed the detection, identification and full quantification of the phenolic compounds present in apricots, sweet cherries, red grapes and sweet oranges by HPLC-ESI-MS/MS **[Manuscripts 1 – 5]**.

The characterisation of the phenolic profile of edible parts of Charisma apricots **[Manuscript 1],** revealed higher quantities of phenolic compounds than in previous studies that used non-optimised and unspecific extraction methodologies (71–73). The phenolic profile of apricots was dominated by hydroxycinnamic acids and flavonols, and neochlorogenic acid and rutin were identified and quantified as the most abundant compounds.

By applying the optimised and specific sweet cherry extraction method, the phenolic composition of Royal Dawn sweet cherries was profiled for the first time. The results demonstrated that the most abundant phenolic compound of this specific variety was rutin instead of cyanidin-3-*O*-rutinoside **[Manuscript 2]**, usually the most abundant phenolic compound in sweet cherry varieties (74–76).

The developed method for red grapes revealed that grape variety and grape culture practise influence the phenolic profile of red grapes **[Manuscripts 3 and 5]**. Particularly, the differences were mainly quantitative rather than qualitative. Organic cultivation system modified the amount of phenolic compounds present in red Grenache grapes, which agrees with different studies in the literature (77–80) and further proves that phenolic compounds are plant stress metabolites (18,81). Red Grenache grapes presented the highest phenolic content and most diverse

phenolic profile of all fruits studied in this thesis. Precisely, organic red Grenache grapes (organic grapes, OG) presented higher concentrations of free flavanols and anthocyanins than their conventional counterparts, while those presented higher dimeric and trimeric concentrations of flavanols. Moreover, the fibre content of conventional red Grenache grapes (conventional grapes, CG) was a 1.6-fold higher than their organic counterpart.

The detection, identification and quantification of the phenolic compounds from sweet orange demonstrated that the phenolic content, but not type of phenolic compounds of sweet orange pulps, depended on geographical growing region **[Manuscript 4]**. These results are in agreement with studies evaluating the effect of geographical growing site on the phenolic content of different fruits (42,82). The phenolic profile of both sweet orange cultivars studied in this thesis was dominated by hesperidin, a potent cardioprotective flavanone (33,34,83). However, their content was a 1.6-fold higher in the cultivar grown in the northern hemisphere (northern oranges, NO) than the cultivar grown in the southern hemisphere (southern orange, SO). NO also reported higher concentrations of caffeic acid derivates and rutin.

All the edible parts of profiled fruits presented high amounts of phenolic compounds with relevant biological effects. However, although the profiling of the phenolic content of a fruit is essential to stablish a link between their phenolic content and the health benefits associated with its consumption, only characterising the fruit polyphenolic profile is not enough for such aim. In this sense, bioavailability and metabolism of phenolic compounds are important limiting factors for polyphenol's bioactivity (23,84). Also, many of the biological effects of polyphenol consumption are attributed to their metabolic products (85–88). Thus, the study of the bioavailability and metabolism of fruit phenolic compounds is of mandatory requirement. There are many factors that can affect the bioavailability of phenolic compounds, which include food matrix,

administration dose and administration length, as well as host's conditions that disrupt their homeostasis, such as MetS or hypertension (23,89–92). This suggests that the modern occidental lifestyle, characterised by a high prevalence metabolic diseases like obesity and changes in traditional fruit intake patterns (3–7), could condition the bioavailability and metabolism of fruit phenolic compounds. Therefore, in this thesis, the effect of organic fruit growing conditions, the consumption of fruits produced at different hemispheres, the consumption of seasonal fruits out of their traditional time of the year, and contribution of MetS on the bioavailability and metabolism of seasonal fruit phenolic compounds was evaluated.

The effect of growing conditions of Navelina sweet oranges and red Grenache grapes on the bioavailability and metabolism of their phenolic compounds was evaluated. Selection of these two fruits was encouraged by its high phenolic content, consumption patterns and their reported differences in phenolic composition depending on growing conditions **[Manuscript 3]** and **[Manuscript 4]**.

Currently, there is a wide commercial availability and consumption of fruits produced non-ecologically (conventionally), which involves their the treatment with pesticides (9–11). However, nowadays, consumption of organically produced fruits is becoming more popular (93). Considering that cultivation system modifies the phenolic content of fruits as previously evidenced for red Grenache grapes **[Manuscript 5]** it seems plausible that the bioavailability and metabolism of phenolic compounds is conditioned by the cultivation system. Thus, we evaluated the effect of organic cultivation system on the bioavailability and metabolism of phenolic compounds by using two different red Grenache grapes: OG and CG. To avoid any external factor that could affect the elucidation of this hypothesis, the selected grapes were from the same variety and were also produced in the same geographical region. Indeed, OG and CG were recollected on the same day (September the 26th, 2015) from contiguous vineyards in the region of Rasquera (Tarragona). Thus, agronomic factors

other than treatment with pesticides (i.e. water availability, soil mineral content or sun exposure) were equal between cultivars.

Therefore, in order to evaluate the effect of red grape cultivation system on the bioavailability and metabolism of their phenolic compounds, an acute intake study under normal housing conditions (light cycle of 12/12 of light/dark; L12) was conducted on adult male Wistar rats **[Manuscript 5]**. It should be highlighted that these grape varieties presented a similar phenolic profile, being the differences merely quantitative. The dose administered to the animals was 65 mg GAE/Kg bw, and its selection was encouraged by previous studies with grape seed flavanols that demonstrated that this dose allowed the detection and quantification of different phenolic metabolites (92). Specifically, this dose used in this study corresponded to the administration of 2.45 mg dw OG/Kg bw and 2.71 mg dw CG/Kg bw. The results of this study demonstrate that cultivation technique conditioned the serum kinetic behaviour of the metabolic products of red grape polyphenols. As a general rule, rats that consumed OG reached higher serum concentrations at 2 hours after grape administration than their counterparts. Remarkably, at this time point, the serum profile of both rat groups reported flavanol phase-II metabolites as the most abundant compounds. Thus, this evidence suggested a higher absorption of OG polyphenols in the small intestine. Contrarily, rats that consumed CG reached higher serum concentrations at 24 h after grape administration than rats that consumed OG. However, in both cases, the main phenolic metabolites were microbial-derived products. This suggests a higher arrival of CG phenolic compounds to the gut and a wider microbial metabolism. Although the type and amount of phenolic compounds present in OC and CG contributes to these effects, dietary fibre, found at higher concentrations in CG **[Manuscript 5]**, could also play an essential role in the modulation of the bioavailability and metabolism of red Grenache grape polyphenols. Indeed, dietary fibre can modulate the bioavailability of phenolic compounds by preventing and/or delaying their

small intestinal absorption, hence promoting a higher arrival of phenolic compounds to the colon (84,94,95).

The effect of organic cultivation system on the bioavailability and metabolism of red Grenache grape polyphenols was also evaluated in a chronic intake study **[Manuscript 7].** In both cultivars, a dose of 100 mg dried fruit/Kg bw was selected, and its selection was encouraged by considering the achievability of its human dietary consumption, which would correspond to eat a portion of fruit a day. Grape cultivation system also influences the bioavailability and metabolism of red Grenache grapes when consumed chronically for 10 weeks. Specifically, the total serum concentration was higher in the rats that consumed OG, as this fruit presented a higher phenolic content. Although the serum profiles of both consumption groups were dominated by microbial-derived metabolites, relevant changes were found in terms of phenolic metabolite families. For example, the contribution of benzoic acid derivates and end-products such as homovanillic acid and valerolactone compounds was reduced in rats that consumed CG. Therefore, a potential modulatory effect of the host microbiota, as well as other physiological factors, by the different cultivars should be considered. In this sense, the different fibre and polyphenol content and profile between the two grape cultivars, which are well-known factors to modulate the host microbiome (86,96,97), could differently modulate host's physiology, hence reporting different serum profiles.

The results using red Grapes of the same variety and same geographical growing region evidenced that the bioavailability and metabolism of red grape phenolic compounds is conditioned by grape cultivation system. Our results suggest that the health benefits associated with the consumption of a fruit variety could vary depending on their growing conditions. In this sense, the consumption of organically- or conventionally-produced grape juices reported different bioactivities in different experimental animal models (80,98). Therefore, the modern

tendency to consume conventional fruits over organic ones could involve relevant changes on the biological effects associated with their consumption.

Traditionally, people consumed fruits that were cultivated in the region in which they lived. However, in modern day societies, there is a huge availability of fruits produced from different regions, countries and even hemispheres (7,8). Importantly, this can affect the phenolic composition of fruits, which has been made evident in Navel sweet oranges **[Manuscript 4]**. Therefore, it is plausible that the consumption of fruits produced at different geographical regions involves a different bioavailability and metabolism of phenolic compounds. To emulate the commercial availability and consumption of fruits produced at different geographical regions on the same season of the year, Navel sweet oranges, produced either in the southern or the northern hemisphere were selected and purchased in September of 2015. SO were originally from Argentina and NO from Spain. Moreover, this selection was encouraged by the high consumption of this fruit and the wide commercial availability of sweet oranges produced at different geographical regions (8,24).

Therefore, the bioavailability and metabolism of sweet orange polyphenols was investigated in two different sweet orange cultivars, namely SO and NO **[Manuscript 6]**. The selected dose in this study was of 100 mg dw orange/Kg bw day for a period of 10 weeks, and this dose approximated to the consumption of a middle size peeled fresh orange a day in humans. Results indicate that the total amount of phenolics in sweet oranges modulates the total bioavailability of metabolic products in the serum of rats that consumed SO or NO. In this sense, the 1.6-fold higher content of flavanones in NO when compared to SO could explain the approximate 2-fold increase on total serum metabolite concentration of rats that consumed NO when compared to rats that consumed SO. This effect has been previously reported orange juice intake (99). Controversially, the consumption of SO involved a higher serum concentration of the native

compounds hesperidin and narirutin, being the changes especially relevant for hesperidin.

All in all, the evaluation of growing conditions, namely cultivation system and region of cultivation, demonstrated that these fruit-related factors associated with modern changes in fruit intake patterns condition the bioavailability and metabolism of red grape and sweet orange phenolic compounds. In the case of sweet oranges, although the metabolism of the orange phenolic compounds was conditioned by the consumption of one cultivar over another, growing conditions affected to a greater extent their bioavailability (serum total concentration). However, in the case of grapes, both the bioavailability and metabolism of their phenolic compounds was conditioned by cultivation system.

Global economy now allows the consumption of fruits out of their traditional consumption season (7). Moreover, humans undergo natural physiological changes in cycles of approximately a year that can significantly modulate the bioavailability and metabolism of phenolic compounds (100–117). For example, gut microbiota changes depending on the season of the year and so does the basal metabolic rate (109,115–117). Also, hormonal levels and kidney glomerular filtration rate present seasonal variations (109,111,112). The factor triggering these changes is none other than light exposure, which can be defined as the total amounts of day light a day (photoperiod) (113,118). All in all, this suggests that the consumption of fruits out of their traditional season could involve important changes in the bioavailability and metabolism of phenolic compounds. To evaluate this, two different fruits were used: Navel Sweet oranges **[Manuscripts 6]** and red Grenache grapes **[Manuscripts 7]**. The selection of these fruits was motivated by its marked traditional seasonal consumption. In this sense, grapes are typically produced, and hence, consumed, from September to December, and oranges from November to April (12). Moreover, these fruits are among the most freshly consumed fruits and their phenolic profile has been linked with important bioactive

functions such as anti-diabetic, anti-inflammatory, anti-oxidant and cardioprotective functions (24,30,83,119–121). To emulate seasonality in the consumption of fruits, three different photoperiods were used, which were L18, L12 and L6 (light hours/day). These photoperiod regimes represent the moments of the year with the highest, intermediate and lowest day light exposure patterns. Thus, L18 corresponds to the summer solstice, L6 to the winter solstice and L12 to the autumn and spring equinox. The animal model used was Fischer 344 rats, and its selection over conventional Wistar rats was encouraged by their sensitivity to photoperiod (122,123). In both fruits, a dose of 100 mg dried fruit/Kg bw was selected, and its selection was encouraged by considering the achievability of its human dietary consumption. Also, in both experimental designs, and considering the long-term supplementation with fruits, rats were sacrificed after 1 h of the last supplementation dose.

The traditional production of oranges ranges from November to April (12). Thus, the photoperiod conditions at which sweet oranges are traditionally consumed are emulated by the L12 and L6 light regimes. The bioavailability and metabolism of Navelina sweet orange polyphenols presented variability depending on photoperiod exposure **[Manuscript 6]**. In this sense, lower bioavailability of sweet orange polyphenols was found in the L6 groups. However, these changes in total bioavailability were most marked in the serum of rats consuming SO. This was mainly attributed to the lack of detection of the principal and microbial-derived metabolite from hesperidin (i.e. 3-(phenyl)propionic acid) (124). Specifically, 3-(phenyl)propionic was the dominating compound in all the experimental conditions, whit the exception of rats that consumed SO in the L6 photoperiod. Precisely, the 3-(phenyl)propionic acid concentration in both orange supplementation groups followed a L12>L6>L18, which agrees with a possible modulation of gut microbiota by the photoperiod (115,116). Although some changes in the presence or absence of phase-II metabolites were found depending on photoperiod exposure, those were

not maintained in SO and NO. In this sense, NO-supplemented rats reported a L6>L12>L18 tendency, while this type of metabolites were only found in SO-supplemented rats in the L6 photoperiod. Nevertheless, the serum concentrations of those metabolites that changed depending on photoperiod exposure were low. As for native compounds (hesperidin and narirutin) in serum, photoperiod was able to modulate their concentrations in SO-supplemented rats, reporting a L18>L6>L12 trend. Importantly, the serum bioavailability of orange phenolic compounds is known to reach their maximum concentrations at later time points (5 – 8 h) than the one used in this study (1 h) (89,99,125). Thus, further modulation of sweet orange polyphenol bioavailability and metabolism by photoperiod exposure at later time points should not be ruled out.

In the case of red Grenache grapes, which are a fruit typically consumed on a narrower season of the year (from September to December) than oranges, the bioavailability and metabolism of their phenolic compounds was demonstrated to be conditioned on photoperiod exposure **[Manuscript 7]**. The serum profile of rats that consumed OG or CG was dominated by microbial-derived metabolites. Considering that microbial.-derived metabolites appear at later times than 1 hour in serum after the administration of phenolic compounds (126), the microbial-derived metabolites found in this study must arise from the dose of the day before the sacrifice. When the effect of photoperiod exposure was evaluated, relevant changes in concentration and serum proportion were found between photoperiod groups. Considering that gut microbiota is responsible for amount and type of microbial-derived metabolites (86), our results are in agreement with the reported changes in gut microbiota depending on photoperiod exposure in animal models and humans (115–117). Morever, the generated serum profile in each photoperiod was conditioned by grape cultivar supplementation, as previously evidenced. Importantly, and regardless of the cultivation system, the highest bioavailability of red Grenache grapes was found in the L6 photoperiod,

which simulates low-light exposure months. Precisely, grapes are typically produced, and, hence consumed, between September and December, and November and December are the months with the lowest day light exposure (12,25). Therefore, our results evidence that the bioavailability of phenolic compounds is higher when red Grenache grapes are consumed at their traditional consumption time.

These results are also of special relevance when considering dietary recommendations. It has been evidenced that deaths by cardiovascular diseases are more predominant during low-light exposure months, and this has been linked with natural physiological changes associated with light exposure such as increases in blood pressure (102). Therefore, given the higher bioavailability of red Grenache grape phenolic compounds under low-light exposure conditions, which is a pre-requisite for their bioactivity, and the wide range of health-promoting effects associated with red grape polyphenol consumption (30,121), the consumption of this fruit should be encouraged during low-light exposure months. Contrarily, there seems not to be metabolically-fonder reason to promote the consumption of sweet oranges specifically during the low-light exposure months.

The study of polyphenol bioavailability and metabolism under homeostatic altered conditions like metabolic diseases such as MetS is of relevance as, precisely, those population groups can greatly benefit for the biological effects associated with fruit polyphenol ingestion (121). In this sense, grape polyphenols have been shown effective to reduce CVR, ameliorate insulin sensitivity and glucose metabolism, and reduce systemic inflammation, among other effects (30). The bioavailability and metabolism of CG polyphenols was evaluated in obesogenic conditions promoted by the consumption of cafeteria diet **[Manuscript 8]**. The selection of this fruit was encouraged by three different reasons. First, CG represents the modern tendency to eat conventional fruits over fruits produced organically (9–11). Second, this fruit presents a marked traditional consumption which includes November and December, which

are the months when more CV deaths are produced (12,102). Third, their content in phenolic compounds is high, and their representatives have reported important bioactivities to prevent and/or manage the development of pathologies associated with MetS and obesity, such as hypertension and diabetes (30,121,127). In order to emulate the current obesogenic dietary pattern, cafeteria diet was chosen. This dietary intervention supplies the animals with food rich in fat and sugar in excess so rats can consume what they prefer, thus simulating human behaviour in food choice (128). More importantly, this diet in known to trigger obesity and MetS, which are predominant pathologies in the modern occidental society (127,129).

The bioavailability and metabolism of conventional red Grenache grapes was altered by cafeteria diet consumption. Remarkably, no phase-II flavan-3-ol metabolites were found, which is in agreement with the 2-hour-delay on their plasma appearance in grape seed flavan-3-ols administration studies to cafeteria-diet-fed rats (90). Total serum concentrations were significantly lower in the L12 and L18 metabolically altered rats when compared to their standard counterparts, demonstrating that health state can modulate polyphenol bioavailability under exposure to different photoperiods. Moreover, the phenolic profile, although dominated by microbial-metabolites, was also significantly different between standard and cafeteria diet consumption. Importantly, rats under the L6 group achieved similar total serum concentrations that their standard counterparts. However, the contribution of the metabolic families was completely different, and the benzoic acid derivates represented a significantly higher proportion of all metabolite in obese rats. This is in agreement with the fact that dietary obesogenic interventions can modulate gut microbiota (130). Hence, this results evidence that health status can modulate the bioavailability and metabolism of red Grenache grape polyphenols. Whether or not these serum profile changes involve different biological activities remains unknown. However, previous studies

in spontaneously hypertensive rats demonstrate that both the plasma an aorta distribution of phenolic metabolites from grape seeds differs from their normotensive counterparts, and this is also involves an anti-hypertensive effect or not (91).

Regardless of dietary intervention or grape cultivar supplementation, the groups under the L6 photoperiod reported the highest serum metabolite concentration. This result should be remarked as the systemic bioactive effects of phenolic compounds highly depend on their bioavailability (84). Moreover, it is during winter when humans are placed at a higher CVR (102). Therefore, this study not only demonstrates for the first time that the bioavailability and metabolism of red grape polyphenols is conditioned by photoperiod exposure but also suggests a potentially higher bioactivity associated with the consumption of whole red grapes during low-light exposure months. However, functional studies should be performed to corroborate whether the changes in the serum profile involve a different bioactivity, and specifically focusing on CVR factors such as vascular function, blood lipid profile or blood glucose levels.

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MODERN CHANGES IN FRUIT INTAKE PATTERNS MODULATE THE BIOAVAILABILITY AND METABOLISM OF FRUIT PHENOLIC COMPOUNDS IN RATS
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CONCLUSIONS:

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CONCLUSIONS

1. A full characterisation by HPLC-MS/MS of the edible parts of Charisma apricots after a specific optimized extraction showed that higher quantities of phenolic compounds were extracted than with other methods in the literature. Apricots were found rich in flavonols and hydroxycinnamic acids.

2. By applying the optimised and specific sweet cherry extraction method, the phenolic composition of Royal Dawn sweet cherries was profiled for the first time by HPLC-MS/MS. The results demonstrated that the most abundant phenolic compound of this specific variety was rutin instead of cyanidin-3-*O*-rutinoside.

3. The specific developed method for the extraction of red grapes polyphenols revealed that grape variety and grape culture system influence the phenolic profile of red grapes. Particularly, the differences were mainly quantitative rather than qualitative. Organic cultivation system modified the amount of phenolic compounds present in red Grenache grapes, presenting the highest phenolic content and most diverse phenolic profile of all fruits studied in this thesis. All three grapes studied in this thesis were rich in flavanols and anthocyanins.

4. The detection, identification and quantification by HPLC-MS/MS of the phenolic compounds from sweet orange demonstrated that the phenolic content, but not type of phenolic compounds of sweet orange pulps, depended on geographical growing region. Specifically, the total polyphenol content of Navelina sweet oranges grown in the northern hemisphere was higher than the one found for Navelina sweet oranges grown in the southern hemisphere.

However, the phenolic profile of both sweet orange cultivars studied in this thesis was dominated by hesperidin, a potent cardioprotective flavanone.

5. The evaluation of fruit growing conditions, namely cultivation system and region of cultivation, demonstrated that these fruit-related factors associated with modern changes in fruit intake patterns condition the bioavailability and metabolism of red Grenache grape and Navelina sweet orange phenolic compounds.
    - Organic grapes presented a higher oligomeric and trimeric flavanol and lower fiber content than their conventional counterparts. This different composition of organic grapes conditions the bioavailability and metabolism of red grape polyphenols. Specifically, organic red grapes showed a higher phenolic phase-II and lower microbial-derived metabolism metabolites than non-ecological (conventional) counterparts.
    - Geographical production site conditioned the bioavailability and metabolism of sweet orange polyphenols by modulating the phenolic content of Navelina sweet orange polyphenols. Rats consuming Navelina sweet oranges from the northern hemisphere reported a higher serum bioavailability of orange polyphenols, but rats consuming Navelina sweet oranges grown in the southern hemisphere reported higher bioavailability of native hesperidin in serum.

6. The intake of red Grenache grapes and Navelina sweet oranges out of their traditional consumption season conditions the bioavailability and metabolism of their phenolic compounds.

- Photoperiod exposure is able to condition the bioavailability and metabolism of Navelina sweet orange polyphenols by changes in 3-(phenyl)propionic and phase-II metabolites
- Photoperiod exposure is able to condition the bioavailability and metabolism of red Grenache grape phenolic compounds mainly by changes in microbial-derived metabolites.
- The intake of specific grape cultivars conditions the effect of photoperiod exposure on the bioavailability and metabolism of red grape polyphenols.
- The highest bioavailability of red grapes was under the L6 photoperiod, which demonstrated higher bioavailability of red grape polyphenols under their traditional consumption season.

7. The alteration of the metabolic and homeostatic state by cafeteria diet influenced the bioavailability and metabolism of Red grape polyphenols.
    - The bioavailability of red Grenache grape polyphenols was lower under disrupted homeostatic control triggered by cafeteria diet, especially under L18 and L12 conditions.
    - Alteration of host's homeostasis by cafeteria diet involved a change in the prevalence of specific microbial-derived metabolic families and the lack of detection of phase-II metabolites.
    - The bioavailability and metabolism of red grapes in healthy and metabolically-altered rats was conditioned differently by photoperiod exposure.

- The bioavailability of red grape polyphenols under alteration of metabolic state by cafeteria diet was higher under light exposure conditions that simulate the traditional grape consumption season (L6).

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