UNIVERSITAT de BARCELONA

Simultaneous determination of phenolic compounds in table olives and plasma by LC-MS/MS. Pharmacokinetic parameters in rats and humans after the consumption of Arbequina variety

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Barcelona, 2020

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Thesis presented by **Ivana Kundisová** to obtain the title of Doctor from the University of Barcelona.

This thesis has been carried out in the group of Experimental Physiology and Nutrition under the supervision of Dr. **Joana Maria Planas Rosselló** and Dr. **Maria Emília Juan Olivé**.

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INFORM THAT:

The thesis entitled *"Simultaneous determination of phenolic compounds in table olives and plasma by LC-MS/MS. Pharmacokinetic parameters in rats and humans after the consumption of Arbequina variety"*, presented by **Ivana Kundisová** to obtain the title of Doctor, was performed under our supervision, and considering it concluded, we authorize her presentation to be judged by the corresponding Commission.

For the corresponding purposes, we hereby sign in Barcelona, December 17th, 2020

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AUC	Area under the plasma concentration time curve
AUC$_{last}$	Area under the concentration curve from time time zero to the last quantified concentration
AUC$_{0-\infty}$	Area under the plasma concentration curve from time zero extrapolated to infinity
AUC$_{extrap\%}$	Percentage of the total AUC, represents the percentage of AUC$_{0-\infty}$ from T$_{last}$ to infinity
BSA	Body surface area
BMI	Body mass index
CE	Collision energy
CEIC	Comité Ético de Investigación Clínica
C$_{max}$	The maximum plasmatic concentration
CXP	Collision cell exit potential
CV	Coefficient of variation
DAD	Diode array detector
3,4-DHPA	3,4-dihydroxyphenylacetate
3,4-DHPEA-EA	Hydroxytyrosol
DMAPP	Dimethylallyl diphosphate
DP	Declustering potential
E4P	Erythrose 4-phosphate
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EMA	European Medicines Agency
EP	Entrance potential
ESI	Electrospray ionization source
FIA	Flow injection analysis
FDA	Food and drug administration
FTICR	Fourier transform ion cyclotron resonance
GPC	Gel permeation chromatography
GPP	Geranyl diphosphate
HL lambda z - t1/2z	The apparent elimination half-life

Abbreviations

4-HPA	4-Hydroxyphenylacetaldehyde
p-**HPAA**	*p*-Hydroxyphenylacetic acid
p-**HPEA**	Tyrosol
p-**HPPA**	Hydroxyphenylpyruvic acid
IPP	Isopentenyl diphosphate
Lambda z – λz	The terminal elimination rate constant
L-DOPA	3,4-Dihydroxyphenylalanine
LC	Liquid chromatography
LC-ESI-MS/MS	Liquid chromatography – electrospray ionization - tandem mass spectrometry
LOQ	Limit of quantification
MS	Mass Spectrometry
MEP	2-C-methyl-d-erythrodiol 4-phosphate
MRM	Multiple reaction monitoring
MRT$_{last}$	The mean residence time, from time zero to the last measurable concentration
MRT$_{0-\infty}$	The mean residence time, from time zero to infinity
MVA	Mevalonate
MVAPP	Mevalonate diphosphate
m/z	Mass/charge ratio
PEP	Phosphoenolpyruvate
RSD (%)	Relative standard deviation
RT	Retention time
SGLT1	Sodium glucose cotransporter 1
T$_{max}$	The time when Cmax is reached
UHPLC	Ultra-high-performance liquid chromatography
UPLC	Ultra pressure liquid chromatography
UV	Ultraviolet

SUMMARY OF THE THESIS

Summary

Table olives are an important source of phenolic compounds, whose beneficial health effects have been widely demonstrated. Therefore, the present thesis aims to assess the different phenolic compounds in Arbequina table olives and their determination in plasma of rats and healthy human volunteers after the consumption of this food. Firstly, the composition of Arbequina table olives was analysed by LC-ESI-MS/MS. In total, 16 phenolic compounds were determined (1.0 g/kg), being hydroxytyrosol, luteolin and verbascoside the most abundant ones (~90%). Subsequently, a new method based on liquid-liquid extraction followed by LC-ESI-MS/MS analysis was developed. Since the validation gave suitable results for linearity, sensibility, accuracy, precision and recoveries, the method could be applied for pre-clinical studies. Hence, Sprague-Dawley rats were orally administered with table olives at 3.85 and 7.70 g/kg. Out of 16 polyphenols found in table olives, only 7 compounds were determined in rat plasma (*p*-coumaric acid, hydroxytyrosol, luteolin, luteolin-7-*O*-glucoside, salidroside, tyrosol, and verbascoside) from whose concentrations pharmacokinetic parameters were evaluated. Results indicated relatively fast absorption from 30 min up to 1 h for all the compounds. At both doses the highest AUC was achieved by *p*-coumaric acid, followed by hydroxytyrosol, and salidroside. Hydroxytyrosol underwent extensive metabolism, being two sulfates the most abundant (~86%), followed by hydroxytyrosol (~10%) and two glucuronides (~4%). Half-life was ranging from 1 h for salidroside up to 5 hours for tyrosol. Then, the first part of clinical trial was performed with human volunteers that received 60 and 120 olives. The method validated in human plasma with satisfactory results allowed the determination of 6 compounds (vanillic acid, hydroxytyrosol, salidroside, luteolin, verbascoside, and hydroxytyrosol acetate) and subsequently the pharmacokinetics was evaluated. Tmax was ranging from 30 min up to 90 min. The highest AUC was reached by vanillic acid, followed by hydroxytyrosol acetate, and hydroxytyrosol. Hydroxytyrosol had similar extensive metabolism as in rats, when two sulfates were the most abundant (~85%), followed by hydroxytyrosol (~10%) and two glucuronides (~5%). Half-life was ranging from 4 h for luteolin, salidroside and hydroxytyrosol acetate up to 7 hours for hydroxytyrosol and verbascoside. Finally, the second part of clinical trial when human volunteers daily ingested 60 olives for 30 days was performed. In total, 9 phenolic compounds were determined. No significant differences were observed within the plasmatic concentrations of vanillic acid, catechol, quercetin, salidroside, apigenin, whereas the plasmatic concentrations of *p*-coumaric acid, hydroxytyrosol (together with two sulfates), luteolin, and hydroxytyrosol acetate increased over time with repeated intake of Arbequina table olives. In conclusion, the present thesis expanded the knowledge about the pharmacokinetics of phenolic compounds contained in Arbequina table olives in rats and humans after their oral administration that might provide a basis for the future use of table olives as a functional food.

Resumen

Las aceitunas de mesa constituyen una fuente importante de compuestos fenólicos, cuyos efectos beneficiosos para la salud han sido ampliamente demostrados. Por tanto, la presente tesis tiene como objetivo evaluar los diferentes compuestos fenólicos presentes en la aceituna de mesa de la variedad Arbequina, así como su determinación en plasma de rata y de voluntarios humanos sanos tras el consumo de este alimento. En primer lugar, se analizó la composición de la aceituna de mesa Arbequina mediante LC-ESI-MS/MS. En total se determinaron 16 compuestos fenólicos (1,0 g/kg), siendo hidroxitirosol, luteolina y verbascósido los más abundantes (~90%). A continuación, se desarrolló un nuevo método basado en extracción líquida y posterior análisis mediante LC-ESI-MS/MS. Dado que la validación arrojó resultados adecuados en cuanto a linealidad, sensibilidad, exactitud, precisión y recuperaciones, el método podría aplicarse a estudios preclínicos. Por consiguiente, se administró por vía oral aceitunas de mesa a razón de 3,85 y 7,70 g/kg a ratas Sprague-Dawley. De los 16 polifenoles hallados en las aceitunas de mesa, solo se determinaron 7 de ellos en plasma de rata (ácido *p*-cumárico, hidroxitirosol, luteolina, luteolina-7-*O*-glucósido, salidrosida, tirosol y verbascósido), a partir de cuyas concentraciones se evaluaron los parámetros farmacocinéticos. Los resultados indicaron una absorción relativamente rápida, desde 30 min hasta 1 h, para todos los compuestos. En ambas dosis, el AUC más alto se obtuvo para ácido *p*-cumárico, seguido de hidroxitirosol y salidrosida. El hidroxitirosol se metabolizó extensivamente, siendo dos sulfatos los más abundantes (~86%), seguidos de hidroxitirosol (~10%) y dos glucurónidos (~4%). La vida media osciló entre 1 h para salidrosida y 5 horas para tirosol. Posteriormente, se realizó la primera parte del ensayo clínico con voluntarios humanos que recibieron 60 y 120 aceitunas. El método, validado en plasma humano con resultados satisfactorios, permitió la determinación de 6 compuestos (ácido vanílico, hidroxitirosol, salidrosida, luteolina, verbascósido y acetato de hidroxitirosol) y seguidamente se procedió a evaluar la farmacocinética. El Tmax osciló entre 30 min y 90 min. El AUC más alto lo alcanzó el ácido vanílico, seguido del acetato de hidroxitirosol y el hidroxitirosol. El hidroxitirosol tuvo un metabolismo extenso similar al de las ratas, donde dos sulfatos fueron los más abundantes (~85%), seguidos de hidroxitirosol (10%) y dos glucurónidos (~5%). La vida media fluctuó entre 4 h para luteolina, salidrosida y acetato de hidroxitirosol; hasta 7 horas para hidroxitirosol y verbascósido. Finalmente, se realizó la segunda parte del ensayo clínico donde voluntarios humanos ingirieron diariamente 60 aceitunas durante 30 días. En total, se determinaron 9 compuestos fenólicos. No se observaron diferencias significativas dentro de las concentraciones plasmáticas de ácido vanílico, catecol, quercetina, salidrosida y apigenina; mientras que las concentraciones plasmáticas de ácido *p*-cumárico, hidroxitirosol (junto con dos sulfatos), luteolina y acetato de hidroxitirosol sí aumentaron con el tiempo con la ingesta repetida de aceitunas de mesa Arbequina. Concluyendo, la presente tesis amplió el conocimiento sobre la farmacocinética de los compuestos fenólicos contenidos en la aceituna de mesa Arbequina en ratas y humanos tras su administración oral, lo que podría sentar las bases para el futuro uso de la aceituna de mesa como alimento funcional.

I. INTRODUCTION

1.1. *OLEA EUROPAEA* L.

Olea europaea L. belongs to the family Oleaceae that includes approximately 30 genera and 600 species. The wild olive (*Olea europaea* subsp. *europaea* var. *sylvestris*) and the cultivated olive (*Olea europaea* subsp. *europaea* var. *europaea*) are the two forms that have been described for this species (Uylaser *et al*., 2014).

Olea europaea L. native to Minor Asia and Syria is nowadays widely cultivated in the entire Mediterranean area for the production of olive oil and table olives. The tree is normally widespread in the areas characterized by dry and hot summers, like coastal areas of the eastern Mediterranean, neighboring coastal areas of south-eastern Europe, western Asia, northern Africa and northern Iran. Although olive is now cultivated in several parts of the world, the Mediterranean region still stands out as the major production area that is accounting for about 98% of the world's olive cultivation (Ghanbari *et al*., 2012, Cabrera-Bañegil *et al*., 2017).

According to estimates, the cultivation of olive tree dates back to the years between 5000 and 1400 BC in the Mediterranean basin. Archaeological evidence indicates that the origin centers where olives were cultivated were in Syria, Israel, Lebanon, Crete and Cyprus. In the beginning of the 20th century BC, paintings showing people consuming olives and using olive oil for cooking and as fuel in lamps were found in Minoan Palace of Kossos in Crete. Many archaeologists still believe the successful trade in olive oil may have been the source of the wealth of the Minoan kingdom (3500-1000 BC) (Kostelenos and Kiritsakis, 2017). Cultivation of olive tree was spread all around Greece. In the 7th century BC, winners of the Olympic Games held in Greek city Olympia were awarded by crowning wreaths (kotinos) that were made of wild olive tree branch. Later (around year 600 BC), olive tree was brought to Italy and to other Mediterranean countries. Olive cultivation was expanded to the entire Roman Empire under occupation by Rome. Romans were the ones that invented hydraulic press (screw press) for processing the olive fruit. Greeks, Romans and Arabs probably brought the olive tree to Spain (Kostelenos and Kiritsakis, 2017).

The olive tree was after widely cultivated in southern Europe. Early Spanish settlers along with the missionaries introduced the olive tree to the newly discovered America, where the olive trees were grown in the past only in restricted areas in Chile, Argentina and California, areas with similar climatic conditions as in the Mediterranean (Kapellakis *et al*., 2008). The trees were first planted in California around 1800 AD. In the 1930s and 1940s, many Californian olive groves were planted for table olives production (Kostelenos and Kiritsakis, 2017). On the other hand, in the 1956 the olive tree was introduced to China for its further cultivation, when Albania sent 30 olive trees as present that were planted in different cities. Seven years later, in 1963, first introduction and cultivation of olive trees grown in modern China was reported to be in the Nanjing botanical garden of Zhongshan (Su *et al*., 2018).

I. Introduction

In modern time, *Olea europaea* L. has been spread all over the world and olive trees are now being grown in about 30 countries for commercial purposes. Nowadays, the Mediterranean area, their ancient home, has the largest number of olive trees and is still considered as the main source of olive oil in the world. The famous olive tree ´Vouves´, being one of the oldest olive trees in the world with age estimated to 2000-3000 years, is still producing some olive fruit and it is located on island Crete (Ghanbari *et al.*, 2012, Kostelenos and Kiritsakis, 2017)

The olive tree has a long history due to its medicinal and nutritional properties. The leaves were already used in ancient Egypt for the mummification of the pharaohs. They were applied also as a folk remedy in the treatment of tropical diseases, such as malaria, and also in the treatment of fever (Ghanbari *et al.*, 2012).

In the context of religious importance, olive tree together with its fruit are mentioned also in the story of the flood in the Old Testament in which Noah released a dove that came back holding an olive branch. It was considered a sign of receding water and a symbol restoring peace between God and human beings (Kapellakis *et al.*, 2008, Kostelenos and Kiritsakis, 2017).

1.2. ARBEQUINA VARIETY

The most important expansion of olive growing on the Catalan coast dates back to the end of the 8th century, when Christians conquered territories from Muslims. Despite the decline in recent years, in Catalonia the olive tree that represents 15% of its agricultural area maintains the first place among the woody trees. A total of 113,069 hectares out of which more than 80% were dry land were registered in 2017. The same year, 33,607 tons that accounted to 99% of the harvested olives of five varieties, namely Arbequina, Morruda, Sevillenca, Empeltre and Farga were used for production of olive oil (Generalitat de Catalunya, 2017, Ninot *et al.*, 2019). The

Figure 1.1. Location of the regions of Catalonia with the most cultivated area of olive trees.

regions with the most cultivated area of olive trees are Garrigues and Baix Ebre, followed by Montsià (Figure 1.1). Nowadays, many varieties are replaced by the Arbequina variety, which, at present occupies more than 50% of the Catalan olive grove area.

Origin of this cultivar is in municipal district Arbeca (Lleida, Catalonia, Spain), where it was grown for the first time. From Arbeca, this variety has spread to different parts around the world and it has become one of the main Spanish olive varieties known in the international

market with an estimated 60000ha planted worldwide, where is consumed as olive oil and in minor proportion as table olives. The reason of this expansion is its frost resistance, adaptability to different climatic and soil conditions, low vigour and high productivity. Moreover, it is not sensitive to the olive fly because the small size and small volume (weight of approximately 1.9 g) of the fruit does not favour the female choosing it to lay eggs (Ninot *et al*., 2019). This reduced size makes mechanical harvesting impossible, but despite this, it is highly regarded because the tree produces a huge amount of olive fruit. The content of oil that is dense and fluid in the olive fruit represents around 20% (Criado *et al*., 2004, Bakhouche *et al*., 2013, Ninot *et al*., 2019). Arbequina table olives are processed as natural green olives. Olives are collected just before they start to change the colour from green to turning colour. A large part of the olives can be harvested as change to ripe, depending on the weather conditions. The final commercial Arbequina table olives can differ in colour, form and other sensorial aspects, due to the variability of processing. In general terms, their desirable characteristics are green/light brown colour, round shape, small size, firm texture, slight acid and bitter taste. The bitter taste is due to the fact that oleuropein is still present in the olive in a certain amount (Hurtado *et al*., 2009).

1.3. THE OLIVE

The fruit of *Olea europaea* L. is the olive, an oval-shaped drupe. Depending on the variety, the typical size is 2–3 cm and the weight is ranging 2-12g, however some varieties may weigh up to 20 g. Structurally, the olive is formed by three anatomically different parts: skin (epicarp), pulp/flesh (mesocarp) and stone (endocarp) that contains the seed (Figure 1.2). All three parts have an influence on the final product. Skin that forms 1.0-3.0% of the olive fruit weight has a protective function against external attacks and it consists mainly of cellulose and cutin. Epicarp is covered by a layer of wax that represents 45-70% of the skin. At the beginning of development, the skin is green because of the chlorophyll content. With time, due to the different concentrations of chlorophylls, carotenoids and anthocyanins, which are the main pigments in olives, the color changes to pale-green, raw yellow, pink, purple-pink and black. Pulp forms the mayor part of the olive and together with skin represent 70-80% of whole fruit. Finally, endocarp that is formed by kernel and woody shell represents 18-22% of the olive weight. The oil content of the seed is 2–4 g oil /100 g (Bianchi, 2003, Ghanbari *et al*., 2012, Conte *et al*., 2020).
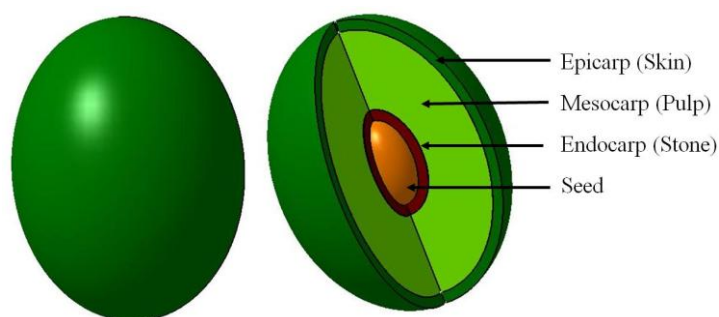
Figure 1.2. The structure of fruit of *Olea europaea* L.

I. Introduction

Olives have a characteristic bitterness which decreases with maturation when the color of the olives changes from green to light-yellow, purple-red and purple-red (Conte *et al.*, 2020). The growth and ripening of olive fruit usually takes around 5 months. The time of harvest of olive cultivars for processing into table olives depends on many factors. Climatic conditions, cropping, amount of pulp, color and olive size must by taking into consideration (Ghanbari *et al.*, 2012).

1.4. TABLE OLIVES

Table olives are the fruits of the *Olea europaea* L. that have been processed to be able to be consumed, fulfilling the descriptions about types, trade preparations and styles and with the essential composition and quality criteria established by the International Olive Council Standard for table olives (Boskou, 2017, IOC, 2004).

Together with olive oil belong among important components of the Mediterranean diet, considered to be one of the healthiest due to its strong ability to reduce some chronic diseases, such as cardiovascular diseases and certain cancers (Charoenprasert and Mitchell, 2012, Ghanbari *et al.*, 2012, Romani *et al.*, 2019). Table olives are extremely rich in compounds exerting biological activities, thus they are considered as "nutraceuticals" that were described by Stephen De Felice in 1989 as "Naturally derived bioactive compounds found in foods, dietary supplements and medical products with health benefits including prevention and/or treatment of disease" (Accardi *et al.*, 2016). Besides providing an important nutritional value, the table olives are important product for national economics (Accardi *et al.*, 2016). In crop year 2018/2019, the total world production of table olives reached 2.569.000 tones, of which 822.000 tones (31%) corresponded to European production. Spain, Greece and Italy together represented 31% of the World and 99% of the European table olives annual production, respectively (Figure 1.3).
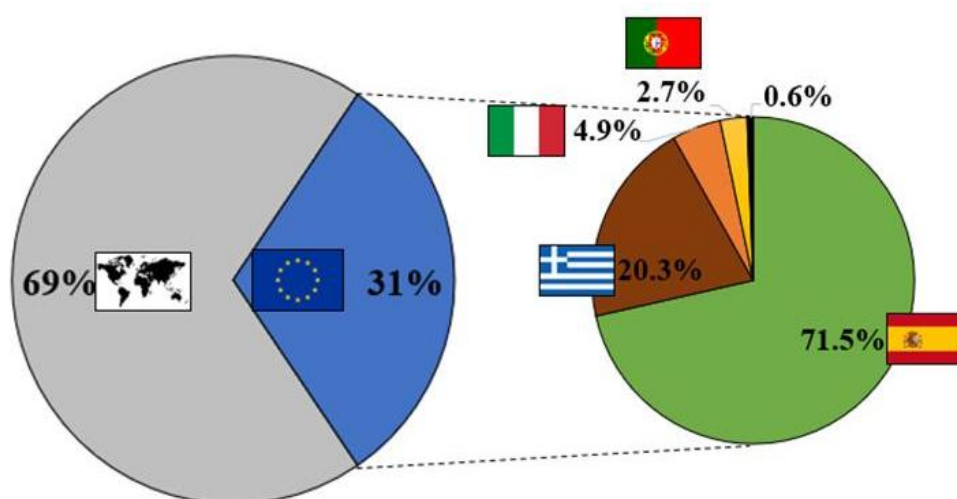
Figure 1.3. World and European table olives annual production in crop year 2018/2019.

Among Europe, Spain was the main producer of table olives with 71.5% that was followed by Greece (20.3%), Italy (4.9%) and Portugal (2.7%) (Figure 1.3). The main exporting countries of table olives to non-EU were Spain (62.7%), followed by Greece (25.1%), Portugal (4.3%), Italy (5.2%) and France (0.6%). In addition, Spain, Italy, France, Greece and Portugal consumed 32.3%, 15.5%, 12.2%, 2.8%, and 1.0% that together represented 64% of consumption in the EU that accounted for 576.000 tons of table olives (IOC, 126 English edition, Cabrera-Bañegil *et al.*, 2017, Durante *et al.*, 2017).

Table olives are classified according to their degree of ripeness into green olives - collected during the ripening period, before the coloring, after reaching the normal size; olives turning color - harvested before the ripening period, at color change; and black olives - harvested when drupes are fully ripe or slightly before full ripeness is reached. Olives can be also classified on the basis of trade preparation (treated olives, natural olives, dehydrated and/or shriveled olives, olives darkened by oxidation, specialties) or styles (whole, stoned (pitted), stuffed, salad and others) (IOC, 2004).

In general, table olives are prepared from varieties that contain oil at low concentrations, since its high levels may damage the consistency and preservation of the processed fruit (Bianchi, 2003, Conte *et al.*, 2020).

1.5. PROCESSING OF TABLE OLIVES

Natural olive fruits have a bitter flavor, and they are inedible due to high content of a glucoside called oleuropein that is formed by glucose, oleanolic acid, and the o-diphenol hydroxytyrosol. The level of oleuropein decreases with maturation and the concentration of this secoiridoid glucoside depends on variety, irrigation, and degree of ripening (Garcia *et al.*, 2008). There are many ways how to make olive fruit palatable, although from economic point of view, there are three main procedures to process the table olives, namely Spanish-style (pickled) green olives in brine, Californian-style (pickled) black olives in brine and Greek-style naturally fermented black olives in brine (Pereira *et al.*, 2006, Garcia *et al.*, 2008, Ghanbari *et al.*, 2012, D´Antuano *et al.*, 2016). Fruits intended for Spanish green and California black olive types are harvested before full maturity is reached, with a greenish-yellow color possessing a strong bitter taste. In production of green and black olives, taste debittering is based on a treatment of the olives with aqueous solution of sodium hydroxide (Garcia *et al.*, 2008, Tufariello *et al.*, 2016).

During the Californian process, olives are kept in brine solution usually for 2-6 months, with acidification to pH 4 with lactic and acetic acids and stored in anaerobic/aerobic conditions. Lactic acid is considered a key step in spontaneous fermentation processes, since in addition to remove the rest of the bitter taste, it also lowers the pH of the brine, which prevents the growth of pathogenic microorganisms and improves the taste and texture characteristics of the final product (Pereira *et al.*, 2006, Charoenprasert and Mitchell, 2012).

I. Introduction

On the other hand, in the Spanish style, green olives are treated with 1.5-4.5% alkaline solution of sodium hydroxide. The olives are rinsed to remove the alkali and placed into brine for several months where they undergo lactic fermentation. In addition, during the process, a big volume of heavily contaminated wastewaters is generated, not only the alkaline solutions but also the further washing waters used to remove the excess alkali from the olive flesh. Because of that, the use of sodium hydroxide is forbidden in many countries (Garcia *et al*., 2008, Charoenprasert and Mitchell, 2012, Ghanbari *et al*., 2012, Tufariello *et al*., 2016, Conte *et al*., 2020).

The table olives used in this thesis were processed by the Greek-style. For Greek-style, the color of the olives can be deep violet, greenish black, reddish black, violet black or deep chestnut, depending on the variety. Olives are placed into 6-14% solution of sodium hydroxide. The addition of acid may prevent the growth of microorganisms. During spontaneous fermentation, oleuropein is degraded. This process may take up to 6-9 months. After the olives have fermented, they are placed to air to obtain the skin color. Then, olives are packed in fresh brine with pH from 3.6 to 4.5 and with the chloride content of around 8−10%. Sorbic acid, potassium sorbate, or pasteurization are used for the preservation (Charoenprasert and Mitchell, 2012).

1.6. COMPOSITION OF TABLE OLIVES

The average composition of olive fruit consists of water (50%); fat (22%); carbohydrates (19.1%); cellulose (5.8%); and proteins (1.6%) (Ghanbari *et al*., 2012). They are also a good source of beneficial fatty acids, especially monounsaturated fat, such as oleic acid (Bianchi *et al*., 2003). Moreover, table olives also contain minerals - calcium, iron, potassium, magnesium, phosphorus, natrium, and selenium; essential amino acids; microelements – manganese, copper, zinc; vitamins - B-complex (thiamin, niacin, pantothenic acid, vitamin B6), pro-vitamin A (betacarotene), vitamin E (13-52 mg/kg). Table olives also provide high dietary fiber content (2.5-5%) and group of minor compounds that includes pentacyclic triterpens and phenolic compounds that are responsible for various biological activities. Organic acids, pigments and pectin are also present in olive fruit. The distribution and structure of the chemical substances strongly depend on the following factors: variety of the olives, geographical origin, cultivation practices and maturation (Bianchi *et al*., 2003, Ghanbari *et al*., 2012, Boskou, 2017).

1.6.1. Phenolic compounds

Although phenolic compounds form only 1-3% of the total olive composition, they play a very important role in human health (Ghanbari *et al*., 2012). They are secondary plant metabolites which serve as a defense mechanism against pathogens and herbivores. Polyphenols are characterized by the presence of at least one hydroxylated aromatic ring in their chemical structure. The largest quantities of phenols are located around the seed and in the skin of the olive. More than 36 structurally different olive oil polyphenols have been identified until now and their structure can range from simple monophenolic to more complex

phenolic substances containing multiple aromatic rings. Modified sugars are often more complex phenolic substances (glycosides). Due to the sugar moieties and multiple hydroxylation sites, most of phenolic compounds are water soluble. The most common classification for the phenolic compounds is into non-flavonoid and flavonoid polyphenols (Figure 1.4) (Romero *et al.*, 2004a, Charoenprasert and Mitchell, 2012, Działo *et al.*, 2016, Durrazo *et al.*, 2019).
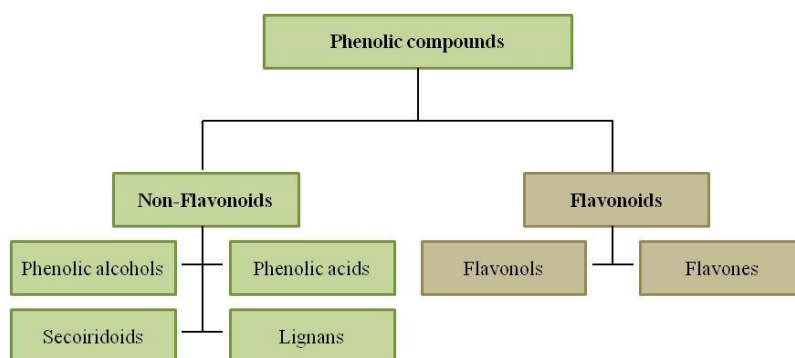
Figure 1.4. Phenolic compounds in *Olea europaea* L. divided into non-flavonoids and flavonoids (Działo *et al.*, 2016).

1.6.1.1. Synthesis of phenolic compounds

Polyphenols form one of the largest groups of secondary metabolites of plants. This group includes substances with various structures, from simple aromatic substances, to more complex ones, such as secoiridoids. For this reason, various pathways are involved in the synthesis of polyphenols in *Olea europaea* L., which act as an interconnected network. These include the 2-C-methyl-d-erythrodiol 4-phosphate (MEP), mevalonate (MVA), shikimate and phenylpropanoid pathways (Figure 1.5 and Figure 1.6).

1.6.1.1.1. Synthesis of the secoiridoids

The complex mechanism of oleuropein biosynthesis in *Olea europaea* L. is still not well understood (Gutierrez-Rosales *et al.*, 2012). In plants, in separate compartments, there are two pathways that lead to the synthesis of isoprenoid precursors, 2-C-methyl-d-erythritol 4-phosphate (MEP) and mevalonic acid (MVA) (Figure 1.5).

Enzymes for the MEP are found in plastids, whereas the ones for MVA pathway are present in cytosol. It has been suggested that oleuropein, a typical secoiridoid of the Oleaceae family is biosynthesized from mevalonic acid (MVA) via a complex metabolic pathway (Alagna *et al.*, 2012, Tetali, 2018).

Secoiridoid biosynthesis begins with isopentenyl diphosphate (IPP). During synthesis, geranyl diphosphate (GPP) is formed from IPP. GPP serves as a substrate for the formation of geraniol, from which deoxyloganic acid is formed. A plausible biosynthetic route from deoxyloganic acid, 7-epiloganic acid, 7-ketologanic acid, 8-epikingisidic acid, oleoside 11-methyl ester, 7-b-1-D-glucopyranosyl 11-methyl oleoside and ligstroside to oleuropein for Oleaceae was proposed by Damtoft *et al.* (1992) (Soler-Rivas *et al.*, 2000).

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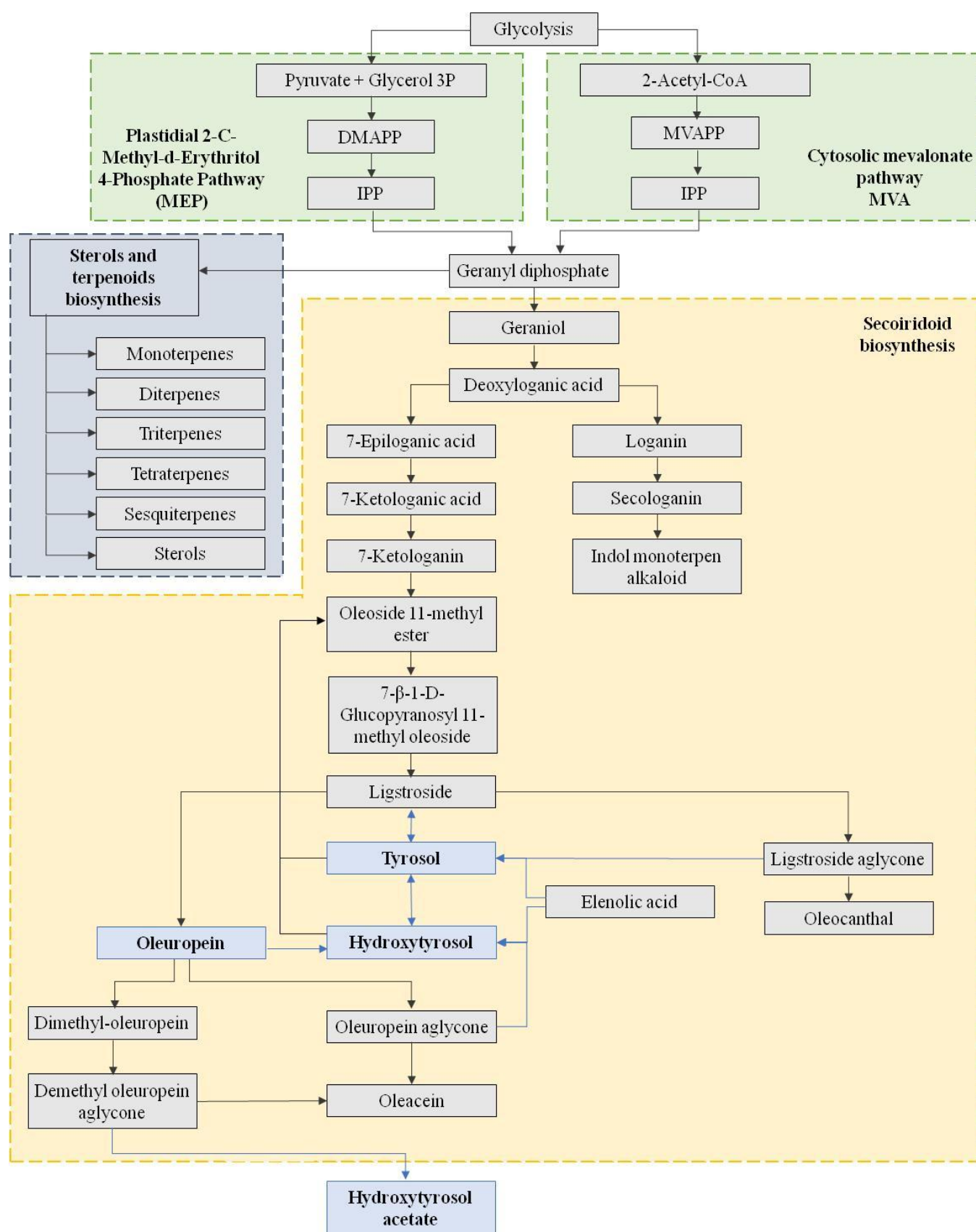
Figure 1.5. Schematic representation of the biosynthesis of secoiridoids in *Olea europaea* L. (Alagna *et al.*, 2012).

Ligstroside and oleuropein accumulate during maturation. Enzyme β-glucosidase is responsible for their hydrolysis into their aglycone forms, where the ligstroside aglycone is the aldehyde form of tyrosol-bound elenolic acid (*p*-HPEA-EA), while the oleuropein aglycone is the aldehyde form of hydroxytyrosol-bound elenolic acid (3,4-DHPEA-EA). The aglycones of ligstroside and oleuropein after subsequent hydrolysis by esterases lead to the formation of elenolic acid, tyrosol and hydroxytyrosol. Ligstroside and oleuropein aglycones can suffer from decarboxylation to form oleocanthal (*p*-HPEA-EDA) and oleacein (3,4-DHPEA-EDA). Moreover, ligstroside and oleuropein aglycones can also undergo direct hydrolysis and form tyrosol and hydroxytyrosol (Johnson *et al*., 2018, Gutierrez-Rosales *et al*., 2012). Obied *et al*. (2008) reported that the demethyloleuropein acts like precursor to form 3,4-DHPEA-EDA. Moreover, demethyloleuropein is behind the formation of hydroxytyrosol acetate (Sivakumar *et al*., 2007).

1.6.1.1.2. Synthesis of phenolic alcohols, phenolic acids, flavonoids and lignans

Phenolic alcohols, phenolic acids, flavonoids and lignans are synthesized through the shikimate and phenylpropanoid biosynthesis (Figure 1.6, Figure 1.7). Shikimate pathway starts with converting phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) into chorismate. PEP and E4P are derived from glycolysis and the non-oxidative branch of the pentose phosphate pathways that connects the shikimate pathway with the central carbon metabolism. In total, this pathway (Figure 1.6) contains seven reactions to form the chorismate.
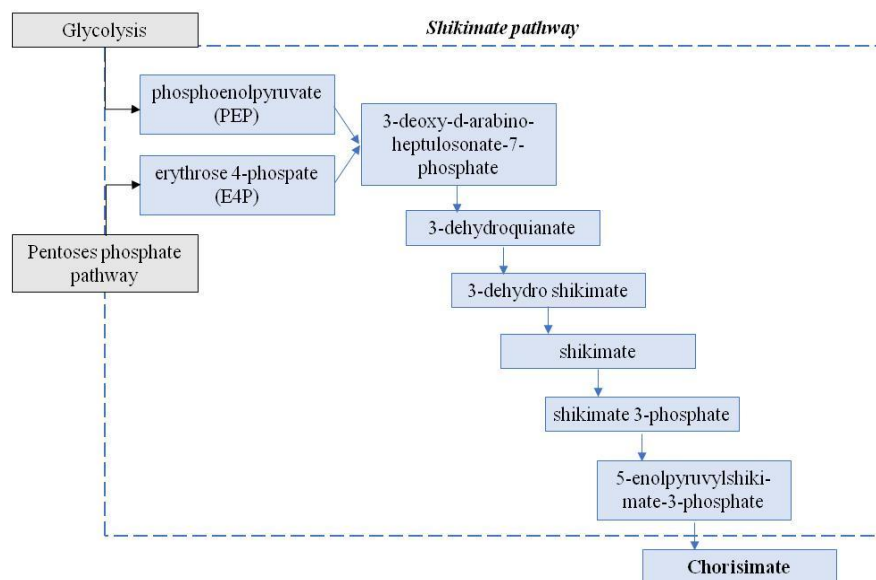
Figure 1.6. The shikimate pathway converting phosphoenolpyruvate and erythrose 4-phosphate into chorismate (Tzin and Galili *et al*., 2010).

Chorismate is a central metabolite in plant cells that serves as a precursor for the synthesis of the aromatic amino acids. Moreover, it is also an initiator substrate for the synthesis of many other metabolites, such vitamin B9 (tetrahydrofolate) (Tzin and Galili *et al*., 2010). After, chorismate is transformed into prephenate and subsequently into arogenate, which leads to the

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formation of tyrosine and phenylalanine. Tyrosine is the key for synthesis of the phenolic alcohols hydroxytyrosol and tyrosol. Endogenous tyrosol is formed from tyrosine in two different ways. The first route is through the hydroxyphenylpyruvic (*p*-HPPA) and *p*-hydroxyphenylacetic (*p*-HPAA) acids, where decarboxylation occurs in the final step that leads to the formation of tyrosol. Second way is through the decarboxylation of tyrosine to give tyramine and later 4-hydroxyphenylacetaldehyde (4-HPA) (Guodong *et al*., 2019, Karković-Marković *et al*., 2019). Salidroside is formed from tyramine via *p*-hydroxyphenyl acetaldehyde and tyrosol (Saimaru *et al*., 2010). The synthesis of hydroxytyrosol from tyrosine happens through 3,4-dihydroxyphenylalanine (L-DOPA), which decarboxylates to dopamine and subsequently to 3,4-dihydroxyphenylacetate (3,4-DHPA) (Guodong *et al*., 2019).
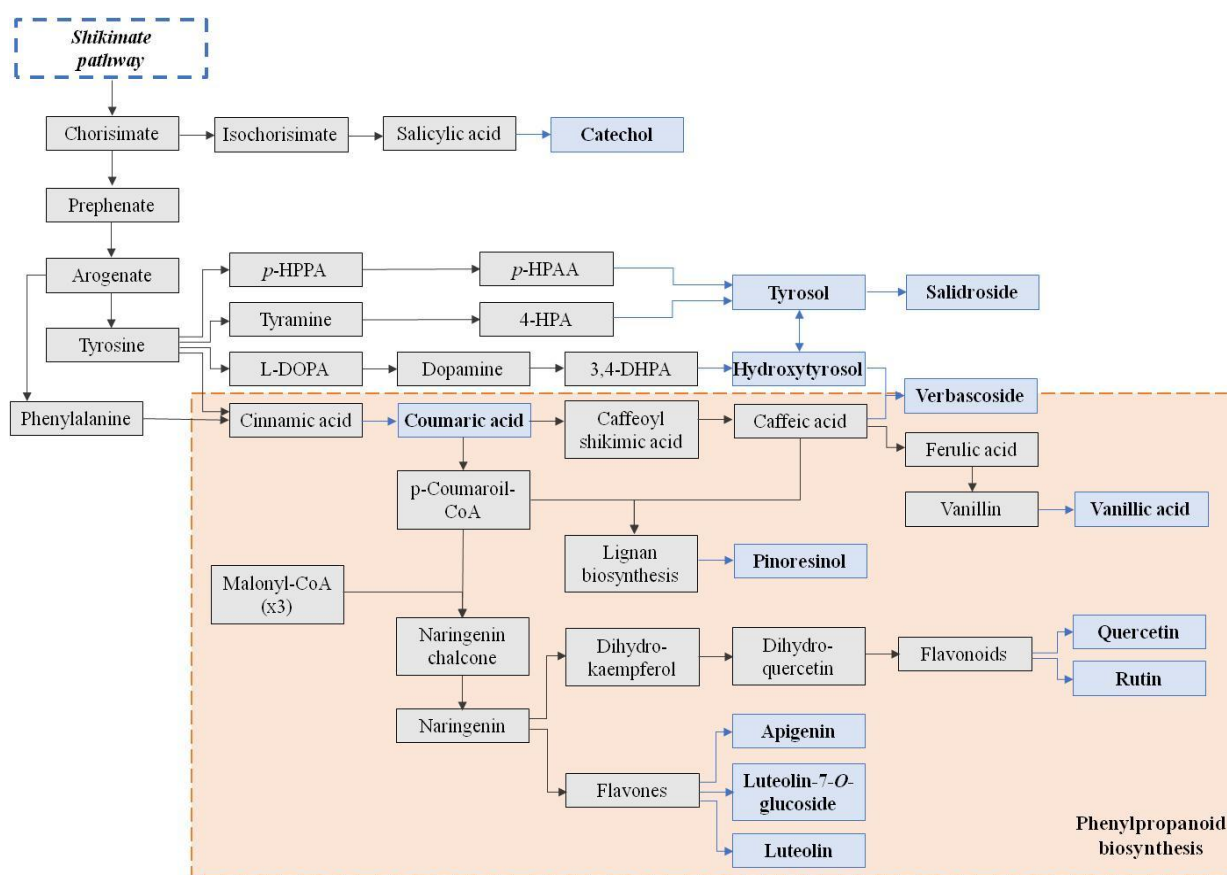
Figure 1.7. Schematic representation of the biosynthetic pathways of phenolic alcohols, phenolic acids, flavones, flavonoids and lignans in *Olea europaea* L.

The major route for biosynthesis of different phenolic acids includes the synthesis of cinnamic acid from phenylalanine. Cinnamic acid is then further transformed by the catalytic action into many types of phenolic acids. Moreover it is also precursor that allows the formation of flavonoids and lignans (Kaushik *et al*., 2015). Cinnamic acid is transformed into coumaric acid and can follow two routes. First way is the transformation of coumaric acid into intermediate caffeoyl shikinic acid, from which caffeic acid is formed. From caffeic acid can

be formed or verbascoside or ferulic acid, which depending on the plant can be transformed through vanillin into vanillic acid (Kaushik *et al.*, 2015, Guodong *et al.*, 2019). The second route of coumaric acid is through *p*-cumaroyl-CoA, which participates in the synthesis of flavonoids and lignans. *p*-Cumaroyl-CoA together with caffeic acid would lead to the synthesis of the lignan pinoresinol, although the metabolic intermediates of this pathway in *Olea europaea* L. have not been studied (Alagna *et al.*, 2012). One unit of *p*-cumaroyl-CoA and three units of malonyl-CoA form the naringenin chalcone which generates naringenin from which flavones like apigenin, luteolin and luteolin-7-*O*-glucoside are formed. Naringenin also produces dihydrokaempferol, from which dihydroquercetin is formed, from which quercetin and rutin that belong to flavonols are obtained (Guodong *et al.*, 2019). In case of catechol, its metabolism in *Olea europaea* L. has not been clarified, although in other plants it is formed from salicylic acid that is derived from chorismate (Akhtar and Pichersky, 2013).

1.6.1.2. Non-flavonoid polyphenols

The group of non-flavonoid polyphenols includes secoiridoids, phenolic alcohols, phenolic acids, and lignans. The group of secoiridoids includes oleuropein, ligstroside, and demethyloleuropein. Phenolic alcohols include hydroxyrosol, tyrosol, salidroside, hydroxytyrosol acetate, and catechol. Caffeic acid, *p*-coumaric acid, vanillic acid and verbascoside belong to the group of phenolic acids. The most important lignan is pinoresinol (Charoenprasert and Mitchell, 2012, Durrazo *et al.*, 2019).

1.6.1.2.1. Secoiridoids

Secoiridoids are monoterpenoids formed by the cleavage of the cyclomethene oxime compounds at C-7 and C-8. In total, 232 secoiridoids (aglycones, glycosides, derivatives and dimers) are isolated from 9 following genus of the family Oleaceae: *Fontanesia, Fraxinus, Jasminum, Ligustrum, Olea, Osmanthus, Phillyrea, Picconia* and *Syringa* (Huang *et al.*, 2019). The most common secoiridoids with related chemical structure include oleuropein, ligstroside, and demethyloleuropein. They are found in the immature and unprocessed olives and while the amount of oleuropein is decreasing with maturation, the concentration of dimethyloleuropein is increasing.

Oleuropein

Oleuropein (2S, 3E, 4S)-3-ethylidene-2-(β-D-glucopyranosyloxy)-3,4-dihydro-5-(methoxycarbonyl)-2H-pyran-4-acetic acid 2-(3,4-dihydroxyphenyl) ethyl ester) is the secoiridoid found at the highest concentrations in unripe olive fruit (Ghanbari *et al.*, 2012). Oleuropein is an ester containing hydroxytyrosol and elenolic acid in its
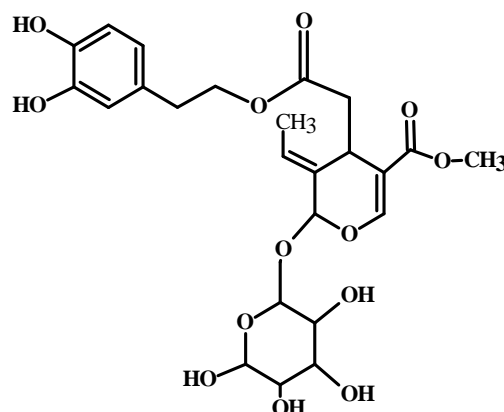
Figure 1.8. Chemical structure of oleuropein.

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chemical structure. The concentration of this compound decreases with olive ripening, irrigation of olive trees, or when the olive is damaged by pathogens, when the enzyme β-glucosidase hydrolyzes oleuropein to form an aglycone. Oleuropein is the most abundant phenol found in olive leaves (*Olea europaea* L.) (Lama-Muñoz *et al*., 2019b).

Moreover, it is also present in olive fruit (up to 14% of the dry weight of olives), olive oil and table olives (Soler-Rivas *et al*., 2000, Ghanbari *et al*., 2012, Bonechi *et al*., 2019). Oleuropein also appears in many other genera from the Oleaceae family. It has been identified in: *Fraxinus excelsior, F angustifolia, F chinensis, F mandshurica* var *japonica, Syringa josikaea* and *S vulgaris, Phillyrea latifolia, Ligustrum ovalifolium* and *L vulgare, Jasminum polyanthum* and *Osmanthus asiaticus* (Soler-Rivas *et al*., 2000).

Oleuropein has shown anti-inflammatory, antiobesity, cardioprotective, anti-infective, antihypertensive, vasodilator, gastroprotective, hepatoprotective, antimicrobial, antiviral, antioxidant and anticancer activities (Bazoti *et al*., 2009, Lemonakis *et al*., 2016, Lama-Muñoz *et al*., 2019b).

1.6.1.2.2. Phenolic alcohols

The group of phenolic alcohols includes hydroxytyrosol, tyrosol, salidroside, hydroxytyrosol acetate, and catechol.

Hydroxytyrosol

Hydroxytyrosol or (2-(3,4-dihydroxyphenyl)ethanol) is a product from the hydrolysis of oleuropein containing catechol moiety in its chemical structure. Hydroxytyrosol is a phenolic alcohol that is found in *Olea europaea* L., grape juice and red wine (Piñeiro *et al*., 2011, Rodríguez-Morató *et al*., 2016).
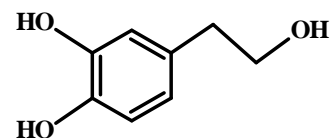
Figure 1.9. Chemical structure of hydroxytyrosol.

Moreover it is product of oxidative metabolism of dopamine (Charoenprasert and Mitchell, 2012, Domínguez-Perles *et al*., 2017). In *Olea europaea* L., hydroxytyrosol is found in fruit, leaves (Cetinkaya and Kulak, 2016, Cifá *et al*., 2018, Lins *et al*., 2018), olive oil and in table olives (Pereira *et al*., 2006, Accardi *et al*., 2016, Durante *et al*., 2018). Hydroxytyrosol is a compound presented in higher content in ripe olives. It is considered to be one of the strongest investigated natural antioxidant among all the polyphenols from olive tree.

According to the European Food Safety Authority (EFSA) health claim, daily intake of 5 mg of hydroxytyrosol and its derivatives (oleuropein complex and tyrosol) provides protection against cardiovascular diseases. Besides, this compound has been widely studied and apart from antioxidant activity, it exerts immunostimulant, antidiabetic, antimicrobial, antihypertensive, cardioprotective, neuroprotective, hypoglycemic, hypocholesterolemic, anticancer, anti-inflammatory, and antiviral properties (Visioli *et al*., 2003, EFSA, 2012, Charoenprasert and Mitchell, 2012, Kotronoulas *et al*., 2013, Durante *et al*., 2018, Karković Marković *et al*., 2019).

Tyrosol

Tyrosol (2-(4-hydroxyphenyl)ethanol) is a product from the hydrolysis of ligstroside. It is usually found in highest concentrations in the fruit (Blekas *et al*., 2002, D´Antuono *et al*., 2016), leaves (Cetinkaya and Kulak *et al*., 2016, Cifá *et al*., 2018), olive oil (Chandramohan *et al*., 2015, Rodríguez-Morató *et al*., 2016, Boronat *et al*., 2018) and table olives (Pereira *et al*., 2006, Cabrera-Bañegil *et al*., 2017) of *Olea europaea* L., but it is also found in white wine, beer and vermouth. Tyrosol is also endogenously generated as byproduct of metabolism of tyramine. Structurally, tyrosol is identical to hydroxytyrosol with the exception that tyrosol lacks the hydroxyl group at C3 in its chemical structure.
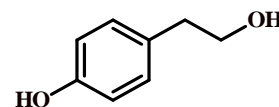
Figure 1.10. Chemical structure of tyrosol.

Tyrosol has multiply biological effects. It exerts antioxidant, cardioprotective, anti-inflammatory, anticancer, antidiabetes, antidepressive, antiatherogenic, antihypertensive, anti-stress, anti-osteoporosis, and neuroprotective activities. Moreover, it also displays antimicrobial, skin protective and anti-aging effects (Berrougui *et al*., 2015, Tundis *et al*., 2015, Angeloni *et al*., 2017, Plotnikov *et al*., 2018).

Salidroside

Salidroside (2-(4-hydroxyphenyl)ethyl β-D-glucopyranoside) is one of the major phenols in the genus *Rhodiola L.(Crassulaceae)* that is usually extracted from the roots of *Rhodiola rosea*. The content of this phenolic compound is one of the main criteria to evaluate the medicinal quality of genus *Rhodiola*. Also, salidroside is one of the components of *Rhodiola rosea extract* that is considered as tonic to increase physical and mental stamina. Salidroside is present in seeds (Obied *et al*., 2008) and table olives of *Olea europaea* L. In Tibetan medicines, salidroside is used as adaptogen to enhance the body's resistance to fatigue. Salidroside shows strong anti-aging, antihypoxia, antioxidant, anticancer, hepatoprotective, anti-fatigue, cardioprotective, anti-inflammatory, and liver, kidney and myocardial protective biological effects (Guo *et al*., 2012, Guo *et al*., 2014, Xie *et al*., 2020). Also, salidroside protects against glutamate-induced neuronal cell death and hypoxia/hypoglycemia (Yu *et al*., 2008).
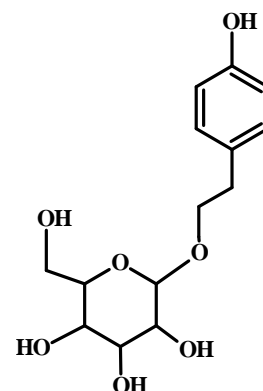
Figure 1.11. Chemical structure of salidroside.

Hydroxytyrosol acetate

Hydroxytyrosol acetate (2-(3,4-dihydroxyphenyl)ethyl acetate) is a derivative of hydroxytyrosol found in the fruit and leaves of *Olea europaea* L. (Goulas *et al*., 2009, Yao *et al*., 2019), as well as in olive oil (Brenes *et al*., 1999) and table olives (Romero *et al*., 2004a). It is more stable and it has
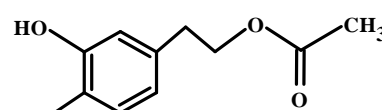
Figure 1.12. Chemical structure of hydroxytyrosol acetate.

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better absorption across intestinal epithelial cell monolayers than free hydroxytyrosol (Rubio *et al*., 2012). Hydroxytyrosol acetate shows strong biological activities, as follows: antioxidant, neuroprotective, antiplatelet, and aggregating. Moreover it has strong anti-inflammatory effects on murine collagen-induced arthritis (Yao *et al*., 2019).

Catechol

Catechol (1,2-benzenediol) is phenolic alcohol that is present in apple, pear, grapes, peach, mango, plum, potato and mushrooms (Corzo-Martinez *et al*., 2012). Moreover, catechol was also found in *Olea europaea* L., in table olives and table olive oils, but it has not been detected in oil from fresh olives (Romero *et al*., 2004a, Romero *et al*., 2004b).
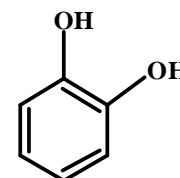
Figure 1.13. Chemical structure of catechol.

This aromatic compound has a use in different applications. It can be used as reagent for photography, dye stuffs, plastic and rubber production, and also as starting material to produce perfumes, some drugs and insecticides. It can be also employed as oxygen scavenger (antioxidant) (Fiege *et al*., 2000).

1.6.1.2.3. Phenolic acids

Phenolic acids (phenolcarboxylic acids) are the simplest forms of phenols in olives and they are characterized by a carboxyl group attached to the benzene ring. Normally they are found at low concentrations, in majority of the cases less than 1%. These compounds are mostly derived from benzoic and cinnamic acids, thus can be divided into benzoic acid derivatives (C6−C1) and cinnamic acid derivatives (C6−C3).

These phytochemicals are expansively distributed in daily intake of food. They are responsible for various physiological activities like enzyme activity, photosynthesis, absorption of nutrients and synthesis of proteins. Phenolic acids are mostly biosynthetically produced via the shikimic acid pathway from L-phenylalanine or L-tyrosine. In plants, phenolic acids are in free, free ester, and conjugated (bound) forms.

Among the most important phenolic acids found in olive belong caffeic acid and chlorogenic acids (ferulic, vanillic, coumaric and syringic acids) as well as more complex sugar ester of caffeic acid – verbascoside (Charoenprasert and Mitchell, 2012, Kaushik *et al*., 2015, Durazzo *et al*., 2019, Zhang *et al*., 2019).

Caffeic acid

Caffeic acid (3,4-dihydroxycinnamic acid) contains two hydroxyl groups in its chemical structure. It is well known hydroxycinnamate and phenylpropanoid derivative in plant tissues. Caffeic acid is the main hydroxycinnamic acid found in the diet of humans where is it mostly present as chlorogenic acid (5-caffeoylquinic acid, an ester of caffeic acid with quinic acid). It is found in cider, coffee beans, fruits - apples,
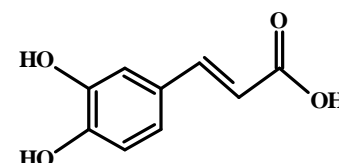
Figure 1.14. Chemical structure of caffeic acid.

blueberries, potatoes, carrots and propolis (Nardini *et al*., 2002, Spagnol *et al*., 2016). Moreover, it is found in fruit, leaves (Charoenprasert and Mitchell., 2012, Cifá *et al*., 2018), olive oil (Bayram *et al*., 2012) and table olives (Boskou *et al*., 2006) of *Olea europaea* L. Caffeic acid exerts antioxidant, antibacterial, antiviral, hepatoprotective, anti-inflammatory, and anti-atherosclerotic activities. This compound is also involved in plants defense mechanism against predators and infections, has an inhibitory effect on the growth of insects, fungi and bacteria. It may also inhibit carcinogenesis and has a positive effect on the leaves protection from ultraviolet radiation (Luo *et al*., 2014, Spagnol *et al*., 2016, Monteiro Espíndola *et al*., 2019).

p-Coumaric acid

p-Coumaric acid (trans-4-hydroxycinnamic acid) is a ubiquitous plant metabolite that can be found in onion, beans, potatoes, tomatoes, apples, pears, grapes, oranges, berries, maize, oats, wheat, chocolate and beverages, as coffee, tea, wine, and beer (Abdel-Wahab *et al*., 2003, Pei *et al*., 2016). In *Olea europaea* L., *p*-coumaric is found in fruit (Bianco and Uccella, 2000),

Figure 1.15. Chemical structure of *p*-coumaric acid.

leaves (Brahmi *et al*., 2013), olive oil (Christophoridou and Dais, 2009, Tasioula-Margari *et al*., 2015) and table olives (Boskou *et al*., 2006).

p-Coumaric acid has antioxidant, chemoprotectant, anti-inflammatory, anti-HIV, antifungal effects, anti-mutagenic, and immunomodulatory effects. In addition, it is involved in the prevention of different pathologies, like cardiovascular diseases or colon cancer (Liu *et al*., 2006, Luceri *et al*., 2007, Navaneethan *et al*., 2014).

Vanillic acid

Vanillic acid (4-hydroxy-3-methoxybenzoic acid), an important derivative of benzoic acid, is oxidized form of vanillin, formed during the conversion of vanillin to ferulic acid. It is used as flavoring agent since it is one of the most important ingredients of 'natural vanilla' flavor (Ghosh *et al*., 2007, Kim *et al*., 2010).
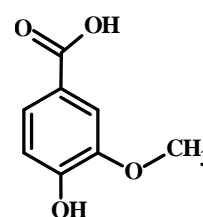
Figure 1.16. Chemical structure of vanillic acid.

Vanillic acid is found at high concentrations in the roots of *Angelica sinensis* that is used in Chinese medicine. In *Olea europaea* L., vanillic acid is found in fruit (Mohamed *et al*., 2018), leaves (Brahmi *et al*., 2013), table olives (Boskou *et al*., 2006) and olive oil (Bayram *et al*., 2012).

Various studies have provided antioxidant, antiviral, hepatoprotective and anti-colitic activities of vanillic acid. Moreover, this compound is effective in treating immune or inflammatory diseases (Ghosh *et al*., 2007, Itoh *et al*., 2009, Kim *et al*., 2010).

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Verbascoside

Verbascoside (acteoside) is structurally characterized by the caffeic acid moiety and 4, 5-hydroxyphenylethanol (hydroxytyrosol) bound to β-(D)-glucopyranoside with a rhamnose in sequence (1–3) to the glucose molecule. The history of verbascoside dates back to 1963 when phenylethanoid glycoside verbascoside was isolated from mullein (*Verbascum sinuatum* L.; Scrophulariaceae). Later, the verbascoside was also isolated from flowers of the common lilac (*Syringa vulgaris*, Oleaceae). The determined structure was 2-(3, 4-dihydroxyphenyl)ethyl-1-O-α-Lrhamnopyranosyl-(1→3)-(4-O-E-caffeoyl)-β-D-glucopyranoside which was named acteoside.
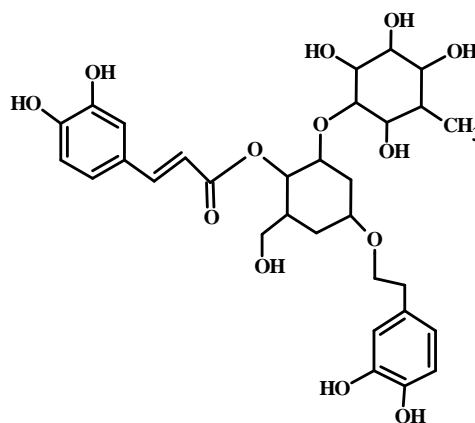
Figure 1.17. Chemical structure of verbascoside.

In 1983, Sakurai and Kato reported the isolation of new phenylethanoid glycoside from tree (*Clerodendron trichotomum Thunb*, Lamiaceae) and the compound was called kusaginin. 50 years after the discovery of verbascoside, there are still doubts about its exact name. Verbascoside, which is one of the most common disaccharides caffeoyl esters was detected in more than 200 plant species that belongs to 23 plant families, like *Buddleja brasiliensis, Striga asiatica, Paulownia tomentosa* var. *tomentosa*, *Lippia javanica, Lantana camara, Lippia citriodora* (Cardinali *et al*., 2013, Alipieva *et al*., 2014, Di Giancamillo *et al*., 2015).

Moreover it was also detected in *Olea europea* L., in olive fruit (D´Antuono *et al*., 2016), leaves (Charoenprasert and Mitchell, 2012) and table olives (Pereira *et al*., 2006). Verbascoside has been also found in primary and secondary roots, stems, leaves and flowers at widely varying levels. Verbascoside possesses many biological activities for human health, like anti-inflammatory, anti-bacterial, antioxidant, antiandrogen, neuroprotective and antineoplastic (Cardinali *et al*., 2013, Alipieva *et al*., 2014, Di Giancamillo *et al*., 2015).

1.6.1.2.4. Lignans

Pinoresinol

Pinoresinol or (4,4′-((1S, 3aR, 4S, 6aR)-hexahydrofuro[3,4-c]furan-1,4-diyl)bis(2-methoxyphenol)) consists of two monolignols. Pinoresinol is normally found in fruits, vegetables and in sesame seeds (*Sesamum indicum*) and in fruit, leaves and also in olive oil of *Olea europaea* L. (Bodoira *et al*., 2016, Olma-Garcia *et al*., 2018a, Yu *et al*., 2019, Olma-Garcia *et al*., 2019a). Pinoresinol exerts multiply supporting effects for
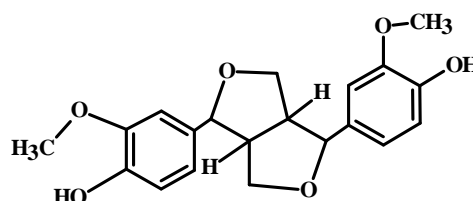
Figure 1.18. Chemical structure of pinoresinol.

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health as anticancer, anti-HIV, chemopreventive, cardioprotective and antifungal. Moreover, pinoresinol improves memory in a model of dementia (López-Biedma *et al*., 2016, Ricklefs *et al*., 2016, Yu *et al*., 2019).

1.6.1.3. Flavonoid polyphenols

Flavonoids consist of more than 4000 types. Mostly, flavonoids in plants are synthesized by shikimic acid pathway. Flavonoids are in most foods of plant origin and they are mainly present in fruits, vegetables, seeds, spices, grains, herbal essences, and also in coffee, tea, wine and cocoa, so a significant amount of them is consumed in our daily diet. In olive they are found at moderately low concentrations (Boyle *et al*., 2000, Charoenprasert&Mitchell., 2012, Działo *et al*., 2016).

Flavonoids contain two aromatic rings connected by a bridge consisting of three carbons - C6-C3-C6 called diphenyl propane structure. Usually, they occur in association with sugar as glycosides dissolved in the vacuolar juice (mainly in the O-glycoside form, rather than C-glycosides). The daily average intake of flavonoids for humans is ranging from 23 mg up to 1 g/day. The group of flavonoids includes e.g. anthocyanins, flavanols, flavanones, flavonols, flavones and isoflavones (Boyle *et al*., 2000, Działo *et al*., 2016, Durrazo *et al*., 2019).

1.6.1.3.1. Flavonols

The most common flavonols in plants and herbs are flavone glycoside and its aglycone rutin and quercetin (Yang *et al*., 2005). Flavonols are the primary pigments in white- and cream-colored flowers (Hostetler *et al*., 2017).

Quercetin

Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one) is ubiquitous flavonol found in many varieties of fruits and vegetables, for example in onion, berries, cherries, apples, red grapes, citrus fruits. At high concentrations it is found kale, broccoli, spinach, asparagus and oregano. It is also found in the tea (*Camellia sinensis*) and in *Matricaria chamomilla* L. (German chamomile). Moreover, quercetin is present in the fruit and leaves of *Olea europaea* L., and also in olive oil and table olives (Soler-

Figure 1.19. Chemical structure of quercetin.

Rivas *et al*., 2000, Anand David *et al*., 2016, Yıldırım *et al*., 2016, Maalej *et al*., 2017, D´Antuono *et al*., 2018).

Dietary quercetin is present mainly as O-glycosidic forms that include quercetin-3-O-glucoside, quercetin-3-O-rutinoside, and quercetin-3,4′-O-diglucoside (Anand David *et al*., 2016, Dong *et al*., 2017, Almeida *et al*., 2018, Dabeek *et al*., 2019). Quercetin has many biological activities including anti-inflammatory, neuroprotective, antioxidant, anti-osteoporosis, anti-aging, antihypertensive, vasodilator, antiobesity, anti-hypercholesterolemic

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and antiatherosclerotic activities (Cao *et al.*, 2015, Anand David *et al.*, 2016, Almeida *et al.*, 2018).

Rutin

Rutin or (quercetin 3-*O*-rutinoside) that is very common quercetin glycoside, contains in its chemical structure 10 hydroxyl groups - four phenolic hydroxyl groups and six sugar hydroxyl groups (Miyake *et al.*, 2000). It is usually found in apples, asparagus, onions, buckwheat, berries, and eucalyptus. It is also present in Fructus Gardeniae (Chinese medicine called Zhizi in Chinese), ruta graveolens, sophora japonica and in plant-based beverages such tea and wine. Rutin is present *Olea europaea* L., in olive fruit, table olives (Pereira *et al.*, 2006) and in olive oil (Blekas *et al.*, 2002, Cardoso *et al.*, 2005, Melliou *et al.*, 2015, Yıldırım *et al.*, 2016).
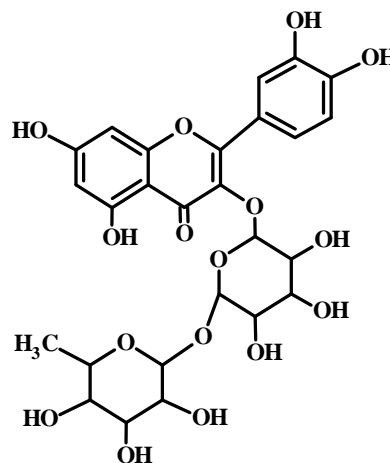
Figure 1.20. Chemical structure of rutin.

Rutin is widely used in clinic since it displays various biological activities: anti-herpes, antioxidant, antidepressant, vasoprotective, anticancer, antidiabetic, antihypertensive, antihaemorrhoids, and antistress (Chen *et al.*, 2014, Zhang *et al.*, 2016, Tuyishime *et al.*, 2018).

1.6.1.3.2. Flavones

There is a difference in the flavonoid skeleton of flanoves comparing to other flavonoids and it is that they have a double bond between C2 and C3, and the compounds are oxidized at the position C4. The absence of a hydroxyl group on C3 distinguishes flavones from flavonols. Flavones together with other flavonoids are generally absorbed in 280- to 315-nm range, so they are able to be used as UVB protectants of the plants from damage. Together with flavonols, they are present as primary pigments in white- and cream- coloured flowers. Flavones in plants provide protection against insects and fungal diseases, thus they act as natural pesticides. Two major sources of flanoves among plants are parsley (*Petroselinum crispum*) and peppers (*Piper nigrum*) (Ali *et al.*, 2016, Hostetler *et al.*, 2017, Aziz *et al.*, 2018).

Apigenin

Apigenin (4′,5,7-trihydroxyflavone) is found in many plants, like onions, parsley, wheat sprouts, grasses, celery, maize, rice, grapefruit, oranges, and chamomile tea prepared from the dried flowers of *Matricaria chamomilla*. Apigenin is also present in wine and beer. Apigenin together with its derivatives are present in many other plants, for example *Acacia farnesianal, Andrographis*
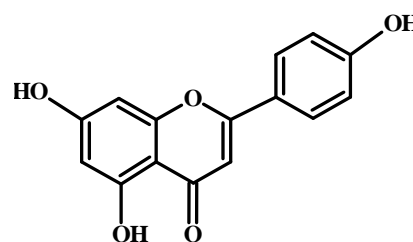
Figure 1.21. Chemical structure of apigenin.

paniculata, Apium graveolens, Daphne genkwa, Euterpe oleracea, Ginkgo biloba, Chrysanthemum morifolium, Lycopodium clavatum, Mentha longifolia, Scutellaria barbata and *Thevetia peruviana* (Ali *et al.*, 2016, Hostetler *et al.*, 2017).

Apigenin in aglycone and in the glycosylated form was found in the fruit, leaves of *Olea europaea* L., as well as in table olives and olive oil (Soler-Rivas *et al.*, 2000, Yorulmaz *et al.*, 2012, Guex *et al.*, 2019).

When apigenin is conjugated with sugars, naturally occurring glycosides in plants as apiin, apigetrin, vitexin, isovitexin, rhoifolin, schaftoside, acacetin and genkwanin are formed. Apigenin is also used to dye wool. It has many biological activities, including antioxidant, antitumor, anti-inflammatory, antidiabetic, anti-HIV, cardioprotective, chemotherapeutic, antiviral, antitoxicant, antigenotoxic, and immunomodulatory effect.

Besides that, apigenin also displays positive preventive and therapeutic effects against Alzheimer's disease, Parkinson's disease, rheumatoid arthritis and autoimmune disorders (Ali *et al.*, 2016, Hostetler *et al.*, 2017).

Luteolin

Luteolin (3′, 4′, 5,7-tetrahydroxyflavone) that generally exists as glucoside is a naturally occurring flavone that is found vegetables, fruits, for example: bell pepper, green pepper, carrots, celery, lettuce, onion, cucumber, broccoli, cabbage, artichokes, perilla, pomegranates, apples, peppermint, thyme, rosemary and oregano. Besides that, luteolin is also found in medicinal plants such as *Flos Chrysanthemi*, the flower of *Dendranthema morifolium* Ramat Tzvel (*Chrysanthemum morifolium* Ramat.) that is traditional in Chinese medicine, *Codariocalyx*

Figure 1.22. Chemical structure of luteolin.

motorius, (Houtt.) H. Ohashi and *Artemisia asiatica* Nakai (also known as *Artemisia dubia* var. *asiatica* Pamp.). Luteolin with its glycosides have been also identified in families Bryophyta, Pteridophyta, Pinophyta and Magnoliophyta (López-Lázaro *et al.*, 2009, Chen *et al.*, 2012, Kure *et al*, 2016, Aziz *et al.*, 2018).

Structurally, the presence of a hydroxyl group at the 3'-position distinguishes this flavone from apigenin (Aziz *et al.*, 2018). It is also present *in Olea europaea* L., in fruit, olive leaves (Charoenprasert and Mitchell, 2012, Mohamed *et al.*, 2018), table olives (Pereira *et al.*, 2006, D'Antuono *et al.*, 2018) and olive oil (López-Lázaro *et al.*, 2009).

Luteolin exerts antioxidant, antimutagenic, antiinflammatory, antidiabetic, anti-allergic, anticancer, antimicrobial, and neuroprotective pharmacological activities (Chen *et al.*, 2012, Kure *et al.*, 2016, Aziz *et al.*, 2018).

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Luteolin-7-*O*-glucoside

Luteolin-7-*O*-glucoside (3', 4′, 5, 7-tetrahydroxyflavone 7-glucoside) is a β-D-glycosylated form of luteolin that is found in parsley, artichokes, celery, salvia, thyme, cumin, cocoa and capers. Moreover, it was also isolated from *Flos Chrysanthemi* (Lin *et al*., 2015). Luteolin-7-*O*-glucoside is present in *Olea europaea* L., in raw olive fruit (Blekas *et al*., 2002, Cardoso *et al*., 2005, D´Antuono *et al*., 2016), leaves (Charoenprasert and Mitchell, 2012, Lama-Muñoz *et al*., 2019a, Lama-Muñoz *et al*., 2019b), table olives (Pereira *et al*., 2006) and in olive oil (Olma-García *et al*., 2019a). Luteolin-7-*O*-glucoside

Figure 1.23 Chemical structure of luteolin-7-*O*-glucoside.

displays antioxidant, anti-inflammatory, anticancer, antimicrobial, gastroprotective, and neuroprotective properties (Lama-Muñoz *et al*., 2019a, Lama-Muñoz *et al*., 2019b).

1.7. ANALYSIS OF PHENOLIC COMPOUNDS BY LIQUID CHROMATOGRAPHY MASS SPECTROMETRY

Liquid chromatography coupled to mass spectrometry (LC-MS) is a combined technique that constitutes a powerful analytical tool that allows the determination and quantification of the compounds in a sample (Figure 1.24). It started to be developed in the beginning of 1970s, the early years were focused into solve the difficulties of coupling both techniques and in the technological innovation of different interfaces (Niessen *et al.*, 2003). The use of this technique has increased spectacularly in analytical studies not only due to its technical advantages but also of its cost reduction, which have made it affordable for more laboratories (Quintela *et al.*, 2005). The main advantage of LC-MS is that combines the powerful separation of LC with the selectivity, sensibility and precision in determining the molecular weight of the mass spectrometry, giving quantitative and qualitative information (Pratima *et al.*, 2014). Moreover, it is possible to study the non-volatile molecules without the need of derivatization reactions, which facilitates sample preparation (Jauregui *et al.*, 2012). Nowadays it is widely used in different fields such as: pharmaceutical, toxicology, forensic analysis, biochemical, food industries, agrochemical, among others (Pratima *et al.*, 2004, Jauregui *et al.*, 2012, Quintela *et al.*, 2005).

There are different analytical techniques for analysis of phenolic compounds described in the literature. The use of LC is the most spread one (Cabrera-Bañegil *et al*., 2017, Romero *et al*., 2017, Moreno et al., 2020*a*), since other methods like Folin-Ciocalteu colorimetric test shows low specificity towards these compounds (Tripoli *et al*., 2005). Although, LC coupled to UV or DAD is widely used, it presents long run times, and moreover these techniques do not allow the determination of the different classes of phenolic compounds from *Olea europaea* L, especially those found at small concentrations, since it presents high limits of

quantification (D´Antuano *et al*., 2016, Cabrera-Bañegil *et al*., 2017). These limitations could be overcome when LC is coupled to MS detection, due to its significant increase in the sensitivity and selectivity (Moreno *et al*., 2020*a*). For all of this, LC-MS has been chosen in this thesis to analyze phenolic compounds and their metabolites in rat and human plasma after oral administration of table olives.
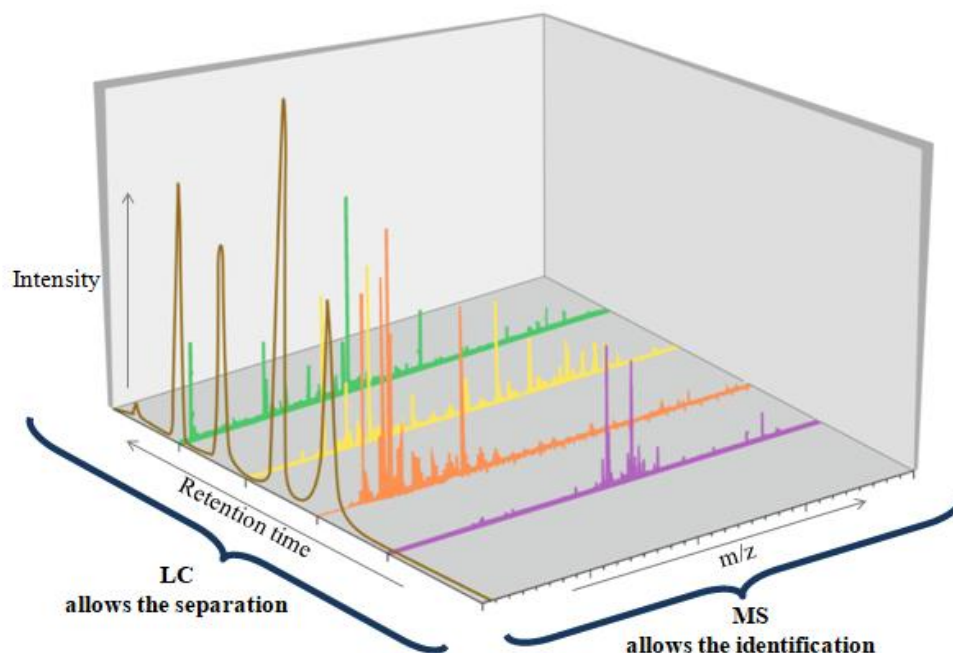
Figure 1.24. Schematic representation of the analysis performed by LC-MS.

1.7.1. Liquid Chromatography - LC

Liquid chromatography is a chromatographic technique that allows the separation of the components of one mixture. The sample contained in a liquid (mobile phase) flows through a column which contains a solid porous material (stationary phase), where the physical/chemical interactions are established with the compounds of the sample causing their separation and the output of the column at different times (retention time). After they are analyzed by detection system placed next to the column, which registers the presence and amount of the analyte coming out of the column. High-performance liquid chromatography (HPLC) or high-performance liquid chromatography is an advanced type of LC, where the difference with the traditional LC is that instead of flowing by the gravity, the mobile phase flows pressured by a pump. Other types of LC are: gel permeation chromatography (GPC), ultra-high-performance liquid chromatography (UHPLC) and flow injection analysis (FIA), among others (Jauregui *et al*., 2012). The main components of HPLC are the following:

- Pump system: it is used to impulse the mobile phase through the column. The three main types of pumps used in HPLC are syringe, reciprocating and constant pressure pumps (Pratima *et al*., 2004).

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- Injector system: it is responsible to introduce the sample to the column. It should work within very small volumes (1µL to 100µL) and withstand the high pressure of the solvent. The injectors can be automatic or manual, but the latter are less accurate, less precise and less user friendly (Pratima *et al*., 2014)**.**

- Separation system: it is formed by a stationary phase packaged inside of a column. There are many different columns that are based on the basis of nature of compounds to be analyzed, being the reverse phase the most common one. The most used solvents in this type of chromatography are Milli-Q water, methanol and acetonitrile. The proportion of the solvents during the process can be constant (isocratic) or can change (gradient). In the present thesis a reverse-phase columns has been used and the mobile phase was delivered with a gradient program.

- Detection system: it registers the presence and amount of the analyte coming out of the column within the time, where the signal can be processed by software when a chromatogram is obtained as a result (Pratima *et al*., 2004).

1.7.2. Mass Spectrometry - MS

LC can be coupled to mass spectrometry (MS) that is a powerful analytical technique for identification and characterization of molecules widely used in medicine, pharmacy, analytical chemistry, and biochemistry. Mass spectrometer causes the ionization of chemical compounds to form charged molecules or fragments of molecules measuring their ratio of relative molecular weight to electric charge (*m/z*).

Sample preparation is a very important step before analysis by LC-MS. Choosing the suitable solvent is necessary in order to get the maximum ionization efficiency of the analyte, since with the mass spectrometers, only analysis of positive and negative ions can be performed. The nature of the analyte together with the ionization source of the spectrometer is crucial in the choice of solvent.

Since many compounds are not ionic, their ionization is not possible or only in a very small degree. Moreover, the solvents employed in the LC-MS/MS analysis must be volatile and evaporable, without crystallization or other solids formations, that could happen in the mobile phase thus preventing plugging the capillaries or entering the mass spectrometer (Berdié *et al*., 2012).

The mass spectrometer (Figure 1.25) is formed by an ion source (ionization), a mass analyzer (separation of ions) and a detector (detects and measures the number of ions formed). Everything is enclosed in a space in which high vacuum conditions are necessary for the mass analyzer, detector, and some ion sources to allow ions to enter the detector without colliding with other gaseous molecules or atoms. The molecules of the sample are introduced into the instrument via the sample inlet (Berdié *et al*., 2012).
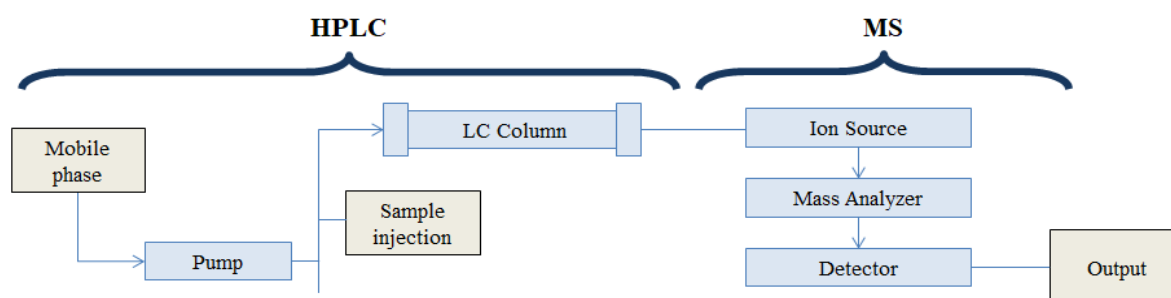
Figure 1.25. Schematic diagram of high-performance liquid chromatography coupled to mass spectrometry.

1. Ionization sources for LC-MS

A. Electrospray Ionization

Nitrogen-assisted electrospray ionization (ESI) is nowadays the most universal ion source in LC-MS. The liquid carrying the analytes is pushed at atmospheric pressure into a nebulizer installed in the chamber that is located in front of a cone with a capillary opening that allows ions to enter the mass spectrometer. The ions to be analyzed are determined by the polarity of the electrostatic field, which is the potential difference observed between the cone and the spray tip. In order to facilitate the evaporation of the solvent, the chamber is heated, which causes the ions present in the solution to be desolvated and attracted to the spectrometer inlet (Berdié *et al*., 2012). ESI ionization source is used for strongly and moderately polar compounds with a large molecular weight (Commiso *et al*., 2017).
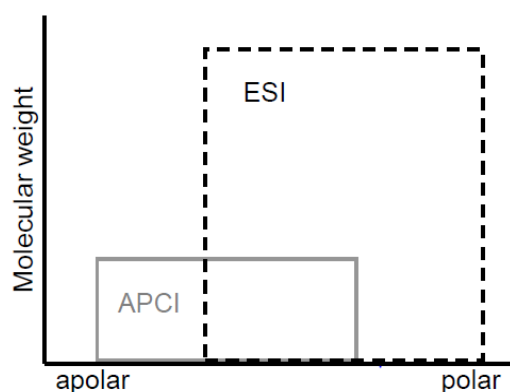
Figure 1.26. Application domain of two ionization interfaces: ESI and APCI (Verplaetse *et al*., 2011).

B. Atmospheric Pressure Chemical Ionization

Atmospheric pressure chemical ionization (APCI) sources form an alternative to the ESI source. APCI is characterized by the presence of a discharge needle in a chamber. A high potential difference is found between the needle and the ground, which creates a corona discharge that ionizes the molecules from the solvent present in the spray. In APCI sources, the formed ions are accelerated in the electrostatic field generated at the end of the cone,

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while the ions in the ESI sources must be present in the sprayed solution. The formed ions affect the neutral analyte and transfer an electric charge to it, making the ions out of analyte molecules that are "visible" to the mass spectrometer (Berdié *et al.,* 2012). APCI ionization source is efficient for non-polar and weakly polar compounds (Commiso *et al.*, 2017).
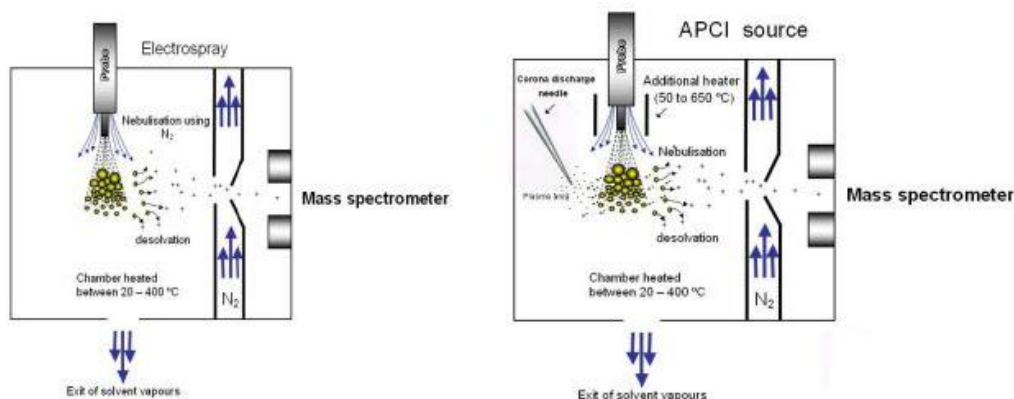
Figure 1.27. Schematic representation of ESI and APCI sources (Berdié *et al.*, 2012)

2, Mass analyzer

The mass analyzer is a part of the mass spectrometer where charged analyte molecules are separated according to their *m/z*. There are several types of mass analyzer: Time-of-flight (TOF), Fourier transform ion cyclotron resonance (FTICR), Quadrupole mass filter, Quadrupole Ion trap analyzer, Linear Ion Trap, and Double-Focusing Magnetic Sector, among others.

QTRAP4000 applied for the analysis of phenolic compounds in rat and human samples in the present thesis is a hybrid system, combined between a triple quadrupole (Figure 1.28) and a linear ion trap.
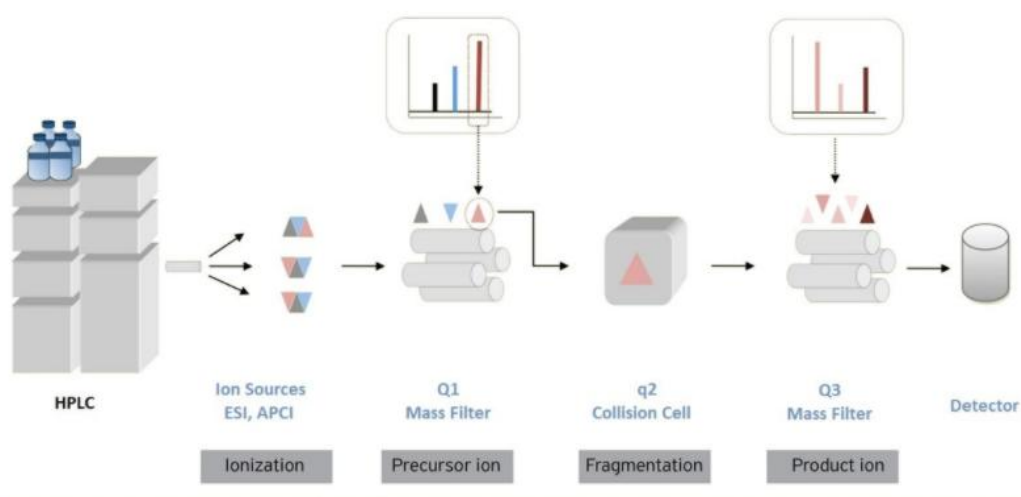
Figure 1.28. The triple quadrupole mass spectrometer (https://www.creative-proteomics.com/technology/triple-quadrupole-mass-spectrometry.htm, consulted December 12th, 2020).

The triple quadrupole mass spectrometer is formed by three quadrupoles arranged in tandem, when the first (Q1) and the third (Q3) act as mass filters, being those that perform the selection and scanning of the sample ions. The second, non-mass filtering central quadrupole (q2) located between them is acting as a collision cell for collision-induced dissociation where fragmentation of the sample occurs.

A linear ion trap is different from a quadropole ion trap (3D) since it restricts ions along the axis of a four-pole mass analyzer by a two-dimensional (2D) radio frequency field (RF) with potentials set to the end electrodes. It has a larger dynamic range and a better quantitative range of quantitative analysis compared to the 3D trap (Berdié *et al*., 2012).

3, Detector

Ion detector system is the last element of the mass spectrometer, which detects the separated ions that have been previously formed and quantifies them. Ion detector produces the electrical signals that are processed to generate a mass spectrum. The detected ions might correspond to the original molecules, fragments of the original molecules, or other species formed during the ionization process. Mass spectrometry plays the role of a qualitative analytical technique with high selectivity because it allows direct identification of molecules based on the mass to charge ratio as well as fragmentation patterns (Urban *et al*., 2016).

II. OBJECTIVES

Mediterranean diet is considered to be one of the healthiest in the world due to the beneficial effects on human health, like its ability to reduce the incidence of several chronic diseases, including cancer, neurodegenerative and cardiovascular ailments (Owen *et al*., 2004, Schwingshackl *et al*., 2017, Carlos *et al*., 2018).

These beneficial properties on the human health are attributed, in part, to the presence in the Mediterranean diet of a high content of bioactive compounds found in minor quantities as pentacyclic triterpenoids and phenolic compounds (Ghanbari *et al*., 2012). Phenolic compounds possess health protecting activities such as antioxidant, antidiabetic, antimicrobial, antiviral, antihypertensive, anti-inflammatory, cardioprotective, antitumoral and neuroprotective (Kano *et al*., 2015, Karković-Marković *et al*., 2019). One of the main sources of phenolic compounds in this diet are table olives together with olive oil, being both nowadays widely consumed worldwide (Uylaser and Yildiz, 2014, Accardi *et al*., 2016). Despite the fact that table olives are a more significant source of polyphenols than olive oil (Charoenprasert and Mitchell, 2012), the majority of previous studies were focused on the olive oil polyphenols, whereas the edible processed fruit of *Olea europaea* L. have been rather overlooked (Boskou, 2017, Cabrera-Bañegil *et al*., 2017).

For all this, the present study is focused on table olives, due to their abundant and unique phenolic profile, including its high content in hydroxytyrosol and derivatives, whose intake at values of 5 mg per day provides protection against cardiovascular diseases according to the EFSA health claim (Reg. EU n° 432/2012).

The general purpose of the thesis is the determination of the different phenolic compounds contained in Arbequina table olives and their concentration in plasma of rats and healthy human volunteers after their consumption. The general aim is divided into partial objectives that are described below:

1. Simultaneous analysis of phenolic compounds in Arbequina table olives by LC-ESI-MS/MS

The first object will be to characterize the composition of 16 phenolic compounds (apigenin, caffeic acid, catechol, *p*-coumaric acid, hydroxytyrosol, hydroxytyrosol acetate, luteolin, luteolin-7-*O*-glucoside, oleuropein, pinoresinol, quercetin, rutin, salidroside, tyrosol, vanillic acid, and verbascoside) contained in table olives that would later allow to study the relation between the concentrations of the individual polyphenols found in the olives and the concentrations found in the plasma after their oral administration. Table olives employed in the study were of the Arbequina variety that comes from the village of Arbeca in Lleida and it is the most important variety in Catalonia. In order to be able to characterize well this food, olives harvested during two seasons (2015/2016 and 2016/2017) will be analyzed using the analytical method previously developed and validated in our research group (Moreno-González *et al*. 2020a).

II. Objectives

2. Simultaneous determination of phenolic compounds in rat plasma by LC-ESI-MS/MS.

Previous studies in the literature assessed the plasmatic concentration of individual phenolic compounds (Ruiz-Gutierrez *et al*., 2000, Miro-Casas *et al*., 2003) or one class of polyphenols (de la Torre-Carbot *et al*., 2007, Pastor *et al*., 2016) after ingestion of olive oil and only two described the determination of polyphenols after oral intake of table olives (Kountouri *et al*., 2007, Goldstein *et al*., 2018). Kountouri *et al*. (2007) was mostly focused on phenolic alcohols and acids, without including luteolin, verbascoside, salidroside, and oleuropein, among others. Goldstein *et al*. (2018) focused their work on plasma catechols after the intake of table olives. Thus, the second objective is to develop a novel analytical method for the concurrent extraction of 16 polyphenols contained in table olives belonging to different classes (secoiridoids, phenolic alcohols, phenolic acids, lignans, and flavonoids) in rat plasma. Moreover, the developed method will be validated in blank rat plasma according to the Guidelines on Bioanalytical Method Validation, established by the European Medicines Agency (2011) (Kundisová *et al*., 2020).

3. Pre-clinical studies: Pharmacokinetics of phenolic compounds in plasma after oral administration of Arbequina table olives to Sprague-Dawley rats.

The third objective will be to evaluate the pharmacokinetics of phenolic compounds contained in Arbequina table olives in rat plasma. To complete this objective, the previously developed analytical method (Kundisová *et al*., 2020) will be verified *in vivo* by determining the plasmatic concentrations of polyphenols after the oral administration of table olives to male Sprague-Dawley rats, since only if the plasma concentrations of each compound are known, it would be possible to establish the relation between dose and effect. Subsequently, the knowledge of pharmacokinetics of individual polyphenols will be extended by evaluating the main pharmacokinetic parameters by non-compartmental analysis from plasma concentrations.

4. Clinical trial: Pharmacokinetics of phenolic compounds in plasma after the consumption of Arbequina table olives by healthy human volunteers.

The next objective will be to evaluate the pharmacokinetics of phenolic compounds in human plasma after the consumption of Arbequina table olives. To achieve this aim, at first, the previously developed analytical method (Kundisová *et al*., 2020) will be fully validated in order to verify if the method could be applied in human samples. After, the method will be applied to human samples obtained in a single centre, randomized, open-label, two-way crossover clinical trial performed with healthy male volunteers that consume 60 and 120 table olives (ClinicalTrials.gov, NCT03886597). It will be followed by the extending the knowledge of pharmacokinetics by evaluation of non-compartmental parameters.

5. Clinical trial: Plasmatic concentrations of phenolic compounds in plasma after the repeated consumption of Arbequina table olives by healthy human volunteers.

The last objective is to evaluate the effect of the repeated intake of 60 Arbequina table olives during 30 days on plasma concentration of phenolic compounds. This objective is part of a single center, randomized, open-label, two-way crossover clinical trial performed with healthy male and female volunteers (ClinicalTrials.gov, NCT03886597). To analyze the samples, previously developed method will be applied (Kundisová *et al*., 2020). Subsequently, the calculated plasma concentrations will be compared in control and olive groups over time to see if phenolic compounds accumulate in the blood with the repeated daily ingestion of table olives.

III. MATERIAL AND METHODS

3.1. CHEMICALS AND REAGENTS

Apigenin (API), luteolin (LUT), luteolin-7-*O*-glucoside (LUT-7-O-GLU), tyrosol (TYR) and verbascoside (VER) were obtained from Extrasynthèse (Genay, France). Hydroxytyrosol (HTY) and hydroxytyrosol acetate (HTY ACE) were acquired from Seprox BIOTECH (Madrid, Spain). Caffeic acid (CA), catechol (CAT), *p*-coumaric acid (PCA), 2-(3-hydroxyphenyl) ethanol (internal standard, IS), oleuropein (OLE), pinoresinol (PIN), quercetin (QUE), rutin (RUT), salidroside (SAL), vanillic acid (VA) were supplied from Sigma-Aldrich (Tres Cantos, Spain). Glacial acetic acid was purchased from Merck KGaA (Darmstadt, Germany) and ethyl acetate (LC-MS grade) from J.T Baker (Deventer, Netherlands). Acetonitrile (LS-MS grade) and methanol (LC-MS grade) were from Panreac (Castellar del Vallès, Spain). Other reagents and solvents were analytical grade and were from Sigma-Aldrich. Millipore, ultrapure water was obtained by passing through a Milli-Q water purification system (18m mΩ) (Millipore, Milan, Italy) and was used in all experiments.

3.2. ARBEQUINA TABLE OLIVES

Table olives that have been employed in the experiments of the present study were of the Arbequina variety and they were produced by the Cooperativa del Camp, (Maials, Lleida, Spain). The orchards of the Arbequina variety were in Ribera d'Ebre (Tarragona, Spain) and cultivated with drip irrigation. Olives were harvested in the green-yellow stage of maturation and in perfect sanitary conditions. Once in the factory, the fruits were separated from the leaves, and those considered of extra quality according to their size were selected. Then, the olives were subjected to a debittering process following the Greek style. The procedure consisted in a natural fermentation with 8% (w/v) of NaCl for a period superior to 2 months followed by washing and placement in the final brine which consisted in a 3.5% (w/v) of NaCl. Then, olives were stored in glass bottles in brine with aromatic herbs with the net content of 465 g. The glass bottles were stored at temperature of 5 °C.

The olives employed in the pre-clinical studies were harvested during the season 2015/2016 whereas the ones consumed by the human volunteers in the clinical trial were collected in the year 2016/2017.

3.3. ANIMALS

Male adult Sprague–Dawley rats–weighing between 250 and 300 g (n = 17) were obtained from breeding colonies from the Animal House Facility at the Faculty of Pharmacy and Food Sciences of the University of Barcelona. Animals were placed in groups of two per cage receiving the standard diet (2014 Teklad Global 14%, Envigo Rms Spain S.L.U., Sant Feliu de Codines, Spain) and water *ad libitum*. Animals were kept in rooms with controlled temperature (22 ± 2 °C), artificial lighting (12h dark: 12 h light) and humidity (50 ± 10%). The animal protocol was in full accordance with the European Community guidelines for the care and management of laboratory animals. The study was approved by the Animal

III. Material and methods

Committee of the University of Barcelona and by the Ethic Committee of Animal Experimentation of the Generalitat de Catalunya with reference number 9468.

Blank plasma was obtained by cardiac puncture from overnight fasted rats (n = 4) that did not receive neither polyphenols nor table olives. In the pre-clinical studies, blank plasma was employed in the development of the new analytical method for the simultaneous determination of phenolic compounds in plasma and for the preparation of calibration standards. Arbequina table olives were orally administered to overnight fasted rats (n = 13) and blood was obtained from the saphenous vein along the experiment. At the end of the experiment, animals were subjected to deep terminal anesthesia by intraperitoneal injection of 90 mg/kg of ketamine (Imalgene 1000, Merial Laboratorios S.A., Barcelona, Spain) and 10 mg/kg of xylazine (Rompun 2%, Química Farmacéutica Bayer S.A., Barcelona, Spain) and blood withdrawn by cardiac puncture. Total loss of pedal withdrawal and palpebral reflexes was carefully checked before blood extraction. Blood was transferred into EDTA-K_3 coated tubes, centrifuged at 1500×g for 15 min at 4 °C (Centrifuge Megafuge 1.0; Heraeus, Boadilla, Spain), and stored at -20 °C until analysis.

3.4. HEALTHY VOLUNTEERS

Eighteen healthy male volunteers with an age of 23.7 ± 0.6 years (range 20-30 years), weight of 75.3 ± 2.0 kg (range 63-94 kg), height of 179.1 ± 2.0 cm (range 167-192 cm) and body mass index (BMI) of 23.4 ± 0.4 kg/m^2 (range 19.7-25.9 kg/m^2) participated in the pharmacokinetics of phenolic compounds after a single oral administration of Arbequina table olives. Inclusion criteria required that volunteers be healthy males between 18 and 45 years of age with a BMI between 19 and 26 kg/m^2.

Forty healthy participants of both genders were included in the second study assessing the plasmatic concentrations of phenolic compound after the daily consumption of Arbequina table olives for 30 days. The 21 male volunteers were 40.2 ± 1.4 years (range 31-51 years), weighted 81.0 ± 2.3 kg (range 62-103 kg), heighted 172.6 ± 1.7 cm (range 160-186 cm) and had a body mass index (BMI) of 27.1 ± 0.5 kg/m^2 (range 23.4-29.9 kg/m^2).

On the other hand, the 19 female volunteers were 40.8 ± 1.6 years (range 30-59 years), weighted 67.2 ± 2.5 kg (range 50.5-91.0 kg), heighted 163.0 ± 1.6 cm (range 154-175 cm) and had a body mass index (BMI) of 25.2 ± 0.8 kg/m^2 (range 19.5-30.0 kg/m^2). For both male and female, the inclusion criteria required that volunteers be between 30 and 60 years of age with a BMI between 19 and 30 kg/m^2

The healthy male and female were selected from the panel volunteers at Centre d'Investigació del Medicament from the Hospital de la Santa Creu i Sant Pau (CIM-Sant Pau, Barcelona). All participants signed a written informed consent before inclusion in the trial. The volunteers were confirmed as healthy by their medical history, physical examination, and routine laboratory test performed before the enrolment.

The exclusion criteria were smoking, drug or alcohol abuse, pregnancy or lactation, chronic diseases, blood donation 4 week prior to the assay, large surgical intervention in the last 6 months, participation in a clinical trial up to 3 months prior to recruitment, high consumption of stimulating drinks (>5 per day) like coffee, different types of tea, chocolate, coca cola or grapefruit juice. Subjects who consumed any medicament during 2 weeks before the start of the intervention (with the exception of use of paracetamol in short-term treatments) included the over-the-counter (OTC) drugs (vitamins, natural food supplements), or any enzymatic inductor/inhibitor within 3 months before the intervention were also excluded.

3.5. METHOD FOR THE SIMULTANEOUS ANALYSIS OF PHENOLIC COMPOUNDS IN ARBEQUINA TABLE OLIVES BY LC-ESI-MS/MS

3.5.1. Extraction of phenolic compounds from Arbequina table olives

Phenolic compounds from Arbequina table olives used both, in the pre-clinical studies and the clinical trial were extracted following the method previously established by Moreno-González *et al.* (2020a).

Briefly, 15.4 g of destoned Arbequina table olives were mixed with 40 mL of mQ water. The sample was carefully grinded with 6 short pulses of 30 s with a Polytron homogenizer (PTA 20 TS rotor, setting 5; Kinematica AG, Lucerne, Switzerland) that yielded a fine and homogeneous olive suspension. The finely grinded destoned olives were mixed in a vortex for 1 min and 1 g of the uniform suspension was placed into 15 mL conical tube. The samples were subjected to the first extraction with the addition of 6 mL of methanol-ethanol (1:1, v/v) containing 2-(3-hydroxyphenyl) ethanol as IS. The tubes were vigorously stirred in the vortex for 5 min and centrifuged at $3345 \times g$ for 30 min at 4 °C (Megafuge 1.0R). Then, the supernatant was taken and placed to new conical tube and the pellet was subjected to two additional extractions with 3 mL ethanol-methanol (1:1, v/v). The supernatants from all three extractions were pooled and centrifuged at $27190 \times g$ for 30 min at 2 °C (Centrifuge 5417R, Eppendorf Ibérica S.L.). After filtration of the supernatant, dilutions of 1/4 and 1/50 were performed in duplicate. The dilution of 1/50 was carried out for the determination of hydroxytyrosol as it is the most abundant phenolic compound in table olive. The dilution 1/4 was carried out to measure other phenolic compounds. Samples were placed into amber vials for immediate LC-ESI-MS/MS analysis.

3.5.2. Determination of phenolic compounds from Arbequina table olives by LC-ESI-MS/MS

3.5.2.1. Instruments

Along the development of the experimental study, two different instruments were used for the analysis of phenolic compounds in Arbequina table olives.

An Agilent 1200 liquid chromatograph (Agilent Technologies, Santa Clara, CA) coupled to a QTRAP 4000 system (AB Sciex, Toronto, Canada) was used for the determination of the phenolic compounds in Arbequina table olives administered to Sprague-Dawley rats (harvest

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2015-2016). The equipment was controlled by the Analyst software version 1.6.2 (AB Sciex) that also executed the data acquisition and analysis.

On the other hand, the determination of phenolic compounds in Arbequina table olives consumed by the human volunteers in the clinical trial (harvest 2016-2017) was performed on an Acquity UPLC system (Waters, Milford, Michigan, USA) coupled to an API3000 triple quadrupole (AB Sciex, Toronto, Canada). The control of the instrument as well as data acquisition and analysis was performed with the Analyst software 1.4.2 (AB Sciex).

Both instruments were available at the Scientific and Technological Centers of the University of Barcelona (CCiTUB).

3.5.2.2. Liquid chromatography conditions

The liquid chromatography conditions were the same in both instruments used that were previously described by Moreno-González *et al.* (2020a) in our laboratory for the analysis of phenolic compounds in table olives, except for the gradient elution.

3.5.2.2.1. Liquid chromatography conditions for the QTRAP instrument

The stationary phase consisted of a Zorbax Eclipse XDB-C18 (Agilent Technologies, Santa Clara, California, USA) reversed-phase column (150 x 4.6 mm, 5 µM) preceded by a Zorbax Eclipse XDB-C18 precolumn (12.5 x 4.6 mm, 5 µM). The mobile phase was composed of an aqueous phase (phase A) formed with Milli-Q water with 0.025% acetic acid and an organic phase (phase B) comprising acetonitrile with 5% acetone. The mobile phase was delivered at a flow rate of 0.8 mL/min with the gradient program indicated in Table 3.1.

Table 3.1. Gradient of elution for the determination of phenolic compounds in table olives.

Time (min)	Aqueous phase (A) (%)	Organic phase (B) (%)
0.0	95	5
1.0	90	10
10.0	35	65
10.5	0	100
15.5	0	100
16.0	95	5
22.0	95	5

The time needed for the acquisition of data was 10.5 min. Then, an ensuing period of 5 min of 100% organic phase was applied to wash the column and prevent carry-over. Finally, mobile phase returned to initial conditions that were maintained for 6 min to equilibrate the equipment before the next injection. Hence, the total injection time was 22 min.

Further carry-over was prevented by washing the injector needle with isopropanol, tetrahydrofuran, and Milli-Q water in a proportion of 1:1:1 (v/v).

The temperature of the column was maintained at 30 °C, while the samples remained at 10 °C to avoid degradation. The automatic injection volume was set at 10 µL.

3.5.2.2.2. Liquid chromatography conditions for the API 3000 instrument

The gradient elution used when the API 3000 equipment was employed is described in Table 3.2.

Table 3.2. Gradient of elution for the determination of phenolic compounds in table olives.

Time (min)	Aqueous phase (A) (%)	Organic phase (B) (%)
0.0	97.5	2.5
2.0	97.5	2.5
3.0	90.0	10.0
8.0	35.5	65.0
8.5	0.0	100.0
13.5	0.0	100.0
14.0	97.5	2.5
20.0	97.5	2.5

Data was acquired within 8.5 min, whereas the total injection time was 20 min, due to the 5 min period of 100% organic phase applied to wash the column, followed by returning to initial conditions for 6 min to ensure equilibration of the equipment prior to the next injection.

3.5.2.3. Mass spectrometry conditions

The mass spectrometry conditions were as previously established by Moreno-González *et al.* (2020a) for the API 3000 equipment and were slightly modified when the QTRAP was employed.

3.5.2.3.1. Mass spectrometry conditions for the QTRAP instrument

Ionization of phenolic compounds in table olives was performed in negative mode using an electrospray ionization source (ESI) with temperature set at 600 °C. Multiple reaction monitoring (MRM) was used to carry out the analysis observing the mass/charge ratio (*m/z*) of the compounds. The MRM experiment was done by specifying the parent mass of the analyte (precursor ion) which was subsequently fragmented into product ions. The dwell times were 60 ms and 30 ms for the quantifier and qualifier transitions, respectively. The ESI source was used with the following settings: curtain gas (N_2), 25 arbitrary units (au); ion source gas 1 (source heating gas, N_2), 50 au; ion source gas 2 (drying gas, N_2): 50 au and ionization spray voltage: -3500 V.

MRM fragmentation and transitions of each compound were established by direct infusion of each polyphenol and IS at 50 μM with the use of a Model 11 syringe (Harvard Apparatus, Massachusetts, USA) at a flow rate of 30 μL/min. Subsequently, the spectrometry parameters were optimized for efficient isolation of precursor ions and their selective fragments.

The quantifier and qualifier transitions, declustering potential, entrance potential, collision energy and collision cell exit potential are displayed in Table 3.3.

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Table 3.3. MRM parameters corresponding to each phenolic compound and the internal standard set or obtained by LC-ESI-MS/MS for the analysis in table olives using the QTRAP equipment.

Analyte	Retention time(min)	Parent ion(*m/z*)	Fragment ion (*m/z*)	Fragment function	DP (V)	EP (V)	CE (V)	CXP (V)
Apigenin	10.65	269.0	117.1	Q	-120	-10	-55	-10
			151.1	I	-100	-10	-35	-10
Caffeic acid	7.13	179.1	135.1	Q	-75	-10	-23	-10
			107.1	I	-75	-10	-30	-15
Catechol	7.38	109.0	91.2	Q	-80	-10	-28	-10
			65.0	I	-82	-10	-40	-10
p-coumaric acid	8.12	163.2	119.2	Q	-80	-10	-22	-15
			93.2	I	-80	-10	-45	-15
Hydroxytyrosol	5.77	153.2	122.8	Q	-78	-10	-20	-10
			94.8	I	-78	-10	-30	-15
HT acetate	8.86	195.0	59.0	Q	-85	-10	-17	-10
			134.7	I	-85	-10	-20	-10
Luteolin	9.79	285.2	133.2	Q	-100	-10	-50	-10
			150.9	I	-110	-10	-75	-10
Luteolin-7-*O*-glu	7.54	447.3	285.2	Q	-130	-10	-40	-15
			327.1	I	-130	-10	-36	-15
Oleuropein	8.37	539.5	275.0	Q	-109	-10	-30	-10
			307.3	I	-100	-10	-30	-10
(+)-Pinoresinol	10.06	357.3	151.1	Q	-97	-10	-27	-10
			136.1	I	-97	-10	-25	-10
Quercetin	9.94	301.2	151.1	Q	-110	-10	-30	-10
			179.1	I	-100	-10	-35	-10
Rutin	7.25	609.5	300.1	Q	-300	-10	-50	-10
			271.0	I	-300	-10	-75	-15
Salidroside	5.74	299.2	119.2	Q	-74	-10	-22	-15
			89.3	I	-74	-10	20	-15
Tyrosol	6.70	137.1	106.2	Q	-70	-10	-20	-15
			118.8	I	-70	-10	-20	-15
Vanillic acid	7.20	167.0	152.0	Q	-70	-10	-20	-10
			157.9	I	-70	-10	-28	-10
Verbascoside	7.	623.5	161.3	Q	-140	-10	-50	-10
			461.3	I	-150	-10	-48	-10
IS	7.13	137.0	107.0	Q	-70	-10	-18	-15

Q, quantifier transition; I, identifier transition; DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, collision cell exit potential; IS, internal standard.

3.5.2.3.2. Mass spectrometry conditions for the API 3000 instrument

When the API 3000 instrument was used, the ESI source operated at 350 °C. The effluent from the chromatographic column was split by means of a T-type phase separator with an approximately 4:1 split ratio before entering the mass spectrometer. The parameters used in the ESI source were nebulizer gas (N_2), 10 arbitrary units (au); curtain gas (N_2), 12 au; collision gas (N_2):4 au and ionization spray voltage: -3500 V.

Table 3.4. MRM parameters corresponding to each phenolic compound and the internal standard set or obtained by LC-ESI-MS/MS for the analysis in table olives in the API3000 instrument.

Analyte	Retention time(min)	Parent ion(*m/z*)	Fragment ion (*m/z*)	Fragment function	DP (V)	EP (V)	CE (V)
Apigenin	8.93	269.1	117.1	Q	-65	-10	-48
			151.1	I	-65	-10	-34
Caffeic acid	6.92	179.1	135.2	Q	-40	-5	-25
			107.1	I	-40	-5	-35
Catechol	7.21	108.8	91.0	Q	-53	-10	-29
			65.0	I	-53	-10	-36
o-Coumaric acid	8.12	163.1	119.2	Q	-40	-4	-20
			93.0	I	-40	-4	-40
p-Coumaric acid	7.57	163.1	119.2	Q	-40	-4	-40
			93.0	I	-40	-4	-20
Hydroxytyrosol	6.23	153.0	123.0	Q	-40	-5	-25
			95.0	I	-40	-5	-25
HT acetate	8.00	195.0	59.1	Q	-40	-4	-25
			135.1	I	-40	-4	-15
Luteolin	8.42	285.0	133.0	Q	-75	-10	-50
			151.0	I	-75	-10	-35
Luteolin-7-*O*-glu	7.05	447.2	285.0	Q	-80	-10	-40
			327.0	I	-80	-10	-35
Oleuropein	7.53	539.5	275.0	Q	-50	-11	-33
			307.0	I	-50	-11	-33
(+)-Pinoresinol	8.61	357.3	151.1	Q	-70	-6	-45
			135.9	I	-70	-6	-25
Quercetin	8.50	301.0	151.0	Q	-55	-4	-30
			179.1	I	-55	-4	-25
Rutin	6.84	609.0	300.1	Q	-55	-10	-50
			271.0	I	-55	-10	-80
Salidroside	6.10	299.1	119.0	Q	-50	-4	-20
			89.3	I	-50	-4	-20
Tyrosol	6.79	137.1	106.0	Q	-45	-4	-25
			118.8	I	-45	-4	-25
Vanillic acid	7.03	167.1	108.0	Q	-50	-6	-25
			152.0	I	-50	-6	-20
Verbascoside	6.91	623.5	161.1	Q	-85	-10	-50
			461.2	I	-85	-10	-40
IS	7.02	137.0	107.0	Q	-40	-5	-20

Q, quantifier transition; I, identifier transition; DP, declustering potential; EP, entrance potential; CE, collision energy; IS, internal standard.

The mass spectrometry parameters were optimized by the direct infusion of each phenolic compound and IS at 50 μM delivered at a flow rate of 10 μL/min using a Model 11 syringe (Harvard Apparatus) as it is shown in Table 3.4. The dwell time for the quantifier transition was 60 ms, and for the qualifier transition it was 10 ms.

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3.5.2.4. Identification of phenolic compounds

The identification of the phenolic compounds was performed by comparing the retention time of each analyte in the sample of Arbequina table olives with those of a standard and considering the quantifier and qualifier transitions obtained with the MRM mode (Tables 3.2 and 3.3).

3.5.2.5. Quantification of phenolic compounds

The quantification of phenolic compounds in Arbequina table olives was carried out using the standard addition method.

3.5.2.5.1. Stock solutions and working solutions

Individual stock solutions of phenolic compound and IS were prepared to a final concentration of 250 µM, were divided into aliquots, placed into 2 mL eppendorfs, and stored at -20 °C. The stock solutions were used for the preparation of mixtures of working standard containing all 17 polyphenols at concentrations of 1 and 10 µM. The stock solution of 2-(3-hydroxyphenyl) ethanol as IS was diluted to produce the working standard of 50 µM. Stock solution and working standards were prepared always employing methanol 80% as solvent. Working solutions of phenolic compounds and IS were freshly prepared before use.

3.5.2.5.2. Calibration curves

Calibration standards were done by directly adding working solutions to the final supernatants of Arbequina table olives at the same dilution of the samples (1/50 or 1/4). Hence, the final concentrations of phenolic compounds in the calibration standards spiked post-extraction were 0, 0.1, 0.25, 0.5, 1, 1.5, and 2 µM.

Table 3.5. Quantification of phenolic compounds in table olives with the standard addition method. Calibration curves were prepared by spiking the samples of Arbequina table olives obtained post-extraction at the dilutions 1/50 and 1/4.with working solutions at 1 and 10 µM.

Concentration	Working solution	Olive extract Dilution 1/50	Methanol 80%	Olive extract Dilution 1/4	Methanol 80%
0 µM	0 µL	20 µL	980 µL	250 µL	750 µL
Calibration standards prepared with the mixture of polyphenols at 1 µM					
0.01 µM	10 µL	20 µL	970 µL	250 µL	740 µL
Calibration standards prepared with the mixture of polyphenols at 10 µM					
0.25 µM	25 µL	20 µL	955 µL	250 µL	725 µL
0.5 µM	50 µL	20 µL	930 µL	250 µL	700 µL
1 µM	100 µL	20 µL	880 µL	250 µL	650 µL
1.5 µM	150 µL	20 µL	830 µL	250 µL	600 µL
2 µM	200 µL	20 µL	780 µL	250 µL	550 µL

Table 3.5 shows the volumes and the concentrations of the working solutions used to obtain the theoretical concentrations for establishment of the calibration curves. All calibration points were prepared in triplicates and methanol 80% was used as dilution solvent.

Within each analytical run, a full set of calibration standards which included reagent blank were injected.

3.6. METHOD FOR THE SIMULTANEOUS DETERMINATION OF PHENOLIC COMPOUNDS IN RAT PLASMA BY LC-ESI-MS/MS

3.6.1. Extraction protocol of phenolic compounds in rat plasma samples

In the process of sample preparation, 200 µL of calibration standard or plasma was taken and placed into 15 mL conical tubes. Then, samples were spiked with 10 µL of freshly prepared 10% ascorbic acid to a final percentage of 0.5%, 10 µL of 0.5% acetic acid to the final percentage of 0.05% and with 10 µL of 10 µM 2-(3-hydroxyphenyl) ethanol as IS to a final concentration of 0.50 µM.

Subsequently, plasma samples were subjected to two liquid-liquid extractions, that consisted in the addition of 2 mL of ethyl acetate, vigorous stirring in a vortex for 5 min followed by 10 min in the ultrasonic bath and centrifugation at $1500 \times g$ for 10 min at 2 °C in a Centrifuge Megafuge 1.0 (Heraeus, Boadilla, Spain). The transparent supernatant was collected with an automatic pipette and was transferred into a new 15 mL conical tube and the pellet was subjected to the same process.

The supernatants of both extractions were pooled before the addition of 10 µL of 10% ascorbic acid. Samples were slightly mixed on the vortex and were evaporated to dryness using a Concentrator 5301 (Eppendorf Ibérica S.L., San Sebastián de los Reyes, Spain) with temperature set to 45 °C. After the evaporation of the solvent, the residue was reconstituted by adding 80 µL of methanol 100% and energetically mixing in a vortex for 5 min. Then, 20 µL of Milli-Q water was added and samples were placed into an ultrasonic bath for 2 min. Finally, a centrifugation at $27190 \times g$ for 30 min at 4 °C (Centrifuge 5417R) was performed to eliminate all the remaining particles. The clear supernatant was placed into vials for immediate LC-ESI-MS/MS analysis.

3.6.2. Liquid chromatography-mass spectrometry analyses of phenolic compounds in rat plasma

3.6.2.1. Instrument

Phenolic compounds in plasma were analyzed in the Agilent 1200 liquid chromatograph coupled to a QTRAP 4000 system that has been described in the section 3.5.2.1.

3.6.2.2. Liquid chromatography-mass spectrometry conditions

The stationary and mobile phases are the same as in the analysis of phenolic compounds in table olives described in section 3.5.2.2.1, with the difference of the injection volume set at 2 µL.

The conditions of the mass spectrometer used for the analysis of phenolic compounds in plasma samples were the same as the ones described in the section 3.5.3.3.1.

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3.6.2.3. Identification and quantification

The identification of phenolic compounds was performed by comparing the retention time of the analytes in plasma samples with those of a standard and taking into account the mass/charge ratio (*m/z*) of the precursor ion and the m/z of the quantifier and qualifier ions obtained with the MRM mode (Table 3.6).

Table 3.6. MRM parameters corresponding to each phenolic compound and the internal standard obtained by LC-ESI-MS/MS for the analysis in plasma samples using the QTRAP equipment.

Analyte	Retention time(min)	Parent ion(*m/z*)	Quantifier ion (*m/z*)	Qualifier ion (*m/z*)
Apigenin	10.58	269.0	117.1	151.1
Caffeic acid	7.02	179.1	135.1	107.1
Catechol	7.24	109.0	91.2	65.0
p-coumaric acid	8.00	163.2	119.2	93.2
Hydroxytyrosol	5.68	153.2	122.8	94.8
HT acetate	8.78	195.0	59.0	134.7
Luteolin	9.73	285.2	133.2	150.9
Luteolin-7-*O*-glu	7.47	447.3	285.2	327.1
Oleuropein	8.34	539.5	275.0	307.3
(+)-Pinoresinol	10.03	357.3	151.1	136.1
Quercetin	9.85	301.2	151.1	179.1
Rutin	7.18	609.5	300.1	271.0
Salidroside	5.70	299.2	119.2	89.3
Tyrosol	6.66	137.1	106.2	118.8
Vanillic acid	7.11	167.0	152.0	157.9I
Verbascoside	7.31	623.5	161.3	461.3I
IS	7.11	137.0	107.0	

The quantification of phenolic compounds in plasma samples was carried out by interpolation of the peak area ratio of the analytes *versus* IS on a calibration curve prepared with calibration standards constructed with blank plasma samples.

3.6.3. Validation of analytical method in rat plasma

The developed analytical method was validated following the Guidelines on Bioanalytical Method Validation of the European Medicines Agency (EMA, 2011), as well as the generally accepted recommendations described by Matuszewski *et al*. (2003).

Validation was carried out in three consecutive day. Three different batches of blank plasma were used to prepare calibration standards that were analyzed in triplicate for each concentration. The parameters evaluated were matrix effect, recovery, linearity, limit of quantification, precision, accuracy, selectivity, and carry-over.

3.6.3.1. Stock solutions, working solutions and calibration standards

Individual stock solutions of phenolic compound and IS were prepared to a final concentration of 250 µM, were divided into aliquots, placed into 2 mL eppendorfs, and stored at -20 °C. The stock solutions were used for the preparation of mixtures of working standard containing all 16 polyphenols at concentrations of 200, 500, 1000, 2000, 3000, and 5000 nmol/L. The stock solution of 2-(3-hydroxyphenyl) ethanol as IS was diluted to produce the working standard of 10 µM. Stock solution and working standards were prepared always employing methanol 80% as solvent.

Calibration standards were prepared with 190 µL of blank plasma spiked with 10 µL of the previously indicated working standards to the final concentrations of phenolic compounds of 10, 25, 50, 100 and 250 nmol/L. Working standards and calibration standards were always freshly prepared before each experiment.

3.6.3.2. Matrix effect

Matrix effect was calculated following the recommendations of Matuszewski *et al*. (2003) at three different concentrations corresponding to 25, 100, and 250 nmol/L. The assessment of matrix effect, expressed as percentage (%) was determined by comparing the analyte peak areas of individual polyphenols and IS spiked in extracted blank rat plasma at the expected concentration at the final volume of 100 µL to standards prepared in methanol 80% at the same concentration.

3.6.3.3. Recovery

The recovery of the analytical method vas evaluated as indicated by Matuszewski *et al*. (2003) at the concentrations of 25, 100, and 250 nmol/L. The recoveries of the analytes and IS were calculated as a percentage (%) by comparing the analyte peak area of blank plasma samples spiked with working standards of polyphenols and IS before extraction to those spiked after extraction with the compounds at the estimated concentrations.

3.6.3.4. Linearity

Linearity is the ability of an analytical method to give results that are directly proportional to the concentration of analyte present in the sample within a given range. Plasma samples were spiked with increasing concentrations of polyphenols and the linearity was evaluated at concentrations of 0, 10, 25, 50, 100, and 150 nmol/L. The calibration curves were constructed by plotting the peak area ratio of the polyphenols to those of the IS (y) against the concentration of analytes (x). A linear regression analysis was performed by the least-squares method and was used to determine slopes, intercepts, and coefficient of correlation. The reproducibility of the test was evaluated by comparing the linear regressions of the three standard plots prepared during three different days.

3.6.3.5. Limit of quantification

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The limit of quantification (LOQ) was the concentration of analyte that yielded a signal five times the signal of a blank sample. The LOQ was validated by carrying out the analysis of six independent blank plasma samples spiked with standards of phenolic compounds at concentrations proximal to the theoretical ones, and their precision and accuracy were below the 20% recommended by the EMA (2011).

3.6.3.6. Precision

Precision was evaluated by replicate analysis (n = 5) of calibration standards spiked with phenolic compounds at six different concentrations as follows: 10, 25, 50, 100, 150, and 250 nmol/L. Intra-day precision was measured by analyzing the calibration standards prepared within a day while the inter-day precision was assessed by the determination of the samples prepared during three different days. The precision, expressed as a relative standard deviation (coefficient of variation) was calculated using the following equation:

$$CV\ (\%) = \frac{Standard\ deviation}{Mean} \times 100$$

Precision should not exceed 15% as indicated by the EMA (2011).

3.6.3.7. Accuracy

Calibration standards spiked with phenolic compounds at the concentrations of 10, 25, 50, 100, 150, and 250 nmol/L were used for the establishment of accuracy. Five replicates were analyzed for each concentration.

The accuracy was assessed as the bias or percentage deviation between the nominal and measured concentrations and was calculated according to the following formula:

$$Accuracy\ (\%) = \frac{Con_T - Con_M}{Con_T} \times 100$$

where Con_T is the theoretical concentration of analyte and Con_M corresponds to the concentration obtained by analytical method. According to EMA (2011), accuracy should be within the $\pm$ 15 % limit.

3.6.3.8. Selectivity

Selectivity was assessed to evaluate if the extraction procedure was able to differentiate individual polyphenols and IS from endogenous compounds in plasma. Thus, six independent double blank plasma samples containing neither analyte nor IS were put into comparison with ones spiked with mixture of polyphenols at 150 nmol/L and IS at 500 nmol/L. The method is considered selective if chromatograms of blank plasma have no peaks at retention times of analytes.

3.6.3.9. Carry-over

The evaluation of carry-over on the LC-ESI-MS/MS instrument was accomplished 6 times in each analytical run by injection of the highest calibration standard followed by a blank sample

at intervals based on the total number of samples per batch. Moreover, as a precautionary measure, two independent blank plasma samples were also set before the first analysis of samples.

3.7. PRE-CLINICAL STUDIES: PHARMACOKINETICS OF PHENOLIC COMPOUNDS IN PLASMA AFTER THE ORAL ADMINISTRATION OF ARBEQUINA TABLE OLIVES TO SPRAGUE-DAWLEY RATS

3.7.1. Selection of the dose of Arbequina table olives

Male Sprague-Dawley rats were orally administered at a dose equivalent to the human intake of 30 or 60 Arbequina table olives. The translation from human to animal dose was calculated using the body surface area normalization method proposed by Reagan-Shaw *et al*. (2007). To this end, we used the following formula:

$$Human\ equivalent\ dose\ (mg/kg) = Animal\ dose\ (mg/kg) \times \frac{Animal\ K_m}{Human\ K_m}$$

The K_m factor is calculated from the body weight (kg) divided by body surface area (m^2) that converts the mg/kg dose used in a study to an mg/m^2 dose. The K_m values based on average BSA calculations for human assuming a weight of 60 kg with a BSA average of 1.6 m^2 is 37. For rats of 0.15 kg with a BSA of 0,025, K_m value is 6 (FDA, 2005). Assuming that the weight of a destoned Arbequina olive is around 1.25 g, the equivalent dose to 30 olives is 3.85 g of destoned olives/kg of rat body weight. In the case of 60 olives, the dose to be administered to the rat is 7.70 g/kg.

3.7.2. Oral administration of Arbequina table olives to rats

Arbequina table olives were administered to male Sprague-Dawley rats as homogeneous suspensions that were prepared considering the volume of administration of 10 mL/kg. Therefore, the doses of 3.85 g/kg (group 30 olives, n = 6) and 7.70 g/kg (group 60 olives, n = 7) were prepared at the concentrations of 384.4 mL/kg and 770.8 mL/kg, respectively. Hence, two different homogenous suspensions were made. On the one hand, the dose equivalent to the human intake of 30 olives was prepared by mixing 15.42 g of destoned Arbequina olives with 40 mL of Milli-Q water. Whereas the dose of 60 olives was produced by mixing 30.84 g of destoned olives with 40 mL of Milli-Q water. The homogenous suspensions were prepared by placing the corresponding amount of destoned olive into 50 mL conical tubes with 20 mL of Milli-Q water. Destoned olives were carefully blended with the help of Polytron® (PTA 20 TS rotor, setting 5, Kinematica AG, Lucerne, Switzerland) coupled to a 20 TS arm. All the process consisted of 6 cycles, each cycle of 30 seconds, at a speed set at 5 and with pause of 1 min in between the cycles. Then, the 20 TS arm was cleaned to recover all the parts of the olive pulp during 2 cycles of 30 seconds at speed 5 with 5 mL of Milli-Q water in each cycle using new 50 mL conical tube. The remains of the triturate were removed from the arm with the use of spatula and added to the triturate. Both cleaning fluids were added to the suspension and vortex stirred for 1 min. In the cleaning process, and additional 20 mL of Milli-Q water

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was added, to a final volume of 40 mL. During all the process, at the time of preparing of the suspensions and during the resting, the conical tube was kept on ice. The final volume was checked by weight. At the end of the preparation process, the suspensions were visually checked to be homogenous and to be able to pass through the cannula used for the administration. The homogenous suspensions were prepared in duplicate. To avoid contamination of subsequent samples, the Polytron® was properly washed (speed 6, cycle of 30 seconds) with Milli-Q water, methanol 100% and NaOH 5%. The freshly prepared homogeneous suspensions of Arbequina table olives were orally administered to overnight fasted male Sprague−Dawley rats by gavage (18-gauge × 76 mm, ref FFSS-185-76, Instech Laboratories, Inc., Plymouth Meeting, PA) at a volume of administration of 10 mL/kg.

3.7.3. Blood sampling

After the oral administration of table olives at the doses of 30 and 60 olives, blood was withdrawn at 30, 60, 90, 120, 180, 240, 360 and 480 min following a sparce sampling design. All blood samples were collected from the lateral saphenous vein following the technique previously reported (Sánchez-González *et al.*, 2014) except for the last point. Briefly, the blood from the saphenous vein was directly collected into Microvette® CB 300 K_2 EDTA-K_2 coated tubes (Sarstedt, Granollers, Spain). At each sampling time, 0.45 mL of blood were withdrawn meaning that a maximum of 1.8 mL was obtained from each animal. This volume represents less than 10% of all circulating blood and do not affect the hematocrit (Mackie *et al*., 2005). The final sampling point was taken by cardiac puncture with the animal under terminal anesthesia. Plasma was immediately obtained by centrifugation at 1500×*g* at 4°C for 15 min (Centrifuge Megafuge 1.0R) and frozen at -20°C until analysis.

3.7.4. Determination of phenolic compounds and its metabolites by LC-ESI-MS/MS

The extraction of phenolic compounds form plasma samples was performed as described in the section 3.6.1, whereas their determination by LC-ESI-MS/MS was carried out as reported in the section 3.6.2.

3.7.4.1. Identification of phenolic compounds and metabolites

Table 3.7. MRM parameters for the determination of metabolites of the main phenolic compounds in table olives obtained by LC-ESI-MS/MS for the analysis in plasma samples using the QTRAP equipment.

Analyte	Parent ion(*m/z*)	Fragment ion (*m/z*)	DP (V)	EP (V)	CE (V)	CXP (V)
Hydroxytyrosol glucuronide	329.1	153.0	-78	-10	-20	-10
Hydroxytyrosol sulfate	233.0	153.0	-78	-10	-20	-10
Hydroxytyrosol sulfo-glucuronide	409.0	153.0	-70	-10	-10	-10
Tyrosol glucuronide	313.0	137.0	-70	-10	-20	-15
Tyrosol sulfate	217.0	137.0	-70	-10	-20	-15

DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, collision cell exit potential.

Concerning the identification of phenolic compounds, in addition to the parent compounds described in section 3.6.2.3, different metabolites of hydroxytyrosol, tyrosol and luteolin were found (Table 3.7).

3.7.4.2. Quantification of phenolic compounds and metabolites

Quantification of phenolic compounds in plasma was achieved by the interpolation of the ratio of the peak area of the analyte and the internal standard interpolated in an external calibration curve.

Table 3.8. Calibration curve prepared with blank rat plasma spiked with increasing concentrations of working solutions of phenolic compounds.

Concentration (nmol/L)	Mixture Polyphenols	Blank plasma
0	0 µL	190 µL
10	10 µL (working solution 0.2 µM)	190 µL
25	10 µL (working solution 0.5 µM)	190 µL
50	10 µL (working solution 1 µM)	190 µL
100	10 µL (working solution 2 µM)	190 µL
150	10 µL (working solution 3 µM)	190 µL

Calibration curves were prepared using blank rat plasma spiked with 10 µL of freshly prepared working solutions at different concentrations to reach the final concentrations: 0, 10, 25, 50, 100, and 150 nmol/L, as indicated in Table 3.8. The calibration standards that were prepared in triplicate were subjected to the same extraction process as the plasma samples (section 3.6.1).

The metabolites were assumed to possess a similar LC-ESI-MS/MS response to that of the parent compounds, thus the concentrations of the sulfate and glucuronides were quantified using the standard curve of hydroxytyrosol, tyrosol and luteolin.

3.7.5. Pharmacokinetic analysis

The plasmatic concentrations of the phenolic compounds and their metabolites found in rat plasma after the oral administration of Arbequina table olives at the doses equivalent to the human intake of 30 and 60 olives were analyzed following a non-compartmental approach using software WinNonlin Professional User's Guide, version 2, Pharsight Corporation (Palo Alto, CA, 1997).

The main pharmacokinetic parameters were evaluated from the mean plasma concentrations *versus* time. Plasmatic concentrations below LOQ were excluded from the analysis.

The following pharmacokinetic parameters were estimated:

- The maximum plasmatic concentration – $\mathbf{C_{max}}$
- The time when C_{max} is reached – $\mathbf{T_{max}}$
- The terminal elimination rate constant, calculated using log-linear regression from the slope of the terminal phase on the graph of the plasma concentration *versus* time curves – **Lambda z - λ_z**
- The apparent elimination half-life - described as $0.693/\lambda z$, is the time needed for the plasma concentration of the analyte to decrease by half of the initial value - **HL lambda z - $t_{1/2z}$**
- The area under the concentration curve from time time zero to the last quantified concentration estimated using the trapezoidal method - **AUC_{last}**
- The area under the plasma concentration curve from time zero extrapolated to infinity – **$AUC_{0-\infty}$**, calculated as: $AUC_{0-\infty} = AUC_{last} + C_{last}/\lambda z$, where C_{last} is the last measurable concentration
- The percentage of the total AUC, represents the percentage of AUC_∞ from T_{last} to infinity: **$AUC_{extrap\%}$**, calculated as: $AUC_{extrap\%} = (1 - AUC_{last}/AUC_\infty) \times 100$
- The mean residence time, from time zero to the last measurable concentration - **MRT_{last}**
- The mean residence time, from time zero to infinity - **$MRT_{0-\infty}$**

3.8. CLINICAL TRIAL: PHARMACOKINETICS OF PHENOLIC COMPOUNDS IN PLASMA AFTER THE CONSUMPTION OF ARBEQUINA TABLE OLIVES BY HEALTHY HUMAN VOLUNTEERS

The clinical intervention was performed at the Centre d'Investigació del Medicament from the Hospital de la Santa Creu i Sant Pau (CIM-Sant Pau), Barcelona.

The protocol used in the clinical trial followed the international recommendations for clinical research, was approved by the Comité Ético de Investigación Clínica (CEIC) from the Hospital de la Santa Creu i Sant Pau (IIBSP-OLI-2016-23) and was registered at the ClinicalTrials.gov (NCT03886597).

Clinical trial was divided into stage I and stage II. Stage I of clinical trial consisted in the pharmacokinetics of phenolic compounds after the single oral ingestion of Arbequina table olives.

3.8.1. Study design

A single center, randomized, open, two-way crossover clinical trial in which two different doses of Arbequina table olives (60 and 120 olives) were administered to 18 healthy young male volunteers was performed.

The study was divided into two periods as displayed in Figure 3.1. In the first period, a group of 9 patients received 60 Arbequina table olives (intervention group I), while the other 9 subjects received 120 Arbequina table olives (intervention group II). The period I was

followed by washout of 7 days and the intervention groups were switched. In this way, all participants received both doses of Arbequina table olives.
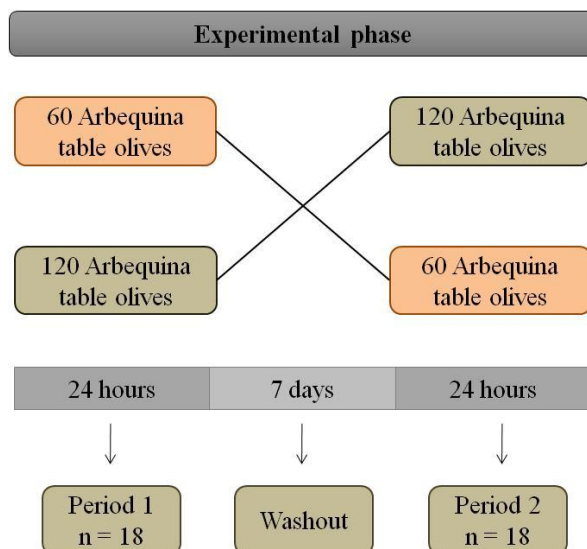
Figure 3.1. Study design of the experimental phase of stage I of nutritional intervention corresponding to the pharmacokinetics of phenolic compounds.

On the day of the experiment, the male volunteers arrived to the CIM-Sant Pau after 10 hours of fasting. The subjects were hospitalized for 12 hours after the intake of table olives under the supervision of qualified personal.

Subsequently, the patients were able to go home and return to the hospital the following day for the withdrawal of the time point of 24 h.

Then, volunteers underwent a 7-day washout period before returning to the hospital to receive a second dose of table olives (Figure 3.1). The study was performed in an appropriate room with heating and air conditioning.

3.8.2. Assignment of the participants

Prior to the study, the participants were randomly divided into 2 intervention groups according to the amount of received olives (AB; BA; A represented intake of 60 table olives, meanwhile B represented intake of 120 table olives).

For the randomization, the program R was used in a balanced way, meaning that equal number of subjects participated in each intervention period. The randomization table (Table 3.9) was generated, and each randomization number corresponded to a sequence of intervention.

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Table 3.9. The randomization table showing how patients were randomly allocated into 2 periods of intervention according to the table olives intake.

Subject	Period	Sequence
1	2	BA
2	2	BA
3	2	BA
4	1	AB
5	1	AB
6	1	AB
7	2	BA
8	1	AB
9	1	AB
10	2	BA
11	1	AB
12	2	BA
13	1	AB
14	1	AB
15	2	BA
16	2	BA
17	1	AB
18	2	BA

3.8.3. Arbequina table olive intake and blood collection

Male healthy volunteers arrived at CIM-Sant Pau early in the morning (around 8 h) after 10 hours of fasting, and blood was collected at 0 h to make the baseline. Then, all subjects (males, n = 18) participating in the study received 60 or 120 olives of Arbequina the variety together with 240 mL of water.

Table olives were weighed prior to the ingestion and the weight of remaining stones was recorded to know the quantity of olive pulp taken by the subject. The volunteers had 5 min to eat 60 olives and 10 min to ingest 120 olives. After the intake, the mouths of the patients were visually checked if the olives were consumed completely and that there were no olive pieces remaining.

During the intervention, the water intake was not allowed from 1 hour prior to administration until 3.5 h post administration, being allowed *ad libitum* from then on. Food consumption was only permitted starting at 4 hours post-administration. Subjects were not allowed to go to the toilet during the first one-hour after the intake of olives.

Food rich in polyphenols and beverages or food containing xanthine such as coffee (decaffeinated coffee included), black tea, coca cola or chocolate were not allowed at the study site.

Blood samples for the determination of phenolic compounds were withdrawn in the following times after the oral intake of Arbequina table olives: 0.5 h, 1 h, 1.5 h, 2 h, 4 h, 6 h, 8 h and 24

h. Blood samples were taken into tubes containing EDTA-K$_2$ that were kept on ice until they were centrifuged at 1900×g at 4°C for 10 min. Plasma was separated from the blood cells, kept in 1.5 mL eppendorf and frozen at – 20 °C until analysis.

3.8.4. Determination of phenolic compounds and its metabolites in human plasma

At the beginning of the sample preparation process, the stored frozen plasma samples were thawed at room temperature. Human plasma samples were treated the same way as rat samples as indicated in section 3.6.1 with the difference that volume of the human plasma was 300 µL instead of 200 µL.

Calibration standards or plasma samples were taken and placed into 15 mL plastic conical tube and spiked with 15 µL of 10% ascorbic acid (always prepared freshly on the day of the experiment) to a final percentage of 0.5%, followed by 15 µL of 10 µM IS to a final concentration of 0.5 µM and 15 µL of 0.5% acetic acid to a final percentage of 0.05%. Samples were mixed in a vortex for 1 min.

Subsequently, two liquid-liquid extractions were performed using ethyl acetate as a solvent. In the first extraction, 3 mL of ethyl acetate was added to the conical tube. Samples were energetically mixed in a vortex for 5 min, placed into an ultrasonic bath for 10 min and centrifuged at 1500×g, 2 °C, 10 min (Centrifuge Megafuge 1.0).

The transparent supernatant was carefully taken with a use of automatic pipette and placed into new 15 mL conical tube. A second extraction of the pellet was performed by adding 3 mL of ethyl acetate. After centrifugation, the supernatant was taken and was added into the conical tube containing the supernatant from the first extraction. Then, 15 µL of 10% ascorbic acid was added to the pooled supernatants, samples were slightly mixed on a vortex and were place to Concentrator 5301 with the temperature set up to 45 °C.

Samples were evaporated to dryness and the residue was dissolved in 120 µL of methanol 100%, vigorously shaken in a vortex for 5 min and 30 µL of Milli-Q water was added. The samples were placed into ultrasonic bath for 2 min, followed by a centrifugation at 27190×g for 30 min at 4 °C (Centrifuge 5417R). The clear supernatant was placed into vials for immediate LC-ESI-MS/MS analysis.

3.8.5. Liquid chromatography-mass spectrometry analyses in human plasma

Phenolic compounds in plasma were analyzed in the Agilent 1200 liquid chromatograph coupled to a QTRAP 4000 system that has been described in the section 3.5.2.1.

The liquid chromatographic conditions were as reported in the section 3.6.2.2 whereas the mass spectrometry conditions were as indicated in the section 3.6.2.3 except for the MRM parameters that are reported in Table 3.10.

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Table 3.10. MRM parameters corresponding to the phenolic compounds and metabolites as well as the internal standard set or obtained by LC-ESI-MS/MS for the analysis of human plasma in Stage I using the QTRAP equipment.

Analyte	Parent ion(m/z)	Fragment ion (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)
Hydroxytyrosol	153.2	122.8	-78	-10	-20	-10
Hydroxytyrosol glucuronide	329.1	153.0	-78	-10	-20	-10
Hydroxytyrosol sulfate	233.0	153.0	-78	-10	-20	-10
Hydroxytyrosol sulfo-glucuronide	409.0	153.0	-70	-10	-10	-10
Hydroxytyrosol acetate	195.0	59.0	-85	-10	-17	-10
Hydroxytyrosol acetate glucuronide	371.0	195.0	-85	-10	-17	-10
Hydroxytyrosol acetate sulfate	275.0	195.0	-85	-10	-17	-10
Luteolin	285.2	133.2	-100	-10	-50	-10
Luteolin-glucuronide	461.2	285.2	-100	-10	-50	-10
Luteolin-sulfate	365.0	285.2	-100	-10	-50	-10
Oleuropein	539.5	275.0	-110	-10	-30	-10
Quercetin	301.2	151.1	-110	-10	-30	-10
Salidroside	299.2	119.2	-74	-10	-22	-15
Tyrosol	137.1	106.2	-70	-10	-20	-15
Tyrosol-glucuronide	313.0	137.0	-70	-10	-20	-15
Tyrosol-sulfate	217.0	137.0	-70	-10	-20	-15
Vanillic acid	167.0	152.0	-70	-10	-20	-10
Verbascoside	623.5	161.3	-140	-10	-50	-10
2-(3-hydroxyphenyl) ethanol (IS)	137.0	107.0	-70	-10	-18	-15

DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, collision cell exit potential.

3.8.5.1. Identification in human plasma

Phenolic compounds were identified in human plasma by comparing the retention time of each analyte in the plasma sample with those of a standard and considering the quantifier and qualifier transitions obtained with the MRM mode (Table 3.10).

3.8.5.2. Quantification in human plasma

The concentrations of phenolic compounds in plasma samples were calculated from the ratio of the peak area of the analyte to the internal standard interpolated in the external calibration curve. The calibration curve was prepared using pooled human blank plasma of the subjects participating in the study obtained at time 0 min (baseline) the day of the intervention.

Blood was taken after 10 hours fasting conditions into EDTA-K_2 tubes and centrifuged at 1900×g at 4°C for 10 min. Plasma was separated from the cells and was divided into 1.5 mL aliquots that were stored at – 20 °C until analysis.

The calibration standards were freshly prepared before each experiment. Tables 3.11 show the volumes and the concentrations of the working solutions used to obtain the theoretical concentrations for establishment of the calibration curves. The calibration standards were prepared in triplicate and they were subjected to two extractions with ethyl acetate as described in section 3.8.1.4.

Table 3.11. Calibration curve prepared with human plasma obtained at time 0 and spiked with increasing concentrations of working solutions of phenolic compounds.

Concentration (nmol/L)	Mixture Polyphenols	Blank plasma
0	0 µL	285 µL
2.5	15 µL (working solution 0.05 µM)	285 µL
5	15 µL (working solution 0.1 µM)	285 µL
10	15 µL (working solution 0.2 µM)	285 µL
25	15 µL (working solution 0.5 µM)	285 µL
50	15 µL (working solution 1 µM)	285 µL
100	15 µL (working solution 2 µM)	285 µL
150	15 µL (working solution 3 µM)	285 µL
200	15 µL (working solution 4 µM)	285 µL
300	15 µL (working solution 6 µM)	285 µL
500	15 µL (working solution 10 µM)	285 µL

3.8.6. Validation of the method

The method developed for the determination of polyphenols in rat plasma was subsequently validated in human plasma following the Guidelines on Bioanalytical Method Validation of the European Medicines Agency (EMA, 2011), as well as the generally accepted recommendations described by Matuszewski *et al*. (2003). The parameters evaluated were matrix effect, recovery, linearity, limit of quantification, precision and accuracy.

The validation of the method was performed using calibration standards prepared in triplicate with blank human plasma as indicated in the section 3.8.1.5.2.

Matrix effect and recovery were evaluated with calibration standards prepared at the concentration of 250 nmol/L, whereas linearity, precision and accuracy were assessed at 10, 25, 50, 100, 150, 200, 300, and 500 nmol/L.

3.8.7. Pharmacokinetic studies

The main pharmacokinetic parameters were evaluated by non-compartmental analysis from mean plasma concentrations of found polyphenols in stage I of the study *versus* time.

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Plasmatic concentrations below LOQ were excluded from the analysis. The following pharmacokinetic parameters were estimated: C_{max}, T_{max}, λ_z, $t_{1/2z}$, AUC_{last}, AUC_{inf}, $AUC_{extrap\%}$, MRT_{last} and $MRT_{0-\infty}$.

3.9. CLINICAL TRIAL: PLASMATIC CONCENTRATIONS OF PHENOLIC COMPOUNDS IN PLASMA AFTER THE REPEATED CONSUMPTION OF ARBEQUINA TABLE OLIVES BY HEALTHY HUMAN VOLUNTEERS

Stage II of the clinical trial that was also performed at the Centre d'Investigació del Medicament from the Hospital de la Santa Creu i Sant Pau (CIM-Sant Pau), Barcelona, assessed the plasmatic concentrations of phenolic compounds after the repeated intake of Arbequina table olives for 30 days. Clinical trial was registered at the ClinicalTrials.gov (NCT03886597).

3.9.1. Study design

The study consisted in a single-center, randomized, open-label, controlled and crossover clinical trial in which 60 table olives of Arbequina variety were consumed by 19 female and 21 male healthy volunteers two times per day (30 olives prior to lunch, 30 olives prior to dinner) during 30 consecutive days.
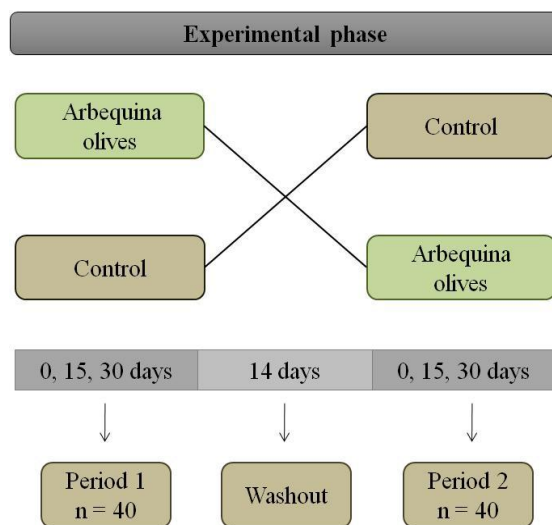
Figure 3.2. Study design of the experimental phase of stage II of nutritional intervention corresponding to plasmatic concentrations after the repeated consumption of Arbequina table olives.

The study was divided into two periods as shown in Figure 3.2. In period I., one group of 20 participants (olive group) received Arbequina table olives, meanwhile the other group of 20 participants (control group) did not receive olives. A washout of 14 days was carried out and the groups of the study were switched for the second period of the intervention.

3.9.2. Assignment of the participants

The program R was used for the random division of the participants into two intervention groups. Labels of CA and AC (where A means Arbequina table olives and C means control)

were used for the identification of the subjects. The randomization schedule of the participants which is shown in Table 3.12. was generated in a balance way, which means that the same number of patients participated in both periods.

Table 3.12. The randomization schedule showing the random assignment of the subjects into 2 periods of intervention.

Subject	Period	Sequence	Subject	Period	Sequence
1	2	AC	21	2	AC
2	2	AC	22	1	CA
3	1	CA	23	2	AC
4	1	CA	24	2	AC
5	2	AC	25	1	CA
6	1	CA	26	1	CA
7	2	AC	27	1	CA
8	1	CA	28	2	AC
9	1	CA	29	2	AC
10	2	AC	30	2	AC
11	2	AC	31	2	AC
12	1	CA	32	2	AC
13	1	CA	33	1	CA
14	2	AC	34	1	CA
15	1	CA	35	2	AC
16	2	AC	36	1	CA
17	2	AC	37	1	CA
18	1	CA	38	2	AC
19	1	CA	39	1	CA
20	1	CA	40	2	AC

3.9.3. Arbequina table olive intake and blood collection

In the second stage of the study, all participants (males, females, n = 40) performed two experimental sessions lasting 30 days with a run-in period of 15 days before the study started and 14 days washout period during the experimental period. During the run-in and washout periods the subjects had to avoid the consumption of table olives or other products containing phenolic compounds. At the beginning and after the inclusion phase, the participants received the amount of table olives corresponding for the first 15 days of the study. All the participants ingested the olives at home following a restrictive diet, low in food containing phenolic compounds. The volunteers received 60 table olives for each day of the intervention and the consumption of Arbequina olives was included in their normal eating habits. The dose of 60 Arbequina olives per day was divided to be taken in two meals, 30 olives were ingested before the lunch and the other 30 olives were consumed prior to dinner. The subjects came back at day 15 when the measurements of variables were taken and received the second part of table olives to be taken in next period. Then participants came at day 30 to complete the final visit. The participants had to follow the strict diet, free of polyphenols, free of drinks or food containing xanthin, such as coffee (decaffeinated coffee as well), black tea, coca cola, cocoa, chocolate or alcoholic beverages. Drinks containing grapefruit and grapefruit juice

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were not allowed as well. In this stage, the participants did not receive neither food nor drinks, as they took table olives to be ingested at home. They were able to drink the water following the normal habits. Blood was collected at CIM-Sant Pau early in the morning (around 8 h) after 10 hours of fasting, at day 0, before starting the intervention with Arbequina table olives, as well as at days 15 and 30. Blood samples were taken into tubes containing EDTA-K_2 that were kept on ice until they were centrifuged at 1900×g at 4°C for 10 min. Plasma was separated from the blood cells, kept in 1.5 mL eppendorf and frozen at – 20 °C until analysis.

3.9.4. Determination of phenolic compounds in plasma by LC-ESI-MS/MS

The extraction of phenolic compounds from human plasma was performed as described in the section 3.8.1.4.

Table 3.13. MRM parameters corresponding to the phenolic compounds and metabolites as well as the internal standard set or obtained by LC-ESI-MS/MS for the analysis in human plasma in Stage II using the QTRAP equipment.

Analyte	Parent ion(*m/z*)	Fragment ion (*m/z*)	DP (V)	EP (V)	CE (V)	CXP (V)
Apigenin	269.0	117.1	-120	-10	-55	-10
Caffeic acid	179.1	135.1	-75	-10	-23	-10
Catechol	109.0	91.2	-80	-10	-28	-10
p-Coumaric acid	163.2	119.2	-80	-10	-22	-15
Hydroxytyrosol	153.2	122.8	-78	-10	-20	-10
Hydroxytyrosol glucuronide	329.1	153.0	-78	-10	-20	-10
Hydroxytyrosol sulfate	233.0	153.0	-78	-10	-20	-10
Hydroxytyrosol sulfo-glucuronide	409.0	153.0	-70	-10	-10	-10
Hydroxytyrosol acetate	195.0	59.0	-85	-10	-17	-10
Hydroxytyrosol acetate glucuronide	371.0	195.0	-85	-10	-17	-10
Hydroxytyrosol acetate sulfate	275.0	195.0	-85	-10	-17	-10
Luteolin	285.2	133.2	-100	-10	-50	-10
Luteolin-glucuronide	461.2	285.2	-100	-10	-50	-10
Luteolin-sulfate	365.0	285.2	-100	-10	-50	-10
Luteolin-7-*O*-glu	447.3	285.2	-130	-10	-40	-15
Oleuropein	539.5	275.0	-110	-10	-30	-10
Pinoresinol	357.3	151.1	-97	-10	-27	-10
Quercetin	301.2	151.1	-110	-10	-30	-10
Rutin	609.5	300.1	-300	-10	-50	-10
Salidroside	299.2	119.2	-74	-10	-22	-15
Tyrosol	137.1	106.2	-70	-10	-20	-15
Tyrosol glucuronide	313.0	137.0	-70	-10	-20	-15
Tyrosol sulfate	217.0	137.0	-70	-10	-20	-15
Vanillic acid	167.0	152.0	-70	-10	-20	-10
Verbascoside	623.5	161.3	-140	-10	-50	-10
2-(3-hydroxyphenyl) ethanol (IS)	137.0	107.0	-70	-10	-18	-15

DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, collision cell exit potential.

The LC-ESI-MS/MS analyses were done as reported in the section 3.8.1.5. The MRM parameters of the phenolic compounds and metabolites analyzed in the Stage II are reported in Table 3.13.

The quantification of phenolic compounds was performed with the calibration curve containing the calibration standards indicated in Table 3.14.

The calibration curve was prepared with calibration standards in the range of concentrations expected in plasma samples, that is, 0.25; 0.5; 0.75; 1; 2.5; 5 and 10 nmol/L. Hence, the working solutions needed for the construction of the calibration standards were 0.005; 0.01; 0.015; 0.2; 0.05; 0.1 and 0.2 µM.

Table 3.14. Calibration curve prepared with human plasma obtained at day 0 and spiked with increasing concentrations of working solutions of phenolic compounds.

Concentration (nmol/L)	Mixture polyphenols	Blank plasma
0	0 µL	285 µL
0.25	15 µL (working solution 0.005 µM)	285 µL
0.5	15 µL (working solution 0.01 µM)	285 µL
0.75	15 µL (working solution 0.015 µM)	285 µL
1	15 µL (working solution 0.2 µM)	285 µL
2.5	15 µL (working solution 0.05 µM)	285 µL
5	15 µL (working solution 0.1 µM)	285 µL
10	15 µL (working solution 0.2 µM)	285 µL

3.9.5. Validation of the method

The method was validated (EMA, 2011) for linearity, limit of quantification, precision and accuracy. The validation of the method was performed using calibration standards prepared in triplicate with blank human plasma as indicated in the section 3.8.5.2. Calibration standards were prepared at the concentrations of 0.25, 0.5, 0.75, 1, 2.5, 5 and 10 nmol/L.

3.10. STATISTICAL ANALYSIS

Results were expressed as mean ± standard error of the mean (SEM). The concentrations of phenolic compounds in table olives were expressed as mg/kg of destoned olives and the concentrations in plasma were expressed as nmol/L. Chauvenet's criterion was applied to discard outliers. Prism version 6 (GraphPad Software Inc., San Diego, USA) was used for the evaluation of data, statistical analysis, and elaboration of the graphs.

In the pharmacokinetics performed in both, the pre-clinical and clinical studies, C_{max}, λ_z, $t_{1/2z}$, AUC_{last}, $AUC_{0-\infty}$, $AUC_{extrap\%}$, MRT_{last} and $MRT_{0-\infty}$ are presented as the mean with its standard deviation, coefficient of variation, the median with its minimum and maximum value and the

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geometric mean with standard error of the geometric mean. The most correct data are expressed as geometric means values ± standard error of the geometric mean. T_{max} is the only parameter expressed as the median with its minimum and maximum value.

In the stage II of the clinical trial, the plasma concentrations at time 0, 15 and 30 days were compared in control and olive groups. Normality of the data was evaluated with the D'Agostino-Pearson omnibus test. When the data fit into the normal distribution, the one-way ANOVA, followed by Bonferroni multiple comparisons test was used. When the data did not fit into the normal distribution, analyses using the non-parametric Kruskal–Wallis test, followed by Dunn's multiple comparisons test were performed. A $p < 0.05$ level was taken as significant.

IV. RESULTS

4.1. SIMULTANEOUS ANALYSIS OF PHENOLIC COMPOUNDS IN ARBEQUINA TABLE OLIVES BY LC-ESI-MS/MS

4.1.1. Analysis of Arbequina table olives harvested in the season 2015/2016

The Arbequina table olives from the crop 2015/2016 that were administered to the Sprague-Dawley rats were analyzed to establish their content of phenolic compounds and calculate the amount administered.

4.1.1.1. Identification of phenolic compounds

The identification of the chromatographic peaks was performed by comparing the retention times of phenolic compounds in the samples of Arbequina table olives, to the ones obtained after the injection of commercial standards. A representative MRM extracted ion chromatogram, showing the presence of 16 compounds from five different classes, namely: phenolic alcohols, phenolic acids, flavonoids, secoiridoids, and lignans is displayed in Figure 4.1.

Salidroside was the phenolic compound that appeared in first place, with a retention time of 5.74 min, followed by hydroxytyrosol at 5.77 min and tyrosol at 6.70 min (Figure 4.1). Most of the analytes eluted between 7.13 min (caffeic acid) and 8.86 min (hydroxytyrosol acetate). Luteolin (9.79 min), quercetin (9.94 min), pinoresinol (10.06 min) and apigenin (10.65 min) were the phenolic compounds that eluted later.

Moreover, the chromatographic method used to analyze phenolic compounds discriminate properly compounds with similar transition as shown in Figure 4.1. That is the case of tyrosol ($137.1 \rightarrow 106.1$) and the internal standard, 2-(3-hydroxyphenyl)-ethanol ($137.0 \rightarrow 107.0$). As it could be appreciated in the extracted ion chromatogram displayed in Figure 4.1 both peaks are well resolved since tyrosol holds a retention time of 6.70 min while the internal standard appears as a small peak at 7.10 min.

Given that hydroxytyrosol was the most abundant compounds, the chromatogram of this analyte in Figure 4.1 corresponds to the dilution 1/50, whereas the rest of the compounds was analyzed at the dilution 1/4. In the second dilution used for the determination of phenolic compounds, luteolin was the analyte displaying the peak with the highest intensity. Finally, although caffeic acid and *p*-coumaric acid were the phenolic compounds with the lowest peak intensities, both could be unambiguously identified and quantified.

4.1.1.2. Quantification of phenolic compounds

Due to the absence of a matrix of table olives without phenolic compounds, calibration curves were prepared with the standard addition method. Calibration standards were prepared by directly adding working solutions to the filtered supernatants at the same dilution of the samples.

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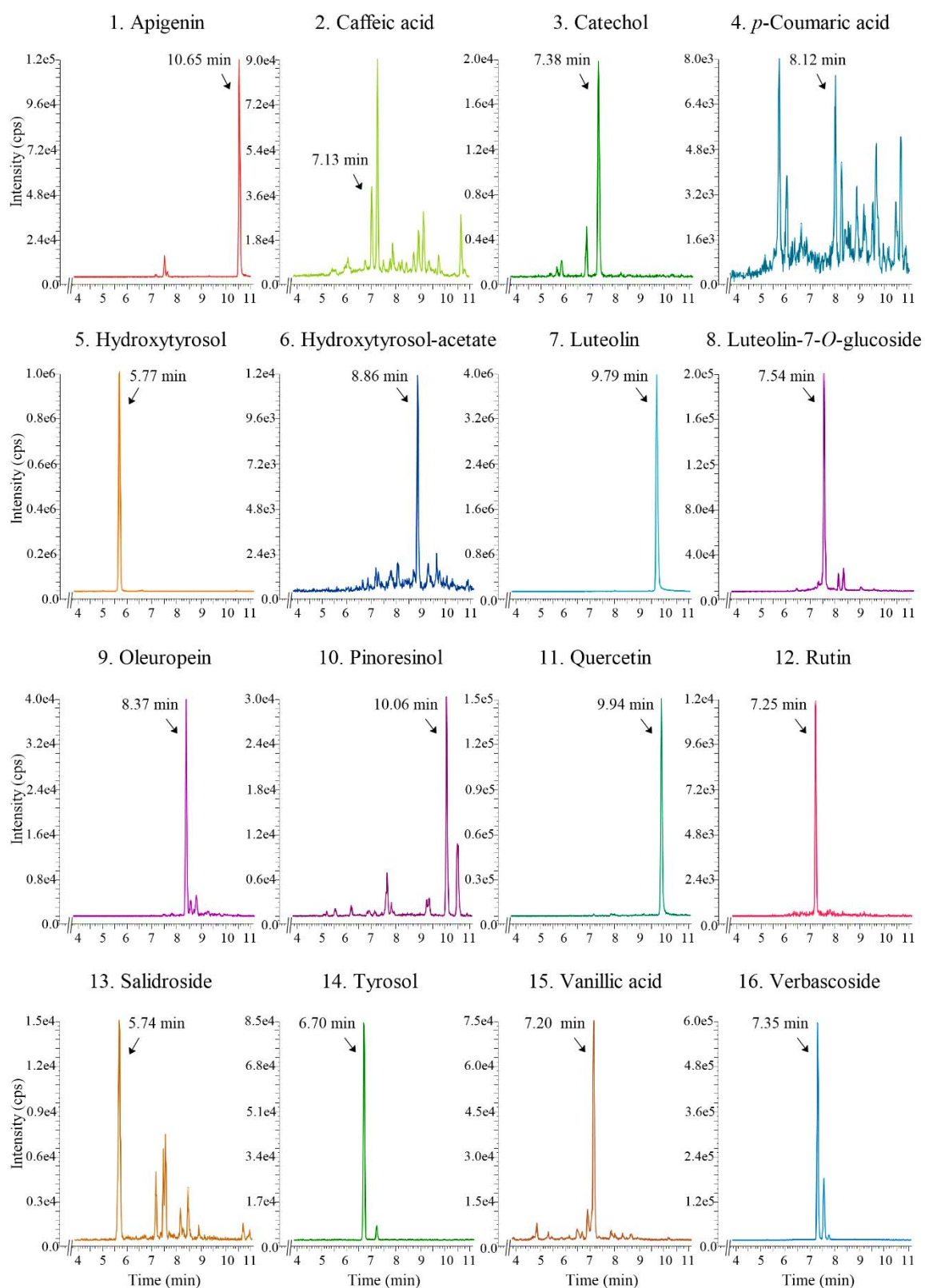
Figure 4.1. Representative LC-ESI-MS/MS extracted ion chromatograms obtained in multiple reaction mode (MRM) of the phenolic compounds from the table olives of the Arbequina variety from the harvest 2015/2016.

Dilution 1/4
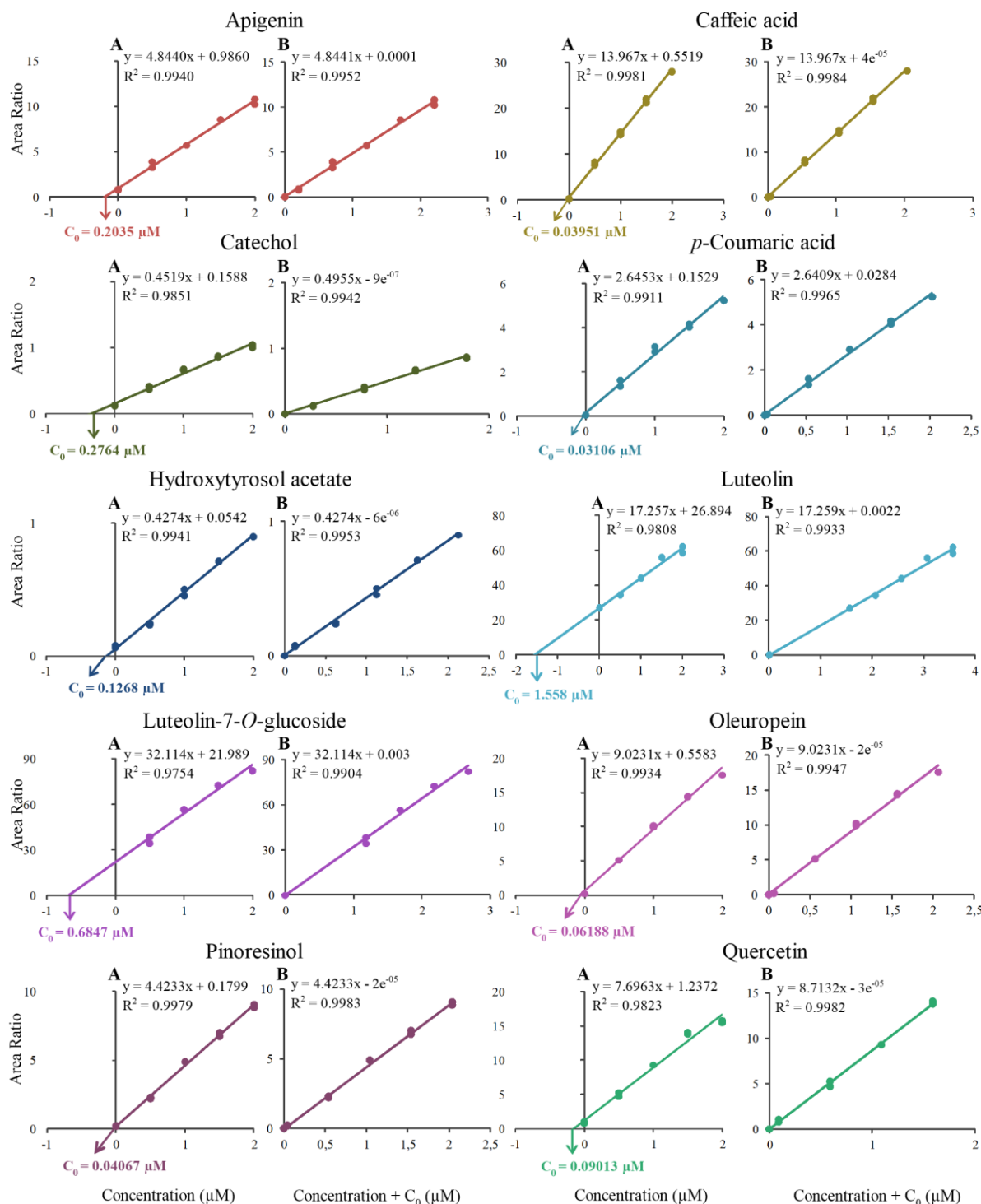
Figure 4.2. (A) Calibration curves of polyphenols in Arbequina table olives obtained by the standard addition method. The curve A depicts the plot that provides the basal concentration (C_0) that is added to the spiked concentration and allows the calculations of calibration curve B. In the figures, individual values were represented. The regression line was calculated by the least square method.

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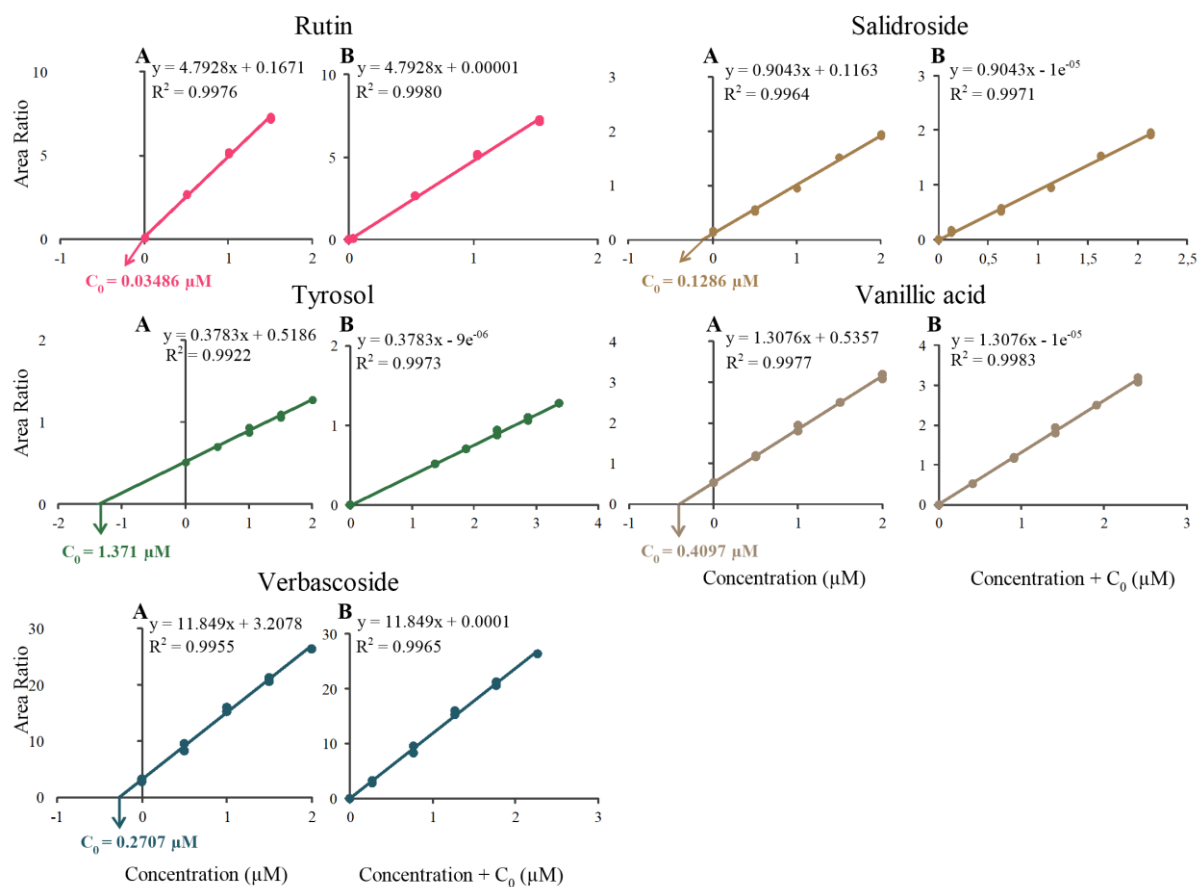
Dilution 1/50
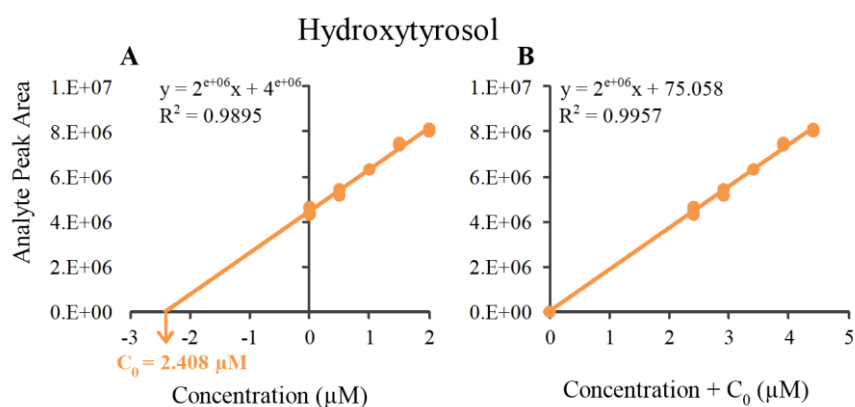
Figure 4.2. (B) Calibration curves of polyphenols in Arbequina table olives obtained by the standard addition method. The curve A depicts the plot that provides the basal concentration (C_0) that is added to the spiked concentration and allows the calculations of calibration curve B. In the figures, individual values were represented. The regression line was calculated by the least square method.

Therefore, two calibration curves were prepared, a first one at the dilution 1/50 for the quantification of hydroxytyrosol, whereas the second one, that employed the samples diluted at 1/4 allowed the analysis of the rest of phenolic compounds (Figure 4.2).

Using the standard addition method, a straight line was obtained for 16 different polyphenols as shown in Figure 4.2, where the cut-off point of the line in the negative part provides the sample concentration, that is, when y = 0, x represents the analyte concentration in the olive sample. This concentration could be calculated through the obtained regression line, since it is true that $0 = ax + b$, being x the concentration to be determined, so it can be calculated using x = b/a, in absolute value. This calculation is provided automatically by GraphPad Prism and allows obtaining the basal concentration (C_0). Then, C_0 was added to the spiked concentrations and enabling the obtention of calibration curve B that was used to calculate the quantity of polyphenols in the samples of Arbequina table olives.

4.1.1.3. Concentrations of phenolic compounds

The concentrations of the phenolic compounds identified in Arbequina table olives cropped in the year 2015/2016 are displayed in Table 4.1.

Table 4.1. Concentrations of phenolic compounds in Arbequina table olives harvested during the season 2015/2016 analyzed by LC-ESI-MS/MS. Amount of each analyte administered to Sprague-Dawley rats at the doses equivalent to the human consumption of 30 and 60 olives.

Analyte	mg polyphenols/kg destoned olive	Dose of 30 olives μg in destoned olives	Dose of 60 olives μg in destoned olives
Apigenin	6.77 ± 0.50	7.2 ± 0.13	15.3 ± 0.48
Caffeic acid	0.48 ± 0.01	0.51 ± 0.03	1.08 ± 0.03
Catechol	4.53 ± 0.28	4.81 ± 0.08	10.2 ± 0.32
p-Coumaric Acid	0.53 ± 0.03	0.56 ± 0.01	1.20 ± 0.04
Hydroxytyrosol	764.25 ± 9.47	813 ± 14.35	1725 ± 54.49
HT acetate	6.67 ± 0.22	7.08 ± 0.13	15.1 ± 0.48
Luteolin	81.43 ± 3.17	86.5 ± 1.53	183 ± 5.81
Luteolin-7-*O*-glu	2.28 ± 0.19	2.42 ± 0.04	5.15 ± 0.16
Oleuropein	1.96 ± 0.21	2.08 ± 0.04	4.43 ± 0.14
Pinoresinol	2.33 ± 0.12	2.47 ± 0.04	5.26 ± 0.17
Quercetin	5.05 ± 0.33	5.36 ± 0.09	11.4 ± 0.36
Rutin	1.47 ± 0.06	1.56 ± 0.03	3.32 ± 0.10
Salidroside	9.29 ± 0.13	9.86 ± 0.17	21.0 ± 0.66
Tyrosol	28.65 ± 1.77	30.4 ± 0.54	64.7 ± 2.04
Vanillic Acid	12.29 ± 0.55	12.9 ± 0.23	27.8 ± 0.88
Verbascoside	26.57 ± 2.74	28.2 ± 0.50	60.0 ± 1.89

Results are expressed as mean ± SEM of three independent samples analyzed by triplicate.

The total amount of phenolic compounds was 954.55 mg per kilogram of destoned olives. Of them, hydroxytyrosol was the most abundant compound with concentrations of 764.25 ± 9.47 mg/kg that represented 80.1% of all the phenolic compounds studied. It was followed by

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luteolin, tyrosol and verbascoside that were found at concentrations of 81.43 ± 3.17; 28.65 ± 1.77 and 26.57 ± 2.74 mg/kg that accounted to 8.53%, 3.00%, 2.79%, respectively. Vanillic acid (1.29%) and salidroside (1.00%) were found at 12.29 ± 0.55 and 9.29 ± 0.13 mg/kg, respectively. Apigenin, hydroxytyrosol acetate, quercetin and catechol gave values of 6.77 ± 0.50 (0.71%), 6.67 ± 0.22 (0.70%), 5.05 ± 0.33 (0.53%) and 4.53 ± 0.28 mg/kg (0.47%), respectively. The rest of the compounds (pinoresinol, luteolin-7-*O*-glucoside, oleuropein, rutin, *p*-coumaric acid and caffeic acid) yielded values lower than 0.2% and their concentrations were ranging from 2.33 ± 0.12 mg/kg for pinoresinol to 0.48 ± 0.01 mg/kg for caffeic acid.

4.1.2. Analysis of Arbequina table olives harvested in the season 2016/2017

The Arbequina table olives from the crop 2016/2017 that were used in the clinical trial were analyzed to establish their content in phenolic compounds. The analysis of the chromatograms obtained in the MRM mode of the extracted ions allowed the identification of 15 polyphenols from different classes, namely, phenolic alcohols, phenolic acids, flavonoids, secoiridoids, and lignans. All the phenolic compounds identified in the season 2015/2016 were detected except for catechol was not detected.

Table 4.2. Concentrations of phenolic compounds in Arbequina table olives harvested during the season 2016/2017 analyzed by LC-ESI-MS/MS. Amount of each analyte ingested by the healthy human volunteers at the doses 60 and 120 olives.

Analyte	mg polyphenols/kg destoned olive[a]	Dose of 60 olives μg in destoned olives	Dose of 120 olives μg in destoned olives
Apigenin	4.52 ± 0.17	299.37 ± 3.18	577.73 ± 6.00
Caffeic acid	4.64 ± 0.14	307.32 ± 3.26	593.07 ± 6.16
Catechol	not detected	--	--
p-Coumaric Acid	5.65 ± 0.10	374.22 ± 3.98	722.16 ± 7.50
Hydroxytyrosol	474.56 ± 11.77	31431.69 ± 333.89	60656.68 ± 630.21
HT acetate	26.95 ± 0.71	1784.99 ± 18.96	3444.66 ± 35.79
Luteolin	89.56 ± 2.97	5931.86 ± 63.01	11447.26 ± 118.93
Luteolin-7-*O*-glu	11.11 ± 1.74	735.85 ± 7.82	1420.04 ± 14.75
Oleuropein	12.59 ± 0.24	833.88 ± 8.86	1609.21 ± 16.72
Pinoresinol	3.08 ± 0.22	204.00 ± 2.17	393.68 ± 4.09
Quercetin	6.49 ± 0.16	429.85 ± 4.57	829.53 ± 8.62
Rutin	25.99 ± 3.14	1721.40 ± 18.29	3321.96 ± 34.51
Salidroside	17.36 ± 0.98	1149.81 ± 12.21	2218.90 ± 23.05
Tyrosol	23.10 ± 0.58	1529.99 ± 16.25	2952.57 ± 30.68
Vanillic Acid	3.56 ± 0.06	235.79 ± 2.50	455.03 ± 4.73
Verbascoside	334.30 ± 30.84	22141.80 ± 235.21	42729.11 ± 443.95

Results are expressed as mean ± SEM of three independent samples analyzed by triplicate.

The concentrations of the phenolic compounds determined in the Arbequina table olives from the season 2016/2017 can be observed in the Table 4.2. The total amount of polyphenols quantified were 1043.46 mg per kilogram of olive pulp. The most abundant compound was

hydroxytyrosol with concentration of 474.56 ± 11.77 mg/kg that accounted to 45.5%. It was followed by verbascoside with concentration of 334.3 mg/kg that accounted to 32.0%. Luteolin formed 8.6% (89.56 ± 2.97 mg/kg). Hydroxytyrosol acetate, rutin, tyrosol and salidroside were found at concentrations of 26.95 ± 0.71 (2.6%), 25.99 ± 3.14 (2.5%), 23.10 ± 0.58 (2.2%) and 17.36 ± 0.98 (1.7%) mg/kg, respectively. Oleuropein and luteolin-7-*O*-glucoside accounted to 1.2% (12.59 ± 0.24 mg/kg) and 0.3% (11.11 ± 1.74 mg/kg). Quercetin (0.6%), *p*-coumaric acid (0.5%) gave values of 6.49 ± 0.16 and 5.65 ± 0.10 mg/kg. Caffeic acid, apigenin, vanillic acid and pinoresinol yielded values lower than 0.4% and their concentrations were ranging from 4.64 ± 0.14 mg/kg for caffeic acid up to 3.08 ± 0.22 mg/kg for pinoresinol.

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4.2. SIMULTANEOUS DETERMINATION OF PHENOLIC COMPOUNDS IN RAT PLASMA BY LC-ESI-MS/MS

The development of the analytical method was performed in blank plasma spiked with a mixture of standards of 16 polyphenols at the concentration of 250 nmol/L. At least three independent calibration standards were tested for recovery and matrix effect for every examined condition.

4.2.1. Optimization of the extraction procedure

4.2.1.1. Extraction solvent

Ethanol-methanol (1:1; v/v), acetonitrile 100%, methanol 100% were employed as protein precipitants, meanwhile ethyl acetate 100% was used as a liquid-liquid extraction solvent to achieve the maximum recoveries and the lowest endogenous interferences. For the selection of the extraction solvent, the results of hydroxytyrosol and tyrosol were reported since they are the most representative phenolic compounds of table olives.

Ethanol-methanol (1:1; v/v) yielded a recovery of $119.76 \pm 6.90\%$ (n = 3) for hydroxytyrosol and $95.45 \pm 1.70\%$ (n = 3). However, ethanol-methanol was discarded due to the suppression of ionization observed for most phenolic compounds with matrix effect of $5.92 \pm 0.60\%$ (n = 3) for hydroxytyrosol and $66.75 \pm 6.50\%$ (n = 3) for tyrosol.

Methanol 100% yielded lower recoveries with values of $84.36 \pm 4.7\%$ (n = 3) for hydroxytyrosol and 76.62 ± 3.00 for tyrosol. This solvent improved the ionization for tyrosol, with a matrix effect of 80.10 ± 3.9 (n = 3), although an important loss of signal was still observed for hydroxytyrosol ($9.45 \pm 0.03\%$; n = 3). The pattern described for ethanol-methanol (1:1; v/v) and methanol 100% for hydroxytyrosol and tyrosol was also observed for most phenolic compounds, and both solvents were discarded.

Acetonitrile 100% did not yield such a strong suppression of the ionization, as shown by the results obtained for hydroxytyrosol ($53.99 \pm 15.50\%$; n = 3) and tyrosol ($80.73 \pm 18,90$; n = 3) but it provided a low recovery for both hydroxytyrosol ($21.85 \pm 0.70\%$; n = 3) and tyrosol (71.58 ± 1.00; n = 3), thus this solvent was also rejected.

Ethyl acetate gave good recoveries for hydroxytyrosol ($92,82 \pm 0,8\%$; n = 3) and tyrosol ($93,03 \pm 0,9$; n = 3), without suppressing the ionization since matrix effect was $111.72 \pm 6.20\%$ (n = 3) for hydroxytyrosol and $119.14 \pm 12.19\%$ (n = 3) for tyrosol. Given that the results for the other phenolic compounds ranged from 50% to 80% for both variables, ethyl acetate was selected as extraction solvents.

4.2.1.2. Volume of solvent

Various volumes of the ethyl acetate, including 1.5 mL, 4 mL and 6 mL were screened. The use of 1.5 mL of solvent decreased the recovery of hydroxytyrosol by a 30% and matrix effect was superior to 140% in comparison with the results obtained when the volume of 6 mL was employed. Thus, the volume of 6 mL of ethyl acetate was considered as appropriate for the

extraction process since it showed the best recoveries and matrix effect for the phenolic compounds assayed.

4.2.1.3. Acidification of the plasma samples

Formic acid in combination with ethyl acetate was assayed at 0.05 and 0.5%. When 0.05% formic acid was used, a good recovery of 88.7 ± 7.7% was reached only for tyrosol, decreasing to 40-70% for apigenin, *p*-coumaric acid, hydroxytyrosol, luteolin, luteolin-7-*O*-glucoside, pinoresinol and vanillic acid. Recoveries of other 8 compounds dropped to values from 37.5 ± 4.0% for oleuropein to 5.4 ± 0.5% for caffeic acid. No matrix effect was observed for apigenin, *p*-coumaric acid, hydroxytyrosol, hydroxytyrosol acetate, luteolin-7-*O*-glucoside, pinoresinol and tyrosol, however, the rest of the polyphenols yielded an increase of ionization of around 140%. The acidification of ethyl acetate with 0.5% formic acid gave recoveries ranging from 80 to 100% for *p*-coumaric acid, tyrosol and vanillic acid. The recovery of the rest of the compounds dropped below 45% for the other 14 analytes. No improvement was found regarding matrix effect, since an increase of ionization of approximately 140% was observed for 11 polyphenols.

In view of the results, the addition of 0.05 and 0.5% formic acid to ethyl acetate to improve the recovery and matrix effect was discarded. Instead, acetic acid was tried at 0.05%, 0.5%, 1%, 2.5% and 10%. The recoveries of hydroxytyrosol were inversely proportional to the percentage of acetic acid used. Hence, the highest concentration of 10% yielded a recovery of 78.8% that increased to 91.2% when 0.05% was added. When acetic acid at 1% was applied, the recovery for hydroxytyrosol was higher than 90%. Recoveries for the other phenolic compounds ranged from 75% to 90% except for caffeic acid, rutin, and salidroside that gave values of 64.0%, 58.1%, and 49.8%, respectively. Recoveries of 77.6% and 75.7% were reached for rutin and caffeic acid, respectively, when acetic acid at 10% was used. Salidroside gave a value of 66.9% when 2.5% acetic acid was used. The use of these amounts of acetic acid was discarded since it was detrimental to the recovery of most phenolic compounds, thus the addition of 0.5% acetic acid to ethyl acetate was selected. This percentage of acid was also adequate to avoid matrix effect, since all the phenolic compounds had values ranging from 80% to 110%.

4.2.1.4. Other modifications of the extraction process

In the optimization process, the addition of ascorbic acid at 1% and 10% used as antioxidant to protect phenolic compounds or sonication to enhance the extraction efficiency were also evaluated. The recovery of phenolic compounds improved when the concentration of ascorbic acid was used at 10%. Moreover, recoveries were enhanced when ascorbic acid at 10% was placed two times, 10 µL to the plasma sample before the extraction and 10 µL to the pooled supernatants to prevent degradation of phenolic compounds during the evaporation to dryness. Moreover, in the process of extraction, the incorporation of a step consisting of the use of an ultrasonic bath for 10 min after the agitation on the vortex for 5 min, increased the recovery of phenolic compounds. These final modifications of the extraction process provided suitable

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recoveries for 12 compounds ranging from 80 up to 115% (Table 4.3). Recoveries of verbascoside, caffeic acid, rutin and salidroside were 78.9, 77.4, 69.3 and 58.2 %, respectively. Moreover, the chosen conditions were also adequate to avoid matrix effect, since 12 polyphenols had values ranging from 80 to 110% and a decrease of ionization of approximately 50% was observed for apigenin and pinoresinol. Matrix effects of hydroxytyrosol acetate and *p*-coumaric acid were 77.6 and 75.2%.

Table 4.3. Recovery and matrix effect for the determination in blank rat plasma spiked with a working solution of phenolic compounds at the concentration of 250 nmol/L.

Analyte	Recovery (%)		Matrix effect (%)	
	Without IS	With IS	Without IS	With IS
Apigenin	69.5 ± 2.8	87.2 ± 4.1	58.0 ± 2.8	55.9 ± 3.3
Caffeic acid	61.6 ± 3.5	77.4 ± 4.7	110.8 ± 0.4	106.7 ± 2.2
Catechol	74.6 ± 3.6	93.7 ± 5.0	94.9 ± 1.6	91.5 ± 3.1
p-Coumaric acid	73.3 ± 3.2	92.2 ± 4.6	78.1 ± 1.3	75.2 ± 1.2
Hydroxytyrosol	91.2 ± 3.1	114.6 ± 4.1	96.0 ± 1.3	92.4 ± 1.1
HT acetate	82.5 ± 3.8	103.6 ± 5.5	80.5 ± 1.4	77.6 ± 2.6
Luteolin	77.6 ± 2.3	97.4 ± 3.5	96.1 ± 3.4	92.7 ± 4.7
Luteolin-7-*O*-glu	91.1 ± 1.7	114.4 ± 2.6	100.6 ± 1.7	97.0 ± 3.4
Oleuropein	85.5 ± 2.5	107.4 ± 3.6	96.2 ± 0.1	92.7 ± 1.7
(+)-Pinoresinol	74.4 ± 2.5	93.3 ± 3.6	42.4 ± 1.9	41.0 ± 2.3
Quercetin	67.9 ± 3.4	85.3 ± 4.8	84.8 ± 4.1	81.8 ± 5.2
Rutin	55.1 ± 3.0	69.3 ± 4.0	94 1 ± 1.9	90.6 ± 1.1
Salidroside	46.3 ± 1.6	58.2 ± 2.3	90.4 ± 0.3	87.1 ± 1.4
Tyrosol	83.2 ± 4.1	104.6 ± 5.8	88.6 ± 0.9	85.4 ± 1.0
Vanillic acid	73.2 ± 0.8	92.0 ± 0.5	100.9 ± 1.8	97.1 ± 1.2
Verbascoside	62.9 ± 2.8	78.9 ± 3.6	96.1 ± 2.6	93.0 ± 3.8
IS	79.6 ± 0.5	-	103.9 ± 2.1	-

4.2.2. Optimization of the liquid chromatography-mass spectrometry conditions

The chromatographic conditions previously established in our group by Moreno-González *et al.* (2020a) were optimized to the analysis of phenolic compounds in plasma samples.

At first place, the influence of the temperature of the electrospray (ESI) source was considered in the LOQ in polyphenols in plasma samples. Thus, a standard of polyphenols prepared at concentration of 1 µM with the use of methanol 80% was injected at 350, 400, 450, 500 and 600°C and the best intensity of the signal was achieved when 600°C was applied.

Various ionization spray voltages (-3000, -3500, -4000, and -4500 V) were tested together with several ion source gas 2 conditions (25, 50, and 70 arbitrary units), with the ion source gas 1 set at 50 arbitrary units. The highest peak intensity was obtained when the ionization spray voltage was set at -3500 V and the ion source gas 2 was set at 50 arbitrary units.

In addition to the optimization of the mass spectrometer, the liquid chromatography

conditions were also tuned. Various elution programs were evaluated, and the best selectivity was obtained when the initial percentage of the aqueous phase was 95%. The selected temperature for the column was 30°C because it yielded satisfactory intensity of the signal.

In the optimization process, plasma samples obtained after oral intake of Arbequina table olives was injected into LC-ESI-MS/MS system were injected to check the performance of the method.

The chromatogram of hydroxytyrosol obtained in MRM mode at the *m/z* 153.2/122.8 Da with the peak of the analyte at retention time of 5.73 min is displayed in Figure 4.3. In the same chromatogram, a non-symmetric bigger peak appeared at the retention time of 5.37 min (Figure 4.3.A). Since no other peak appeared in chromatogram at the same retention time when blank plasma samples were analyzed (Figure 4.3), it was considered a metabolite of hydroxytyrosol. To improve the chromatographic separation of peaks obtained with an injection volume of 10 µL, the modification of the volume of injection was tried and volumes of 2 and 5 µL were assayed. The smaller volume of 2 µL allowed the correct separation of the peaks of metabolites, thus being chosen as the injection volume (Figure 4.3.B).
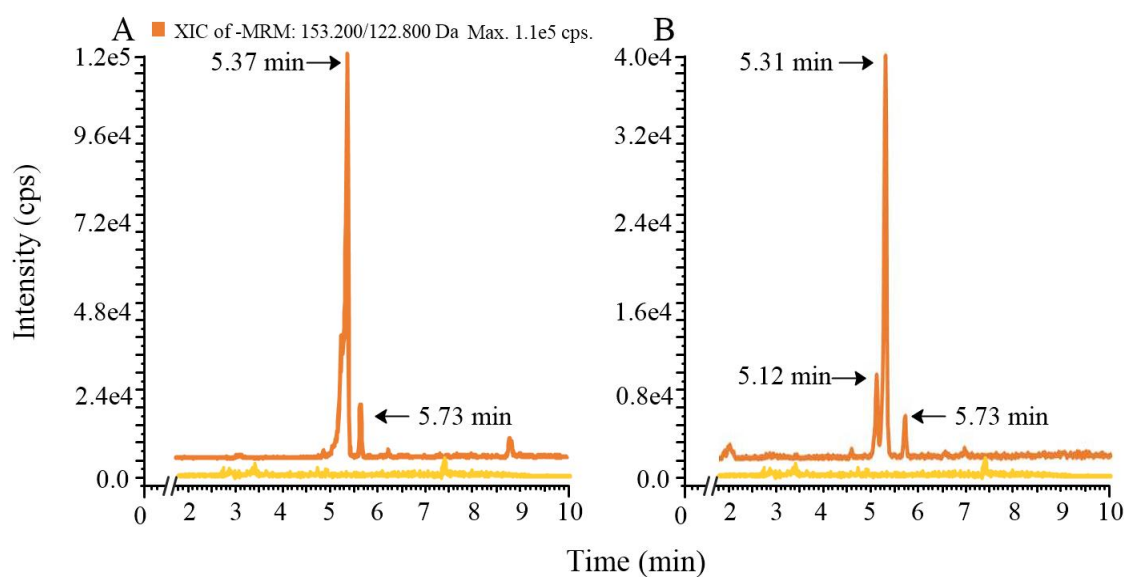
Figure 4.3. Representative LC-ESI-MS/MS chromatograms of hydroxytyrosol obtained in multiple reaction mode (MRM) at the m/z 153.2/122.8. (A) Rat blank plasma (yellow line), rat plasma obtained 30 min after the administration of Arbequina table olives at dose equivalent to a human consumption of 30 olives after injecting a volume of 10 µL (orange line), (B) Rat blank plasma (yellow line), the same sample injected at 2 µL (orange line).

4.2.3. Validation of the method

4.2.3.1. Matrix effect

The developed method did not enhance or decrease the ionization of phenolic compounds, since the values of matrix effect were within 80-120% as it can be observed in Table 4.4. Remarkably, 2-(3-hydroxyphenyl) ethanol (IS) hold a mean value of 100.5 ± 3.3 nmol/L,

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which demonstrated its lack of matrix effect. Moreover, the coefficient of variation (CV) expressed as a percentage was less than 15%, which met the requirements set by the EMA (2011).

Table 4.4. Matrix effect in blank rat plasma samples spiked at three concentrations of phenolic compounds and analyzed by LC-ESI-MS/MS.

| Analyte | Matrix effect (%) | | | | | |
| | 25 nmol/L | | 100 nmol/L | | 250 nmol/L | |
	Without IS	With IS	Without IS	With IS	Without IS	With IS
Apigenin	95.7 ± 10.5	88.8 ± 7.0	86.7 ± 9.9	83.4 ± 10.3	80.7 ± 3.7	81.3 ± 3.4
Caffeic acid	112.3 ± 7.9	98.5 ± 2.7	116.2 ± 5.5	112.5 ± 6.0	110.8 ± 0.4	106.7 ± 2.2
Catechol	109.1 ± 4.1	105.1 ± 5.4	104.7 ± 5.0	97.9 ± 3.7	90.6 ± 2.2	87.4 ± 3.5
p-Coumaric acid	118.8 ± 2.9	116.8 ± 7.1	95.4 ± 8.2	93.6 ± 8.5	85.3 ± 2.2	83.3 ± 1.3
Hydroxytyrosol	98.7 ± 2.1	92.3 ± 5.7	100.5 ± 3.5	103.3 ± 2.4	96.0 ± 1.3	92.4 ± 1.1
HT acetate	92.7 ± 6.0	85.0 ± 3.6	87.7 ± 3.9	82.9 ± 3.2	82.1 ± 1.6	80.8 ± 4.0
Luteolin	114.1 ± 4.5	110.9 ± 1.4	107.7 ± 3.1	103.8 ± 3.5	92.7 ± 4.3	89.4 ± 5.4
Luteolin-7-*O*-glucoside	118.4 ± 6.2	118.8 ± 7.4	114.4 ± 4.0	113.2 ± 3.7	100.6 ± 1.7	100.5 ± 3.3
Oleuropein	103.7 ± 3.8	97.4 ± 3.8	105.4 ± 2.5	107.2 ± 4.2	96.2 ± 0.1	96.1 ± 1.5
(+)-Pinoresinol	83.5 ± 10.7	80.8 ± 8.3	99.9 ± 3.3	90.0 ± 15.1	80.8 ± 1.0	82.5 ± 3.8
Quercetin	114.0 ± 9.9	106.0 ± 4.2	104.1 ± 7.5	99.0 ± 7.0	84.8 ± 4.1	81.8 ± 5.2
Rutin	118.1 ± 7.4	107.4 ± 6.8	112.2 ± 6.9	108.4 ± 4.4	94.1 ± 1.9	90.6 ± 1.1
Salidroside	99.9 ± 7.8	95.7 ± 3.9	92.9 ± 2.3	89.6 ± 2.9	90.4 ± 0.3	87.1 ± 1.4
Tyrosol	99.5 ± 2.9	95.8 ± 6.1	88.5 ± 2.7	88.4 ± 3.0	88.8 ± 1.0	86.0 ± 0.7
Vanillic acid	114.3 ± 7.1	114.7 ± 7.1	113.6 ± 4.7	113.6 ± 4.7	100.5 ± 1.3	97.3 ± 1.2
Verbascoside	119.5 ± 7.9	111.7 ± 6.8	119.4 ± 3.1	115.7 ± 5.4	93.7 ± 2.5	90.7 ± 3.8
IS	108.6 ± 5.1	--	99.6 ± 3.3	--	91.7 ± 7.3	--

4.2.3.2. Recovery

Among the different phenolic compounds, the highest recovery corresponded to hydroxytyrosol with an average value of 93.6 ± 2.0% (Table 4.5), for the three concentrations evaluated. Significant recoveries were also obtained for tyrosol (87.9 ± 1.7%), oleuropein (87.1 ± 1.6%), luteolin-7-*O*-glucoside (86.0 ± 1.5%) and vanillic acid (85.9 ± 1.9%). Pinoresinol, catechol, *p*-coumaric acid and luteolin gave recoveries slightly superior to 80%.

Hydroxytyrosol acetate, apigenin, verbascoside and quercetin made acceptable recoveries of approximately 77%. The lowest recoveries were obtained for caffeic acid, rutin and salidroside with values of 64.0 ± 1.5%, 58.1 ± 2.0%, 49.8 ± 1.3%, respectively. The internal standard, 2-(3-hydroxyphenyl)-ethanol provided a recovery of 85.0 ± 2.9% which is like the phenolic compounds. Subsequently, when the results were normalized by IS, recoveries were acceptable (80-100%) for all the polyphenols except salidroside. Hence, recoveries ranging between 90 and 100% were obtained for 13 phenolic compounds. The recoveries of caffeic acid and rutin were 88.5 ± 2.3 and 80.0 ± 2.6%, respectively. The lowest recovery of 63.7 ±

2.1% was corresponded to salidroside.

Table 4.5. Recovery in blank rat plasma samples spiked at three concentrations of phenolic compounds and analyzed by LC-ESI-MS/MS.

Analyte	Recovery (%)					
	25 nmol/L		100 nmol/L		250 nmol/L	
	Without IS	With IS	Without IS	With IS	Without IS	With IS
Apigenin	77.7 ± 5.8	95.7 ± 4.2	78.9 ± 2.1	93.9 ± 5.6	75.7 ± 1.9	93.1 ± 3.8
Caffeic acid	66.6 ± 1.9	82.9 ± 3.1	64.3 ± 3.2	84.7 ± 2.1	59.9 ± 2.3	81.0 ± 3.6
Catechol	81.2 ± 1.7	98.9 ± 3.0	83.4 ± 2.5	91.0 ± 4.0	81.8 ± 0.7	91.8 ± 4.8
p-Coumaric acid	78.2 ± 3.3	98.4 ± 7.0	84.4 ± 2.3	99.6 ± 9.2	78.2 ± 1.7	95.7 ± 3.0
Hydroxytyrosol	91.3 ± 3.2	97.0 ± 2.3	100.5 ± 2.5	99.1 ± 6.0	91.0 ± 2.3	99.3 ± 2.0
HT acetate	78.3 ± 1.9	99.3 ± 5.4	77.5 ± 3.7	90.4 ± 5.0	77.4 ± 3.0	95.7 ± 3.9
Luteolin	80.0 ± 2.0	99.4 ± 2.5	80.1 ± 1.8	94.4 ± 3.3	80.6 ± 1.3	99.8 ± 2.2
Luteolin-7-*O*-glu	85.6 ± 2.6	97.0 ± 2.8	85.1 ± 3.2	94.7 ± 5.5	87.5 ± 2.0	98.8 ± 3.0
Oleuropein	87.1 ± 3.5	98.2 ± 3.3	89.3 ± 1.4	96.0 ± 5.1	84.5 ± 2.0	99.6 ± 3.2
(+)-Pinoresinol	83.4 ± 2.3	99.4 ± 4.8	82.5 ± 3.1	93.0 ± 4.4	80.3 ± 1.4	93.3 ± 3.9
Quercetin	79.4 ± 2.6	99.4 ± 3.2	72.4 ± 2.5	87.4 ± 5.2	70.6 ± 0.7	91.8 ± 1.2
Rutin	56.4 ± 1.9	80.0 ± 1.3	62.9 ± 5.8	84.3 ± 10.5	64.4 ± 1.7	80.9 ± 3.8
Salidroside	48.6 ± 0.7	65.3 ± 1.7	54.7 ± 2.7	68.9 ± 5.5	53.6 ± 1.4	66.2 ± 1.8
Tyrosol	89.3 ± 2.8	99.5 ± 7.3	84.0 ± 1.6	94.1 ± 4.3	91.0 ± 3.9	98.0 ± 3.6
Vanillic acid	84.3 ± 2.5	103.5 ± 6.9	91.8 ± 2.8	97.7 ± 9.5	80.7 ± 3.1	97.4 ± 3.1
Verbascoside	85.8 ± 11.0	97.2 ± 2.7	73.3 ± 1.2	92.9 ± 2.7	72.5 ± 2.8	90.3 ± 3.9
IS	81.5 ± 1.9	--	93.2 ± 7.4	--	80.1 ± 1.2	--

4.2.3.3. Linearity

The linearity of the analytical method was evaluated by spiking the blank rat plasma with mixture of polyphenols at increasing concentrations of 0, 10, 25, 50, 100, and 150 nmol/L that is the range of application of the analytical method. A straight-line fit was performed through the data points by least square regression analysis. The calibration curves indicated that the analytical procedure was linear for all the studied phenolic compounds as can be seen in Figure 4.4.

The extraction process followed by LC-ESI-MS/MS analysis allowed an accurate detection of phenolic compounds from different classes at the concentrations analyzed. Hence, for all the analytes, the correlation coefficients (R^2) achieved yielded satisfactory results, being all higher than 0.9954 that was obtained for tyrosol (Table 4.6 and Figure 4.4).

Quercetin gave an R^2 values 0.9978, while salidroside was 0.9979. The calibration curves obtained for apigenin and hydroxytyrosol produced an R^2 of 0.9985. Then, the analysis of the calibration standards for most of phenolic compounds produced curves with R^2 superior to 0.999. The calibration curves for luteolin and vanillic acid presented R^2 of 0.9990, luteolin-7-*O*-glucoside delivered an R^2 of 0.9992, caffeic acid was 0.9993 while pinoresinol provided an R^2 of 0.9994. The highest R^2 of 0.9997 was found for catechol, *p*-coumaric acid and oleuropein (Table 4.6 and Figure 4.4).

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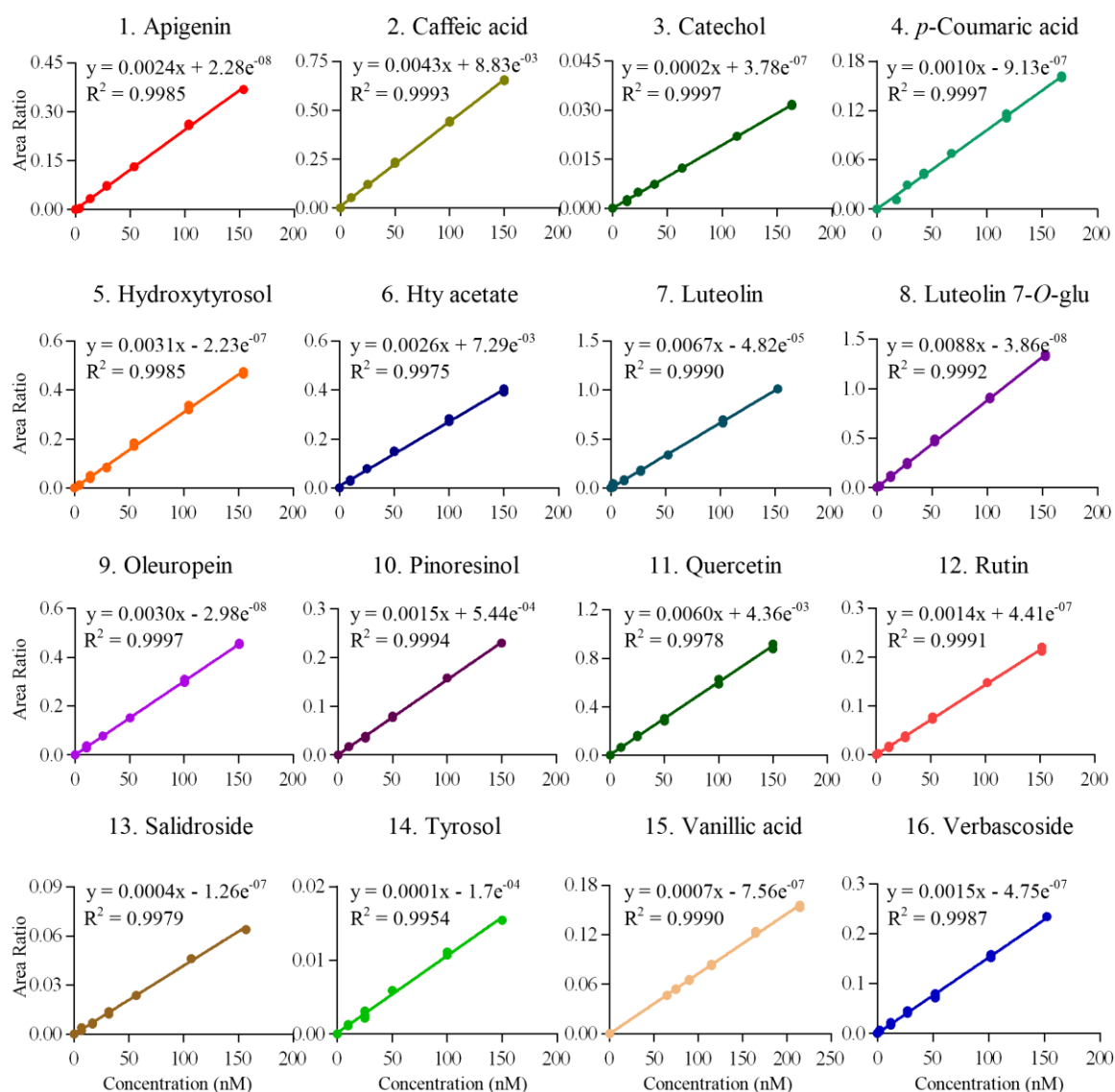
Figure 4.4. Representative calibration curves of phenolic compounds spiked in rat blank plasma and determined by LC-ESI-MS/MS. The figure shows the individual values for each of the analytes. The regression line had been calculated using the least square method.

4.2.3.4. Limit of quantification

The sensitivity of the analytical method expressed as limit of quantification (LOQ) is displayed in Table 4.6. The lowest LOQ was found for luteolin-7-*O*-glucoside with a concentration of 0.04 nmol/L. Oleuropein and verbascoside produced similar LOQ with values of 0.06 nmol/L. Then the results were nearly double for rutin (0.11 nmol/L), and hydroxytyrosol acetate and luteolin, both with LOQ of 0.12 nmol/L. These compounds are followed by apigenin (0.15 nmol/L) and hydroxytyrosol (0.19 nmol/L). LOQ below 1.00 nmol/L were found for pinoresinol, quercetin and salidroside with concentrations of 0.32 nmol/L, 0.40 nmol/L and 0.63 nmol/L, respectively.

Vanillic acid yielded an LOQ of 1.08 nmol/L, whereas *p*-coumaric acid, tyrosol and caffeic acid hold values of 1.75 nmol/L, 1.95 nmol/Land 2.01 nmol/L. Finally, the highest LOQ was found for catechol with a concentration of 2.51 nmol/L.

Table 4.6. Linearity and limit of quantification (LOQ) of phenolic compounds spiked in blank plasma and analyzed by LC-ESI-MS/MS.

| Analyte | Linearity | | LOQ |
	Equations	R^2	(nmol/L)
Apigenin	$y = (0.00259 \pm 0.00014)x + (-1.12e^{-7} \pm 1.35e^{-7})$	0.9985	0.15
Caffeic acid	$y = (0.00341 \pm 0.00092)x + (3.31e^{-4} \pm 8.50e^{-3})$	0.9993	2.01
Catechol	$y = (0.00027 \pm 0.00008)x + (5.59e^{-4} \pm 5.59e^{-3})$	0.9997	2.51
p-Coumaric acid	$y = (0.00081 \pm 0.00015)x + (-5.05e^{-7} \pm 4.07e^{-7})$	0.9979	1.75
Hydroxytyrosol	$y = (0.00287 \pm 0.00022)x + (2.21e^{-6} \pm 2.43e^{-6})$	0.9981	0.19
HT acetate	$y = (0.00138 \pm 0.00125)x + (3.67e^{-3} \pm 3.62e^{-3})$	0.9975	0.12
Luteolin	$y = (0.01245 \pm 0.00579)x + (1,25e^{-3} \pm 1,25e^{-3})$	0.9990	0.12
Luteolin-7-*O*-glu	$y = (0.01616 \pm 0.00735)x + (-5.73e^{-7} \pm 3.29e^{-6})$	0.9992	0.04
Oleuropein	$y = (0.00466 \pm 0.00164)x + (6.61e^{-3} \pm 6.62e^{-3})$	0.9997	0.06
(+)-Pinoresinol	$y = (0.00195 \pm 0.00041)x + (2.00e^{-3} \pm 1.45e^{-3})$	0.9994	0.32
Quercetin	$y = (0.00401 \pm 0.00199)x + (5.08e^{-4} \pm 3.85e^{-3})$	0.9978	0.40
Rutin	$y = (0,00204 \pm 0,00059)x + (4,08e^{-3} \pm 4,07e^{-3})$	0.9991	0.11
Salidroside	$y = (0.00060 \pm 0.00019)x + (9.44e^{-4} \pm 9.44e^{-4})$	0.9979	0.63
Tyrosol	$y = (0.00012 \pm 0.00002)x + (4.06e^{-4} \pm 2.36e^{-4})$	0.9954	1.95
Vanillic acid	$y = (0.00074 \pm 0.00001)x + (-1.09e^{-6} \pm 3.30e^{-7})$	0.9990	1.08
Verbascoside	$y = (0.00328 \pm 0.00176)x + (2.74e^{-7} \pm 2.01e^{-7})$	0.9987	0.06

4.2.3.5. Precision

The results for intra-day precision evaluated in blank rat plasma spiked at 10, 20, 50, 100, 150 and 250 nmol/L are displayed in Table 4.7. The phenolic compounds with the lowest intra-day precision were vanillic acid and lutenoli-7-*O*-glucoside since the mean value for the 6 spiked concentration were 4.40 ± 1.07% and 5.05 ± 0.92%. Most phenolic compounds provided intra-day precision with a mean value within 6% and 7%, Only quercetin and luteolin gave values of relative standard deviation (RSD%) superior to 7%, with means of 7.15 ± 0.49% and 7.17 ± 0.60%. Finally, salidroside showed a mean intra-day precision of 8.83 ± 1.40%, mainly due to the high value of 14.91% observed at the lowest concentration of 10 nmol/L. Consequently, intra-day precision was inferior to 15% for all the phenolic compounds at all the concentrations, thus fulfilling the criteria established by EMA guidelines (2011).

The developed analytical method also showed satisfactory inter-day precision as shown in Table 4.8. The phenolic compound with the lowest values were vanillic and caffeic acids with means of 3.67 ± 0.80% and 3.75 ± 0.87%. *p*-Coumaric acid, verbascoside, pinoresinol, luteolin-7-*O*-glucoside and catechol yielded mean inter-day precisions of approximately 5%. Whereas oleuropein, hydroxytyrosol, apigenin, luteolin, hydroxytyrosol acetate and rutin produced mean inter-day precisions that ranged from 6 to 7%. Finally, tyrosol and salidroside

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gave an inter-day precision of $8.08 \pm 1.01\%$ and $8.50 \pm 1.44\%$, respectively.

Table 4.7. Intra-day precision of phenolic compounds spiked in rat blank plasma and analyzed by LC-ESI-MS/MS.

Analyte	Intra-day precision (% RSD)					
	10 nmol/L	20 nmol/L	50 nmol/L	100 nmol/L	150 nmol/L	250 nmol/L
Apigenin	6.91	9.70	4.82	4.12	2.91	8.18
Caffeic acid	5.56	6.38	6.35	5.69	3.43	7.31
Catechol	8.23	6.65	7.06	7.19	1.12	6.60
p-Coumaric acid	8.06	9.39	6.42	8.10	1.14	6.40
Hydroxytyrosol	9.36	9.92	9.03	4.22	1.79	3.46
HT acetate	6.68	7.33	4.12	9.56	2.38	7.69
Luteolin	6.01	8.45	6.38	9.27	7.42	5.50
Luteolin-7-*O*-glu	7.93	6.46	2.57	6.73	3.35	3.25
Oleuropein	7.96	8.29	3.16	8.63	6.92	4.29
(+)-Pinoresinol	8.62	8.45	2.86	5.95	4.61	9.23
Quercetin	8.44	5.66	8.32	6.74	5.95	7.76
Rutin	9.63	8.78	4.31	7.20	3.03	7.70
Salidroside	14.91	7.67	7.10	9.90	8.58	4.82
Tyrosol	9.03	9.94	9.25	3.03	0.65	6.94
Vanillic acid	6.79	6.80	3.27	6.39	2.51	0.62
Verbascoside	6.39	9.75	5.26	9.63	1.34	5.69

Table 4.8. Inter-day precision of phenolic compounds spiked in rat blank plasma and analyzed by LC-ESI-MS/MS.

Analyte	Inter-day precision (% RSD)					
	10 nmol/L	20 nmol/L	50 nmol/L	100 nmol/L	150 nmol/L	250 nmol/L
Apigenin	6.71	8.19	4.27	5.37	3.05	9.91
Caffeic acid	7.33	2.25	2.18	2.73	2.64	5.38
Catechol	7.78	5.20	6.26	6.39	1.08	5.00
p-Coumaric acid	5.97	8.52	2.28	6.34	1.00	6.01
Hydroxytyrosol	12.18	7.64	4.01	7.77	1.67	3.78
HT acetate	9.80	9.60	1.98	8.85	1.62	8.55
Luteolin	3.80	8.58	5.12	8.02	7.11	6.43
Luteolin-7-*O*-glu	8.26	5.38	2.14	8.24	3.51	3.49
Oleuropein	9.59	7.74	2.58	6.37	5.13	4.06
(+)-Pinoresinol	6.47	5.56	2.12	4.63	3.61	8.40
Quercetin	4.62	4.30	6.53	3.35	7.23	8.43
Rutin	9.46	9.41	3.84	8.83	3.06	7.12
Salidroside	14.08	9.12	8.43	6.47	9.49	3.41
Tyrosol	11.23	9.59	9.72	7.06	4.82	6.04
Vanillic acid	4.44	5.80	3.06	5.48	2.63	0.58
Verbascoside	7.17	4.92	5.30	7.69	1.01	4.43

4.2.3.6. Accuracy

The accuracy of the developed analytical method evaluated at 6 concentrations of phenolic compounds spiked in blank rank plasma are displayed in Table 4.9. The method provided a good accuracy even for the lowest concentration of 10 nmol/L. In this case, only vanillic acid (12.31%) and catechol (-13.79%) gave results superior to 10%. At the concentrations of 20, 50, 100, 150 and 250 nmol/L the different phenolic compounds generated results for accuracy lower than 5%.

Results were acceptable since the deviation between the theoretical and calculated concentrations were inferior to 15%. Accuracy fulfilled the acceptable criteria established by EMA guidelines (2011).

Table 4.9. Accuracy of phenolic compounds spiked in rat blank plasma and analyzed by LC-ESI-MS/MS.

Analyte	Accuracy (% RSD)					
	10 nmol/L	20 nmol/L	50 nmol/L	100 nmol/L	150 nmol/L	250 nmol/L
Apigenin	-2.23	0.64	-1.99	-2.20	3.52	0.01
Caffeic acid	-8.82	2.23	0.71	-0.11	0.69	1.90
Catechol	-13.79	-4.50	-2.21	-0.57	7.81	0.02
p-Coumaric acid	3.06	-1.06	-2.67	-0.50	1.22	0.01
Hydroxytyrosol	-3.12	1.40	2.79	-4.19	1.10	0.03
HT acetate	-0.88	-1.31	0.94	3.17	-0.32	0.04
Luteolin	-0.99	1.99	1.57	-1.23	5.73	0.05
Luteolin-7-*O*-glu	-0.42	4.83	-2.92	-1.25	3.32	0.02
Oleuropein	-3.37	0.11	0.58	-1.71	2.91	0.88
(+)-Pinoresinol	-8.34	4.67	-0.91	-1.59	4.65	0.02
Quercetin	-5.92	-0.11	5.70	0.38	-1.30	-3.94
Rutin	4.38	3.14	-6.79	-0.44	2.00	2.12
Salidroside	3.48	2.82	-3.25	-4.66	-2.18	0.04
Tyrosol	-1.28	-0.21	-4.42	-2.72	4.83	0.05
Vanillic acid	12.31	0.39	-1.69	-1.90	2.12	0.06
Verbascoside	-1.34	0.51	4.34	4.95	0.24	0.02

4.2.3.7. Selectivity

Selectivity was evaluated to determine if the developed extraction procedure was able to distinguish 16 studied polyphenols and 2-(3-hydroxyphenyl) ethanol used as IS from endogenous compounds in the blank rat plasma.

The developed method showed high selectivity as no interferences from endogenous compounds were detected at the retention times of the individual polyphenols and IS.

The comparison between blank plasma samples where the absence of peaks can be observed in the retention times of the analytes displayed in the chromatograms of blank plasma spiked with mixture of phenolic compounds at 150 nmol/L is shown in Figure 4.5.

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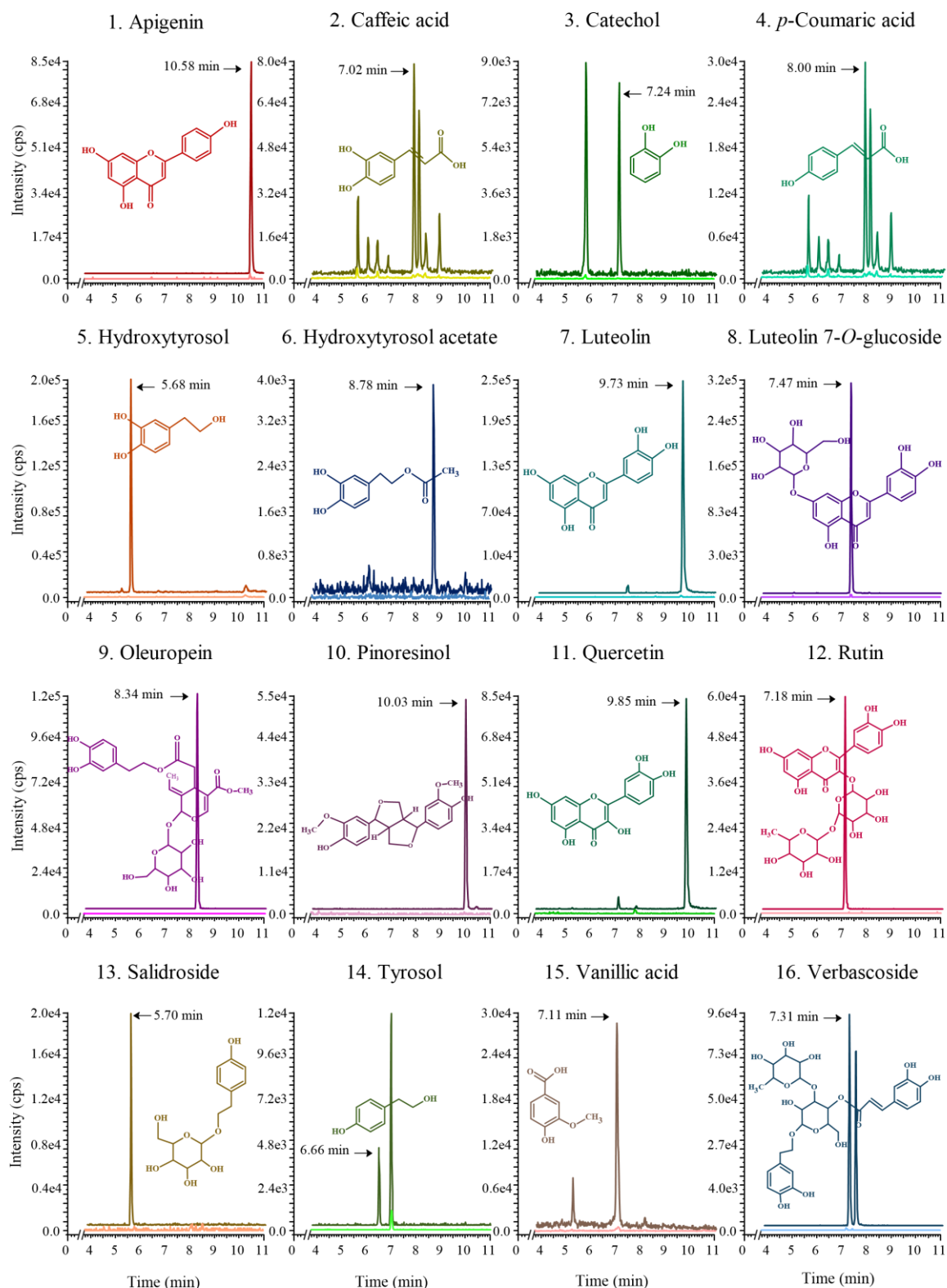
Figure 4.5. Representative LC-ESI-MS/MS extracted ion chromatograms of phenolic compounds obtained in multiple reaction mode (MRM). Chromatograms show blank rat plasma (pale line) and blank rat plasma spiked with standards at 150 nmol/L (darker line).

Moreover, the developed method allowed the separation of 16 polyphenols in a short analysis time of less than 11 min. Under our experimental conditions, hydroxytyrosol (*m/z* 153.2-122.8, RT: 5.68 min) and salidroside (*m/z* 299.2-119.2, RT: 7.70 min) were polyphenols which eluted at first place and apigenin elutes as last compound at 10.58 min. Tyrosol was detected at *m/z* of 137.10-106.0 which is really similar to the *m/z* of the IS (*m/z* 137.00-107.00). Both compounds appear in different retention times, tyrosol at 6.66 min and IS at 7.13 min what ensures their adequate determination.

4.2.3.8. Carry-over

The assessment of the carry-over on the LC-ESI-MS/MS instrument was performed 6 times in each analytical run by sequential injection of the highest calibration standard followed by a blank sample with interval based on the number of samples in the batch. No enhancement in the retention time of the studied polyphenols and IS was observed in blank samples that were analyzed immediately after the injection of highest concentration of calibration standard. Hence, the absence of carry-over ensured the reliability of the method at low concentrations.

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4.3. PRE-CLINICAL STUDIES: PHARMACOKINETICS OF PHENOLIC COMPOUNDS IN PLASMA AFTER THE ORAL ADMINISTRATION OF ARBEQUINA TABLE OLIVES TO SPRAGUE-DAWLEY RATS

Once validated, the developed method was applied to the determination of phenolic compounds in rat plasma after the oral administration of table olives of the Arbequina variety harvested in the season 2015/2016. Subsequently, the plasmatic concentrations of the different analytes found in plasma were subjected to a pharmacokinetic analysis.

4.3.1. Identification of phenolic compounds in rat plasma

The analysis of the chromatograms of plasma samples obtained after the oral administration of Arbequina table olives at two doses revealed the presence of 7 phenolic compounds. The representative extracted ion chromatograms obtained at 30 min after the administration of the dose equivalent to the human consumption of 30 olives is shown in Figure 4.6, whereas Figure 4.7 depicts those obtained after the intake of 60 olives at the same sampling time. The figures display the chromatograms of 16 polyphenols, which correspond to the ones determined in Arbequina table olives.

From the 16 phenolic compounds found in Arbequina table olives, only 7 analytes hold concentrations above the LOQ (Figures 4.6 and 4.7), namely, *p*-coumaric acid, hydroxytyrosol, luteolin, luteolin-7-*O*-glucoside, salidroside, tyrosol, verbascoside. All these polyphenols were identified in all extraction times except for salidroside that was found until 360 min.

The analysis of the chromatograms obtained by LC-ESI-MS/MS revealed the presence of apigenin, oleuropein, pinoresinol, quercetin, and vanillic acid at concentrations below the LOQ. Finally, caffeic acid, catechol, hydroxytyrosol acetate, and rutin were not detected in any of the samples analyzed (Figures 4.6 and 4.7).

A targeted metabolomic approach was used for the identification of the metabolites of hydroxytyrosol, which is the main phenolic compound in Arbequina table olives. The analysis of hydroxytyrosol (M0) was performed in MRM mode characterized by the *m/z* pair 153.2/122.8 Da with a retention time of 5.72 min that coincides with the one of standard. In the same chromatogram it could be seen the presence of four more peaks, two bigger ones identified as sulfate derivatives (M1-a and M1-b) while two small peaks corresponded to the glucuronide derivatives (M2-a and M2-b) (Figure 4.8).

Hydroxytyrosol sulfates that appeared at 5.16 (M1-a) and 5.35 min (M1-b) were characterized for an increase in its molecular weight in 80 Da. This metabolite was analyzed using 2 transitions, the first one characterized by the *m/z* pair 233/153 (quantification transition) and the second one at the *m/z* 153.0/122.8 (qualifier transition) (Figure 4.8).
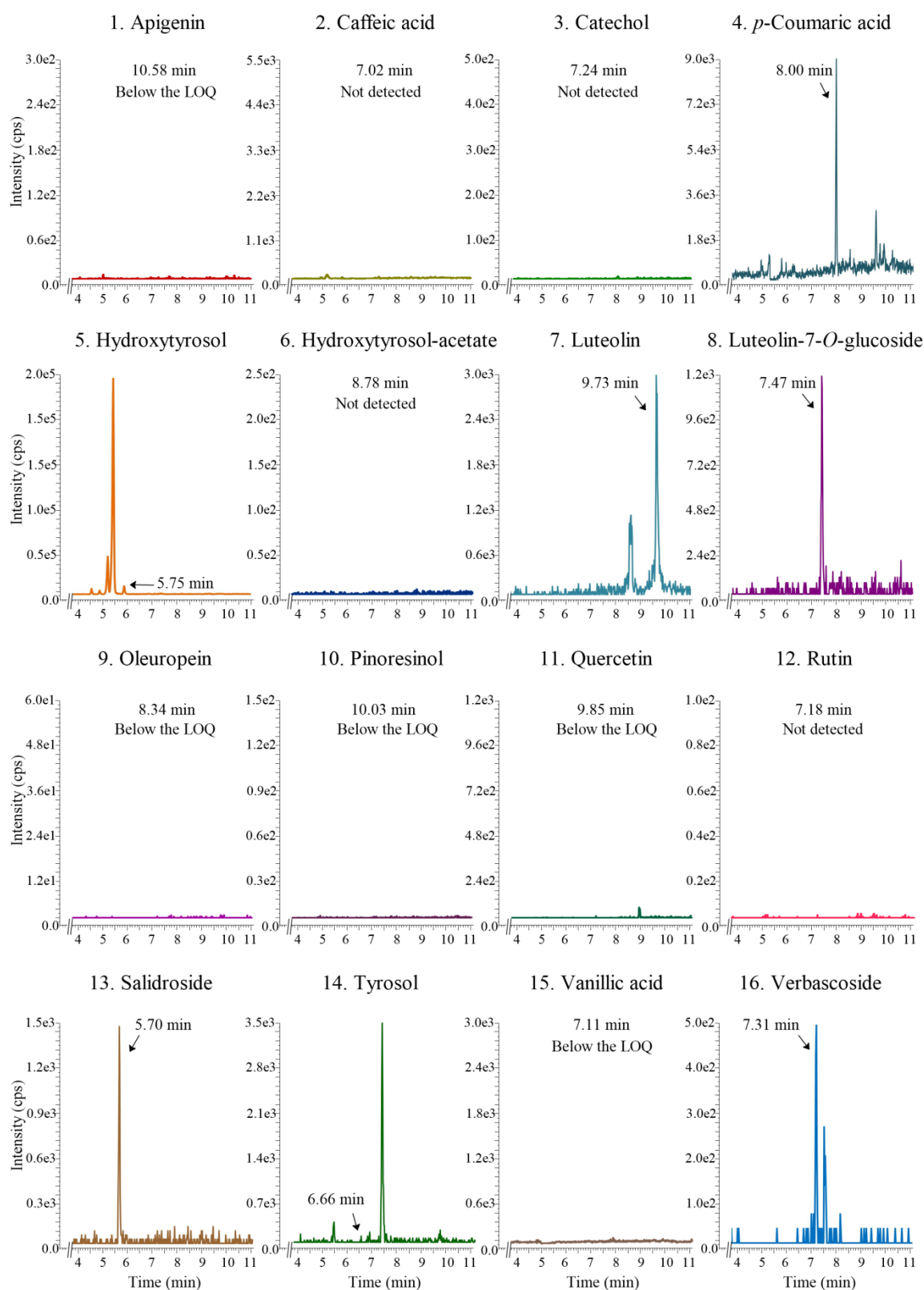
Figure 4.6. Representative LC-ESI-MS/MS chromatograms of Sprague-Dawley rats plasma obtained 30 min after the oral administration of a dose equivalent to the human intake of 30 Arbequina table olives obtained in multiple reaction monitoring mode (MRM).

IV. Results
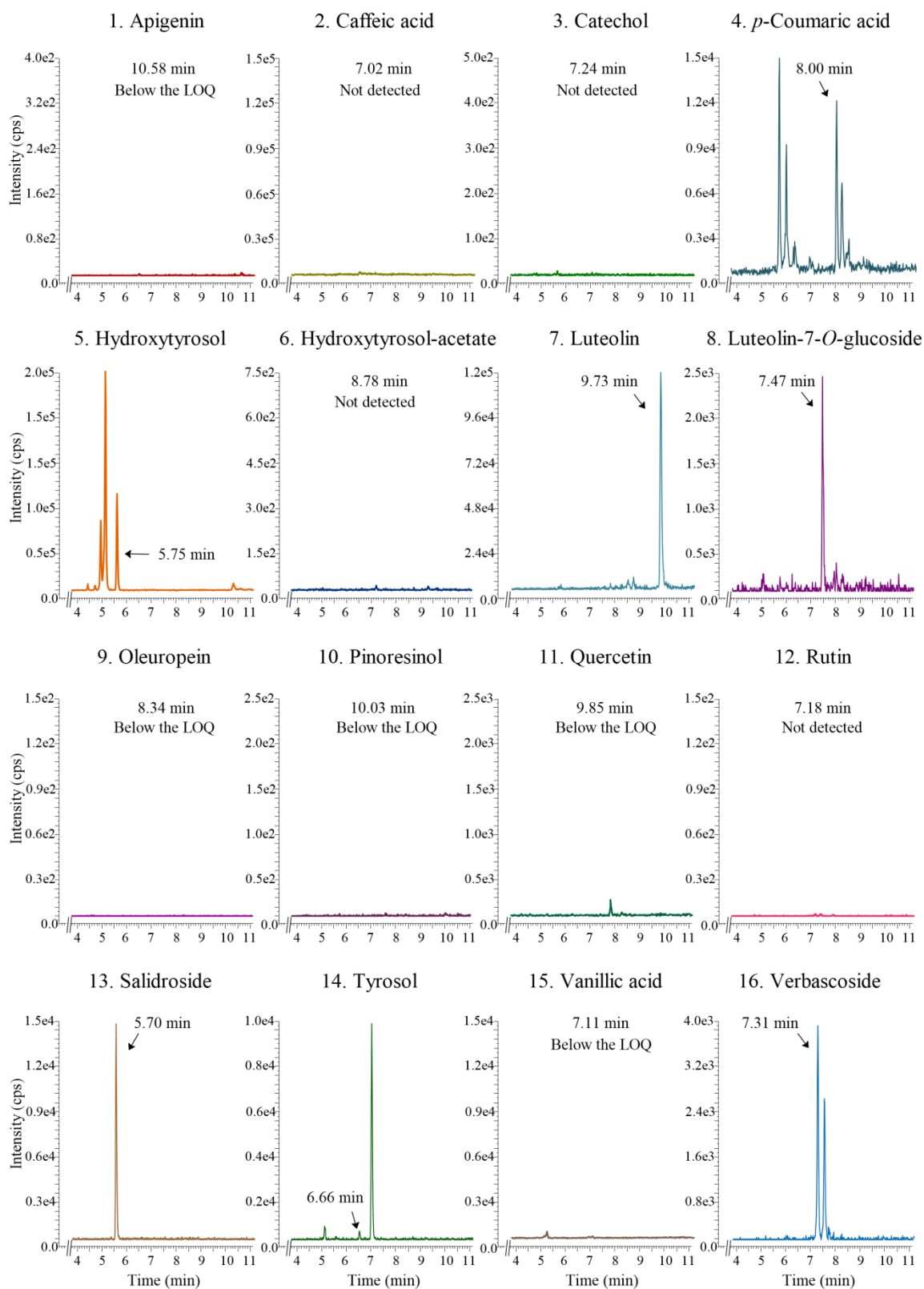
Figure 4.7. Representative LC-ESI-MS/MS chromatograms of Sprague-Dawley rats plasma obtained 30 min after the oral administration of a dose equivalent to the human intake of 60 Arbequina table olives obtained in multiple reaction monitoring mode (MRM).

The glucuronide of hydroxytyrosol appeared at 4.60 (M2-a) and 4.88 min (M2-b), and they were identified by an increase of 176 Da in the mass of the parent compound. Thus, the product ion was detected at 329.0 Da in the negative mode. Hence the glucuronide metabolites were analyzed at 2 transitions, the first at m/z 329/153 (quantification transition) and the second at m/z 153.0/122.8 (qualifier transition). The representative LC-ESI-MS/MS chromatograms of M0 and its metabolites M1-a, M1-b, M2-a, and M2-b obtained 30 minutes after oral administration of table olives to rats at dose equivalent to human intake of 30 and 60 table olives are shown in Figure 4.8. The identity of both groups of metabolites was confirmed since they were also present at the chromatogram of hydroxytyrosol (Figure 4.8A and D) at the same retention times (Figure 4.8B, C, E and F). The targeted analysis revealed no traces of the sulfo-glucuronide in any of the chromatograms analyzed.
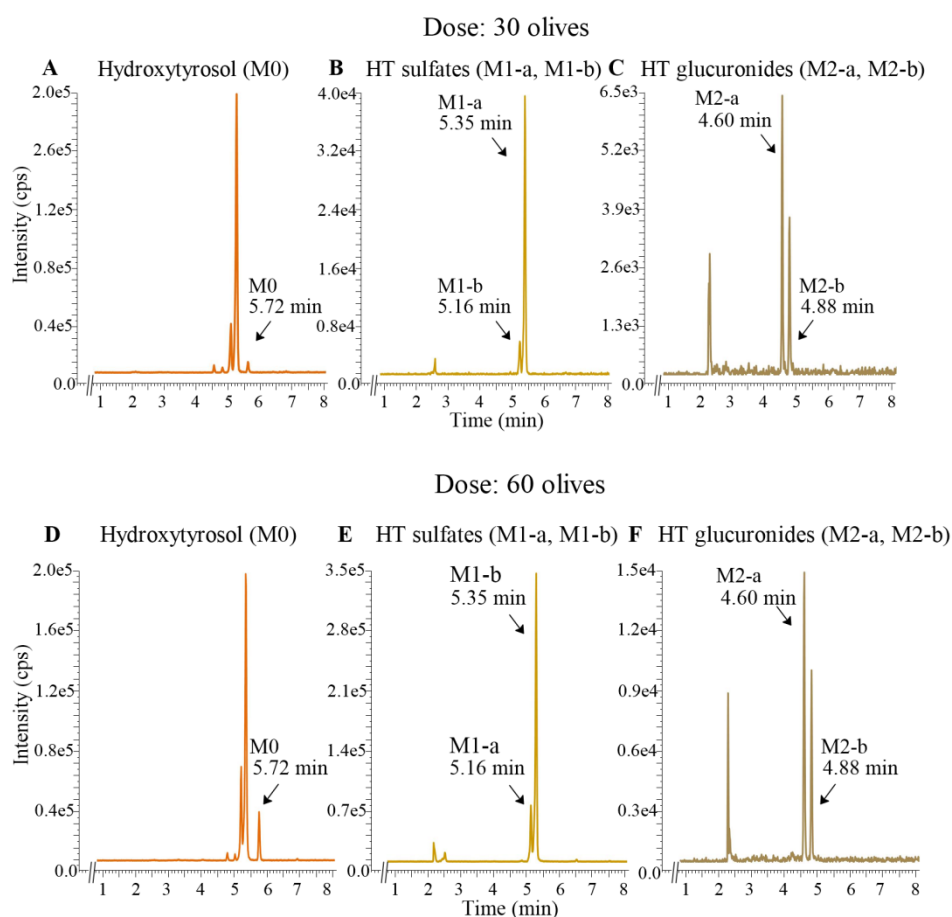
Figure 4.8. Representative LC-ESI-MS/MS chromatograms of hydroxytyrosol (M0) and its metabolites (hydroxytyrosol sulfates: M1-a, M1-b and hydroxytyrosol glucuronides: M2-a, M2-b) obtained in multiple reaction monitoring mode (MRM) 30 minutes after oral administration of Arbequina table olives to Sprague-Dawley rats at dose equivalent to human intake of 30 a 60 table olives. Hydroxytyrosol (A, D) appeared at 5.72 min (m/z 153.2/122.8), hydroxytyrosol sulfates (B, E) at 5.16 and 5.35 min (m/z 233.0/153.2) and hydroxytyrosol glucuronides (C, F) at 4.60 and 4.88 min (m/z 329.0/153.2).

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4.3.2. Plasmatic concentrations of phenolic compounds in rat plasma

After the identification of phenolic compounds, the plasmatic concentrations were calculated using the calibration curves. Blank plasma samples were checked for the presence of polyphenols. Apigenin, *p*-coumaric acid, luteolin, luteolin-7-*O*-glucoside, salidroside, vanillic acid, and verbascoside were found in low concentrations. The plasmatic concentrations obtained after the oral administration of table olives, were subtracted with the amounts found in blank plasmas. Consequently, no increase in the concentrations of apigenin and vanillic acid were found after the intake of table olives compared to ones observed in blank plasma. Hence, the phenolic compounds found in plasma from the highest concentration to the lowest were salidroside, *p*-coumaric acid, hydroxytyrosol, verbascoside, tyrosol, luteolin and luteolin-7-*O*-glucoside.

4.3.2.1. Salidroside

The analysis of blank plasma samples indicated the presence of salidroside at a concentration of 2.61 ± 0.52 (n = 6) that was subtracted from all the values obtained after the oral administration of table olives. Salidroside reached the maximum plasma concentrations at 30 min after the oral administration of both doses (Figure 4.9). Rats received an amount of 9.86 ± 0.17 µg of salidroside (n = 6) in the dose equivalent to the human intake of 30 Arbequina table olives (Figure 4.9). At 30 min, a value of 26.2 ± 6.0 nmol/L was achieved that dropped to 6.60 ± 1.1 nmol/L at 60 min and diminished to amounts lower than 5 nmol/L at 90, 120 and 240 min. At this dose, salidroside was not detected neither at 360 min nor at 480 min.

Figure 4.9. Plasma concentrations of salidroside. Results were obtained after oral administration of table olives at doses of 3.85 and 7.70 g/kg to Sprague-Dawley rats. Values are presented as mean ± SEM.

After the oral administration of the dose equivalent to 60 olives, the rats received an amount of 21.0 ± 0.66 µg of salidroside (n = 7). This compound was found at 30 min at 166 ± 28.5 nmol/L that was value 6.33-fold higher than when the dose of 30 olives was administered (Figure 4.9). The plasma concentrations were progressively decreasing with values of 83.9 ± 14.0 nmol/L, 49.4 ± 10.7 nmol/L and 23.4 ± 8.8 nmol/L at 60, 90 and 120 min, respectively. From 180 min up to 360 min, the concentrations were below 10 nmol/L, and no salidroside

was detected 8 hours after the intake of olives.

4.3.2.2. *p*-Coumaric acid

Blank plasma samples presented a concentration of *p*-coumaric acid of 12.9 ± 1.08 nmol/L (n = 6), that was subtracted of all the samples obtained after the oral administration of Arbequina table olives.

The maximum plasmatic concentrations of *p*-coumaric acid were reached at 60 min when the doses of 3.85 and 7.70 g/kg were used (Figure 4.10). This analyte was found at all sampling times, from 30 min up to 480 min after the oral administration of table olives at both doses.

When the animals were given the low dose of 3.85 g of destoned olives/kg, *p*-coumaric accounted for 0.56 ± 0.01 µg (n = 6). The plasmatic curve followed the same pattern as for the dose of 7.70 g destoned olives/kg, but with lower concentrations. In this sense, at 30 min, *p*-coumaric accounted for 19.7 ± 3.2 nmol/L that rose to 35.3 ± 4.6 nmol/L at 60 min, lowering to 24.7 ± 2.4 nmol/L at 90 min (Figure 4.10). Then, the concentrations diminished in a progressive way, encountering values of 18.3 ± 4.5 nmol/L; 15.3 ± 0.6 nmol/L; 10.5 ± 2.2 nmol/L and 5.58 ± 2.96 nmol/L at 120; 240; 360 and 480 min.
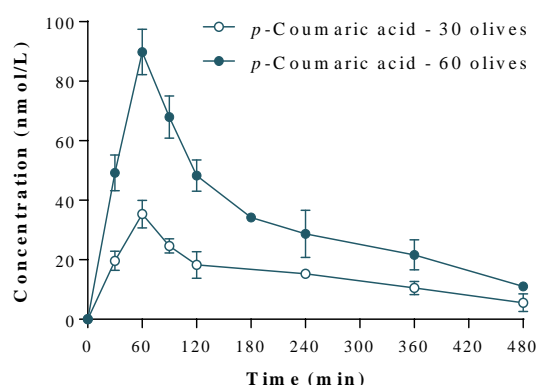
Figure 4.10. Plasma concentrations of *p*-coumaric acid. Results were obtained after the oral administration of table olives at doses of 3.85 and 7.70 g/kg to Sprague-Dawley rats. Values are presented as mean ± SEM.

The rats received 1.20 ± 0.04 µg of *p*-coumaric acid (n = 7) when the dose of 60 olives was orally administered. This compound was already detected in plasma at 30 min with concentrations of 49.2 ± 6.0 nmol/L that increased to 89.9 ± 7.6 nmol/L at 60 min that was 2.55 times higher than when the dose of 30 olives was administered, and started to lessen at 90 min with 68.0 ± 7.1 nmol/L (Figure 4.10). The plasmatic concentrations steadily decrease from 48.3 ± 5.2 nmol/L at 120 min up to 11.0 ± 0.5 at 480 min.

4.3.2.3. Hydroxytyrosol and its metabolites

The analysis of the chromatograms obtained from blank plasma samples showed no traces of hydroxytyrosol or the sulfate and glucuronide metabolites.

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4.3.2.3.1. Hydroxytyrosol

The oral administration of 3.85 g/kg of destoned Arbequina table olives provided the animals with 813 ± 14.35 μg of hydroxytyrosol (n = 6). This phenolic compound yielded a peak plasmatic concentration of 22.4 ± 4.0 nmol/L at 30 min. Afterwards, a decrease with time was observed. The concentrations encountered at 60, 90 and 120 min were 19.6 ± 8.1 nmol/L; 15.0 ± 2.6 nmol/L and 12.6 ± 1.6 nmol/L, respectively. At 240 min hydroxytyrosol was 4.88 ± 0.87 nmol/L. Finally, the phenolic compound reached concentrations of approximately 3.5 nmol/L at 6 and 8 hours (Figure 4.11).

When the Sprague-Dawley rats were given the higher dose of 7.70 g/kg of destoned Arbequina table olives, the animals received an amount of 1725 ± 54.49 μg of hydroxytyrosol (n = 7). Hydroxytyrosol was found at 30 min at 45.0 ± 6.6 nmol/L that was value 2-fold higher than when the dose of 30 olives was administered, showing a gradual decrease with time, since the concentrations found at 60, 90 and 120 min were 35.0 ± 4.6 nmol/L; 30.9 ± 6.6 nmol/L and 25.3 ± 3.2 nmol/L, respectively. At 180 and 240 min the values of hydroxytyrosol were approximately 18 nmol/L. Finally, the hydroxytyrosol dropped to 8.57 ± 1.61 nmol/L at 6 hours and 6.05 ± 0.58 nmol/L at 8 hours (Figure 4.11).
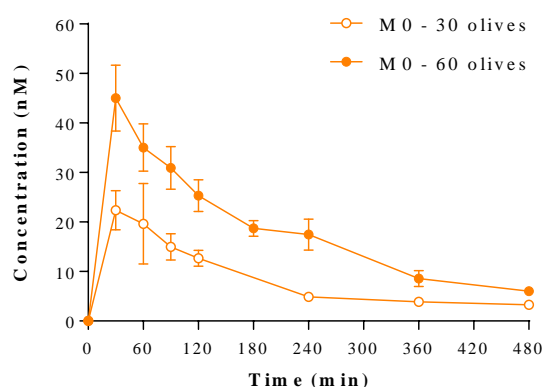
Figure 4.11. Plasma concentrations of hydroxytyrosol. Results were obtained after the oral administration of table olives at doses of 3.85 and 7.70 g/kg to Sprague-Dawley rats. Values are presented as mean ± SEM.

4.3.2.3.2. Hydroxytyrosol sulfate (M1-a and M1-b)

The analysis of the chromatograms already reported the presence of the parent compound along with two sulfate metabolites that exhibited higher concentrations than the parent compound. The metabolite M1-a eluted at 5.16 whereas the derivative M1-b had a retention time of 5.35 min and hold the highest concentrations.

After, the oral administration of a dose equivalent to a human intake of 30 olives, the hydroxytyrosol sulfate M1-a achieved the highest values of 53.6 ± 14.6 nmol/L at 30 min, was reduced to nearly the half at 60 min (24.4 ± 7.74 nmol/L) and dropped to 15.6 ± 6.0 nmol/L at 90 min. Subsequently, this metabolite was found at 18.4 ± 5.7 nmol/L at 120 min

and 6.81 ± 2.18 nmol/L at 240 min that decreased to 1.37 ± 0.35 nmol/L at 480 min (Figure 4.12.A).

At the same dose of 30 olives, the hydroxytyrosol sulfate that eluted at 5.35 min (M1-b) was the derivative with the highest plasmatic concentrations. This metabolite peaked at 30 min with amounts of 352 ± 85.3 nmol/L. At 60 min, the concentrations dropped to half, since the values found were 149 ± 42.1 nmol/L. Then, the concentrations achieved for this compound were 86.6 ± 30.0 nmol/L at 90 min, 107 ± 28.8 nmol/l at 120 min and were reduced to 42.8 ± 11.8 nmol/L at 120 min. This compound was still detected at 8 hours with values of 5.28 ± 0.73 nmol/L (Figure 4.12.B).
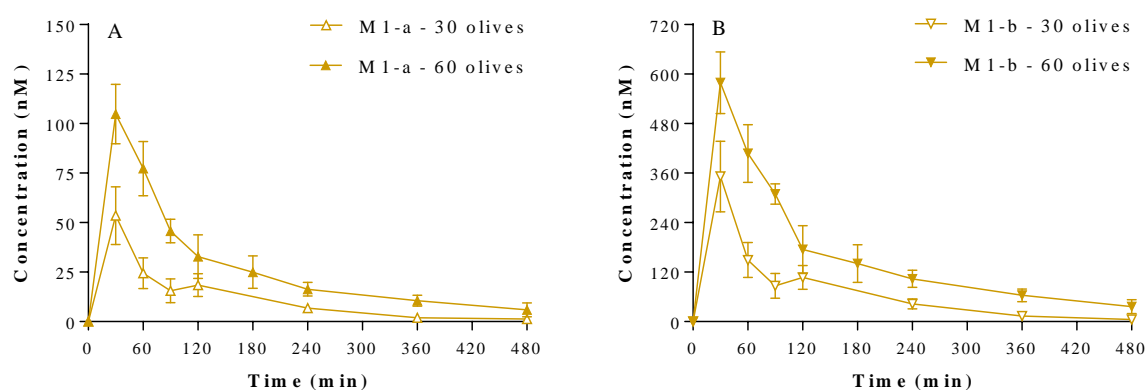
Figure 4.12. Plasma concentrations of hydroxytyrosol sulfates. A) depicts the isomer M1-a with retention time of 5.16 whereas B shows the metabolite M1-b eluting at 5.35 min. Results were obtained after the oral administration of table olives at doses of 3.85 and 7.70 g/kg to Sprague-Dawley rats. Values are presented as mean ± SEM.

When, experimental rats received the dose equivalent to the human consumption of 60 Arbequina table olives, the hydroxytyrosol sulfate M1-a attained a peak concentration of 105 ± 15.0 nmol/L at 30 min that was 1.96 times higher than when the dose of 30 olives was administered. Concentrations decreased to 77.3 ± 13.7 nmol/L at 60 min and dropped to 45.7 ± 5.9 nmol/L at 90 min. Then, M1-a hold concentrations of 32.8 ± 10.9 nmol/L at 120 min and 24.9 ± 8.2 nmol/L at 180 min that dropped to 5.95 ± 3.46 nmol/L at 480 min (Figure 4.12.A). On the other hand, and at the same dose of 60 olives, hydroxytyrosol sulfate M1-b, exhibited much higher plasmatic values, since the concentrations reached at 30 min were of 579 ± 74.7 nmol/L that was value 1.64-fold higher than when the dose equivalent to human consumption of 30 olives was administered. From this time on, the amounts found for this compound lessened although, the values were still quite high. M1-b was 407 ± 69.8 nmol/L at 60 min that diminished to 309 ± 24.4 nmol/l at 90 min and were reduced to nearly half at 120 min (175 ± 57.8 nmol/L). This metabolite was still found at 8 hours yielding concentrations of 36.2 ± 16.8 nmol/L (Figure 4.12.B).

4.3.2.3.3. Hydroxytyrosol glucuronide (M2-a and M2-b)

Finally, the hydroxytyrosol glucuronide also displayed two isomers, one appearing at 4.60 (M2-a) and the other 4.88 min (M2-b) (Figure 4.13). At the dose of 30 olives, the first

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metabolite (M2-a) was detected already at 30 min with concentrations of 8.46 ± 2.41 nmol/L. Then, M2-a lessened to 6.10 ± 2.01 nmol/L, 5.53 ± 1.88 nmol/L and 4.58 ± 1.28 nmol/L at 60, 90 and 120 min. At 240 min, M2-a was found at 1.64 ± 0.41 nmol/L and decreased to concentrations lower to 0.5 nmol/L at 6- and 8-hours post-administration (Figure 4.13.A). The second isomer (M2-b) hold the peak plasmatic concentrations of 5.33 ± 1.48 at 30 min. Then, at the extraction times of 60 and 90 min the values were of approximately 3.5 nmol/L. Concentrations reduced to 2.80 ± 0.64 nmol/L at 240 min and were still detected at 8 hours with values of 0.09 ± 0.01 nmol/L (Figure 4.13.B).

The oral administration of a dose equivalent to the human consumption of 60 olives, yielded the presence of both glucuronide derivatives. The metabolite M2-a was found already at 30 min with values of 14.4 ± 2.2 nmol/L that was value 1.7 times higher than when the dose equivalent to human intake of 30 olives was administered. Concentrations decreased to 12.0 ± 1.7 nmol/L, 9.54 ± 1.3 nmol/L and 7.08 ± 2.45 nmol/L at 60, 90 and 120 min. From this time on, the concentrations were lower than 5 nmol/L and was still detected at 8 hours post-administration (Figure 4.13.A). The isomer M2-b followed a similar profile, but the concentrations were slightly lower. The peak plasmatic concentrations of 9.52 ± 1.36 nmol/L were detected at 30 min that was a value 1.79-fold higher that when the dose equivalent to human intake of 30 olives was administered, and lowered to 7.10 ± 1.14 nmol/L and 6.05 ± 0.82 nmol/L at 60- and 90 min. Concentrations lowered to 4.33 ± 1.05 nmol/L at 120 min and were still detected at 480 min with values of 0.35 ± 0.12 nmol/L (Figure 4.13.B).
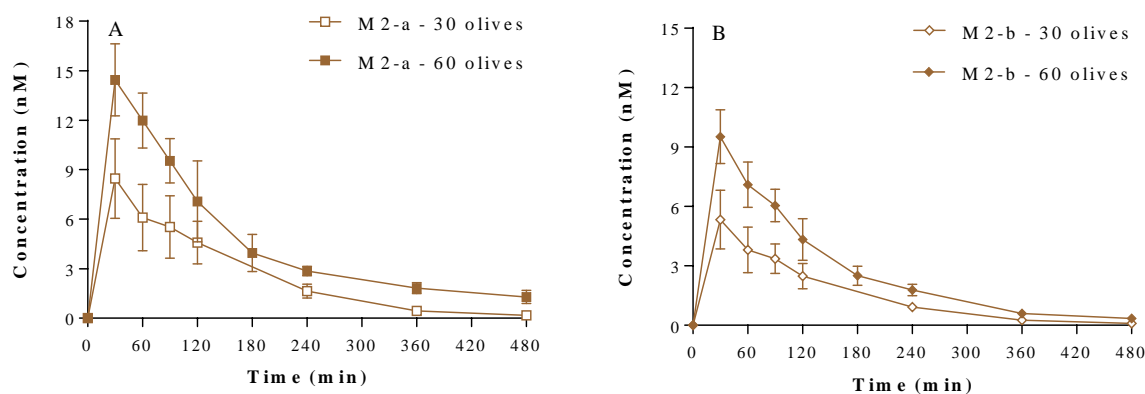
Figure 4.13. Plasma concentrations of hydroxytyrosol glucuronides. A) depicts the isomer M2-a with retention time of 4.60 whereas B shows the metabolite M2-b eluting at 4.88 min. Results were obtained after the oral administration of table olives at doses of 3.85 and 7.70 g/kg to Sprague-Dawley rats. Values are presented as mean ± SEM.

4.3.2.4. Verbascoside

Verbascoside was found in blank plasma at 0.37 ± 0.02 nmol/L (n = 6). This result was subtracted from all the concentrations obtained after the oral administration of Arbequina table olives.

The peak plasmatic concentrations of verbascoside after the oral administration of both doses of Arbequina table olives were found at 30 min (Figure 4.14). When Sprague-Dawley rats received the dose of 3.85 g/kg, the amount of verbascoside given to the animals was 28.2 ± 0.50 µg (n = 6). Then, verbascoside was already found in plasma at 30 min at 2.32 ± 0.36 nmol/L, lowering to 1.26 ± 0.39 nmol/L and 0.87 ± 0.22 nmol/L at 60 and 90 min, respectively (Figure 4.14). Later, concentrations decreased by half both at 120 min (0.42 ± 0.13 nmol/L) and at 240 min (0.21 ± 0.01 nmol/L). Although the plasmatic concentrations of this compound decreased, it was still detected at 360 and 480 min with values of 0.19 ± 0.06 nmol/L and 0.12 ± 0.06 nmol/L, respectively.

The treatment with 7.70 g/kg supplied the experimental animals with 60.0 ± 1.89 µg of verbascoside (n = 7). This phenolic compound achieved a concentration of 9.94 ± 1.59 nmol/L at 30 min that was 4.28-fold higher than when the dose equivalent to human intake of 30 olives was administered, and gradually diminished to 7.74 ± 1.13 nmol/L, 5.41 ± 0.44 nmol/L and 3.71 ± 0.86 nmol/L at 60, 90 and 120 min, respectively (Figure 4.14). Then, the values were approximately 1.5 nmol/L from 180 min to 360 min and decreased to 1.14 ± 0.70 nmol/L at 8 hours.
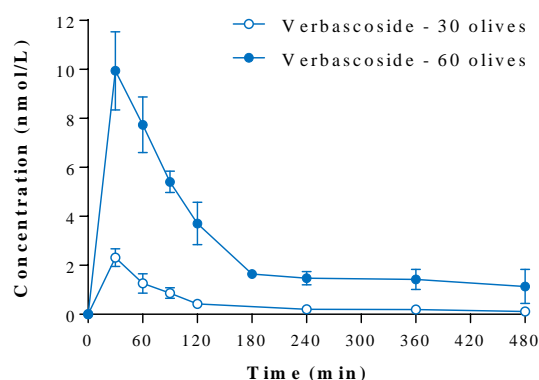
Figure 4.14. Plasma concentrations of verbascoside. Results were obtained after oral administration of table olives at doses of 3.85 and 7.70 g/kg to Sprague-Dawley rats. Values are presented as mean ± SEM.

4.3.2.5. Tyrosol

Tyrosol was not found in any of the plasma samples withdrawn from rats that were not orally administered with Arbequina table olives (n = 6).

The oral administration of experimental animals with a dose equivalent to the human consumption of 30 Arbequina table olives provided an amount of 30.4 ± 0.54 µg of tyrosol (n = 6). This phenolic compound achieved high plasmatic concentrations at both 30 (3.64 ± 1.08 nmol/L) and 60 min (4.14 ± 0.49 nmol/L) that decreased to nearly half at 90 (2.39 ± 0.71 nmol/L), 120 (2.10 ± 0.45 nmol/L) and 240 min (1.91 ± 0.42 nmol/L) (Figure 4.15). Tyrosol was also quantified at 360 and 480 min with concentrations of 1.48 ± 0.31 nmol/L and 1.16 ±

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0.02 nmol/L, respectively.

When Sprague-Dawley rats were given the dose equivalent to the human consumption of 60 Arbequina table olives, rats received 64.7 ± 2.04 µg of tyrosol (n = 7). At this dose, the plasmatic concentrations of this phenolic compound followed a similar pattern than in the administration of 30 olives (Figure 4.15). Hence, the major values were found at 30 and 60 min, with results of 8.07 ± 1.50 nmol/L and 9.16 ± 2.32 nmol/L, that were values 2.22 and 2.21-fold higher than when the dose equivalent to human ingestion of 30 olives was administered. Then, plasmatic concentrations dropped to 6.00 ± 1.46 nmol/L, 4.97 ± 1.02 nmol/L and 4.55 ± 0.97 nmol/L at 90, 120 and 180 min, respectively. However, from this time on, values followed a more steeply decrease since the concentrations found at 240, 360 and 480 min were 3.46 ± 0.69 nmol/L, 2.23 ± 0.27 nmol/L and 1.53 ± 0.09 nmol/L.
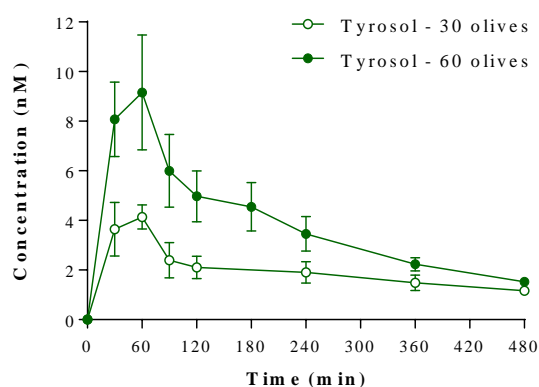
Figure 4.15. Plasma concentrations of tyrosol. Results were obtained after the oral administration of table olives at doses of 3.85 and 7.70 g/kg to Sprague-Dawley rats. Values are presented as mean ± SEM.

4.3.2.6. Luteolin

The analysis of blank rat plasma showed that luteolin was present with values of 0.70 ± 0.06 nmol/L (n = 6). This concentration was subtracted from the results calculated in plasma withdrawn from rats that received Arbequina table olives.

Plasma concentrations of luteolin when table olives were orally administered at doses of 3.85 and 7.70 g/kg are shown in Figure 4.16.

Experimental animals were given 86.5 ± 1.53 µg of luteolin (n = 6) after the oral administration of 3.85 g of destoned olives/kg of body weight. The major plasmatic concentrations were quantified at 30 min with values of 1.98 ± 0.18 nmol/L (Figure 4.16). After the peak, the results showed a marked decrease since at 60 min, the concentrations were 1.38 ± 0.36 nmol/L, at 90 min diminished to 1.01 ± 0.13 nmol/L and at 120 min lowered to 0.81 ± 0.20 nmol/L. This decrease could also be observed at 240 (0.58 ± 0.04 nmol/L), 360 (0.32 ± 0.03 nmol/L) and 480 min (0.24 ± 0.07 nmol/L).
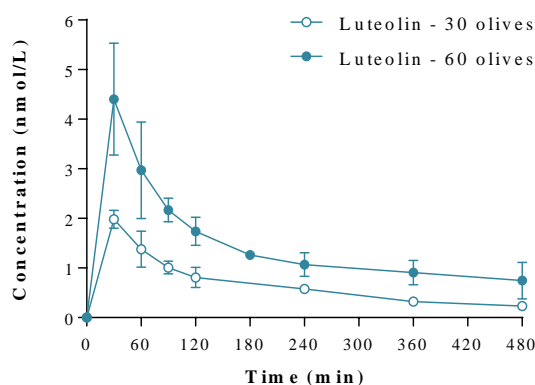
Figure 4.16. Plasma concentrations of luteolin. Results were obtained after oral administration of table olives at doses of 3.85 and 7.70 g/kg to Sprague-Dawley rats. Values are presented as mean ± SEM.

The oral administration of the dose of 7.70 g/kg, supplied the Sprague-Dawley rats with 183 ± 5.81 µg of luteolin (n = 7). The plasmatic concentrations obtained followed a similar profile as have been described for the dose of 3.85 g/kg (Figure 4.16). Therefore, a maximal concentration was found at 30 min (4.40 ± 1.13 nmol/L), value 2.22-fold higher than when the dose of 30 olives was administered, followed by a pronounced decrease at 60 (2.97 ± 0.97 nmol/L), 90 (2.17 ± 0.24 nmol/L), 120 (1.74 ± 0.28 nmol/L) and 180 min (1.26± 0.08 nmol/L). From 240 to 480 min, the decrease in the curve was slower (Figure 4.16). Hence, the plasmatic concentrations encountered were 1.07 ± 0.24 nmol/L, 0.91 ± 0.25 nmol/L and 0.75 ± 0.37 nmol/L at 4, 6 and 8 hours, respectively.

4.3.2.7. Luteolin-7-*O*-glucoside

Luteolin-7-*O*-glucoside was found in the plasma of rats that were not administered table olives at a concentration of 0.31 ± 0.02 nmol/L (n = 6). The results presented corresponded to the ones obtained after the subtraction of the concentration found in bank plasma.

Analysis of the obtained chromatograms allowed the identification and quantification of luteolin-7-*O*-glucoside at all sampling times when table olives were administered at doses of 3.85 and 7.70 g/kg (Figure 4.17).

The dose equivalent to the human consumption of 30 Arbequina table olives supplied the rats with 2.42 ± 0.04 µg of luteolin-7-*O*-glucoside (n = 6). This flavonoid exhibited the major plasmatic concentration of 0.58 ± 0.18 nmol/L at 30 min that in the next sampling time of 60 min gave a result of 0.29 ± 0.05 nmol/L (Figure 4.17).

After this sharp decline, this compound was found at 0.20 ± 0.06 nmol/L and 0.17 ± 0.06 nmol/L at 90 and 120 min. From this time on, luteolin-7-*O*-glucoside was quite stable in plasma with values of 0.12 ± 0.03 nmol/L and 0.11 ± 0.02 nmol/L at 240 and 360 min. Finally, this phenolic compound was still detected at 8 hours with a concentration of 0.05 ± 0.01 nmol/L.
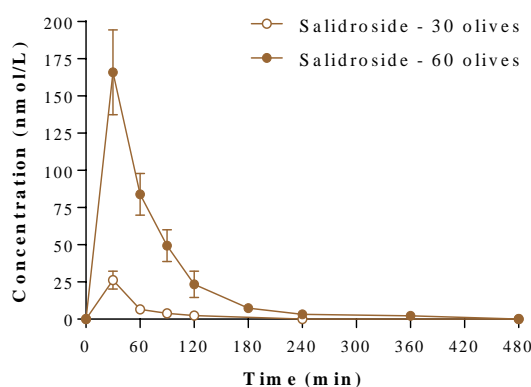
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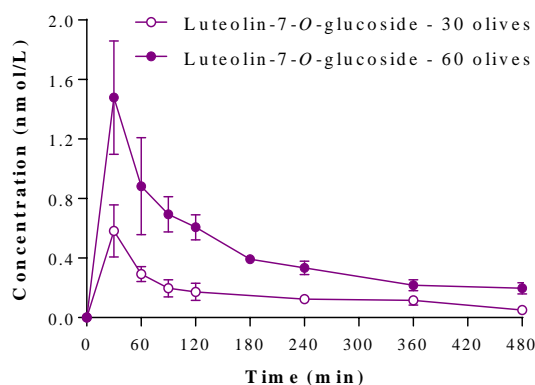
Figure 4.17. Plasma concentrations of luteolin-7-*O*-glucoside. Results were obtained after oral administration of table olives at doses of 3.85 and 7.70 g/kg to Sprague-Dawley rats. Values are presented as mean ± SEM.

The Sprague-Dawley rats that were orally administered with the dose equivalent to the human intake of 60 Arbequina table olives were given 5.15 ± 0.16 µg of luteolin-7-*O*-glucoside (n = 7). This phenolic compound reached a peak at 30 min with a concentration of 1.48 ± 0.38 nmol/L (2.55 times higher than when the dose equivalent to human intake of 30 olives was administered) and was followed by a sharp decline, since the values at 60 min were 0.88 ± 0.32 nmol/L (Figure 4.17). Afterwards, plasmatic values showed an slight decrease to keep rather similar at 90 and 120 min with results of 0.69 ± 0.12 nmol/L and 0.61 ± 0.09 nmol/L. Plasmatic concentrations dropped to 0.39 ± 0.02 nmol/L and 0.33 ± 0.04 nmol/L at 180 and 240 min. Finally, this flavonoid diminished slowly since the amounts quantified were 0.22 ± 0.04 nmol/L and 0.20 ± 0.04 nmol/L at 6 and 8 hours, respectively.

4.3.3. Pharmacokinetic analysis of phenolic compounds

The main pharmacokinetic parameters were calculated using a non-compartmental approach from the plasma concentrations of 7 polyphenols found in rat plasma after oral administration of table olives at doses of 3.85 and 7.70 g/kg.

4.3.3.1. Salidroside

The descriptive statistics of the pharmacokinetic constants of salidroside are shown in Table 4.10. Non-compartmental analysis of plasma concentrations of salidroside over time showed that this compound reached a maximum concentration of 18.9 ± 2.0 and 145.4 ± 1.5 nmol/L when table olives at doses of 3.85 and 7.70 g/kg were administered. Pharmacokinetic analysis gave a median values of time to peak concentrations of 30.5 min (min: 30 min, max: 62 min) and of 38 min (min: 30 min, max: 100 min) when the doses of 30 and 30 Arbequina table olives were administered. This parameter was indicative of a rapid absorption of salidroside. Lambda (λz) was 0.0170 ± 1.3 and 0.0134 ± 1.4 min^{-1} for both doses.

Plasma concentration of salidroside was reduced by half (elimination half-life) at 40.8 ± 1.3 and 51.7 ± 1.4 min after the oral administration of the doses of 30 and 60 olives. The areas

under the curve from time 0 until the last measured time (360 min) were 898.6 ± 1.8 and 9260.8 ± 1.5 nmol/L·min for 30 and 60 olives. The values of $AUC_{0-\infty}$ were calculated (1188.8 ± 1.5 nmol/L·min - low dose, 9858.1 ± 1.5 nmol/L·min - high dose).

When the area under the concentration curve extrapolated to infinity was calculated and expressed as a percentage of the total AUC ($AUC_{extrap\%}$), a values of 3.9 ± 5.1 and 1.1 ± 2.8 % were obtained for the doses of 30 and 60 olives, respectively. Since the $AUC_{extrap\%}$ values were below 20%, in case of salidroside this result of $AUC_{0-\infty}$ is considered as reliable. Finally, the mean residence time from time 0 min up to the time when the last concentration was quantified (MRT_{last}). The obtained values of MRT_{last} (30 olives: 54.4 ± 1.2 min, 60 olives: 76.0 ± 1.2 min), indicates the average permanence of salidroside in the body in accordance with the result obtained in half-life.

$MRT_{0-\infty}$ was calculated from time 0 min up to infinity and the obtained values were 63.7 ± 1.1 and 77.5 ± 1.2 min, for the doses of 30 and 60 olives, respectively.

Table 4.10. Pharmacokinetic parameters of salidroside estimated by non-compartmental analysis.

Parameters	Units	Mean	SD	CV %	Min	Median	Max	Geom. Mean	Geom. SD
Salidroside 3.85 g/kg (30 olives)									
T_{max}	min	--	--	--	30	30.5	62	--	--
C_{max}	nmol/L	22.8	14.6	64.0	6.0	21.3	48.4	18.9	2.0
λ_z	min^{-1}	0.0175	0.0042	23.9	0.0106	0.0186	0.0208	0.0170	1.3
t1/2z	min	42.2	13.5	31.9	33.3	37.2	65.6	40.8	1.3
AUC_{last}	nmol/L·min	1026.3	544.2	53.0	340.4	839.6	1817.3	898.6	1.8
$AUC_{0-\infty}$	nmol/L·min	1282.2	562.3	43.9	792.8	1038.2	2023.5	1188.8	1.5
$AUC_{extrap\%}$	%	8.0	7.1	89.2	0.6	10.2	16.8	3.9	5.1
MRT_{last}	min	55.2	10.2	18.4	44.7	53.7	70.1	54.4	1.2
$MRT_{0-\infty}$	min	64.1	7.3	11.5	55.0	65.9	73.7	63.7	1.1
7.70 g/kg (60 olives)									
T_{max}	min	--	--	--	30	38	100	--	--
C_{max}	nmol/L	157.1	67.8	43.1	84.2	137.3	261.5	145.4	1.5
λ_z	min^{-1}	0.0141	0.0043	30.4	0.0071	0.0145	0.0189	0.0134	1.4
t1/2z	min	54.7	22.4	40.9	36.7	48.5	97.5	51.7	1.4
AUC_{last}	nmol/L·min	9887.4	4128.7	41.8	5987.5	8309.1	17392.1	9260.8	1.5
$AUC_{0-\infty}$	nmol/L·min	10527.2	4322.0	41.1	6028.6	8917.5	17581.6	9858.1	1.5
$AUC_{extrap\%}$	%	1.7	1.7	96.9	0.3	0.9	4.0	1.1	2.8
MRT_{last}	min	77.2	14.8	19.2	60.0	78.8	102.7	76.0	1.2
$MRT_{0-\infty}$	min	78.4	13.1	16.7	61.4	78.0	94.8	77.5	1.2

4.3.3.2. *p*-Coumaric acid

Non-compartmental approach of plasma concentrations of *p*-coumaric acid over time showed that *p*-coumaric acid reached a maximum concentration of 30.8 ± 1.5 and 81.3 ± 1.3 nmol/Lwhen table olives at doses of 3.85 and 7.70 g/kg were administered (Table 4.11).

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Median values of T_{max} were 60 min (min: 60 min, max: 120 min) and 65 min (min: 60 min, max: 100 min) when the doses equivalent to the human consumption of 30 and 60 Arbequina table olives were given. This parameter indicated a relatively rapid absorption of *p*-coumaric acid. The values of lambda (λz) were 0.0040 ± 1.5 and 0.0044 ± 2.0 min^{-1} for both doses.

Elimination values of half-life were 173.9 ± 1.5 and 155.9 ± 2.0 min for the doses of 30 and 60 olives (Table 4.11). AUC_{last} and $AUC_{0-\infty}$ were 5473 ± 1.6 and 8931 ± 1.3 nmol/L·min for the dose of 30 olives and 13667 ± 1.3 and 19676 ± 1.4 nmol/L·min when the double dose was administered. Values of $AUC_{extrap\%}$ were $20.5 \pm 2.3\%$ for both doses. When $AUC_{extrap\%}$ is greater than 20%, it is suggested that the $AUC_{0-\infty}$ extrapolated from the experimental values has some unreliability. This unreliability is not due to a calculation error, but to the fact that in our experimental conditions more sampling points are missing, mostly in the terminal phase.

The estimated values for MRT_{last} were 161.8 ± 1.2 and 147.8 ± 1.3 min and of $MRT_{0-\infty}$ were 286.7 ± 1.4 and 264.2 ± 1.8 min for the doses of 30 and 60 olives, respectively (Table 4.11).

Table 4.11. Pharmacokinetic parameters of *p*-coumaric acid estimated by non-compartmental analysis.

Parameters	Units	Mean	SD	CV %	Min	Median	Max	Geom Mean	Geom SD
_p_-Coumaric acid 3.85 g/kg (30 olives)									
T_{max}	Min	--	--	--	60	60	120	--	--
C_{max}	nmol/L	32.7	11.2	34.3	17.7	37.1	43.3	30.8	1.5
λ_z	min^{-1}	0.0043	0.0019	45.3	0.0027	0.0031	0.0070	0.0040	1.5
t1/2z	Min	186.3	71.1	38.2	98.4	221.9	253.8	173.9	1.5
AUC_{last}	nmol/L•min	5883	2155	36.6	2483	6350	8517	5473	1.6
$AUC_{0-\infty}$	nmol/L•min	9142	2170	23.7	6682	9186	11377	8931	1.3
$AUC_{extrap\%}$	%	26.1	18.7	71.6	8.5	23.0	52.6	20.5	2.3
MRT_{last}	Min	163.5	25.1	15.4	124.2	166.8	192.7	161.8	1.2
$MRT_{0-\infty}$	Min	298.4	88.7	29.7	180.1	336.4	392.7	286.7	1.4
7.70 g/kg (60 olives)									
T_{max}	Min	--	--	--	60	65	100	--	--
C_{max}	nmol/L	84.1	22.8	27.1	49.8	83.3	118.1	81.3	1.3
λ_z	min^{-1}	0.0053	0.0029	54.1	0.0014	0.0054	0.0095	0.0044	2.0
t1/2z	Min	195.0	155.0	79.5	73.2	128.7	496.1	155.9	2.0
AUC_{last}	nmol/L•min	14048	3782	26.9	11059	12062	21279	13667	1.3
$AUC_{0-\infty}$	nmol/L•min	20611	6943	33.7	13576	18394	31909	19676	1.4
$AUC_{extrap\%}$	%	27.2	21.3	78.6	6.0	21.8	65.0	20.5	2.3
MRT_{last}	Min	151.4	33.1	21.9	95.0	168.4	186.2	147.8	1.3
$MRT_{0-\infty}$	Min	315.9	227.7	72.1	140.0	216.1	766.8	264.2	1.8

4.3.3.3. Hydroxytyrosol and its metabolites

4.3.3.3.1. Hydroxytyrosol

The plasma concentrations of hydroxytyrosol assessed by non-compartimental analysis,

yielded plasmatic concentrations that are displayed in Table 4.12.

Hydroxytyrosol was relatively rapidly absorbed with fast conversion to its metabolites. Median values of time to peak concentrations (T_{max}: 76 min and 41 min for the doses of 30 and 60 olives, respectively) were indicative of a relatively rapid absorption process. Although a trend towards higher T_{max} values has been observed after a low dose was administered, one should be cautious since T_{max} is a categorical variable whose discriminating power strongly depends on the sampling frequency.

Hydroxytyrosol reached C_{max} of 23.4 ± 1.7 and 46.4 ± 1.4 nmol/Lwhen the doses of 30 and 60 olives were administered. Terminal elimination rate were 0.0042 ± 1.7 and 0.0063 ± 1.4 min^{-1} and apparent elimination half-life gave a value of 166.2 ± 1.7 and 109.7 ± 1.4 min for the doses of 30 and 60 olives. AUC_{last} and $AUC_{0-\infty}$ were 3363 ± 1.3 and 4293 ± 1.1 nmol/L·min when 3.85 g/kg was administered and 7912 ± 1.3 and 8919 ± 1.3 nmol/L·min when h7.70 g/kg was applied. $AUC_{extrap\%}$ were below 20% in both doses.

The average permanence of hydroxytyrosol in the body was approximately 2.5 h that is described by MRT_{last}.

Table 4.12. Pharmacokinetic parameters of hydroxytyrosol estimated by non-compartmental analysis.

Parameters	Units	Mean	SD	CV %	Min	Median	Max	Geom. Mean	Geom. SD
Hydroxytyrosol (M0)									
3.85 g/kg (30 olives)									
T_{max}	min	--	--	--	31	76	120	--	--
C_{max}	nmol/L	26.3	14.5	55.0	11.8	22.0	52.1	23.4	1.7
λ_z	min^{-1}	0.0047	0.0024	50.4	0.0025	0.0043	0.0078	0.0042	1.7
t1/2z	min	185.7	90.9	48.9	88.6	178.5	282.1	166.2	1.7
AUC_{last}	nmol/L·min	3439	811	23.6	2477	3375	4807	3363	1.3
$AUC_{0-\infty}$	nmol/L·min	4315	493	11.4	3672	4242	5136	4293	1.1
$AUC_{extrap\%}$	%	21.0	11.3	53.8	6.4	21.3	33.5	17.9	1.9
MRT_{last}	min	156.3	34.7	22.2	118.3	150.3	218.8	153.4	1.2
$MRT_{0-\infty}$	min	276.2	115.8	41.9	142.1	273.5	442.9	255.5	1.6
7.70 g/kg (60 olives)									
T_{max}	min	--	--	--	33	41	68	--	--
C_{max}	nmol/L	45.0	14.6	32.5	26.4	44.8	68.0	43.0	1.4
λ_z	min^{-1}	0.0067	0.0027	40.3	0.0039	0.0059	0.0124	0.0063	1.4
t1/2z	min	115.3	36.9	32.0	56.0	118.4	179.3	109.7	1.4
AUC_{last}	nmol/L·min	8218	2540	30.9	5033	7882	13196	7912	1.3
$AUC_{0-\infty}$	nmol/L·min	9217	2634	28.6	5805	8659	14303	8919	1.3
$AUC_{extrap\%}$	%	11.2	4.9	43.7	3.9	10.2	17.4	10.1	1.7
MRT_{last}	min	161.3	24.0	14.9	127.7	151.4	194.7	159.8	1.2
$MRT_{0-\infty}$	min	209.8	43.0	20.5	158.7	207.8	279.7	206.1	1.2

4.3.3.3.2. Hydroxytyrosol sulfate (M1-a and M1-b)

Descriptive statistics of the pharmacokinetic constants of hydroxytyrosol sulfates are shown

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in Table 4.13 for the metabolite M1-a and Table 4.14 for the derivative M1-b (a,b).

Table 4.13. Pharmacokinetic parameters of M1-a estimated by non-compartmental analysis.

Parameters	Units	Mean	SD	CV%	Min	Median	Max	Geom. Mean	Geom. SD
Hydroxytyrosol sulfate M1-a									
3.85 g/kg (30 olives)									
T_{max}	min	--	--	--	30	45.5	120	--	--
C_{max}	nmol/L	53.1	28.6	53.8	26.6	44.1	99.7	47.3	1.7
λ_z	min^{-1}	0.0064	0.0043	66.9	0.0023	0.0053	0.0137	0.0053	2.0
t1/2z	min	154.6	93.9	60.7	50.6	140.6	297.8	130.1	2.0
AUC_{last}	nmol/L·min	4484	1311	29.2	3002	4466	6112	4321	1.4
$AUC_{0-\infty}$	nmol/L·min	4937	1398	28.3	3181	4977	6366	4765	1.3
$AUC_{extrap\%}$	%	9.0	7.8	86.4	1.5	5.8	19.2	6.3	2.7
$MRT_{0-\infty}$	min	181.0	58.3	32.2	109.5	180.8	263.4	173.0	1.4
7.70 g/kg (60 olives)									
T_{max}	min	--	--	--	30	36	42	--	--
C_{max}	nmol/L	104.8	39.7	37.8	47.3	100.3	155.1	97.4	1.5
λ_z	min^{-1}	0.0061	0.0041	66.9	0.0038	0.0050	0.0152	0.0054	1.6
t1/2z	min	139.7	46.8	33.5	45.7	138.1	184.1	129.4	1.6
AUC_{last}	nmol/L·min	11760	2954	25.1	6644	12974	14700	11376	1.3
$AUC_{0-\infty}$	nmol/L·min	13664	3879	28.4	7913	14067	18051	13133	1.4
$AUC_{extrap\%}$	%	13.1	8.1	62.0	0.7	16.0	24.7	9.0	3.4
$MRT_{0-\infty}$	min	194.5	47.1	24.2	101.1	197.0	255.1	188.3	1.3

Table 4.14. Pharmacokinetic parameters of M1-b estimated by non-compartmental analysis.

Parameters	Units	Mean	SD	CV%	Min	Median	Max	Geom. Mean	Geom. SD
Hydroxytyrosol sulfate M1-b									
3.85 g/kg (30 olives)									
T_{max}	min	--	--	--	30	30.5	120	--	--
C_{max}	nmol/L	340.0	174.4	51.3	179.2	307.4	642.0	306.4	1.6
λ_z	min^{-1}	0.0082	0.0020	23.9	0.0059	0.0079	0.0114	0.0080	1.3
t1/2z	min	88.4	19.9	22.6	60.6	88.5	116.9	86.4	1.3
AUC_{last}	nmol/L·min	27850	7832	28.1	17846	27306	38094	26907	1.3
$AUC_{0-\infty}$	nmol/L·min	29160	8525	29.2	18221	29150	40259	28084	1.4
$AUC_{extrap\%}$	%	4.2	2.2	51.8	2.1	3.5	7.9	3.8	1.6
$MRT_{0-\infty}$	min	143.3	26.4	18.5	115.9	137.8	193.1	141.4	1.2
7.70 g/kg (60 olives)									
T_{max}	min	--	--	--	30	38	100	--	--
C_{max}	nmol/L	581.2	193.8	33.3	266.1	596.7	855.6	548.4	1.5
λ_z	min^{-1}	0.0064	0.0047	74.2	0.0028	0.0051	0.0166	0.0054	1.8
t1/2z	min	147.7	73.5	49.8	41.8	136.9	245.6	129.3	1.8
AUC_{last}	nmol/L·min	69175	14548	21.0	39505	71215	87431	67548	1.3
$AUC_{0-\infty}$	nmol/L·min	81495	16316	20.0	54688	81504	102273	80006	1.2
$AUC_{extrap\%}$	%	14.8	12.2	82.2	0.6	12.2	33.9	8.8	4.0
$MRT_{0-\infty}$	min	211.3	77.5	36.7	100.8	190.3	332.7	198.8	1.5

The exposure to the metabolites given by C_{max} and AUC_{last} and the values were higher for the sulfate M1-b. (30 olives: C_{max}: 306.4 ± 1.6 nmol/L, AUC_{last}: 26907 ± 1.3 nmol/L·min, 60 olives: C_{max}: 548.4 ± 1.5 nmol/L, AUC_{last}: 67548 ± 1.3 nmol/L·min), followed by the sulfate M1-a. (low dose: C_{max}: 47.3 ± 1.7 nmol/L, AUC_{last}: 4321 ± 1.4 nmol/L·min, high dose: C_{max}: 97.4 ± 1.5 nmol/L, AUC_{last}: 11376 ± 1.3 nmol/L·min).

Values of $AUC_{0-\infty}$ were as follows: 4765 ± 1.3 and 13133 ± 1.4 nmol/L·min for M1-a, 28084 ± 1.4 and 67548 ± 1.3 nmol/L·min for M1-b, when the rats received the doses of 3.85 or 7.70 g/kg. The results obtained for both sulfates at the two doses for $AUC_{extrap\%}$ were lower than 20%, thus $AUC_{0-\infty}$ are considered as reliable.

Median of T_{max} was for M1-a and M1-b between 30.5 and 45.5 min, meaning that after the absorption of hydroxytyrosol, this compound rapidly transformed to both sulfates. The values of $MRT_{0-\infty}$ describing the permanence of sulfates in the body were around 2.5 - 3 h.

4.3.3.3.3. Hydroxytyrosol glucuronide (M2-a and M2-b)

Once in the organism, hydroxytyrosol not only underwent a transformation to sulfate but also to glucuronide.

The plasma concentrations of both hydroxytyrosol glucuronides were evaluated by non-compartmental analysis and the estimated pharmacokinetic parameters are displayed in Table 4.15 for the derivative M2-a and Table 4.16 for the metabolite M2-b.

The exposure to the metabolites given by C_{max} and AUC_{last} and values were higher for the glucuronide M2-a than the glucuronide M2-b.

For the hydroxytyrosol glucuronide M2-a, C_{max} was 9.5 ± 1.5 nmol/L and AUC_{last}: 1017 ± 1.2 nmol/L·min when the animals were given a dose equivalent to the human consumption of 30 olives. In the case of the oral administration of the double dose, the C_{max} was 14.2 ± 1.6 nmol/L and AUC_{last} was 1953 ± 1.2 nmol/L·min.

On the other hand, the values obtained for hydroxytyrosol glucuronide M2-b for the dose of 30 olives are characterized by a C_{max} of 5.4 ± 1.6 nmol/L and a AUC_{last} of 642 ± 1.3 nmol/L·min. While the estimates obtained for this compound at the dose of 60 olives, were a C_{max} of 9.0 ± 1.5 nmol/L and a AUC_{last} of 1161 ± 1.3 nmol/L·min.

The values of $AUC_{0-\infty}$ for M2-a were 1059 ± 1.2 and 2234 ± 1.2 nmol/L·min and the results of $AUC_{0-\infty}$ for M2-b were 642 ± 1.3 and 1239 ± 1.3 nmol/L·min, when the doses of 3.85 and 7.70 g/kg were administered. $AUC_{extrap\%}$ were below 20%, for the two isomers at both doses.

Median of T_{max} for both glururonides was between 38 and 45.5 min, meaning that both isomers were formed fast after hydroxytyrosol reached the organism. Values of $MRT_{0-\infty}$ values for both glucuronides were between 2 and 3 h.

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Table 4.15. Pharmacokinetic parameters of M2-a estimated by non-compartmental analysis.

Parameters	Units	Mean	SD	CV %	Min	Median	Max	Geom. Mean	Geom. SD
Hydroxytyrosol glucuronide M2-a									
3.85 g/kg (30 olives)									
T_{max}	min	--	--	--	30	45.5	120	--	--
C_{max}	nmol/L	10.2	4.3	41.7	5.3	9.7	16,0	9.5	1.5
λ_z	min^{-1}	0.0085	0.0031	36.3	0.0040	0.0094	0.0123	0.0080	1.5
t1/2z	min	94.3	45.0	47.7	56.3	73.9	174.0	86.9	1.5
AUC_{last}	nmol/L·min	1030	180	17.4	796	1023	1278	1017	1.2
$AUC_{0-\infty}$	nmol/L·min	1076	211	19.6	825	1041	1332	1059	1.2
$AUC_{extrap\%}$	%	3.9	3.7	94.4	1.3	2.7	11.1	2.9	2.2
$MRT_{0-\infty}$	min	144.3	23.3	16.2	121.7	140.3	179.0	142.8	1.2
7.70 g/kg (60 olives)									
T_{max}	min	--	--	--	30	41	123	--	--
C_{max}	nmol/L	15.3	5.9	38.3	7.1	16.7	22.4	14.2	1.6
λ_z	min^{-1}	0.0070	0.0040	57.0	0.0028	0.0062	0.0153	0.0062	1.7
t1/2z	min	124.5	63.1	50.7	45.2	111.4	249.7	111.8	1.7
AUC_{last}	nmol/L·min	1987	390	19.6	1491	1990	2444	1953	1.2
$AUC_{0-\infty}$	nmol/L·min	2267	411	18.1	1629	2147	2919	2234	1.2
$AUC_{extrap\%}$	%	12.3	7.9	64.7	5.3	9.0	28.4	10.6	1.7
$MRT_{0-\infty}$	min	197.4	83.6	42.4	117.4	168.5	374.9	185.6	1.4

Table 4.16. Pharmacokinetic parameters of hydroxytyrosol glucuronide M2-b estimated by non-compartmental analysis.

Parameters	Units	Mean	SD	CV %	Min	Median	Max	Geom. Mean	Geom. SD
Hydroxytyrosol glucuronide M2-b									
3.85 g/kg (30 olives)									
T_{max}	min	--	--	--	30	45.5	120	--	--
C_{max}	nmol/L	5.9	2.8	47.9	3.2	5.3	10.5	5.4	1.6
λ_z	min^{-1}	0.0088	0.0031	34.9	0.0035	0.0099	0.0121	0.0082	1.6
t1/2z	min	93.6	52.7	56.3	57.2	70.3	197.1	84.8	1.6
AUC_{last}	nmol/L·min	632	158	25.0	456	600	840	616	1.3
$AUC_{0-\infty}$	nmol/L·min	659	165	25.0	468	652	850	642	1.3
$AUC_{extrap\%}$	%	3.9	4.7	118.6	1.1	2.3	13.3	2.6	2.5
$MRT_{0-\infty}$	min	141.8	32.0	22.6	103.8	136.9	183.4	138.8	1.3
7.70 g/kg (60 olives)									
T_{max}	min	--	--	--	30	38	91	--	--
C_{max}	nmol/L	9.6	3.6	37.3	4.8	9.3	14.1	9.0	1.5
λ_z	min^{-1}	0.0087	0.0051	58.2	0.0040	0.0072	0.0197	.0078	1.6
t1/2z	min	96.6	40.8	42.2	35.1	96.2	172.6	88.6	1.6
AUC_{last}	nmol/L·min	1193	289	24.3	772	1192	1491	1161	1.3
$AUC_{0-\infty}$	nmol/L·min	1287	369	28.7	799	1289	1836	1239	1.3
$AUC_{extrap\%}$	%	6.2	6.2	100.5	0.3	3.6	18.8	3.6	3.7
$MRT_{0-\infty}$	min	152.0	33.3	21.9	110.1	149.9	218.0	149.2	1.2

4.3.3.4. Verbascoside

Non-compartmental approach of plasma concentrations of verbascoside over time showed that verbascoside reached a maximum concentration of 2.2 ± 1.5 nmol/L and 9.3 ± 1.5 nmol/L, when table olives were administered at the doses of 3.85 and 7.70 g/kg (Table 4.17).

When Arbequina table olives were administered, verbascoside was absorbed fast since median of T_{max} was 31 and 38 min after the intake of 3.85 g/kg and 7.70 g/kg. Lambda (λz) gave a value of 0.0049 ± 1.5 and 0.0058 ± 1.3 min^{-1} for both doses (Table 4.17).

Plasma concentration of salidroside was reduced by half (t1/2z) at 140.3 ± 1.5 and 118.8 ± 1.3 min. These values agreed with the MRT_{last} of approximately 2 h.

The estimates of AUC_{last} were 174.0 ± 1.5 and 1199.7 ± 1.5 nmol/L·min and $AUC_{0-\infty}$ 199.4 ± 1.4 and 1340.7 ± 1.5 nmol/L·min when the doses of 30 and 60 olives were administered. $AUC_{extrap\%}$ was below 20%, thus confirming the reliability of the results of the $AUC_{0-\infty}$.

Table 4.17. Pharmacokinetic parameters of verbascoside estimated by non-compartmental analysis.

Parameters	Units	Mean	SD	CV%	Min	Median	Max	Geom. Mean	Geom. SD
Verbascoside 3.85 g/kg (30 olives)									
T_{max}	min	--	--	--	30	31	60	--	--
C_{max}	nmol/L	2.4	0.8	35.8	1.2	2.6	3.2	2.2	1.5
λ_z	min^{-1}	0.0053	0.0024	45.0	0.0028	0.0051	0.0096	0.0049	1.5
t1/2z	min	151.4	63.5	42.0	72.1	136.7	250.7	140.3	1.5
AUC_{last}	nmol/L·min	184.5	64.5	35.0	100.6	201.1	269.8	174.0	1.5
$AUC_{0-\infty}$	nmol/L·min	209.1	64.7	31.0	123.8	240.4	276.9	199.4	1.4
$AUC_{extrap\%}$	%	12.5	6.6	52.6	2.6	11.5	21.0	10.5	2.1
MRT_{last}	min	115.4	5.2	4.5	109.4	114.4	124.0	115.3	1.0
$MRT_{0-\infty}$	min	183.4	47.9	26.1	120.8	175.7	263.1	178.4	1.3
7.70 g/kg (60 olives)									
T_{max}	min	--	--	--	30	38	92	--	--
C_{max}	nmol/L	10.0	3.7	37.4	5.3	11.0	15.0	9.3	1.5
λ_z	min^{-1}	0.0060	0.0013	21.5	0.0042	0.0059	0.0074	0.0058	1.3
t1/2z	min	121.4	28.0	23.0	93.1	118.0	165.5	118.8	1.3
AUC_{last}	nmol/L·min	1293.2	558.4	43.2	681.9	1055.3	2312.8	1199.7	1.5
$AUC_{0-\infty}$	nmol/L·min	1438.8	634.0	44.1	878.4	1135.4	2701.8	1340.7	1.5
$AUC_{extrap\%}$	%	10.3	6.3	61.2	3.2	8.8	22.4	8.8	1.9
MRT_{last}	min	138.9	20.6	14.8	111.3	136.3	163.1	137.5	1.2
$MRT_{0-\infty}$	min	185.5	44.2	23.8	143.1	168.0	253.3	181.3	1.3

4.3.3.5. Tyrosol

The analysis of the plasma concentration of tyrosol obtained at two doses allowed the obtention of the descriptive statistics of the pharmacokinetic constants of verbascoside shown in Table 4.18. Following the administration of table olives, the peak plasma concentrations of

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tyrosol (4.5 ± 1.5 and 11.5 ± 1.2 nmol/L) were achieved at median T_{max} of 61 and 62 min for doses of 30 and 60 olives, respectively.

Plasma concentrations declined with a half-life of approximately 4.5 h. AUC_{last} were 719.4 ± 1.3 and 1505.7 ± 1.3 nmol/L·min, when the experimental animals were administered with 3.85 and 7.70 g/kg. Since $AUC_{extrap\%}$ was 35.2 and 36.2% for both doses, the result obtained for $AUC_{0-\infty}$ (low dose: 1236.6 ± 1.6 nmol/L·min, high dose: 2538.2 ± 1.5 nmol/L·min) cannot be considered as reliable. The average permanence of tyrosol in the body characterized by the estimated values of MRT_{last} was approximately 2.5-3 h.

Table 4.18. Pharmacokinetic parameters of tyrosol estimated by non-compartmental analysis.

Parameters	Units	Mean	SD	CV %	Min	Median	Max	Geom. Mean	Geom. SD
Tyrosol 3.85 g/kg (30 olives)									
T_{max}	min	--	--	--	31	61	120	--	1.6
C_{max}	nmol/L	4.7	1.3	27.8	3.3	4.4	6.6	4.5	1.3
λ_z	min^{-1}	0.0034	0.0028	82.4	0.0011	0.0018	0.0072	0.0025	2.3
$t1/2z$	min	352.7	234.3	66.4	95.9	392.8	627.4	275.6	2.3
AUC_{last}	nmol/L·min	734.3	154.4	21.0	486.7	754.2	909.8	719.4	1.3
$AUC_{0-\infty}$	nmol/L·min	1339.9	546.7	40.8	725.2	1678.9	1800.4	1236.7	1.6
$AUC_{extrap\%}$	%	41.1	19.5	47.4	11.1	49.5	60.2	35.5	2.0
MRT_{last}	min	172.7	45.5	26.4	109.7	165.9	230.4	167.5	1.3
$MRT_{0-\infty}$	min	522.6	321.0	61.4	195.5	532.6	926.7	434.6	2.0
7.70 g/kg (60 olives)									
T_{max}	min	--	--	--	36	64	100	--	1.5
C_{max}	nmol/L	11.8	2.4	20.3	8.5	11.4	14.4	11.5	1.2
λ_z	min^{-1}	0.0027	0.0008	29.5	0.0021	0.0025	0.0040	0.0026	1.3
$t1/2z$	min	270.7	67.2	24.8	173.7	279.0	337.2	262.6	1.3
AUC_{last}	nmol/L·min	1556.0	414.1	26.6	904.4	1607.3	2201.0	1505.7	1.3
$AUC_{0-\infty}$	nmol/L·min	Lut	1374.7	49.9	1767.0	2158.2	5669.4	2538.2	1.5
$AUC_{extrap\%}$	%	38.8	15.7	40.5	23.0	34.5	61.2	36.2	1.5
MRT_{last}	min	155.3	29.6	19.0	130.0	141.6	205.2	153.1	1.2
$MRT_{0-\infty}$	min	391.0	100.1	25.6	248.6	435.3	505.9	378.6	1.3

4.3.3.6. Luteolin

Descriptive statistics of the pharmacokinetic constants of verbascoside are shown in Table 4.19. Although Figure 4.16 shows a peak at 30 min when both doses were administered, the pharmacokinetic analysis calculated following a non-compartmental approach gave a T_{max} of 45.5 min, with a minimum value of 30 min and a maximum value of 91 min for the dose of 30 olives and T_{max} of 66 min, with a minimum value of 30 and maximum value of 91 min, when the dose of 60 olives was administered Table 4.19.

Elimination values of half-life were 193.6 ± 1.2 and 177.2 ± 1.5 min for the doses of 3.85 and 7.70 g/kg, respectively. AUC_{last} were 276.6 ± 1.2 and 548.5 ± 1.6 nmol/L·min. $AUC_{extrap\%}$

were higher than 20% when dose of 30 olives was administered (Table 4.19). Although at a higher dose, the value of geometric mean is just below 20%, when taking results in account individually, only two rats out of 7 had a value below 20%. Thus, in case of both doses, the results of $AUC_{0-\infty}$ are not reliable.

The estimates of MRT_{last} were 158.6 ± 1.2 min and 138.0 ± 1.2 min, for the doses of 30 and 60 olives. The results indicate a mean permanence of luteolin in the body in accordance with the result obtained for the half-life.

Table 4.19. Pharmacokinetic parameters of luteolin estimated by non-compartmental analysis.

		Luteolin 3.85 g/kg (30 olives)							
Parameters	**Units**	**Mean**	**SD**	**CV %**	**Min**	**Median**	**Max**	**Geom. Mean**	**Geom. SD**
T_{max}	min	--	--	--	30	45.5	91	--	1.7
C_{max}	nmol/L	1.9	0.7	35.8	1.0	2.1	2.8	1.8	1.5
λ_z	min^{-1}	0.0036	0.0006	15.9	0.0029	0.0036	0.0045	0.0036	1.2
t1/2z	min	195.6	30.5	15.6	152.6	192.8	239.6	193.6	1.2
AUC_{last}	nmol/L·min	280.2	48.2	17.2	208.0	277.3	354.3	276.6	1.2
$AUC_{0-\infty}$	nmol/L·min	367.7	65.4	17.8	268.2	364.8	467.0	362.7	1.2
$AUC_{extrap\%}$	%	23.6	5.5	23.4	13.4	24.4	28.6	22.9	1.3
MRT_{last}	min	160.6	29.3	18.3	131.6	154.3	216.9	158.6	1.2
$MRT_{0-\infty}$	min	283.6	54.3	19.1	237.6	268.7	388.7	279.8	1.2
		7.70 g/kg (60 olives)							
T_{max}	min	--	--	--	30	66	91	--	1.6
C_{max}	nmol/L	5.1	3.0	57.9	1.6	5.3	10.1	4.4	1.9
λ_z	min^{-1}	0.0042	0.0020	47.9	0.0022	0.0038	0.0085	0.0039	1.5
t1/2z	min	189.5	70.8	37.4	81.3	181.3	314.1	177.2	1.5
AUC_{last}	nmol/L·min	596.2	254.6	42.7	286.6	637.9	1010.5	548.5	1.6
$AUC_{0-\infty}$	nmol/L·min	807.8	291.6	36.1	448.0	824.1	1211.5	759.2	1.5
$AUC_{extrap\%}$	%	26.3	15.7	59.6	1.6	28.4	49.7	18.8	3.1
MRT_{last}	min	140.5	28.9	20.6	107.4	135.8	188.9	138.0	1.2
$MRT_{0-\infty}$	min	273.1	89.2	32.7	115.4	277.2	379.2	257.1	1.5

4.3.3.7. Luteolin-7-*O*-glucoside

The non-compartmental analysis of the plasma concentrations of luteolin-7-*O*-glucuronide obtained after the oral administration of Arbequina table olives at 3.85 and 7.70 g/kg allowed the estimation of the pharmacokinetic constants described in Table 4.20.

The peak plasma concentration of luteolin-7-*O*-glucoside was 0.5 ± 1.6 nmol/L and 1.3 ± 1.9 nmol/L after the oral administration of the doses equivalent to the human intake of 30 and 60 Arbequina table olives. This C_{max} was achieved at median T_{max} of 45.5 and 41 min, for the doses of 30 and 60 olives, even though Figure 4.17 shows a peak at 30 min when both doses were applied.

AUC_{last} were 57.3 ± 1.4 and 184.0 ± 1.4 nmol/L·min, at the doses of 30 and 60 olives,

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respectively (Table 4.20). $AUC_{extrap}\%$ were above 20%, thus the obtained results of $AUC_{0-\infty}$ are not considered as reliable. The average permanence of luteolin-7-O-glucoside in the body was approximately 2.5 h that is described by MRT_{last} (Table 4.20).

Table 4.20. Pharmacokinetic parameters of luteolin-7-O-glucoside estimated by non-compartmental analysis.

Parameters	Units	Mean	SD	CV %	Min	Median	Max	Geom. Mean	Geom. SD
Luteolin-7-O-glucoside 3.85 g/kg (30 olives)									
T_{max}	min	--	--	--	30	45.5	62	--	--
C_{max}	nmol/L	0.5	0.3	59.7	0.3	0.4	1.1	0.5	1.6
λ_z	min^{-1}	0.0038	0.0018	47.0	0.0020	0.0033	0.0058	0.0034	1.6
t1/2z	min	220.8	101.2	45.8	119.4	210.3	339.1	201.6	1.6
AUC_{last}	nmol/L·min	60.2	19.7	32.7	30.9	62.1	91.1	57.3	1.4
$AUC_{0-\infty}$	nmol/L·min	91.8	36.7	40.0	41.4	107.0	126.4	84.7	1.6
$AUC_{extrap\%}$	%	28.7	18.1	63.1	8.5	25.3	49.0	23.5	2.1
MRT_{last}	min	155.3	22.5	14.5	133.3	148.2	193.1	154.0	1.1
$MRT_{0-\infty}$	min	323.8	138.2	42.7	193.0	271.7	491.7	300.9	1.5
7.70 g/kg (60 olives)									
T_{max}	min	--	--	--	30	41	128	--	--
C_{max}	nmol/L	1.6	1.1	66.4	0.6	1.4	3.5	1.3	1.9
λ_z	min^{-1}	0.0032	0.0013	41.1	0.0018	0.0027	0.0050	0.0030	1.5
t1/2z	min	250.1	99.5	39.8	137.3	255.9	390.7	232.6	1.5
AUC_{last}	nmol/L·min	191.2	56.8	29.7	125.9	166.5	268.8	184.0	1.4
$AUC_{0-\infty}$	nmol/L·min	276.8	51.7	18.7	200.6	302.0	324.4	272.3	1.2
$AUC_{extrap\%}$	%	30.1	18.1	60.3	11.0	22.3	58.2	25.9	1.8
MRT_{last}	min	148.2	14.5	9.8	124.6	149.6	163.9	147.6	1.1
$MRT_{0-\infty}$	min	340.7	134.8	39.6	208.1	310.9	568.0	319.8	1.5

4.4. CLINICAL TRIAL: PHARMACOKINETICS OF PHENOLIC COMPOUNDS IN PLASMA AFTER THE CONSUMPTION OF ARBEQUINA TABLE OLIVES BY HEALTHY HUMAN VOLUNTEERS

Stage I of clinical trial studied the pharmacokinetics of phenolic compounds after the single oral intake of 60 and 120 Arbequina table olives harvested during the season 2016/2017.

Previously developed analytical method was validated prior to the determination of the plasma concentrations in order to confirm that the concentrations were calculated accurately.

Apart from linearity, limit of quantification, precision and accuracy, the validation also included recovery and matrix effect in order to verify the applicability of the method to human samples.

The pharmacokinetic study was performed with plasma obtained at 0, 30, 60, 90, 120, 240, 360, 480 and 1440 min after the ingestion of table olives.

4.4.1. Determination of phenolic compounds in human plasma in the pharmacokinetic study

4.4.1.1. Validation of the analytical method in stage I of clinical trial

Previously developed analytical method was validated for its use in human plasma. Matrix effect and recovery were analyzed at 250 nmol/L for 16 polyphenols.

Linearity, limit of quantification, precision and accuracy were assessed at 2.5, 5, 10, 25, 50, 100, 150, 200, 300, 500 nmol/L for 9 polyphenols: hydroxytyrosol, hydroxytyrosol acetate, luteolin, oleuropein, quercetin, salidroside, tyrosol, vanillic acid and verbascoside.

4.4.1.1.1. Matrix effect

Matrix effect was evaluated according to the recommendations of Matuszewski *et al.* (2003). The values obtained for 16 polyphenols with and without normalization by IS are shown in Table 4.21.

The results ranged from 80 to 120% for 13 of the studied polyphenols. Salidroside, pinoresinol and hydroxytyrosol acetate ranged from 82.9% and 88.6%.

Hydroxytyrosol, *p*-coumaric acid and apigenin gave results of $90.1 \pm 0.6\%$; $98.5 \pm 0.6\%$; 3.5% and $98.2 \pm 7.0\%$. Tyrosol and catechol displayed a matrix effect of approximately 100%.

Finally, oleuropein, vanillic acid, luteolin, luteolin-7-*O*-glucoside and quercetin had a matrix effect lower than 120%.

An enhancement of ionization of 140.0 ± 2.7, 144.8 ± 1.7 and $153.2 \pm 4.3\%$ was observed only in case of rutin, caffeic acid and verbascoside, respectively. IS gave a value of $103.6 \pm 2.0\%$, thus was considered as suitable.

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Table 4.21. Matrix effect and recovery in blank human plasma samples spiked with polyphenols at 250 nmol/L and analyzed by LC-ESI-MS/MS. The results are expressed as mean ± standard error (n = 3).

Analyte	Matrix effect (%)		Recovery (%)	
	Without IS	**With IS**	**Without IS**	**With IS**
Apigenin	98.2 ± 7.0	94.7 ± 5.2	70.9 ± 2.9	80.8 ± 3.1
Caffeic acid	149.8 ± 4.2	144.8 ± 1.7	69.2 ± 3.1	82.9 ± 3.2
Catechol	102.4 ± 2.5	98.8 ± 1.7	83.7 ± 5.0	95.2 ± 5.2
p-Coumaric acid	95.9 ± 3.5	92.5 ± 1.6	84.4 ± 12.0	94.7 ± 6.7
Hydroxytyrosol	90.1 ± 0.6	87.1 ± 1.4	93.5 ± 4.7	106.1 ± 3.4
HT acetate	88.6 ± 2.9	85.5 ± 1.4	81.0 ± 0.4	88.8 ± 3.9
Luteolin	117.8 ± 0.5	115.9 ± 0.1	77.3 ± 0.3	84.2 ± 4.0
Luteolin-7-*O*-glucoside	118.4 ± 2.0	116.4 ± 1.4	85.5 ± 3.6	97.3 ± 3.9
Oleuropein	109.3 ± 3.3	105.5 ± 1.2	87.9 ± 4.5	99.9 ± 3.2
(+)-Pinoresinol	86.9 ± 3.3	83.9 ± 1.5	82.5 ± 1.7	89.6 ± 3.0
Quercetin	115.1 ± 1.8	113.2 ± 2.3	73.0 ± 1.0	83.4 ± 3.2
Rutin	144.9 ± 4.2	140.0 ± 2.7	56.4 ± 3.9	64.1 ± 3.3
Salidroside	82.9 ± 2.3	80.0 ± 0.6	47.9 ± 2.7	54.4 ± 1.3
Tyrosol	100.2 ± 2.9	96.7 ± 1.1	87.6 ± 3.8	100.1 ± 7.5
Vanillic acid	119.5 ± 4.1	115.4 ± 3.8	83.0 ± 7.2	93.8 ± 4.4
Verbascoside	158.6 ± 6.8	153.2 ± 4.3	65.3 ± 4.9	74.2 ± 4.2
IS	103.6 ± 2.0	--	88.3 ± 6.1	--

4.4.1.1.2. Recovery

Recoveries analyzed at 250 nmol/L are displayed in Table 4.21. The analysis of the results without being normalized for the IS indicated that the best recovery was achieved for hydroxytyrosol (93.5 ± 4.7%). The analytical method yielded a good recovery for most of the polyphenols since, oleuropein, tyrosol, luteolin-7-*O*-glucoside, *p*-coumaric acid, catechol, vanillic acid, pinoresinol and hydroxytyrosol acetate gave results that ranged from 87.9% to 81%. Luteolin, quercetin, apigenin and caffeic acid gave values of 77.3 ± 0.3%, 73.0 ± 1.0%, 70.9 ± 2.9% and 69.2 ± 3.1% respectively. The lowest recoveries were observed for verbascoside (65.3 ± 4.9%), rutin (56.4 ± 3.9%) and salidroside (47.9 ± 2.7%).

The value of recovery for IS was 88.3 ± 6.1%, that is value similar to other polyphenols, thus confirming its suitable use as IS.

4.4.1.1.3. Linearity

The calibration curves indicated that the analytical method was linear for all the studied polyphenols with correlation coefficients (R^2) higher than 0.9992 that was obtained for hydroxytyrosol (Figure 4.18 and Table 4.22).

The rest of polyphenols obtained the values ranging from 0.9993 for hydroxytyrosol acetate up to 0.9998 for verbascoside. The results of equations and R^2 are shown in Table 4.22.
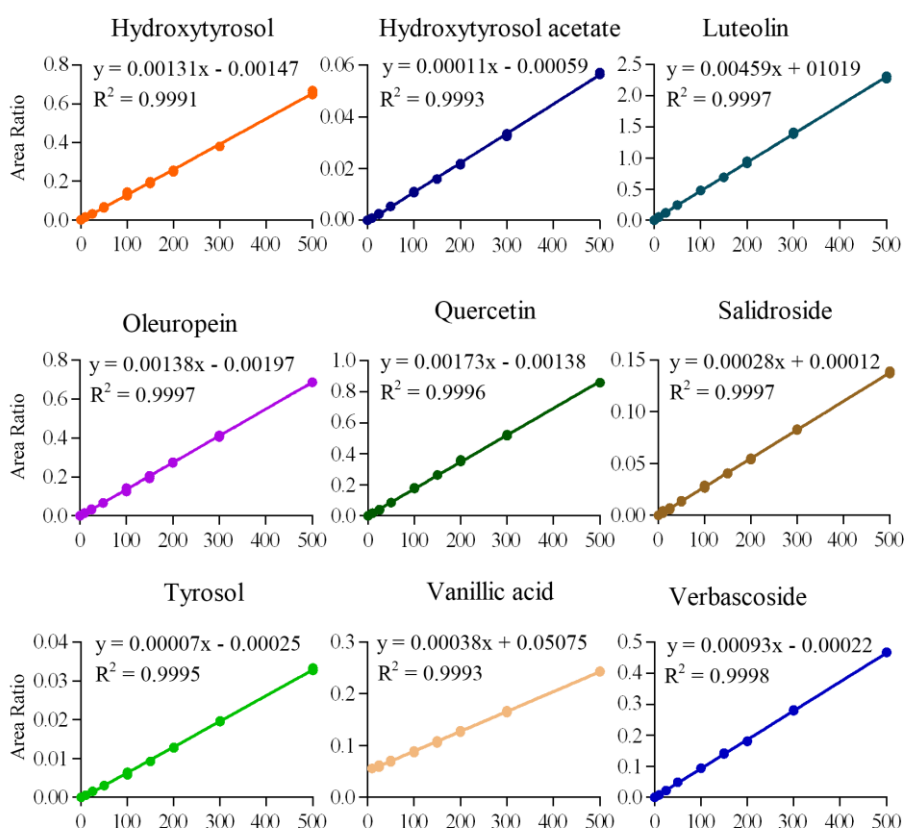
Figure 4.18. Representative calibration curves of phenolic compounds spiked in blank human plasma and determined by LC-ESI-MS/MS. The figure shows the individual values for each analyte. The regression line had been calculated using the least square method.

Table 4.22. Linearity and limit of quantification (LOQ) of phenolic compounds spiked in blank plasma and analyzed by LC-ESI-MS/MS.

Analyte	Linearity		Sensitivity
	Equations	R^2	LOQ (nmol/L)
Hydroxytyrosol	$y = 0.00131x - 1.30e^{-3}$	0.9992	0.53
HT acetate	$y = 0.00011x - 4.67e^{-4}$	0.9993	1.58
Luteolin	$y = 0.00460x - 3.33e^{-7}$	0.9997	0.04
Oleuropein	$y = 0.00138x - 1.36e^{-3}$	0.9997	0.04
Quercetin	$y = 0.00173x + 7.06e^{-9}$	0.9996	0.16
Salidroside	$y = 0.00028x - 7.18e^{-5}$	0.9997	0.20
Tyrosol	$y = 0.00007x - 2.92e^{-4}$	0.9994	0.99
Vanillic acid	$y = 0.00038x + 5.51e^{-6}$	0.9996	0.61
Verbascoside	$y = 0.00093x - 3.38e^{-4}$	0.9998	0.04

4.4.1.1.4. Limit of quantification

Results of sensitivity of the analytical method expressed as LOQ are displayed in Table 4.22. The calculated values of 8 polyphenols were below 1 nmol/L, ranging from 0.04 nmol/L for luteolin, oleuropein as well as verbascoside and up to 0.99 nmol/L for tyrosol. The value of the LOQ for hydroxytyrosol acetate was 1.58 nmol/L.

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4.4.1.1.5. Precision

Intra-day precision had been evaluated in blank human plasma spiked with polyphenols at 10 concentrations. Results expressed as relative standard deviation (RSD%) are displayed in Table 4.23. Values of intra-day precision were inferior to 15% for all the polyphenols at all concentrations as established in the Guidelines for the validation of Bioanalytical Methods (EMA, 2011).

Table 4.23. Intra-day precision of phenolic compounds spiked in human blank plasma and analyzed by LC-ESI-MS/MS.

Analyte	Intra-day precision (%RSD) Concentration (nmol/L)									
	2.5	5	10	25	50	100	150	200	300	500
Hydroxytyrosol	11.23	12.13	11.02	3.40	3.63	7.39	2.67	10.14	6.87	1.54
HT acetate	6.23	13.63	4.82	3.58	4.13	2.66	0.53	1.58	1.48	0.67
Luteolin	9.51	6.71	7.69	2.13	1.40	1.50	0.92	1.77	1.16	0.93
Oleuropein	2.22	0.49	1.56	4.40	2.31	6.13	3.30	0.74	0.86	0.35
Quercetin	9.06	2.42	3.03	6.05	3.00	1.85	0.34	1.55	1.02	0.36
Salidroside	2.00	5.25	12.82	12.24	4.66	4.57	0.74	3.77	0.76	0.76
Tyrosol	15.60	2.81	10.48	3.96	4.22	5.19	2.04	0.54	0.51	0.90
Vanillic acid	-11.11	-13.94	7.23	9.23	7.54	3.98	3.72	1.61	1.56	0.35
Verbascoside	9.56	1.37	8.90	3.59	2.71	1.03	1.01	0.73	0.84	0.52

4.4.1.1.6. Accuracy

Accuracy had been evaluated at 2.5, 5, 10, 25, 50, 100, 150, 200, 300, and 500 nmol/L. The results expressed as relative standard deviation (RSD%) are displayed in Table 4.24. Accuracy of analytical method was acceptable since the values of accuracy were inferior to 15% for all the polyphenols at all concentrations. Guidelines for the validation of Bioanalytical Methods established by the European Medicines Agency were fulfilled (EMA, 2011).

Table 4.24. Accuracy of phenolic compounds spiked in blank human plasma and analyzed by LC-ESI-MS/MS.

Analyte	Accuracy (%RSD) Concentration (nmol/L)									
	2.5	5	10	25	50	100	150	200	300	500
Hydroxytyrosol	-5.67	-4.21	-4.05	5.28	-3.55	-3.27	1.96	-3.04	-4.43	-0.74
HT acetate	8.93	5.79	8.20	-3.45	1.92	0.76	3.04	2.79	1.40	-1.24
Luteolin	-6.73	2.35	-2.46	-0.18	-2.96	-4.62	1.29	-0.27	-0.62	0.34
Oleuropein	0.15	1.00	-0.42	2.53	-0.20	1.36	2.48	0.30	0.34	-0.41
Quercetin	1.66	-4.49	-7.07	-2.25	5.50	-2.43	-1.09	-2.70	-0.35	0.68
Salidroside	-10.69	-5.98	-9.99	5.17	3.23	1.12	1.38	3.12	-0.01	-0.20
Tyrosol	10.31	-12.01	8.15	-2.62	3.42	2.49	3.55	1.39	-0.08	-0.63
Vanillic acid	-3.68	0.98	4.58	-4.42	-2.50	3.29	2.21	0.20	-0.03	-0.29
Verbascoside	12.85	5.46	7.37	-2.17	-2.28	-0.55	-1.61	2.13	-0.15	-0.09

4.4.1.2. Identification of phenolic compounds in human plasma in the pharmacokinetic study

The LC-ESI-MS/MS analysis of human plasma samples obtained after the oral ingestion of 60 and 120 Arbequina table olives confirmed the presence of 6 polyphenols. The representative extracted ion chromatograms of 9 polyphenols obtained 30 min after the oral ingestion of 60 Arbequina table olives are represented in Figure 4.19, while Figure 4.20 displays the ones obtained after oral intake of 120 table olives. The analysis of chromatograms allowed the identification of vanillic acid, hydroxytyrosol, salidroside, luteolin and verbascoside in human plasma after oral intake of both doses of table olives, whereas hydroxytyrosol acetate was found in plasma only when the dose of 120 olives was ingested. Oleuropein, quercetin and tyrosol were not detected.
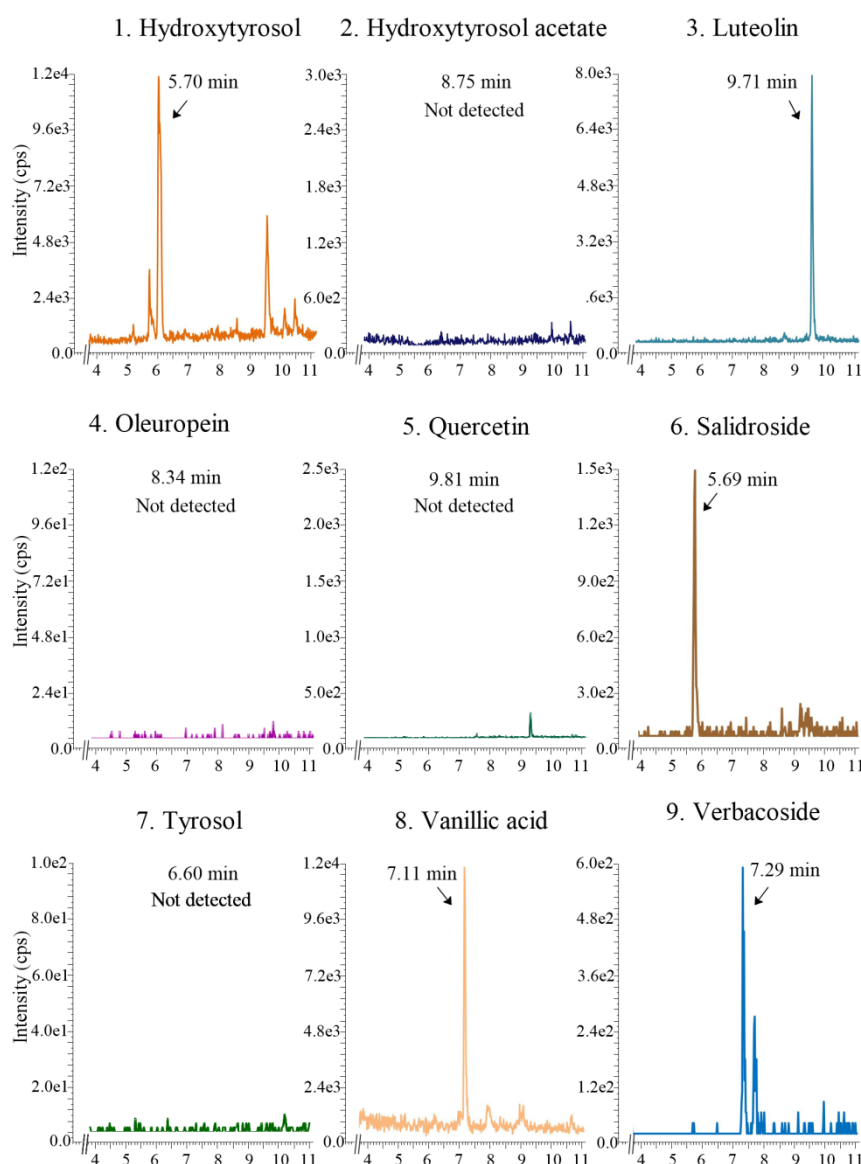
Figure 4.19. Representative LC-ESI-MS/MS chromatograms of polyphenols found in human plasma 30 min after the oral intake of 60 Arbequina table olives.

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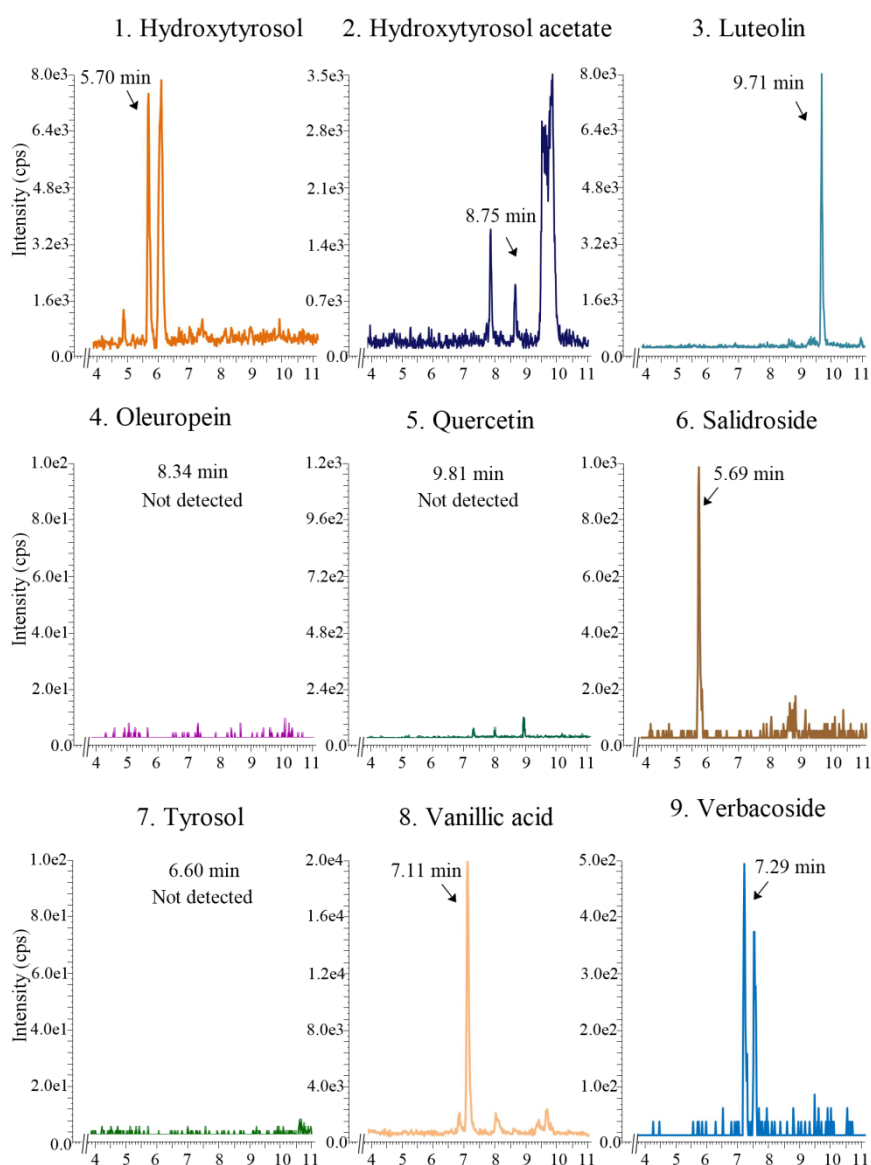
Figure 4.20. Representative LC-ESI-MS/MS chromatograms of polyphenols found in human plasma 30 min after the oral intake of 120 Arbequina table olives.

A targeted metabolomic approach was used to identify the metabolites of hydroxytyrosol, hydroxytyrosol acetate, luteolin and tyrosol.

Hydroxytyrosol (M0) underwent phase II reactions and sulfate derivatives (M1-a and M1-b) were identified as the main metabolites along with two glucuronide derivatives (M2-a and M2-b) that were found in minor amounts. The analysis of hydroxytyrosol (M0) was performed in MRM mode at the *m/z* 153.2/122.8 Da. Retention time of M0 was 5.70 min that coincides with the one of the standard. Hydroxytyrosol sulfates were detected at *m/z* 233.0/153.0 Da and they appeared at 5.79 min (M1-a) and 6.05 min (M1-b). Sulfate derivatives were analyzed using 2 transitions, the first one characterized by the *m/z* pair 233/153 (quantification transition) and the second one at the *m/z* 153.0/122.8 (qualifier transition).

In the case of hydroxytyrosol glucuronides with retention times of 4.73 min (M2-a) and 5.05 min (M2-b), an increase of 176 Da was observed. So, the product ion was detected at m/z of 329.0 in the negative mode. Glucuronide metabolites were analyzed by 2 transitions, the first at m/z 329/153 (quantification transition) and the second at m/z 153.0/122.8 (qualifier transition). The representative LC-ESI-MS/MS chromatograms of M0, M1-a, M1-b, M2-a, and M2-b obtained 30 minutes after ingestion of 60 and 120 Arbequina table olives are shown in Figure 4.21. Both isomers of hydroxytyrosol sulfates can be seen in the Figure 4.21 B and E. Hydroxytyrosol glucuronides were also present in two isomers (Figure 4.21 C and F). The identity of hydroxytyrosol sulfate M1-a and hydroxytyrosol sulfate M1-b was confirmed since they were also present at the chromatogram of hydroxytyrosol at the same retention time.
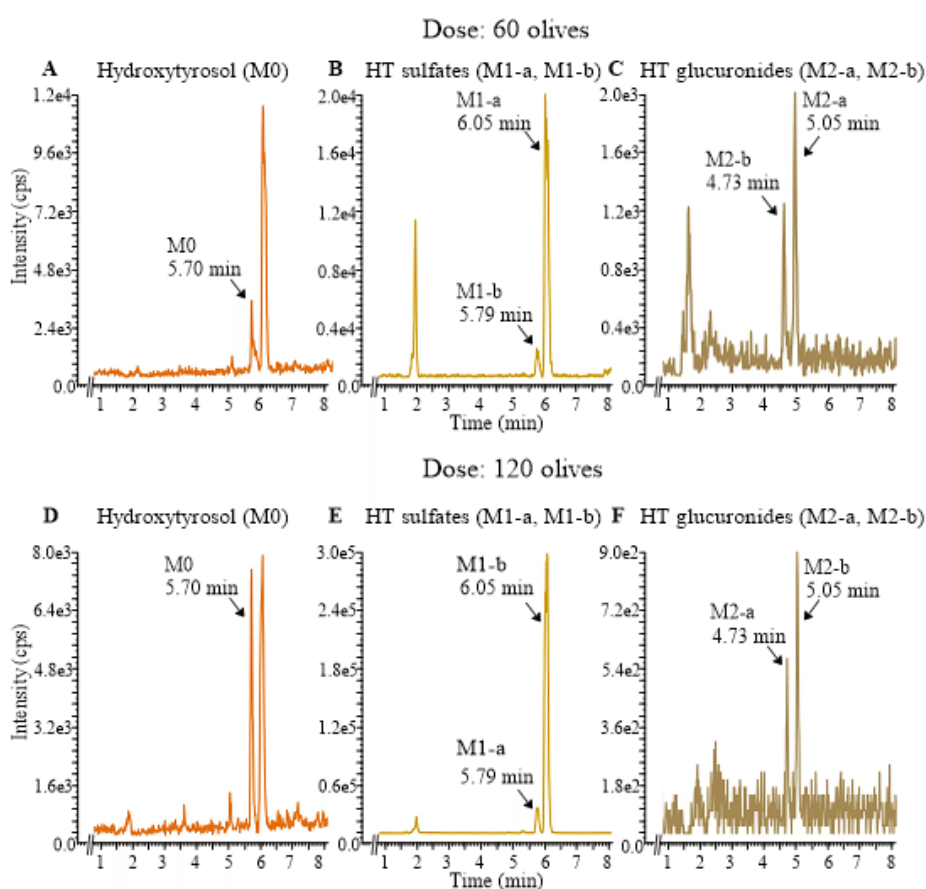
Figure 4.21. Representative LC-ESI-MS/MS chromatograms of hydroxytyrosol (M0) and its metabolites (hydroxytyrosol sulfates: M1-a, M1-b and hydroxytyrosol glucuronides: M2-a, M2-b) obtained in multiple reaction monitoring mode (MRM) 30 minutes after oral ingestion of 60 and 120 Arbequina table olives. Hydroxytyrosol (A) appeared at 5.70 min (m/z 153.2/122.8), hydroxytyrosol sulfates (B) at 5.79 and 6.05 min (m/z: 233.0/153.2) and hydroxytyrosol glucuronides (C) at 4.73 and 5.05 min (m/z 329.0/153.2).

Metabolites of hydroxytyrosol acetate (hydroxytyrosol acetate glucuronide and hydroxytyrosol acetate sulfate), luteolin (luteolin glucuronide and luteolin sulfate) and tyrosol (tyrosol glucuronide and tyrosol sulfate) were not detected in plasma samples.

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4.4.1.3. Plasmatic concentrations of phenolic compounds in human plasma in the pharmacokinetic study

Once the polyphenols were identified, the plasmatic concentrations were calculated using the calibration curves previously described in the section 4.4.1.1.3. Blank plasma samples were checked for the presence of polyphenols and traces of hydroxytyrosol, luteolin, oleuropein, quercetin, salidroside, vanillic acid and verbascoside were found at low concentrations, in some of the patients. For each patient, the concentration found at time 0 min was subtracted from the ones obtained in plasma after the oral ingestion of table olives.

After oral intake of table olives, in total, 6 polyphenols were quantified. The most abundant compound after the ingestion of 60 and 120 Arbequina table olives was vanillic acid, followed by hydroxytyrosol, salidroside, luteolin and verbascoside. Hydroxytyrosol acetate was found only after the intake of 120 table olives. The values used for the calculations were always above LOQ.

4.4.1.3.1. Vanillic acid

At the dose of 60 table olives, the mean value of vanillic acid found in blank plasma of human participants was 7.90 ± 0.52 nmol/L (n = 18). When the dose of 120 table olives was applied, vanillic acid in blank plasma was found at 9.60 ± 1.06 nmol/L (n = 18).

The concentration of vanillic acid found in blank plasma was individually subtracted for each patient from all the concentrations obtained after the oral ingestion of Arbequina table olives.

The vanillic acid reached maximum plasma concentrations at 30 min after the oral intake of both doses (Figure 4.22).

Figure 4.22. Plasma concentrations of vanillic acid in human plasma. Results were obtained after oral intake of 60 and 120 table olives. Values are presented as mean $\pm$ SEM (n = 18).

The ingestion of 60 table olives corresponded to the intake of 235.8 ± 2.5 µg of vanillic acid. At 30 min, this compound was found at 30.4 ± 2.3 nmol/L, decreasing to 22.9 ± 2.1 nmol/L at 60 min and 13.1 ± 1.5 nmol/L at 90 min. From 120 min up to 1440 min, the compound was found at concentrations below 10 nmol/L.

With the intake of 120 table olives, the patients received an amount of vanillic acid of 455.0 ± 4.73 µg. At 30 min, vanillic acid was found at 55.7 ± 4.1 nmol/L, that is concentration 1.83-fold higher than when the dose of 60 olives was ingested. Since that time on, the plasma concentrations were progressively decreasing at 60, 90 and 120 min with values of 41.7 ± 3.1 nmol/L, 29.8 ± 2.6 nmol/L and 20.9 ± 2.0 nmol/L, respectively. At 240 min, the value dropped to 7.3 ± 1.1 nmol/L. From 360 min up to 1440 min, the concentrations were below 5 nmol/L. Vanillic acid was still detected at 24 h with the plasmatic concentration of 3.6 ± 1.5 nmol/L.

4.4.1.3.2. Hydroxytyrosol and its metabolites

At the dose of 60 table olives, the concentration of hydroxytyrosol found in blank human plasma was 0.95 ± 0.17 nmol/L (n = 5). Hydroxytyrosol sulfate M1-b was found in 3 samples with the mean value of 1.38 ± 0.48 nmol/L. In these three patients, the plasma concentration at time 0 h was subtracted from the ones obtained after the intake of olives. Hydroxytyrosol glucuronide M2-b was found only in one patient at 2.07 nmol/L. Hydroxytyrosol sulfate M1-a and hydroxytyrosol glucuronide M2-a were not found in any of the blank plasma samples.

At the dose of 120 olives, hydroxytyrosol was found in blank plasma at 1.21 ± 0.10 nmol/L (n = 9) and hydroxytyrosol sulfate M1-b at 1.46 ± 0.36 nmol/L (n = 2). Neither hydroxytyrosol sulfate M1-a nor hydroxytyrosol glucuronides (M2-a and M2-b) were found in any of the blank samples. Concentrations of hydroxytyrosol and its metabolites found in blank plasma were subtracted individually from all the patients for all the concentrations obtained after the consumption of olives.

4.4.1.3.2.1. Hydroxytyrosol

The peak plasmatic concentrations of hydroxytyrosol after the oral intake of 60 and 120 Arbequina table olives were found at 30 min (Figure 4.23).

With the intake of 60 table olives, the amount of ingested hydroxytyrosol accounted for 31431.69 ± 333.89 µg.

Figure 4.23. Plasma concentrations of hydroxytyrosol in human plasma. Results were obtained after oral intake of 60 and 120 table olives. Values are presented as mean $\pm$ SEM (n = 18).

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The maximum plasma concentration for hydroxytyrosol of 3.4 ± 0.3 nmol/L was reached at 30 min and decreased to 2.4 ± 0.4, 2.1 ± 0.2 and 1.9 ± 0.2 nmol/L at 60, 90, and 120 min, respectively. From 240 min to 1440 min, the substance was found ranging from 1.7 ± 0.2 to 0.9 ± 0.1 nmol/L, respectively.

The dose of 120 table olives contained hydroxytyrosol at 60656.68 ± 630.21 µg. Hydroxytyrosol at 30 min reached a maximum plasma concentrations of 7.1 ± 0.7 nmol/L that was 2.09 times higher than when 60 olives were taken. The plasma concentrations were progressively decreasing with values of 5.4 ± 0.7, 4.7 ± 0.2, 3.2 ± 0.3 at 60, 90 and 120 min, respectively. From 240 min, hydroxytyrosol was found at values lower than 2.5 nmol/L and at 1440 min, it was still detected at minor concentration of 1.0 ± 0.2 nmol/L.

4.4.1.3.2.2. Hydroxytyrosol sulfates (M1-a and M1-b)

The analysis of the chromatograms confirmed the presence of hydroxytyrosol along with two sulfate metabolites. Both sulfates, that eluted at 5.79 (M1-a) and 6.05 (M1-b) min were found at concentrations higher than the one of parent compound, being the sulfate derivate M1-b the most abundant one. After the oral ingestion of 60 table olives, the maximum concentration for hydroxytyrosol sulfate M1-a of 3.6 ± 0.5 nmol/L was reached at 30 min. Plasmatic concentrations of M1-a decreased to 2.4 ± 0.7 and 1.9 ± 0.3 nmol/L at 60 and 90 min. At 120 min, the concentration was 2.2 ± 0.2 nmol/L. M1-a was found up to 480 min and the results were ranging from 1.5 ± 0.2 nmol/L (240 min) up to 1.2 ± 0.2 nmol/L (480 min). Plasmatic concentrations are shown in Figure 4.24 A.

At the same dose, hydroxytyrosol sulfate M1-b was found at 39.2 ± 5.8 nmol/L at 30 min. The values of hydroxytyrosol sulfate M1-b dropped to 29.8 ± 9.4 nmol/L at 60 min, 25.2 ± 8.4 nmol/L at 90 min and 22.6 ± 3.2 nmol/L at 120 min. A decrease in concentration was observed up to 360 min (5.2 ± 0.9 nmol/L). At 480 min, the concentration of M1-b increased to 8.5 ± 1.4 nmol/L and at 1440 min the concentration dropped to 2.0 ± 0.6 nmol/L (Figure 4.24 B).
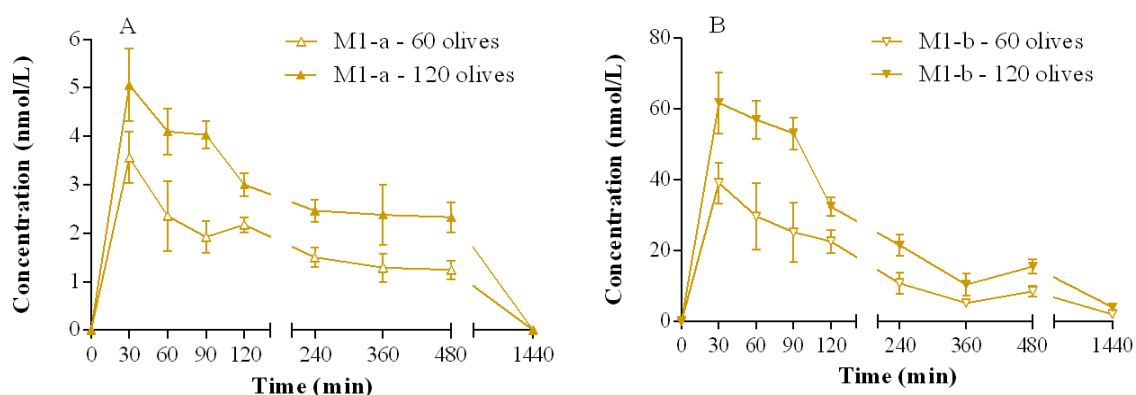
Figure 4.24. Plasma concentrations of hydroxytyrosol sulfates. A) depicts the isomer M1-a with retention time of 5.79 min whereas B shows the metabolite M1-b eluting at 6.05 min. Results were obtained after oral intake of 60 and 120 table olives. Values are presented as mean ± SEM (n = 18).

With the intake of 120 table olives, the peak concentration of hydroxytyrosol sulfate M1-a (5.1 ± 0.7 nmol/L) was found at 30 min, and the values were 1.42-fold higher than when the dose of 60 table olives was consumed (Figure 4.24 A). At 60 and 90 min, results decreased and M1-a were 4.1 ± 0.5 and 4.0 ± 0.3 nmol/L. From 120 min, M1-a was present in amounts below 3 nmol/L and the compound was detected up to 480 min (2.3 ± 0.3 nmol/L).

At the same dose, the plasmatic values of hydroxytyrosol sulfate M1-b were progressively decreasing after the maximum of 61.8 ± 8.6 nmol/L (1.58 times higher than 60 olives) was reached at 30 min. At 60 and 90 min, M1-b dropped to 57.1 ± 5.4 and 53.2 ± 4.5 nmol/L. Plasma concentrations for M1-b were decreasing from 32.4 ± 2.6 nmol/L at 120 min up to 10.5 ± 3.2 at 360 min. At 480 min, the concentration of M1-b increased to 15.6 ± 2.1 nmol/L and at 1440 min again decreased to 4.0 ± 0.5 nmol/L (Figure 4.24 B).

4.4.1.3.2.3. Hydroxytyrosol glucuronides (M2-a and M2-b)

Both hydroxytyrosol glucuronides were found only in few samples. Hydroxytyrosol glucuronide M2-a was found only in 11 and 14 samples when doses of 60 and 120 Arbequina table olives were ingested. Hydroxytyrosol glucuronide M2-b was detected in 21 and 32 samples, when the doses of 60 and 120 olives were applied.

4.4.1.3.3. Salidroside

Salidroside, when the dose of 60 table olives was ingested, was found in blank human plasma at 0.97 ± 0.10 nmol/L (n = 4). At the dose of 120 olives, the concentration of salidroside in blank plasma was 1.16 ± 0.25 nmol/L (n = 3). The concentrations of salidroside in blank plasma were individually subtracted for all the human volunteers from the concentrations obtained in all sampling times after oral intake of table olives.

Salidroside was found in human plasma from 30 min up to 480 min after the oral intake of 60 and 120 Arbequina table olives (Figure 4.25).
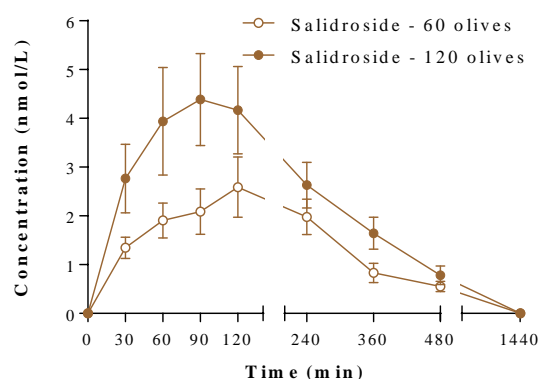
Figure 4.25. Plasma concentrations of salidroside in human plasma. Results were obtained after oral intake of 60 and 120 table olives. Values are presented as mean ± SEM (n = 18).

The healthy human volunteers ingested an amount of 1149.81 ± 12.21 µg of salidroside with the intake of 60 table olives. At 30 min, salidroside was found at 1.3 ± 0.2 nmol/L. The

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concentrations were progressively increasing up to 120 min, when the maximum concentration of 2.6 ± 0.6 nmol/L was achieved. Salidroside was found in minor amounts of 0.8 ± 0.2 and 0.6 ± 0.1 nmol/L at 360 and 480 min and at 1440 min this compound was not detected.

With the intake of 120 table olives, human participants received 2218.90 ± 23.05 µg of salidroside. This compounds was found at 30 min at 2.8 ± 0.7 nmol/L and the concentration increased at 60 min (3.9 ± 1.1 nmol/L) and 90 min (4.4 ± 0.9 nmol/L) when the maximum concentration was attained. From 120 min to 480 min, plasma concentration of salidroside decreased and the values were below 4.2 nmol/L. At 1440 min, salidroside was not detected.

4.4.1.3.4. Luteolin

Luteolin was found in blank human plasma at concentration of 0.48 ± 0.06 nmol/L (n = 11) when the dose of 60 table olives was ingested. At the dose of 120 table olives, luteolin in blank plasma gave concentration of 0.45 ± 0.7 nmol/L (n = 10). Each patient was subtracted the concentration of luteolin found in blank plasma from the values obtained at all sampling points after oral ingestion of table olives.

Analysis of the obtained chromatograms allowed the quantification of luteolin in all sampling times, up to 1440 min after the oral intake. Luteolin reached maximum plasma concentrations at 240 min and at 360 min after oral intake of 60 and 120 olives, respectively (Figure 4.26).
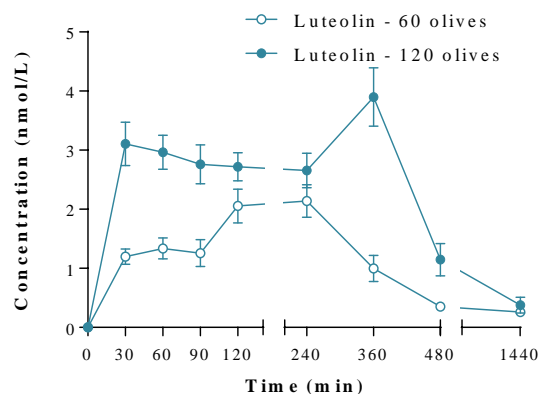
Figure 4.26. Plasma concentrations of luteolin in human plasma. Results were obtained after oral intake of 60 and 120 table olives. Values are presented as mean ± SEM (n = 18).

With the ingestion of 60 table olives the amount of luteolin consumed by the human participants was 5931.86 ± 63.01 µg. Plasmatic concentration of 1.2 ± 0.1 nmol/L was found in 30 min. The maximum concentration was of 2.2 ± 0.2 at 240 min. After that time on, the concentrations diminished and luteolin was found in minor amounts ranging from 0.9 ± 0.2 (360 min) up to 0.3 ± 0.1 (1440 min) nmol/L.

The oral intake of 120 table olives provided an amount of luteolin of 11447.26 ± 118.93 µg. Luteolin was detected already at 30 min at 3.1 ± 0.4 nmol/L. Then, values dropped to 3.0 ± 0.3, 2.8 ± 0.3, 2.7 ± 0.2 and 2.7 ± 0.3 nmol/L at 60, 90, 120 and 240 min, respectively. At 360

min, an increase was observed and the maximum plasmatic concentration of 3.9 ± 0.5 nmol/L was reached. It was followed by a repeated decrease in concentrations and at 480 and 1440 min, luteolin was found in minor amounts.

4.4.1.3.5. Verbascoside

Traces of verbascoside were found in blank plasma of human participants with mean concentration of 0.58 ± 0.05 nmol/L (n = 10) at the dose of 60 table olives. At the dose of 120 table olives, no traces of verbascoside were observed. The concentrations of verbascoside in blank plasma were subtracted individually from the all concentrations obtained after the oral ingestion of Arbequina table olives.

Verbascoside reached maximum plasma concentrations at 60 min and was detected in all sampling times when the doses of 60 and 120 olives were consumed (Figure 4.27).
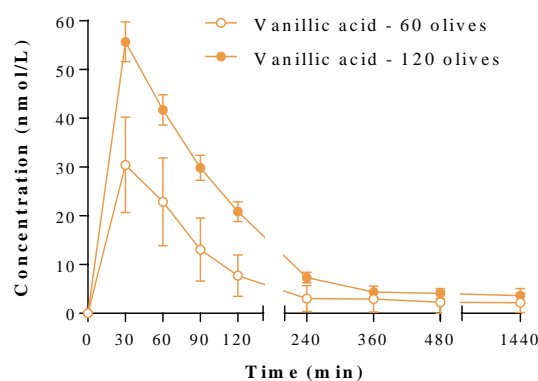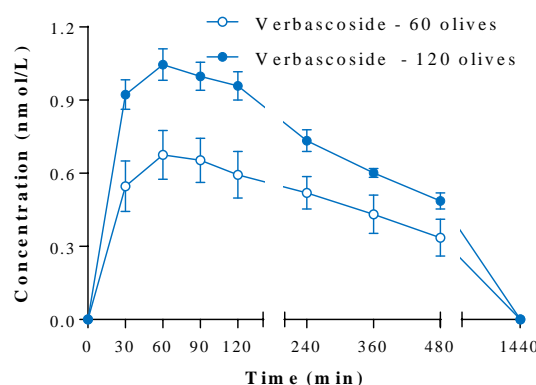

Figure 4.27. Plasma concentrations of verbascoside in human plasma. Results were obtained after oral intake of 60 and 120 table olives. Values are presented as mean ± SEM (n = 18).

The dose of 60 table olives contained verbascoside at 22141.80 ± 235.21 µg. This compound was already detected at 30 min with concentration of 0.55 ± 0.10 nmol/L, that increased to 0.68 ± 0.10 nmol/L at 60 min. The concentrations started to lessen at 90 min (0.65 ± 0.09 nmol/L) and at 480 min, verbascoside was still detected at 0.34 ± 0.08 nmol/L.

Participants received 42729.11 ± 443.95 µg of verbascoside with the ingestion of 120 table olives. Verbascoside was found at 0.92 ± 0.06 nmol/L at 30 min. At 60 min, the maximum plasma concentration of 1.05 ± 0.06 nmol/L was reached, which was 1.54-fold higher than when 60 olives were ingested. At 90, 120 and 240 min, verbascoside gave values of 1.00 ± 0.06, 0.96 ± 0.06 and 0.73 ± 0.04, respectively.

At 360 min and 480 min, the compound was found in minor concentration of 0.60 ± 0.02 and 0.48 ± 0.03 nmol/L. At 24 h, verbascoside was not detected.

4.4.1.3.6. Hydroxytyrosol acetate

No traces of hydoxytyrosol acetate were found in plasma of any of human participants prior to oral intake of Arbequina table olives (n = 36). Hydroxytyrosol acetate was found after oral

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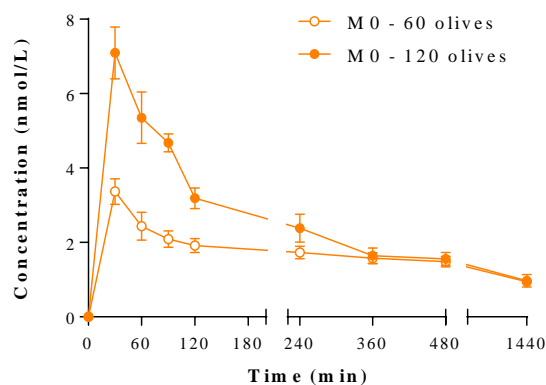intake of 120 table olives with maximum plasmatic concentration at 90 min (Figure 4.28).
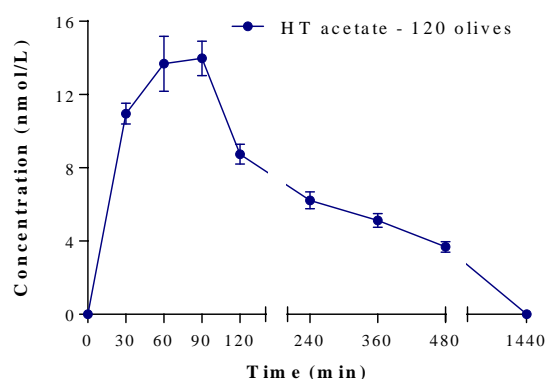
Figure 4.28. Plasma concentrations of hydroxytyrosol acetate in human plasma. Results were obtained after oral intake of 120 table olives. Values are presented as mean ± SEM (n = 18).

With the intake of 120 table olives, participants ingested 3444.66 ± 35.79 μg of hydroxytyrosol acetate. At 30 min, the compound was found at 11.0 ± 0.6 nmol/L. Then, the concentration increased and gave the values of 13.7 ± 1.5 and 14.0 ± 0.9 nmol/L at 60 and 90 min, when the maximum concentration was reached. The plasmatic concentrations steadily decrease from 8.7 ± 0.5 nmol/L at 120 min up to 3.7 ± 0.3 at 480 min and at 1440 min, hydroxytyrosol acetate was not detected.

4.4.1.4. Pharmacokinetic analysis of phenolic compounds

From the plasmatic concentrations of 6 polyphenols that were found in human plasma at different times after single oral intake of 60 and 120 Arbequina table olives, the following pharmacokinetic parameters were estimated following a non-compartmental approach, T_{max}, C_{max}, λ_z, $t_{1/2z}$, AUC_{last}, $AUC_{0-\infty}$, $AUC_{extrap\%}$, MRT_{last} and $MRT_{0-\infty}$.

4.4.1.4.1. Vanillic acid

Results of descriptive statistics of the pharmacokinetic constants of vanillic acid are shown in Table 4.25. Non-compartmental analysis of plasma concentrations over time showed that maximum plasma concentration of vanillic acid when 60 and 120 olives were ingested were 30.0 ± 1.4 and 55.1 ± 1.3 nmol/L.

Median values of time to peak concentrations (T_{max}: 30 min for both doses) were indicative of a relatively rapid absorption process for vanillic acid. Lambda (λ_z) gave a values of 0.0033 ± 3.0 and 0.0044 ± 3.1 min^{-1} for both doses. Elimination half-life was 210.7 ± 3.0 and 156.8 ± 3.1 min for dose of 60 and 120 table olives.

The areas under the curve from time 0 until 1440 min were 3721 ± 1.8 and 6988 ± 1.6 nmol/L·min, meanwhile the values of $AUC_{0-\infty}$ were 4502 ± 1.9 and 7911 ± 1.8 nmol/L·min. Since the estimated values of $AUC_{extrap\%}$ were 2.3 and 5.2%, the results of $AUC_{0-\infty}$ are considered as reliable.

The obtained values of MRT_{last} of 210.7 ± 2.3 and 156.3 ± 2.0 min indicates the average permanence of vanillic acid in the body in accordance with the result obtained in half-life. MRT from time 0 up to infinity gave values of 316.7 ± 2.6 and 217.4 ± 2.6 min.

Table 4.25. Pharmacokinetic parameters of vanillic acid estimated by non-compartmental analysis.

Parameters	Units	Mean	SD	CV%	Min	Median	Max	Geom. Mean	Geom. SD
Vanillic acid **DOSE: 60 olives**									
T_{max}	min	--	--	--	30	30	60	--	--
C_{max}	nmol/L	31.4	9.3	29.6	16.9	29.8	49.5	30.0	1.4
λ_z	min^{-1}	0.0060	0.0086	142.8	0.0006	0.0027	0.0373	0.0033	3.0
$t_{1/2z}$	min	338.0	320.4	94.8	18.6	256.4	1068.5	210.7	3.0
AUC_{last}	nmol/L·min	4416	2916	66.0	1418	3767	13422	3721	1.8
$AUC_{0-\infty}$	nmol/L·min	5491	4265	77.7	1448	4142	20167	4502	1.9
$AUC_{Extrap}\%$	%	13.6	20.1	148.4	0.0	5.6	77.0	2.3	54.9
MRT_{last}	min	282.0	198.5	70.4	43.9	269.4	630.2	210.7	2.3
$MRT_{0-\infty}$	min	466.5	432.0	92.6	46.1	379.7	1619.0	316.7	2.6
DOSE: 120 olives									
T_{max}	min	--	--	--	30	30	60	--	--
C_{max}	nmol/L	57.0	15.1	26.4	38.2	52.8	80.4	55.1	1.3
λ_z	min^{-1}	0.0071	0.0065	91.3	0.0004	0.0074	0.0264	0.0044	3.1
$t_{1/2z}$	min	295.1	391.2	132.6	26.2	94.8	1579.0	156.8	3.1
AUC_{last}	nmol/L·min	7783	3836	49.3	2994	6455	15378	6988	1.6
$AUC_{0-\infty}$	nmol/L·min	9487	7136	75.2	3254	7279	33182	7911	1.8
$AUC_{Extrap}\%$	%	10.4	13.3	127.7	0.5	5.1	53.7	5.2	3.5
MRT_{last}	min	201.3	163.2	81.1	52.9	124.3	639.2	156.3	2.0
$MRT_{0-\infty}$	min	375.8	533.1	141.9	61.3	133.8	2291.1	217.4	2.6

4.4.1.4.2. Hydroxytyrosol and its metabolites

4.4.1.4.2.1. Hydroxytyrosol

Values of descriptive statistics of the pharmacokinetic constants of hydroxytyrosol are displayed in Table 4.26.

Pharmacokinetic analysis gave a median values of time to peak concentrations of 60 (min: 30 and max 360 min) and 30 min (min: 30 min, max: 240 min) when 60 and 120 olives were taken, respectively. These results meant that hydroxytyrosol was absorbed relatively fast with fast conversion to its metabolites.

Hydroxytyrosol reached C_{max} of 2.8 ± 1.8 and 7.0 ± 1.5 nmol/L when the doses of 60 and 120 table olives were ingested. Terminal elimination rate was 0.0009 ± 1.9 and 0.0022 ± 2.3 min^{-1}. Apparent elimination half-life gave values of 754.1 ± 1.9 and 319.1 ± 2.3 min for 60 and 120 olives.

AUC_{last} and $AUC_{0-\infty}$ were 1121 ± 2.4 and 2528 ± 1.8 nmol/L·min and 1436 ± 1.7 and 2019 ± 1.9 nmol/L·min when the doses of 60 and 120 table olives were administered, respectively.

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$AUC_{extrap\%}$ was above 20% in both doses, thus results of $AUC_{0-\infty}$ cannot be considered as reliable. The average permanence of hydroxytyrosol in the body described by MRT_{last} was approximately 6 and 4 h for when 60 and 120 table olives were taken.

Table 4.26. Pharmacokinetic parameters of hydroxytyrosol estimated by non-compartmental analysis.

Parameters	Units	Mean	SD	CV%	Min	Median	Max	Geom. Mean	Geom. SD
Hydroxytyrosol (M0) DOSE: 60 olives									
T_{max}	min	--	--	--	30	60	360	--	--
C_{max}	nmol/L	3.2	1.4	45.1	0.7	3.1	5.8	2.8	1.8
λ_z	min^{-1}	0.0012	0.0011	93.4	0.0005	0.0009	0.0050	0.0009	1.9
$t_{1/2z}$	min	881.2	435.8	49.5	138.3	800.4	1450.4	754.1	1.9
AUC_{last}	nmol/L·min	1433	798	55.7	92.0	1255.2	2508.4	1121	2.4
$AUC_{0-\infty}$	nmol/L·min	2897	1294	44.7	590	2577	4777	2528	1.8
$AUC_{Extrap}\%$	%	46.0	16.9	36.8	11.8	47.5	76.3	42.0	1.6
MRT_{last}	min	412.6	215.8	52.3	153.9	408.7	640.2	354.5	1.8
$MRT_{0-\infty}$	min	1288.9	610.0	47.3	249.4	1170.4	2111.4	1123.2	1.8
DOSE: 120 olives									
T_{max}	min	--	--	--	30	30	240	--	--
C_{max}	nmol/L	7.5	3.2	42.5	3.5	6.6	15.7	7.0	1.5
λ_z	min^{-1}	0.0030	0.0025	84.3	0.0005	0.0022	0.0107	0.0022	2.3
$t_{1/2z}$	min	438.5	361.7	82.5	65.0	310.3	1270.8	319.1	2.3
AUC_{last}	nmol/L·min	1653	905	54.7	523.0	1243.6	3415.6	1436	1.7
$AUC_{0-\infty}$	nmol/L·min	2421	1441	59.5	579	2120	5242	2019	1.9
$AUC_{Extrap}\%$	%	26.7	15.9	59.5	6.9	27.8	71.6	22.5	1.9
MRT_{last}	min	279.5	183.3	65.6	91.8	185.2	596.8	230.5	1.9
$MRT_{0-\infty}$	min	612.8	474.0	77.3	115.3	443.7	1556.7	459.5	2.2

4.4.1.4.2.2. Hydroxytyrosol sulfates (M1-a and M1-b)

The descriptive statistics of the pharmacokinetic constants of sulfate metabolites of hydroxytyrosol are shown in Table 4.27 for metabolite M1-a and Table 4.28 for metabolite M1-b.

The most abundant metabolite was M1-b that gave values of C_{max} and AUC_{last} as follows: dose: 60 olives, C_{max}: 45.7 ± 1.9 nmol/L, AUC_{last}: 8347 ± 1.6 nmol/L·min; dose: 120 olives, C_{max}: 70.5 ± 1.4 nmol/L, AUC_{last}: 18252 ± 1.4 nmol/L·min. M1-a reached the maximum plasma concentration of 4.1 ± 1.7 and 5.5 ± 1.5 nmol/L and AUC_{last} gave values of 614 ± 1.4 and 1150 ± 1.4 nmol/L·min, when doses of 60 and 120 were given.

Values of $AUC_{0-\infty}$ were 1421 ± 1.7 and 2772 ± 3.0 nmol/L·min for M1-a and 10482 ± 2.0 and 21047 ± 1.4 nmol/L·min for M1-b, when 60 and 120 table olives were ingested. $AUC_{extrap\%}$ were above 20% for M1-a and below 20% in case of M1-b.

Median of T_{max} for both sulfates was between 30 and 60 min, meaning that both sulfates were formed relatively fast.

Table 4.27. Pharmacokinetic parameters of M1-a estimated by non-compartmental analysis.

Parameters	Units	Mean	SD	CV%	Min	Median	Max	Geom. Mean	Geom. SD
Hydroxytyrosol sulfate M1-a **DOSE: 60 olives**									
T_{max}	min	--	--	--	30	60	360	--	--
C_{max}	nmol/L	4.6	2.8	61.4	2.1	3.6	13.6	4.1	1.7
λ_z	min^{-1}	0.0020	0.0011	55.0	0.0004	0.0017	0.0043	0.0017	1.9
$t_{1/2z}$	min	509.1	392.3	77.1	162.5	397.7	1635.0	413.0	1.9
AUC_{last}	nmol/L·min	648	205	31.6	200	644	1223	614	1.4
$AUC_{0-\infty}$	nmol/L·min	1622	916	56.5	731	1344	3668	1421	1.7
$AUC_{Extrap}\%$	%	49.6	19.3	38.9	17.2	46.9	83.2	45.7	1.5
$MRT_{0-\infty}$	min	739.5	570.2	77.1	209.0	520.5	2403.3	601.1	1.9
DOSE: 120 olives									
T_{max}	min	--	--	--	30	60	480	--	--
C_{max}	nmol/L	5.9	2.3	38.0	3.0	5.3	10.3	5.5	1.5
λ_z	min^{-1}	0.0023	0.0016	72.2	0.00002	0.0018	0.0074	0.0016	3.5
$t_{1/2z}$	min	2351.5	8106.6	344.7	93.9	396.0	33799.6	440.4	3.5
AUC_{last}	nmol/L·min	1220	397	32.6	519	1293	1859	1150	1.4
$AUC_{0-\infty}$	nmol/L·min	11850	34963	322.2	919	2121	146465	2772	3.0
$AUC_{Extrap}\%$	%	46.7	24.4	52.2	15.2	44.9	99.1	40.8	1.7
$MRT_{0-\infty}$	min	3424.1	11693.3	341.5	254.8	595.8	48787.0	696.0	3.2

Table 4.28. Pharmacokinetic parameters of M1-b estimated by non-compartmental analysis.

Parameters	Units	Mean	SD	CV%	Min	Median	Max	Geom. Mean	Geom. SD
Hydroxytyrosol sulfate M1-b **DOSE: 60 olives**									
T_{max}	min	--	--	--	30	30	240	--	--
C_{max}	nmol/L	56.5	43.2	76.5	18.3	41.5	161.6	45.7	1.9
λ_z	min^{-1}	0.0023	0.0011	48.9	0.0001	0.0021	0.0046	0.0019	2.3
$t_{1/2z}$	min	722.2	1646.3	228.0	150.4	326.6	7298.9	371.0	2.3
AUC_{last}	nmol/L·min	9177	4096	44.6	3808	8252	18637	8347	1.6
$AUC_{0-\infty}$	nmol/L·min	14805	20594	139.1	4278	9932	95672	10482	2.0
$AUC_{Extrap}\%$	%	15.4	19.6	127.0	0.4	10.4	86.5	8.9	3.2
$MRT_{0-\infty}$	min	994.0	2365.5	238.0	163.8	424.2	10445.0	480.5	2.4
DOSE: 120 olives									
T_{max}	min	--	--	--	30	60	360	--	--
C_{max}	nmol/L	75.2	29.0	38.5	40.4	65.0	146.3	70.5	1.4
λ_z	min^{-1}	0.0014	0.0005	32.4	0.0007	0.0013	0.0023	0.0014	1.4
$t_{1/2z}$	min	532.7	186.0	34.9	305.6	532.2	945.2	504.3	1.4
AUC_{last}	nmol/L·min	19170	6141	32.0	9499	18382	31273	18252	1.4
$AUC_{0-\infty}$	nmol/L·min	22326	8206	36.8	10982	20289	43659	21047	1.4
$AUC_{Extrap}\%$	%	12.9	7.6	59.0	4.8	12.3	28.4	11.7	1.8
$MRT_{0-\infty}$	min	679.9	229.8	33.8	361.0	628.0	1178.1	646.9	1.4

4.4.1.4.2.3. Hydroxytyrosol glucuronides (M2-a and M2-b)

The descriptive statistics of the pharmacokinetic constants of the glucuronide metabolites of

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hydroxytyrosol is displayed in Table 4.29 for metabolite M2-a and Table 4.30 for metabolite M2-b.

Since both glucuronides were found only in several samples of human plasma after oral intake of 60 and 120 olives, not all the constants could be calculated.

Glucuronide derivatives were found in minor amount with C_{max} of 1.0 ± 2.9 nM for M2-a for both doses and 4.0 ± 2.1 and 2.1 ± 2.7 nmol/L for M2-b when 60 and 120 table olives were taken.

Median values of T_{max} were 60 and 90 for M2-a and 240 and 360 min for M2-b for the dose of 60 and 120 table olives, respectively. AUC_{last} gave values of 41.2 ± 5.8 (60 olives) and 50.6 ± 3.8 nmol/L·min (120 olives) for M2-a and 497 ± 4.3 (60 olives) and 718 ± 4.6 nmol/L·min (120 olives) for M2-b.

Table 4.29. Pharmacokinetic parameters of M2-a estimated by non-compartmental analysis.

Parameters	Units	Mean	SD	CV %	Min	Median	Max	Geom. Mean	Geom. SD
**Hydroxytyrosol glucuronide M2-a** **DOSE: 60 olives**								
T_{max}	min	--	--	--	30	60	360	--	--
C_{max}	nmol/L	1.6	1.4	86.5	0.2	1.0	4.1	1.0	2.9
AUC_{last}	nmol/L·min	128	177	138.3	2.7	37.8	484.2	41.2	5.8
	DOSE: 120 olives								
T_{max}	min	--	--	--	30	90	480	--	--
C_{max}	nmol/L	1.8	2.2	122.9	0.3	0.7	6.5	1.0	2.9
AUC_{last}	nmol/L·min	101	105	103.4	8.3	51.5	292.5	50.6	3.8

Table 4.30. Pharmacokinetic parameters of M2-b estimated by non-compartmental analysis.

Parameters	Units	Mean	SD	CV%	Min	Median	Max	Geom. Mean	Geom. SD
	Hydroxytyrosol glucuronide 2 M2-b **DOSE: 60 olives**								
T_{max}	min	--	--	--	30	240	480	--	--
C_{max}	nmol/L	4.9	2.5	51.0	0.6	5.9	7.8	4.0	2.1
AUC_{last}	nmol/L·min	851	624	73.2	9.6	900	1870	497	4.3
	DOSE: 120 olives								
T_{max}	min	--	--	--	30	360	1440	--	--
C_{max}	nmol/L	3.4	4.0	117.5	0.7	1.5	13.2	2.1	2.7
AUC_{last}	nmol/L·min	1468	1749	119.2	17.0	922	6971	718	4.6

4.4.1.4.3. Salidroside

Results of descriptive statistics of the pharmacokinetic constants of salidroside are shown in Table 4.31. Non-compartmental analysis of plasma showed that salidroside reached a maximum concentration of 2.3 ± 1.9 and 4.5 ± 1.7 nmol/L after oral intake of 60 and 120 table olives.

Pharmacokinetic analysis gave a median values of time to peak concentrations of 120 min (min: 30 min, max: 240 min) and of 90 min (min: 60 min, max: 360 min) when the doses of 60 and 120 table were applied, respectively.

Lambda (λz) gave values of 0.0042 ± 1.8 and 0.0043 ± 2.1 min^{-1} for both doses. Concentration of salidroside in plasma was reduced by half at 163.2 ± 1.8 and 163.0 ± 2.1 min for 60 and 120 olives.

The values of AUC$_{last}$ were 480 ± 2.0 and 919 ± 1.6 nmol/L·min and of AUC$_{0-\infty}$ were 756 ± 2.1 and 1252 ± 1.8 nmol/L·min, when 60 and 120 table olives were ingested. Geometric means of AUC$_{extrap\%}$ were below 20%.

Finally, the obtained values of MRT$_{last}$ (60 olives: 176.5 ± 1.2 min, 120 olives: 173.6 ± 1.3 min), indicated the average permanence of salidroside of approximately 3 h in the body that was in accordance with the results obtained in half-life.

Obtained values of MRT$_{0-\infty}$ were 292.6 ± 1.6 and 287.3 ± 1.9 min, for doses of 60 and 120 table olives, respectively.

Table 4.31. Pharmacokinetic parameters of salidroside estimated by non-compartmental analysis.

Parameters	Units	Mean	SD	CV %	Min	Median	Max	Geom. Mean	Geom. SD
Salidroside DOSE: 60 olives									
T_{max}	Min	--	--	--	30	120	240	--	--
C_{max}	nmol/L	2.9	2.3	81.2	0.7	2.3	10.9	2.3	1.9
λ_z	min^{-1}	0.0049	0.0030	60.4	0.0019	0.0048	0.0126	0.0042	1.8
$t_{1/2z}$	Min	187.8	100.6	53.5	55.0	144.5	363.1	163.2	1.8
AUC$_{last}$	nmol/L·min	615	531	86.4	145	500	2460	480	2.0
AUC$_{0-\infty}$	nmol/L·min	989	816	82.5	193	598	2937	756	2.1
AUC$_{extrap\%}$	%	22.8	19.6	85.8	1.4	17.6	72.1	15.5	2.7
MRT$_{last}$	Min	180.0	34.7	19.3	106.9	189.0	236.0	176.5	1.2
MRT$_{0-\infty}$	Min	320.9	142.8	44.5	111.4	271.2	590.7	292.6	1.6
DOSE: 120 olives									
T_{max}	Min	--	--	--	60	90	360	--	--
C_{max}	nmol/L	5.3	4.2	79.8	2.4	4.3	20.9	4.5	1.7
λ_z	min^{-1}	0.0053	0.0036	67.6	0.0005	0.0049	0.0166	0.0043	2.1
$t_{1/2z}$	Min	232.3	308.6	132.9	41.7	141.9	1395.2	163.0	2.1
AUC$_{last}$	nmol/L·min	1035	595	57.5	292	959	3109	919	1.6
AUC$_{0-\infty}$	nmol/L·min	1510	1127	74.6	308	1246	5085	1252	1.8
AUC$_{extrap\%}$	%	22.4	20.9	93.1	3.7	15.2	84.1	15.4	2.5
MRT$_{last}$	Min	177.7	38.4	21.6	103.2	178.8	250.9	173.6	1.3
MRT$_{0-\infty}$	Min	374.9	436.4	116.4	113.2	258.5	2023.5	287.3	1.9

4.4.1.4.4. Luteolin

The results of the descriptive statistics of the pharmacokinetic constants obtained for luteolin are represented in Table 4.32. C_{max} of 2.6 ± 1.5 and 4.8 ± 1.3 nmol/L were obtained.

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Median values of T_{max} were 120 min (min: 30 min, max: 360 min) and 60 min (min: 30 min, max: 360 min). Lambda (λ_z) gave values of 0.0036 ± 2.9 and 0.0044 ± 2.5 min^{-1} for both doses.

Plasma concentration of luteolin was reduced by half at 191.0 ± 2.9 and 155.8 ± 2.5 min for the dose of 60 and 120 table olives.

Areas under the curve from time 0 until the last measured time (1440 min) were 609 ± 1.9 and 1319 ± 1.5 nmol/L·min for 60 and 120 olives. The values of $AUC_{0-\infty}$ were calculated (60 olives: 836 ± 1.6 nmol/L·min, 120 olives: 1653 ± 1.5 nmol/L·min).

Results of $AUC_{0-\infty}$ are considered as reliable, since the mean values of $AUC_{extrap\%}$ were below 20%.

Average permanence of luteolin in the body was around 4-4.30 h in case of both doses.

Table 4.32. Pharmacokinetic parameters of luteolin estimated by non-compartmental analysis.

Parameters	Units	Mean	SD	CV %	Min	Median	Max	Geom. Mean	Geom. SD
Luteolin DOSE: 60 olives									
T_{max}	min	--	--	--	30	120	360	--	--
C_{max}	nmol/L	2.8	0.9	32.5	0.9	2.9	4.5	2.6	1.5
λ_z	min^{-1}	0.0057	0.0050	87.6	0.0006	0.0042	0.0176	0.0036	2.9
$t_{1/2z}$	min	320.8	331.8	103.4	39.4	165.3	1204.9	191.0	2.9
AUC_{last}	nmol/L·min	705	321	45.5	69.4	670	1337	609	1.9
$AUC_{0-\infty}$	nmol/L·min	924	446	48.2	410	782	1940	836	1.6
$AUC_{Extrap}\%$	%	15.8	14.8	93.9	0.9	10.6	52.1	9.4	3.1
MRT_{last}	min	289.5	147.7	51.0	65.6	221.2	511.4	252.1	1.8
$MRT_{0-\infty}$	min	499.7	385.2	77.1	177.2	287.2	1473.9	394.3	2.0
DOSE: 120 olives									
T_{max}	min	--	--	--	30	60	360	--	--
C_{max}	nmol/L	5.0	1.4	27.9	2.8	4.9	7.7	4.8	1.3
λ_z	min^{-1}	0.0067	0.0067	99.8	0.0013	0.0034	0.0264	0.0044	2.5
$t_{1/2z}$	min	218.7	166.5	76.1	26.2	207.1	539.5	155.8	2.5
AUC_{last}	nmol/L·min	1419	570	40.2	650	1314	2819	1319	1.5
$AUC_{0-\infty}$	nmol/L·min	1789	732	40.9	699	1632	3434	1653	1.5
$AUC_{Extrap}\%$	%	17.8	18.7	105.0	0.6	7.3	53.7	8.8	3.9
MRT_{last}	min	279.2	124.8	44.7	113.9	223.7	562.2	256.5	1.5
$MRT_{0-\infty}$	min	407.2	194.9	47.9	189.7	390.4	847.6	366.6	1.6

4.4.1.4.5. Verbascoside

Descriptive statistics of verbascoside is displayed in Table 4.33. Maximum plasmatic concentration gave values of 0.8 ± 0.5 and 1.1 ± 1.3 nmol/L when 60 and 120 olives were taken. Median values of time to peak concentrations (T_{max}: 90 min and 75 min for the doses of 60 and 120 olives, respectively) were indicative of a relatively slow absorption process.

Values of λz were 0.0036 ± 1.9 and 0.0018 ± 1.7 min^{-1} for both doses. Elimination half-life

was 193.1 ± 1.9 and 376.6 ± 1.7 min when 60 and 120 table olives were ingested.

AUC_{last} and $AUC_{0-\infty}$ were 104.7 ± 2.9 and 234.8 ± 2.5 nmol/L·min for the dose of 60 table olives and 288.7 ± 1.2 and 623.9 ± 1.4 nmol/L·min when the dose of 120 table olives was applied.

The average permanence of verbascoside in the body described by MRT_{last} was aprox. 2.4 and 3 h when 60 and 120 table olives were ingested.

Table 4.33. Pharmacokinetic parameters of verbascoside estimated by non-compartmental analysis.

Parameters	Units	Mean	SD	CV %	Min	Median	Max	Geom. Mean	Geom. SD
Verbascoside DOSE: 60 olives									
T_{max}	min	--	--	--	0	90	240	--	--
C_{max}	nmol/L	0.8	0.5	59.7	0.0	0.7	1.3	--	--
λ_z	min^{-1}	0.0044	0.0034	77.4	0.0015	0.0030	0.0121	0.0036	1.9
$t_{1/2z}$	min	226.7	117.7	51.9	57.1	233.6	463.5	193.1	1.9
AUC_{last}	nmol/L·min	156.9	117.2	74.7	7.5	126.0	362.6	104.7	2.9
$AUC_{0-\infty}$	nmol/L·min	317.6	196.6	61.9	49.1	395.2	576.6	234.8	2.5
AUC_{Extra} %	%	30.9	16.4	53.2	6.3	30.6	61.4	26.0	2.0
MRT_{last}	min	149.8	42.1	28.1	76.8	147.5	221.4	143.8	1.4
$MRT_{0-\infty}$	min	352.0	158.5	45.0	135.4	371.9	689.4	319.0	1.6
DOSE: 120 olives									
T_{max}	min	--	--	--	30	75	120	--	--
C_{max}	nmol/L	1.1	0.3	23.4	0.8	1.1	1.6	1.1	1.3
λ_z	min^{-1}	0.0020	0.0008	41.3	0.0004	0.0020	0.0035	0.0018	1.7
$t_{1/2z}$	min	440.2	325.1	73.9	200.8	353.4	1602.3	376.7	1.7
AUC_{last}	nmol/L·min	293.3	55.3	18.9	223.2	287.0	428.0	288.7	1.2
$AUC_{0-\infty}$	nmol/L·min	666.7	321.2	48.2	416.5	608.2	1862	623.9	1.4
AUC_{Extrap}%	%	50.9	14.6	28.7	28.4	51.0	86.4	49.0	1.3
MRT_{last}	min	181.2	22.3	12.3	157.4	174.7	235.0	180.0	1.1
$MRT_{0-\infty}$	min	661.3	467.6	70.7	322.9	528.5	2334.2	574.0	1.6

4.4.1.4.6. Hydroxytyrosol acetate

Descriptive statistics of the pharmacokinetic constants of hydroxytyrosol acetate is shown in Table 4.34.

Non-compartmental analysis of plasma concentrations over time showed that maximum plasma concentration when 120 olives were ingested was 14.8 ± 1.3 nmol/L. Median values of T_{max} were 90 min, with minimum at 30 min and maximum at 120 min.

Lambda (λz) gave a value of 0.0035 ± 2.1 min^{-1} and apparent elimination half-life was 195.4 ± 2.1 min.

Areas under the curve from time 0 until 1440 min was 2282 ± 1.5 nmol/L·min, meanwhile the value of $AUC_{0-\infty}$ was 4043 ± 1.6 nmol/L·min. Since calculated value of $AUC_{extrap\%}$ was greater than 20% (37.8%) the result of $AUC_{0-\infty}$ is not considered as reliable. The permanence

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of hydroxytyrosol acetate in the body was around 2.15 h that was described by MRT_{last}.

Table 4.34. Pharmacokinetic parameters of hydroxytyrosol acetate estimated by non-compartmental analysis.

Parameters	Units	Hydroxytyrosol acetate DOSE: 120 olives							
		Mean	SD	CV %	Min	Median	Max	Geom. Mean	Geom. SD
T_{max}	min	--	--	--	30	90	120	--	--
C_{max}	nmol/L	15.3	4.4	28.5	8.4	14.6	26.5	14.8	1.3
λ_z	min-1	0.0046	0.0037	80.4	0.0007	0.0033	0.0137	0.0035	2.1
$t_{1/2z}$	Min	254.3	213.3	83.9	50.7	208.6	952.2	195.4	2.1
AUC_{last}	nmol/L·min	2445	913	37.3	1110	2183	4201	2282	1.5
$AUC_{0-\infty}$	nmol/L·min	4461	2204	49.4	1797	4665	11626	4043	1.6
$AUC_{Extrap}\%$	%	40.2	15.8	39.2	20.0	37.4	86.7	37.8	1.4
MRT_{last}	min	140.4	42.3	30.2	68.2	145.7	200.3	133.6	1.4
$MRT_{0-\infty}$	min	386.4	302.5	78.3	114.1	327.0	1418.9	316.0	1.9

4.5. CLINICAL TRIAL: PLASMATIC CONCENTRATIONS OF PHENOLIC COMPOUNDS IN PLASMA AFTER THE REPEATED CONSUMPTION OF ARBEQUINA TABLE OLIVES BY HEALTHY HUMAN VOLUNTEERS

Stage II of clinical trial assessed the plasmatic concentrations of phenolic compounds after daily intake of 60 Arbequina table olives from the season 2016/2017 during a period of 30 days. Prior to analyses of obtained plasma, the method was subjected to a brief validation.

4.5.1. Determination of phenolic compounds in human plasma after the repeated consumption of Arbequina table olives

4.5.1.1. Validation of the analytical method in stage II of clinical trial

Prior to the determination of phenolic compounds in human plasma obtained at 0, 15 and 30 days, the method was subjected to a brief validation which included linearity, limit of quantification, accuracy and precision to confirm the correct analysis of the data. Parameters were validated at 0.25, 0.5, 0.75, 1, 2.5, 5, 10, 25, 50, 100, and 200 nmol/L, using blank plasma samples.

4.5.1.1.1. Linearity

The calibration curves of 16 polyphenols indicated that the analytical method was linear for all the compounds (Figure 4.29). Correlation coefficients (R^2) were higher than 0.9972 that was obtained for caffeic acid.

The values for the rest of the polyphenols were ranging from 0.9973 for hydroxytyrosol acetate up to 0.9998 for rutin. The results of equations and R^2 are shown in Table 4.35.
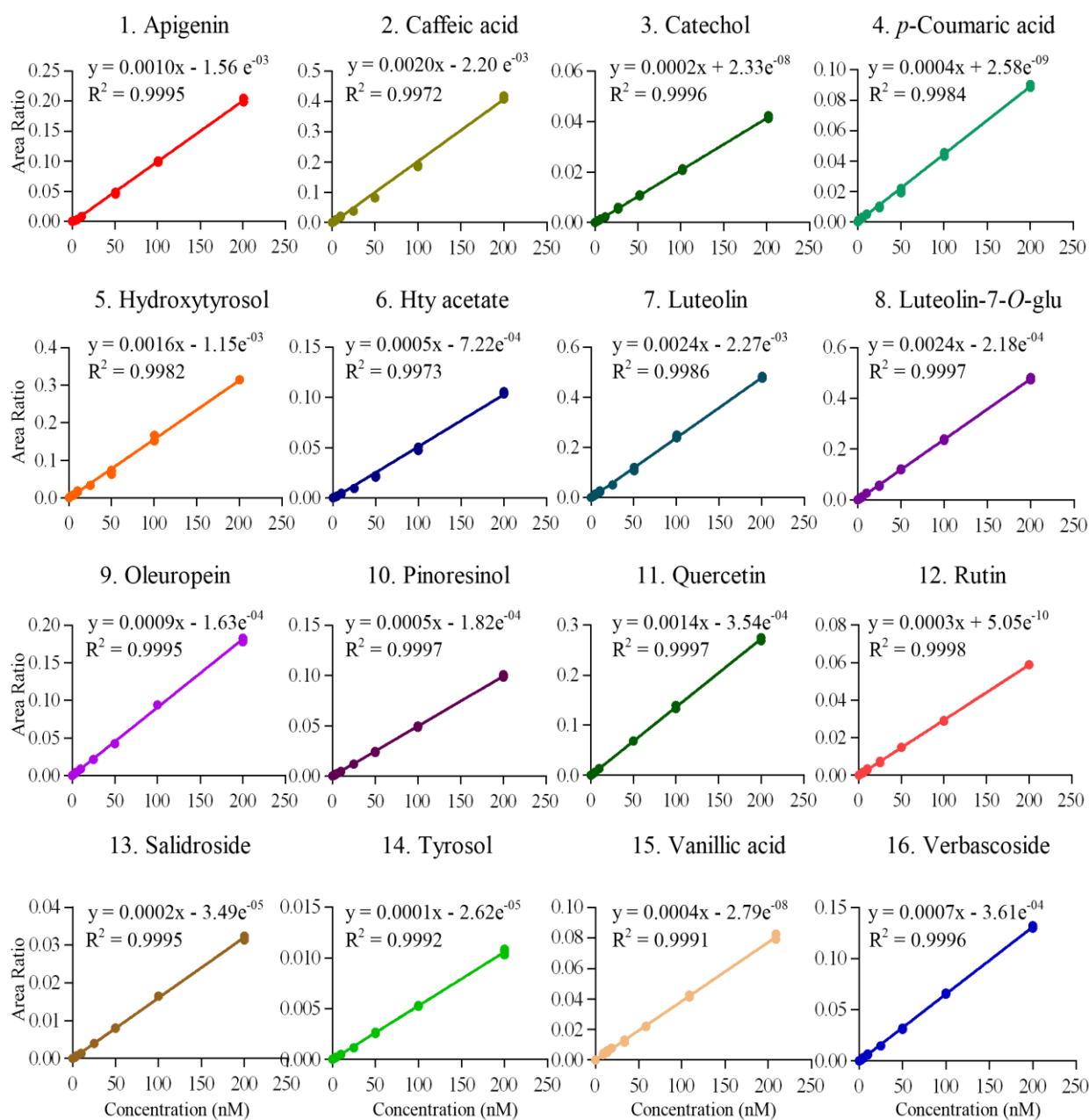
Figure 4.29. Representative calibration curves of phenolic compounds spiked in blank human plasma and determined by LC-ESI-MS/MS. The figure shows the individual values for each of the analytes. The regression line had been calculated using the least square method.

4.5.1.1.2. Limit of quantification

The results of sensitivity of the analytical method expressed as LOQ are shown in Table 4.35.

The calculated values of 15 polyphenols were below 0.50 nmol/L ranging from 0.02 nmol/L for luteolin-7-O-glucoside up to 0.60 nmol/L for tyrosol. The LOQ of catechol was 2.00 nmol/L.

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Table 4.35. Linearity and limit of quantification (LOQ) of phenolic compounds spiked in blank human plasma and analyzed by LC-ESI-MS/MS.

Analyte	Linearity Equations	R^2	Sensitivity LOQ (nmol/L)
Apigenin	$y = (0.00097 \pm 0.00004) + (-7.82e^{-4} \pm 6.38e^{-4})$	0.9995	0.12
Caffeic acid	$y = (0.00165 \pm 0.0004) + (-1.04e^{-3} \pm 1.16e^{-3})$	0.9972	0.31
Catechol	$y = (0.00019 \pm 0.00001) + (1.53e^{-8} \pm 7.95e^{-9})$	0.9996	2.00
p-Coumaric acid	$y = (0.00036 \pm 0.00009) + (-4.82e^{-8} \pm 5.08e^{-8})$	0.9984	0.25
Hydroxytyrosol	$y = (0.00151 \pm 0.0001) + (-5.77e^{-4} \pm 4.71e^{-4})$	0.9982	0.14
HT acetate	$y = (0.00046 \pm 0.00005) + (-3.35e^{-4} \pm 3.87e^{-4})$	0.9973	0.22
Luteolin	$y = (0.00215 \pm 0.0003) + (-1.14e^{-3} \pm 9.28e^{-4})$	0.9986	0.05
Luteolin-7-*O*-glu	$y = (0.00270 \pm 0.0003) + (-1.09e^{-4} \pm 8.90e^{-5})$	0.9997	0.02
Oleuropein	$y = (0.00076 \pm 0.00015) + (-8.14e^{-5} \pm 6.65^{-5})$	0.9995	0.25
Pinoresinol	$y = (0.00052 \pm 0.00002) + (-9.09e^{-5} \pm 9.10e^{-5})$	0.9997	0.25
Quercetin	$y = (0.00109 \pm 0.0003) + (-1.77e^{-4} \pm 1.45e^{-4})$	0.9997	0.25
Rutin	$y = (0.00032 \pm 0.00003) + (-5.67e^{-5} \pm 5.68e^{-5})$	0.9998	0.09
Salidroside	$y = (0.00015 \pm 0.00002) + (2.39e^{-5} \pm 5.87e^{-5})$	0.9995	0.26
Tyrosol	$y = (0.00006 \pm 0.000002) + (-7.47e^{-6} \pm 1.87e^{-5})$	0.9992	0.47
Vanillic acid	$y = (0.00036 \pm 0.00003) + (-3.79e^{-7} \pm 3.51e^{-7})$	0.9991	0.24
Verbascoside	$y = (0.00047 \pm 0.00019) + (-1.81e^{-4} \pm 1.80^{-4})$	0.9996	0.06

4.5.1.1.3. Precision

Intra-day precision had been evaluated in blank human plasma spiked with polyphenols at 12 concentrations. Results expressed as relative standard deviation (RSD%) are shown in Table 4.36.

Table 4.36. Intra-day precision of phenolic compounds spiked in blank human plasma analyzed by LC-ESI-MS/MS.

Analyte	Inter-day precision (% RSD) Concentration (nmol/L)											
	0.25	0.5	0.75	1	2	2.5	5	10	25	50	100	200
Apigenin	2.31	10.50	5.82	12.42	4.91	6.75	4.79	9.03	12.14	3.91	2.41	1.55
Caffeic acid	7.28	12.31	3.65	6.87	5.12	12.20	13.40	7.87	12.37	14.10	1.98	1.23
Catechol	--	--	--	--	4.11	13.81	8.12	10.18	6.62	2.21	3.07	3.82
p-Coumaric acid	5.36	13.97	4.90	12.73	7.08	13.90	11.80	6.74	10.24	7.10	2.80	1.36
Hydroxytyrosol	10.10	13.73	8.27	11.60	7.63	12.65	7.80	9.04	7.36	7.02	6.84	0.37
HT acetate	4.00	12.22	9.28	10.80	9.16	14.06	8.33	6.69	14.40	9.00	10.80	1.12
Luteolin	9.96	8.85	5.06	13.12	10.94	6.78	13.69	10.91	10.77	5.30	2.32	3.03
Luteolin-7-*O*-glu	6.30	8.15	10.21	5.87	7.73	6.98	8.89	4.70	6.18	1.41	7.06	1.01
Oleuropein	14.33	8.09	8.77	3.35	4.65	5.90	9.68	5.53	4.35	4.44	9.77	1.63
Pinoresinol	14.23	8.43	2.35	10.96	6.98	6.59	4.71	5.29	4.53	2.47	1.80	1.21
Quercetin	--	12.52	3.57	16.50	4.28	9.13	4.77	6.44	12.09	1.11	2.59	2.85
Rutin	0.41	12.56	14.19	5.80	5.10	10.62	7.82	9.12	4.23	1.36	1.10	1.33
Salidroside	9.23	13.81	2.55	5.80	4.96	13.20	4.17	10.24	7.53	7.02	1.08	1.63
Tyrosol	--	8.46	6.96	14.97	0.94	11.12	6.13	11.78	11.71	3.73	1.63	2.60
Vanillic acid	3.63	11.17	9.74	14.75	1.76	6.18	10.10	8.00	5.64	2.87	7.42	3.76
Verbascoside	0.46	12.24	3.71	14.75	8.20	5.82	9.61	7.72	5.04	4.10	1.78	0.91

Values of intra-day precision were inferior to 15% for all the polyphenols at all concentrations. Guidelines for the validation of Bioanalytical Methods established by the European Medicines Agency were followed (EMA, 2011).

4.5.1.1.4. Accuracy

Accuracy had been evaluated at 0.25, 0.5, 0.75, 1, 2.5, 5, 10, 25, 50, 100, and 200 nmol/L. Results expressed as relative standard deviation (RSD%) are shown in Table 4.37.

Table 4.37. Accuracy of phenolic compounds spiked in blank human plasma and analyzed by LC-ESI-MS/MS.

Analyte	Accuracy (% RSD) Concentration (nmol/L)											
	0.25	0.5	0.75	1	2	2.5	5	10	25	50	100	200
Apigenin	7.39	-5.42	-2.32	-9.50	7.81	6.10	-11.35	2.21	10.25	4.03	1.95	-0.31
Caffeic acid	-7.87	9.19	-8.25	-4.95	3.82	14.89	0.95	4.88	10.94	12.61	-3.58	-1.75
Catechol	--	--	--	--	9.16	5.19	1.38	0.19	1.23	-0.44	-0.96	1.92
p-Coumaric Acid	2.06	-10.56	7.11	3.88	3.01	4.24	4.02	-4.23	8.09	5.38	0.33	-0.86
Hydroxytyrosol	14.23	6.23	7.95	1.20	30.42	-6.58	-0.82	-11.77	4.11	8.09	7.32	-0.48
HT acetate	-7.43	12.04	-0.43	-9.89	11.74	8.49	3.89	-3.34	12.99	7.73	-1.72	-1.55
Luteolin	-14.35	5.64	2.93	-10.11	14.82	-2.82	-4.72	-6.16	5.08	3.73	-2.38	-1.68
Luteolin-7-*O*-glu	-0.98	-5.41	-4.08	-9.78	10.18	3.08	-1.54	-7.46	3.68	-0.47	-3.77	-0.11
Oleuropein	-7.65	-3.92	0.20	-10.14	7.46	3.96	-3.80	-4.21	6.75	4.07	-8.99	0.20
Pinoresinol	-8.00	-0.88	-0.45	-8.01	11.77	3.09	2.07	-4.35	3.46	1.15	0.08	-0.20
Quercetin	--	-11.33	11.69	9.36	2.63	8.87	1.20	-3.88	7.27	0.51	-0.68	1.65
Rutin	-3.29	1.78	-9.91	-10.54	11.98	6.04	0.19	-0.26	1.50	-0.97	0.95	-1.03
Salidroside	10.83	-7.02	-5.87	-7.18	2.16	3.82	1.26	-0.87	0.27	3.41	7.69	0.60
Tyrosol	--	-14.97	-14.17	-16.99	7.24	6.55	6.06	-4.08	8.17	2.70	-0.38	-0.18
Vanillic acid	1.61	11.42	-4.14	11.03	0.81	-0.31	1.74	-0.78	3.51	3.02	-4.39	-2.39
Verbascoside	0.07	-0.18	5.90	-4.26	-4.05	0.07	-8.97	2.03	5.03	3.29	-1.56	0.11

Accuracy of analytical method was acceptable since the values of accuracy were inferior to 15% for almost all the polyphenols at all concentrations. Guidelines for the validation of Bioanalytical Methods established by the European Medicines Agency were fulfilled (EMA, 2011).

4.5.1.2. Identification of polyphenols in human plasma after repeated consumption of Arbequina table olives

The representative extracted ion chromatograms from human plasma samples obtained on day 15 after daily intake of 60 table olives are displayed in Figure 4.30.

From the 16 phenolic compounds quantified in Arbequina table olives, 2 were not detected (oleuropein and rutin), 5 were detected at concentrations below the LOQ or in very few plasma samples (luteolin-7-*O*-glucoside, pinoresinol, caffeic acid, verbascoside and tyrosol) and 9 were found at concentrations above the LOQ and in nearly all the samples (vanillic acid, catechol, *p*-coumaric acid, quercetin, hydroxytyrosol, hydroxytyrosol acetate, salidroside, apigenin and luteolin).

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Concerning luteolin-7-*O*-glucoside, pinoresinol, caffeic acid, verbascoside and tyrosol, it is worth mentioning that they were found in only half or less of the analyzed samples at concentrations that were at or slightly above the LOQ.

Although luteolin-7-*O*-glucoside was found in approximately half of the samples in control and olive groups, the plasma concentrations were on the verge of the LOQ. Pinoresinol was found in 22 samples in control group and in 18 samples from olive group. Caffeic acid was detected in 20 samples in control group and in 14 samples in olive group. Verbascoside was found in 10 and 14 samples from control and olive group, respectively. Tyrosol was found only in 2 samples in control group and in 4 in olive group.

In addition of being found in not all the samples, luteolin-7-*O*-glucoside, pinoresinol, caffeic acid, verbascoside and tyrosol were found at plasma concentrations below the LOQ thus not being reliably quantified. Finally, oleuropein and rutin were not identified in any of the analyzed samples.

A targeted metabolomic approach was used for identification of the metabolites of hydroxytyrosol, hydroxytyrosol acetate, luteolin and tyrosol. Only derivatives of hydroxytyrosol were found in human plasma, whereas, metabolites of hydroxytyrosol acetate (hydroxytyrosol acetate glucuronide and hydroxytyrosol acetate sulfate), luteolin (luteolin glucuronide and luteolin sulfate) and tyrosol (tyrosol glucuronide and tyrosol sulfate) were not found in human plasma samples.

Hydroxytyrosol (M0) underwent phase II reactions and two sulfate derivatives (M1-a and M1-b) were formed. The analysis of hydroxytyrosol was performed in MRM at the *m/z* 153.2/122.8 Da. Retention time of M0 was 5.78 min that coincides with the one of the standard.

Hydroxytyrosol sulfates were detected at *m/z* 233.0/153.0 Da and they appeared at 5.26 min (M1-a) and 5.45 min (M1-b).

The representative LC-ESI-MS/MS chromatograms of M0, M1-a and M1-b obtained on 15th day of the study after daily ingestion of 60 table olives by human volunteers are shown in Figure 4.31.

Both isomers of hydroxytyrosol sulfates can be seen in Figure 4.31 and the identity of M1-a and M1-b was confirmed since they were also present at the chromatogram of hydroxytyrosol at the same retention time.

Chromatograms were searched for hydroxytyrosol glucuronide (M2-a and M2-b) at *m/z* 329.1/153.0 Da, and no derivatives were found at any of the plasma samples analyzed.
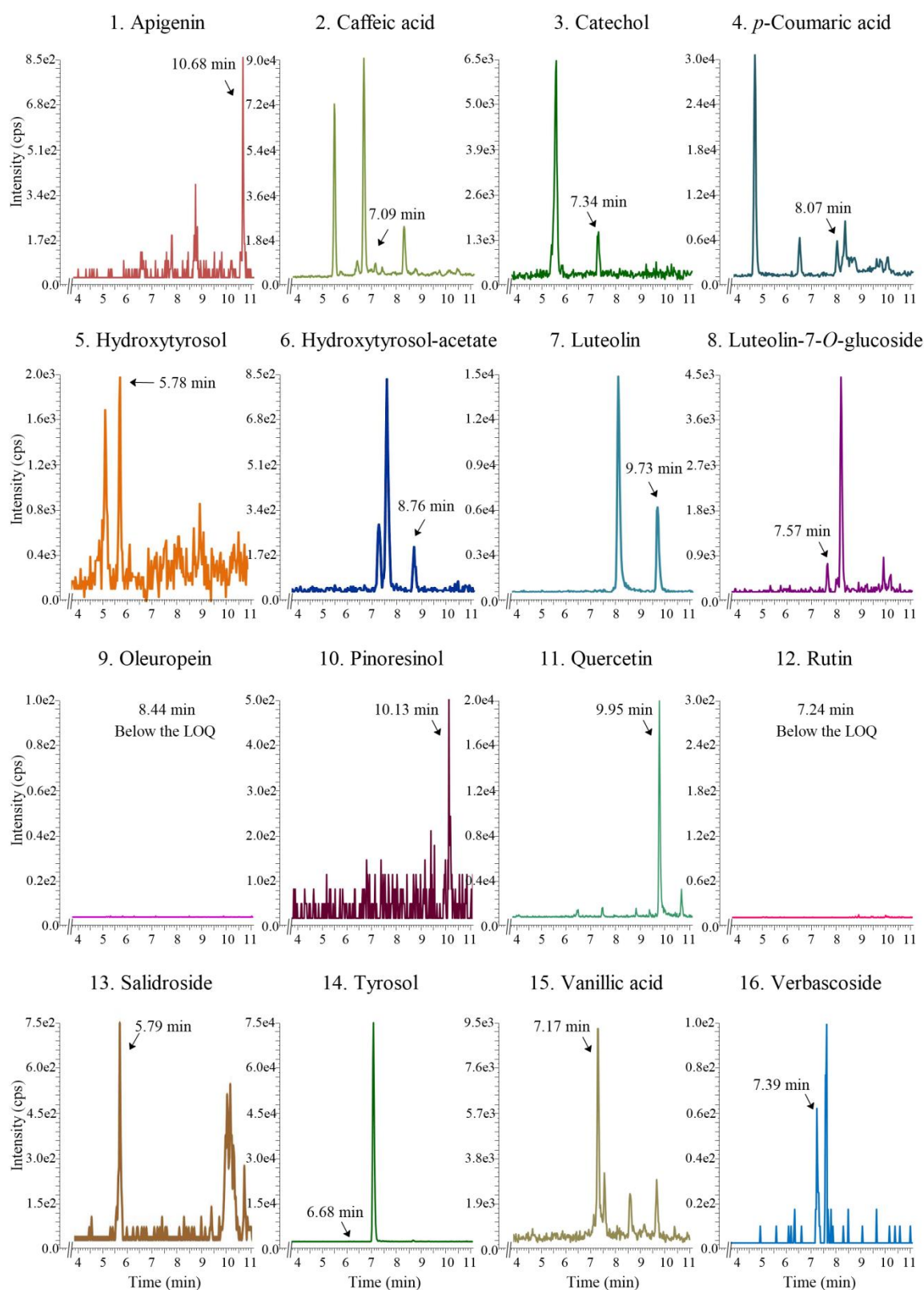
Figure 4.30. Representative LC-ESI-MS/MS chromatograms of polyphenols found in human plasma at 15 days obtained in multiple reaction monitoring mode (MRM).

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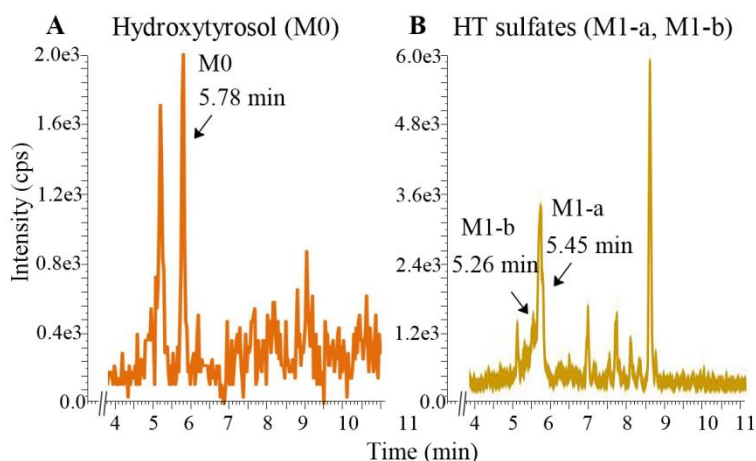
Figure 4.31. Representative LC-ESI-MS/MS chromatograms of hydroxytyrosol (M0) and its metabolites (hydroxytyrosol sulfates: M1-a, M1-b) obtained in multiple reaction monitoring mode (MRM) on day 15 after 60 table olives were daily ingested by human volunteers. Hydroxytyrosol (A) appeared at 5.78 min (*m/z* 153.2/122.8), hydroxytyrosol sulfate M1-a and M1-b (B) at 5.26 and 5.45 min (*m/z* 233.0/153.2).

4.5.1.3. Plasmatic concentrations of phenolic compounds in human plasma after the repeated consumption of Arbequina table olives

After the identification of the polyphenols, the plasmatic concentrations were calculated using the calibration curves described in the section 4.4.2.1.1. The presence of polyphenols was checked at day 0, and traces of apigenin, caffeic acid, catechol, *p*-coumaric acid, hydroxytyrosol, luteolin, luteolin-7-*O*-glucoside, pinoresinol, quercetin, salidroside, tyrosol, vanillic acid, verbascoside were found at low concentrations. Over time, in control and in olive groups, in total 9 polyphenols were quantified in human plasma, being vanillic acid the most abundant compound. It was followed by catechol, *p*-coumaric acid, quercetin, hydroxytyrosol, hydroxytyrosol acetate, salidroside, apigenin and luteolin.

4.5.1.3.1. Vanillic acid

Plasmatic concentrations of vanillic acid reached the values of 10.23 ± 0.97 (n = 36), 9.74 ± 0.82 (n = 37) and 8.84 ± 0.75 (n = 36) nmol/L at 0, 15 and 30 days in control group. No statistically significant difference was observed between day 0 and the concentrations found at 15 and 30 days in the control group ($p > 0.05$).

In the olive group, the plasma concentrations of vanillic acid when the human volunteers ingested daily 60 Arbequina table olives were as follows: 10.02 ± 1.08 (n = 40), 9.07 ± 0.71 (n = 39) and 8.94 ± 0.67 (n = 38) nmol/L at days 0, 15 and 30 day, respectively. No significant differences was observed between the values obtained at day 0 and after the consumption of Arbequina table olives for 15 and 30 days ($p > 0.05$).

The plasmatic concentrations of vanillic acid at all three time points for both, control and olive groups are shown in Figure 4.32.
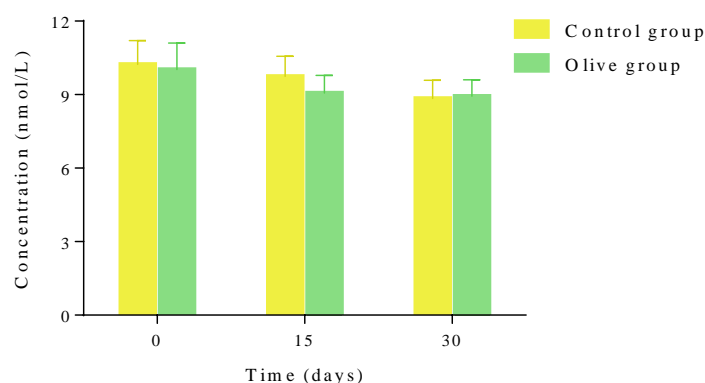
Figure 4.32. Plasma concentration of vanillic acid in human plasma in the control and olive groups at 0, 15 and 30 days determined by LC-ESI-MS/MS. Results are expressed as means ± SEM and within groups, differences from day 0 were evaluated with the non-parametric Kruskal–Wallis test, followed by Dunn's multiple comparisons test. No differences were found.

4.5.1.3.2. Catechol

In the control group, catechol was found at concentrations of 3.86 ± 0.60 (n = 25), 3.96 ± 0.30 (n = 26) and 4.06 ± 0.34 (n = 31) nmol/L at day 0, 15 and 30, respectively. Between the times, no statistically significant difference was observed ($p > 0.05$).

Concentrations of catechol obtained in the olive group were 3.95 ± 0.51 (n = 25), 3.90 ± 0.39 (n = 28), 4.25 ± 0.32 (n = 32) nmol/L at 0, 15 and 30 days, respectively. No significant difference was observed through the times, thus catechol did not accumulate in humans after daily ingestion of 60 table olives for 30 days ($p > 0.05$).

The plasmatic concentration of catechol in human plasma in control and olive group at 3 different times are displayed in Figure 4.33.
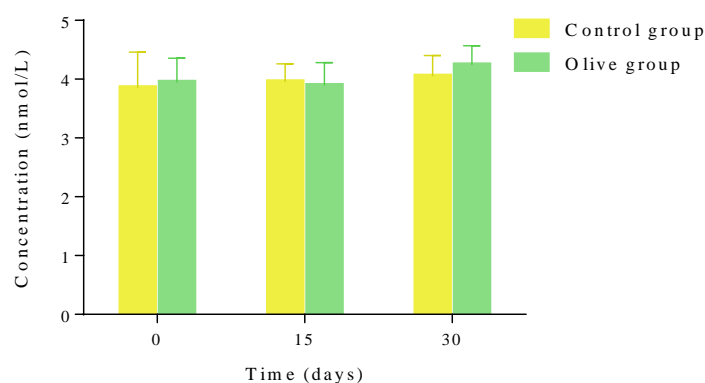
Figure 4.33. Plasma concentration of catechol in human plasma in control and olive groups at 0, 15 and 30 days determined by LC-ESI-MS/MS. Results are expressed as means ± SEM and within groups, differences from day 0 were evaluated with the non-parametric Kruskal–Wallis test, followed by Dunn's multiple comparisons test. No differences were found.

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4.5.1.3.3. *p*-Coumaric acid

p-Coumaric acid was found at 2.36 ± 0.28 (n = 28), 2.37 ± 0.17 (n = 22) and 2.44 ± 0.23 (n = 18) nmol/L at 0, 15 and 30 days, respectively, in control group. No statistically significant difference was observed over the time ($p > 0.05$). On the contrary, in the olive group, *p*-coumaric acid at 0, 15 and 30 days gave the concentrations of 2.34 ± 0.19 (n = 18), 3.44 ± 0.31 (n = 28) and 3.50 ± 0.29 (n = 26) nmol/L, respectively. Significant difference ($p < 0.05$) through the time was observed, since the obtained concentrations at 15 and 30 days were approximately 1.5-fold higher in comparison with the concentration found at day 0. Given that, the daily intake of 60 Arbequina table olives during period of 30 days has an effect on the accumulation of *p*-coumaric acid in the human body. Plasma concentrations of *p*-coumaric acid found in control and olive group are represented in Figure 4.34.

Figure 4.34. Plasma concentration of *p*-coumaric acid in human plasma in control and olive groups at 0, 15 and 30 days determined by LC-ESI-MS/MS. Results are expressed as means ± SEM and within groups, differences from day 0 were evaluated with the one-way ANOVA, followed by Bonferroni multiple comparisons test. Different from day 0: *$p < 0.05$.

4.5.1.3.4. Quercetin

Plasma concentrations of quercetin found in humans are represented in Figure 4.35.

Quercetin was found in human plasma of the control group at 0.65 ± 0.10 (n = 23), 0.65 ± 0.08 (n = 34), 0.67 ± 0.08 (n = 32) nmol/L at 0, 15 and 30 days, respectively. Results did not show any statistically significant difference ($p > 0.05$).

Quercetin in the olive group presented plasma concentrations of 0.69 ± 0.10 (n = 24), 0.69 ± 0.07 (n = 25), 0.68 ± 0.10 (n = 26) at 0, 15 and 30 days, respectively. The consumption of 60 Arbequina table olives for 30 days did not increment de plasmatic concentrations of this flavonoid, since no significant difference was observed in the olive group over the time ($p > 0.05$).

Figure 4.35. Plasma concentration of quercetin in human plasma in control and olive groups at 0, 15 and 30 days determined by LC-ESI-MS/MS. Results are expressed as means ± SEM and within groups, differences from day 0 were evaluated with the non-parametric Kruskal–Wallis test, followed by Dunn's multiple comparisons test. No differences were found.

4.5.1.3.5. Hydroxytyrosol and its metabolites

4.5.1.3.5.1. Hydroxytyrosol

Hydroxytyrosol in the control group reached the values of 0.50 ± 0.03 (n = 40), 0.47 ± 0.03 (n = 40), 0.53 ± 0.02 (n = 40) nmol/L at days 0, 15 and 30, respectively. No significant difference was found between the concentrations determined at any of the 3 sampling points ($p > 0.05$).

Figure 4.36. Plasma concentration of hydroxytyrosol in human plasma in control and olive groups at 0, 15 and 30 days determined by LC-ESI-MS/MS. Results are expressed as means ± SEM and within groups, differences from day 0 were evaluated with the non-parametric Kruskal–Wallis test, followed by Dunn's multiple comparisons test. Different from day 0: ***$p < 0.001$, and ****$p<0.0001$.

On the other hand, significant differences was observed over the time when plasma concentrations of hydroxytyrosol were calculated in the olive group ($p < 0.05$). Obtained values were as follows: 0.48 ± 0.05 (n = 40), 0.71 ± 0.06 (n = 39), 0.63 ± 0.03 (n = 40) nmol/L at 0, 15 and 30 days, respectively. Concentrations at 15 and 30 days were 1.48 and

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1.31-fold higher in comparison with the value obtained at day 0, which means that table olives intake had an influence on the concentrations of hydroxytyrosol in human plasma. Plasmatic concentrations of hydroxytyrosol in human plasma after daily intake of 60 Arbequina table olives are displayed in Figure 4.36.

4.5.1.3.5.2. Hydroxytyrosol sulfates

The concentration of hydroxytyrosol sulfate M1-a in the human volunteers belonging to the control group were as follows: 0 day: 0.30 ± 0.09 (n = 2) nmol/L, 15 day: 0.31 ± 0.07 (n = 7) nmol/L, 30 day: 0.31 ± 0.06 (n = 8) nmol/L (Figure 4.37). No significant differences were observed ($p > 0.05$). On the contrary, significant differences were observed in the olive group ($p < 0.05$). The concentrations at 15 (0.47 ± 0.05 nmol/L; n = 24) and 30 days (0.56 ± 0.08 nmol/L; n = 27) were 2.04 and 2.43 times higher than at day 0 (0.23 ± 0.04 nmol/L; n = 5).
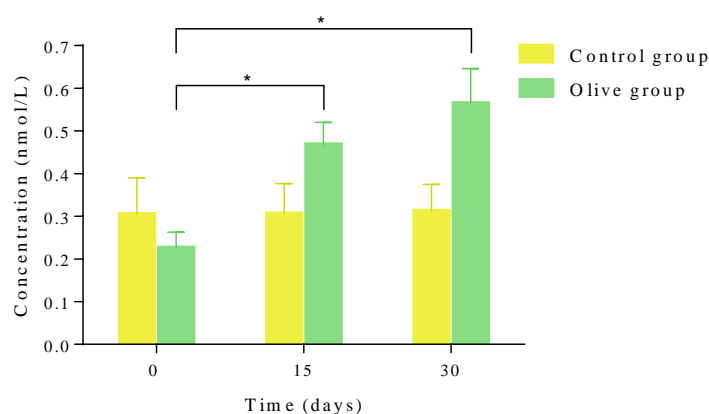
Figure 4.37. Plasma concentration of hydroxytyrosol sulfate M1-a in human plasma in control and olive groups at 0, 15 and 30 days determined by LC-ESI-MS/MS. Results are expressed as means ± SEM and within groups, differences from day 0 were evaluated with the non-parametric Kruskal–Wallis test, followed by Dunn's multiple comparisons test. Different from day 0: *$p < 0.05$.
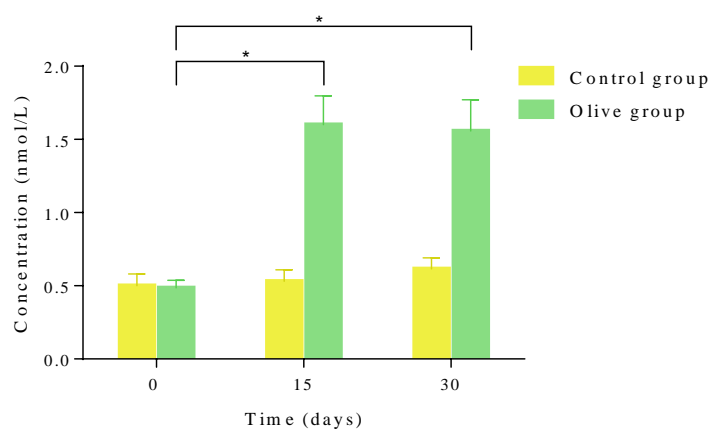
Figure 4.38. Plasma concentration of hydroxytyrosol sulfate M1-b in human plasma in control and olive groups at 0, 15 and 30 days determined by LC-ESI-MS/MS. Results are expressed as means ± SEM and within groups, differences from day 0 were evaluated with the one-way ANOVA, followed

by Bonferroni multiple comparisons test. Different from day 0: *$p < 0.05$.

Hydroxytyrosol sulfate M1-b in human volunteers participating in the control group of the study was found at 0.50 ± 0.08 (n = 8), 0.53 ± 0.08 (n = 12), 0.62 ± 0.08 (n = 8) nmol/L (Figure 4.38). No statistically significant differences were found over the time (p > 0.05). In the olive group, significant differences were observed between times (p < 0.05). The plasma concentrations obtained for day 0 was 0.48 ± 0.05 (n = 10), that increased 3.33 and 3.25-fold on days 15 and 30 compared to day 0. The results for day 15 and 30 were 1.60 ± 0.20 (n = 35), 1.56 ± 0.21 (n = 37) nmol/L, respectively. The daily intake of Arbequina table olives yielded higher plasma concentration of this metabolite over time.

4.5.1.3.6. Hydroxytyrosol acetate

Hydroxytyrosol acetate was not found in any samples in the control group, nor at day 0 in olive group. In the olive group, hydroxytyrosol acetate reached plasma concentrations of 0.67 ± 0.11 (n = 11) and 0.62 ± 0.06 (n = 10) nmol/L at 15 and 30 days, respectively. Thus, daily intake of 60 table olives during 30 days caused an increase in the concentration of this substance in the human plasma. The plasmatic concentration of hydroxytyrosol acetate after oral intake of table olives are shown in Figure 4.39.
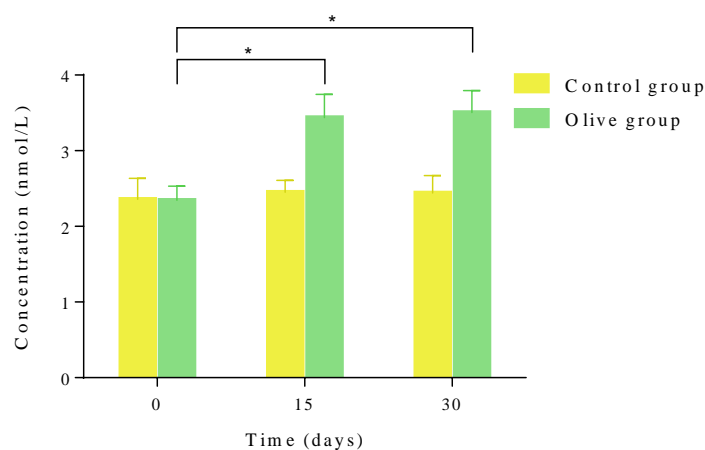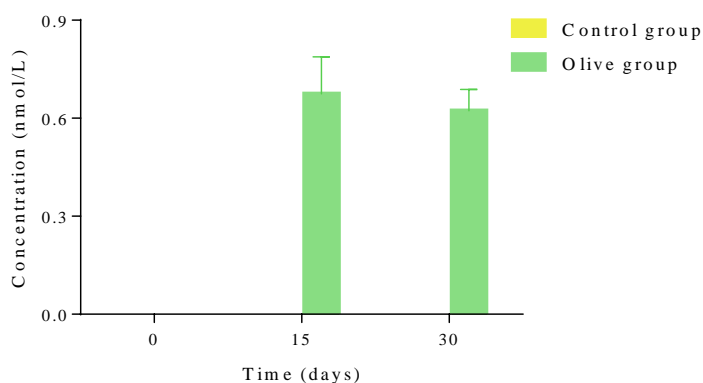

Figure 4.39. Plasma concentration of hydroxytyrosol acetate in human plasma in control and olive groups at 0, 15 and 30 days determined by LC-ESI-MS/MS. Results are expressed as means $\pm$ SEM.

4.5.1.3.7. Salidroside

The plasmatic concentrations of salidroside during the study are shown in Figure 4.40.

The concentration of salidroside detected in human plasma was 0.47 ± 0.07 (n = 5), 0.49 ± 0.06 (n = 4) and 0.53 ± 0.18 (n = 5) nmol/L in the control group at days 0, 15 and 30. Results did not show statistically significant difference between concentrations ($p > 0.05$).

The concentrations in the olive group gave values of 0.67 ± 0.07 (n = 3), 0.70 ± 0.12 (n = 10) and 0.64 ± 0.11 (n = 9) nmol/L at 0, 15 and 30 days. No significant difference was observed in olive group ($p > 0.05$).
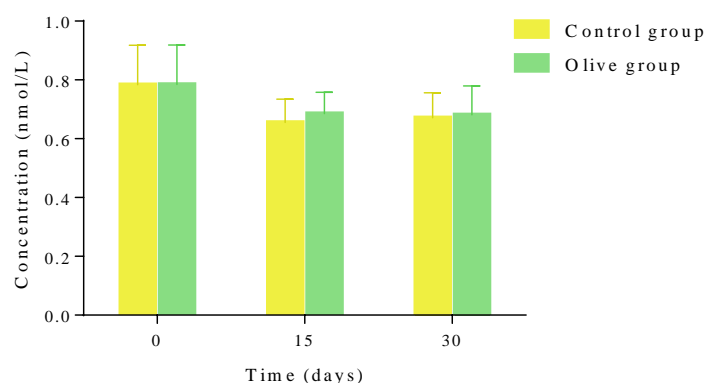
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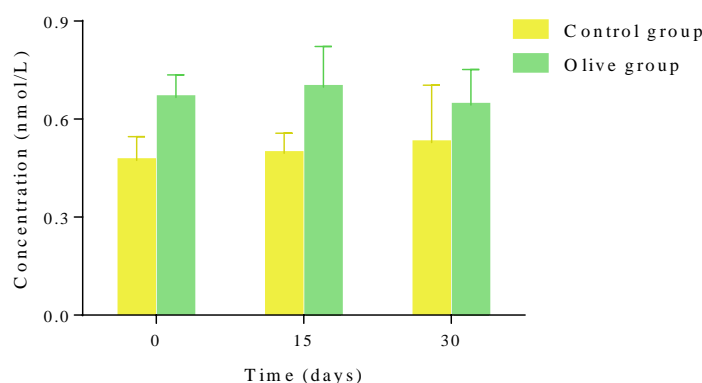
Figure 4.40. Plasma concentration of salidroside in human plasma in control and olive groups at 0, 15 and 30 days determined by LC-ESI-MS/MS. Results are expressed as means ± SEM and within groups, differences from day 0 were evaluated with the non-parametric Kruskal–Wallis test, followed by Dunn's multiple comparisons test. No differences were found.

4.5.1.3.8. Apigenin

In the control group of the study, apigenin was found at 0.27 ± 0.03 (day 0; n = 24), 0.31 ± 0.03 (day 15; n = 19), 0.26 ± 0.06 (day 30; n = 12) nmol/L. No significant differences were observed over time ($p > 0.05$). In the olive group, apigenin reached plasma concentrations of 0.31 ± 0.06 (n = 27), 0.30 ± 0.05 (n = 22) and 0.28 ± 0.07 (n = 14) nmol/L at 0, 15 and 30 days, respectively. Results did not show any significant differences over time ($p > 0.05$). Plasma concentrations of apigenin obtained during the study are represented in Figure 4.41.
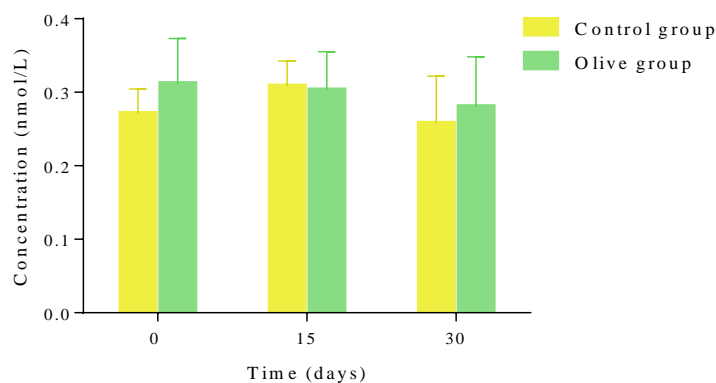
Figure 4.41. Plasma concentration of apigenin in human plasma in control and olive groups at 0, 15 and 30 days determined by LC-ESI-MS/MS. Results are expressed as means ± SEM and within groups, differences from day 0 were evaluated with the non-parametric Kruskal–Wallis test, followed by Dunn's multiple comparisons test. No differences were found.

4.5.1.3.9. Luteolin

The plasma concentrations of luteolin in humans over the time are shown in Figure 4.42.

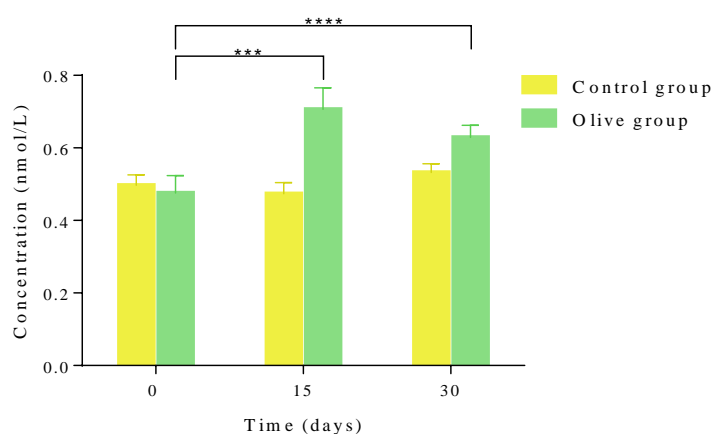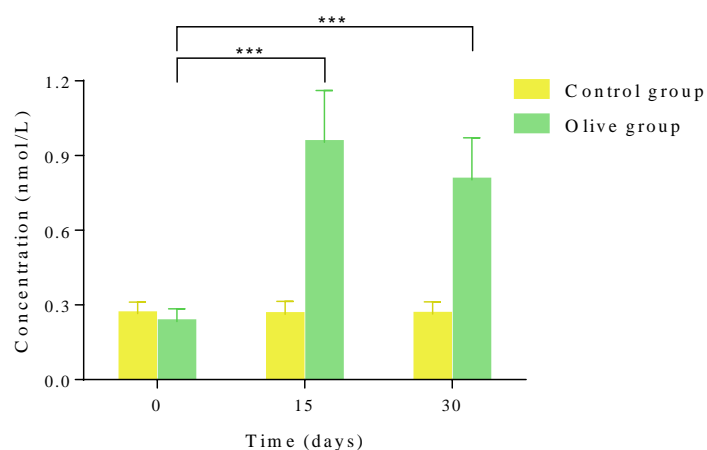**Figure 4.42.** Plasma concentration of luteolin in human plasma in control and olive groups at 0, 15 and 30 days determined by LC-ESI-MS/MS. Results are expressed as means ± SEM and within groups, differences from day 0 were evaluated with the non-parametric Kruskal–Wallis test, followed by Dunn's multiple comparisons test. Different from day 0: *** $p<0.001$.

In the control group, luteolin was found at 0.26 ± 0.05 (n = 37), 0.26 ± 0.05 (n = 31) and 0.26 ± 0.05 (n = 37) nmol/L at day 0, 15 and 30, thus no significant difference was observed through the time (p > 0.05).

However, in the case of the olive group, a significant difference was observed, since the concentration of 0.23 ± 0.05 (n = 36) nmol/L were obtained on the day 0, that increased to 0.95 ± 0.21 (n = 36) and 0.80 ± 0.17 (n = 35) nmol/L at days 15 and 30 ($p < 0.05$).

The values obtained at 15 and 30 min were 4.13 and 3.48-fold higher in comparison with the one on day 0. Thus, daily intake of table olives increased the plasmatic concentrations of luteolin in the human body.

V. DISCUSSION

Table olives together with olive oil obtained from the fruit of *Olea europaea* L. are important food components of the Mediterranean diet. Table olives are not only a significant source of nutrients, but also of compounds with nutraceutical value. This food is characterized by an average composition which includes water, unsaturated fatty acids, cellulose, vitamin E, minerals, carotenoids, pectin, organic acids, pigments, and minor compounds like pentacyclic triterpenoids and phenolic compounds (Ghanbari *et al*., 2012, Boskou, 2017). The latter represents only 1-3% of all the compounds and they are found mostly in the fresh olive pulp (Tuck *et al*., 2001, Charoenprasert and Mitchell, 2012). Phenolic compounds are secondary plant metabolites characterized by the presence of at least one hydroxylated aromatic ring. Besides they exert a protective role against pathogens, insects and ultraviolet radiation, they have been described as compounds with antioxidant (Visioli *et al*., 2003), anticancer (Owen *et al*., 2000), and anti-inflammatory activities. Moreover, they have been associated with protective effect on neurodegenerative diseases, like dementia or Alzheimer's disease (Bazoti *et al*., 2009).

Concerning cardiovascular disease, it is noteworthy that data from epidemiological studies have linked a regular consumption of polyphenols with a lower incidence of cardiovascular events in the populations that accomplish a Mediterranean-type dietary pattern, where olive oil is the major source of fat (Bazoti *et al*., 2009, Dominguez-Perles *et al*., 2015). In this sense, and according to the EFSA health claim (Reg. EU nº 432/2012), in a balanced diet the daily consumption of olive oil containing 5 mg of hydroxytyrosol and its derivatives (tyrosol, oleuropein) protects blood lipids from oxidative damage (EFSA 2012). However, the concentrations in some olive oils may be too low to reach the amounts of hydroxytyrosol and derivatives stated in the claim (Pedret *et al*., 2018). Therefore, table olives emerge as a putative functional food since their content of phenolic compounds is higher to the one described in olive oil (Boskou 2017). Owen *et al*. (2003) already appointed that in comparison to extra virgin olive oil, the total content of phenolic compounds, is approximately 40 and 10 times greater for black and green olives, respectively. These authors pointed out that the intake of 50 g of pericarp of black olives, would provide an amount of phenolic compounds of around 400 mg, while the same amount of extra virgin olive oil would contribute with only 12 mg of polyphenols (Owen *et al*. 2003). Consequently, the consumption of table olives could be an efficient dietary source of phenolic compounds that would strengthen the health promoting effects of the Mediterranean diet (Owen *et al*., 2003).

Notwithstanding their potential beneficial effects on health, the studies on the absorption, distribution, and metabolism of the phenolic compounds from table olives are scarce (Kountouri *et al*., 2007; Golstein *et al*., 2018). Both studies described the plasmatic profile after the consumption of Kalamata table olives by healthy volunteers but focusing only on a few compounds, lacking the assessment of the plasmatic concentration of all the phenolic compounds that come from the same food after its ingestion. Moreover, the knowledge of the content of polyphenols in foods, although providing valuable nutritional information, does not guarantee a prediction of their bioavailability *in vivo*. Consequently, only when the plasmatic

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concentrations of each compound are known, it could be possible to establish the relationship between dose and effect and then recommend a daily intake of a determined food. Bearing all these in mind, the present thesis aims at broadening the knowledge on the pharmacokinetics of the phenolic compounds in both rats and humans after the consumption of Arbequina table olives.

Hence, the first objective of the present thesis consisted of the characterization of the phenolic content of the table olives of the Arbequina variety, that was selected due to its high content of polyphenols (Moreno-González *et al*., 2020b). The table olives used in the present thesis were processed using the Greek-style that consisted in immersing the fruit in 8% solution of sodium hydroxide (NaCl) where spontaneous fermentation occurs. Subsequently, olives were placed in fresh brine. In the present work, two different harvest of table olives were employed for the assessment of the pharmacokinetics of phenolic compounds in rats and humans after the consumption of the Arbequina variety. On the one side, in the pre-clinical study olives from the season 2015/2016 were used, while in the clinical trial, the fruits were from the cop of 2016/2017. Given that, the content of phenolic compounds in table olives could be affected by several factors such as the cultivar, genetic factors, climate, degree of ripeness, and the method of processing (Bianchi *et al*., 2003, Boskou *et al*., 2017), the content of phenolic compounds in both seasons was established. The extraction of phenolic compounds from table olives was performed by a procedure previously described in our research group (Moreno-González *et al*., 2020a). Briefly, destoned olives were homogenized causing the cells disrupt, which facilitated the isolation of the phenolic compounds in a second step that included three consecutive extractions with ethanol:methanol (1/1; v/v). The applied method has minimal sample treatment, whereby the olives are ground to release the phenolic compounds prior to extraction (Moreno-González *et al*., 2020a).

When analyses are performed by LC-MS/MS, the linear range of the method (standard calibration curve) should contain at least five standard points, constructed in the sample matrix, according to the EMA guidelines (2011). Preparation of a calibration curve in methanol 80% would not yield the compounds that co elute with phenolic compounds in the olive sample and that could interfere with the ionization process in the MS detector, which would suppress or enhance ionization and consequently affect the accuracy, reproducibility and sensitivity of the analysis (Matuszewski *et al*., 2003, Honor, 2011). Calibration curve was developed by the standard addition method, since there are no blind matrices available for the analysis of table olives (Thakare *et al*., 2016).

Olives included in the preclinical study that were administered to Sprague-Dawley rats were cropped during the season 2015/2016. Analysis permitted the identification of 16 polyphenols. The total amount of phenolic compounds accounted for 954.55 mg per kilogram of destoned olives. Hydroxytyrosol that represented 80.1% (764.25 ± 9.47 mg/kg), was followed by luteolin, tyrosol and verbascoside that accounted for 8.53%; 3.00% and 2.79%. Vanillic acid and salidroside represented 1.29% and 1.00%. Apigenin, hydroxytyrosol acetate, quercetin and catechol yielded values lower than 1%, whereas pinoresinol, luteolin-7-*O*-

glucoside, oleuropein, rutin, *p*-coumaric acid and caffeic acid represented values lower than 0.2%.

Olives included in the clinical trial that were ingested by healthy human volunteers were collected during the season 2016/2017. The total amount of phenolic compounds was 1043.46 mg per kilogram of olive pulp. Hydroxytyrosol that accounted to 45.5% (474.56 ± 11.77 mg/kg), was followed by verbascoside and luteolin that formed 32.0% and 8.6%. Hydroxytyrosol acetate, rutin, tyrosol, salidroside, oleuropein, and luteolin-7-*O*-glucoside accounted for 2.6%, 2.5%, 2.2%, 1.7%, 1.2%, and 1.1%. Finally, quercetin, and *p*-coumaric acid formed 0.6% and 0.5% of phenolic compounds.

The total amount of phenolic compounds from the two different crops analyzed in the present thesis, were quite similar, being in both cases around 1 g/kg. But the concentrations of the individual polyphenols were slightly different between the two crops, being hydroxytyrosol and verbascoside the compounds with the highest variability. Despite the variability observed for hydroxytyrosol, our results for this compound were in accordance with several studies (Blekas *et al*., 2002; D'Antuono *et al*., 2016, D'Antuono *et al*., 2018), where it was also found as principal polyphenol. Blekas *et al*. (2002) reported that the amount of hydroxytyrosol found in Kalamata table olives ranged between 250-760 mg/kg (Blekas *et al*., 2002). Our results of total polyphenols were higher than the amount of 0.19 g/kg reported by Cabrera-Bañegil *et al*. (2017) in Arbequina olives processed following the Spanish style that were collected in season 2014/2015. Treatment of raw olives to remove the bitter taste of the fruit can affect the final profile of polyphenols. Although the Greek-style retains more polyphenols in the fruit than the Spanish-style or Californian-style (Boskou, 2017; D'Antuono *et al*., 2018), the hydrolysis of glycosides decreases oleuropein, ligstroside, verbascoside, as well as glycosides of hydroxytyrosol and tyrosol which leads to an increase in the content of the aglycones (Boskou, 2017). Apart from the treatment, the phenolic content in olives is also influenced by the different washings applied to the fruit, since polyphenols are water-soluble compounds that can easily diffuse in brine and they can get lost during the washing (Romero *et al*., 2004a, Boskou, 2017). In an another study published by Romero *et al*. (2017), raw Arbequina olives cropped at different stages of ripening during the 2012/2013 contained approximately 12.0–14.0 g/kg of phenolic compounds. These values are much higher to the ones we found for the same variety processed as natural olives in brine.

The analysis of Arbequina table olives showed its high content of phenolic compounds. Hence, the results of the present thesis validate table olives as a source of phenolic compounds that could meet the health claim from the European Union (Reg. EU nº 432/2012) more reliably than extra virgin olive oil. The results of hydroxytyrosol, oleuropein and tyrosol were 794.86 mg/kg and 510.25 mg/kg in season 2015/2016 and 2016/2017, respectively. Arbequina olives are small sized fruits with an average weight of 1.55 ± 0.03 g/fruit (n = 83) and a destoned weight of 1.10 g ± 0.02 g (Moreno-González *et al*., 2020b). Taking into account the calculated amount of hydroxytyrosol together with its derivatives and the weight of the studied olives, we can conclude that daily consumption of 6 olives from season

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2015/2016 and 9 olives of season 2016/2017 would provide an amount of polyphenols of about 5 mg, the amount necessary to achieve a beneficial health effects according to the EFSA health claim (Reg. EU nº 432/2012).

The second objective of the present thesis was to develop and validate an analytical method that would allow a simultaneous determination of 16 phenolic compounds belonging to different chemical groups, namely secoiridoids, phenolic alcohols, phenolic acids, lignans and flavonoids, in rat plasma after the oral administration of table olives. For the development of the method, blank plasma from rats that had never received either table olives or the phenolic compounds were used. Blank rat plasma was spiked with a mixture of the investigated polyphenols.

The use of internal standard (IS) in chromatographic techniques coupled to mass spectrometry is necessary since it allows the elimination of possible fluctuations of the device and systematic or randomized errors. As IS, the compound with similar chemical structure as the analytes with the same behaviour in either extraction process or analytical determination was used. In this way, anything that could affect the analyte will also affect the internal standard (Bansal and DeStefano, 2007). In the present thesis, 2-(3-hydroxyphenyl) ethanol which under our experimental conditions eluted at 7.16 min and did not appear in blank plasma was used as internal standard (IS).

For the development of the method, recovery and matrix effect were considered. Recovery is highly rated in terms of evaluation of the efficiency of the extraction process, while matrix effect appears to be a major issue during the analysis of samples by LC-ESI-MS/MS (Taylor *et al.*, 2005). These parameters were calculated following the recommendations of Matuszewski *et al.* (2003). The development of a new analytical method involved several critical processes, including extraction prior to LC-ESI-MS/MS analysis. To extract the phenolic compounds and IS without endogenous interference and to achieve high recoveries, it was necessary to evaluate the type of solvent, the volume of the solvent, the acidification with formic acid or acetic acid and the use of ascorbic acid to prevent the degradation of phenolic compounds. In the first place of the optimization process, the most suitable solvent for the extraction of hydroxytyrosol was evaluated among ethanol-methanol (1:1; v/v), acetonitrile 100% (Lin *et al.*, 2015, Kure *et al.*, 2016, Ni *et al.*, 2016, Pang *et al.*, 2016), methanol 100% (Li *et al.*, 2004, Sun *et al.*, 2013, Luo *et al.*, 2014, Cao *et al.*, 2016, Kure *et al.*, 2016, Lee *et al.*, 2016) that were employed as protein precipitants, and ethyl acetate 100%, investigated as a liquid-liquid extraction solvent to achieve the maximum recoveries and the lowest endogenous interferences (del Boccio *et al.*, 2003, Chen *et al.*, 2007, Chen *et al.*, 2012, Guan *et al.*, 2014, Zhou *et al.*, 2015). Nevertheless, the recovery could be strengthened by involving a step of acidification which is used for disruption of the binding of the analyte with plasma proteins. Acidification facilitates the release of the analyte from the plasma proteins and its solubilization in the extraction solvent. In terms of acidification of the samples, formic acid and acetic acid which are regularly used in sample preparation prior to LC-ESI-MS/MS were tested (Kushnir *et al.*, 2010). To disrupt protein binding, acetic acid at

0.05%, 0.5%, 1%, 2.5%, 10% and formic acid at 0.05% and 0.5% were applied (Pereira-Caro *et al*., 2016, Kushnir *et al*., 2010). Formic acid yielded very low recovery of hydroxytyrosol at the two concentrations applied. Hence, this acid was discarded. On the contrary, acetic acid improved the recoveries for hydroxytyrosol as well as the other polyphenols and the concentration of 1% was selected since it provided the best recovery for most of the polyphenols. Since the recovery could be low also due to a degradation of polyphenols, ascorbic acid was employed to protect the polyphenols during the extraction process. In the beginning, ascorbic acid 1% was added only prior to evaporation to dryness (Ruiz-Gutierrez *et al*., 2000). After more literature was revised, plasma was spiked with this antioxidant twice, first time in the beginning of extraction process and second time prior to evaporation to dryness at concentration 10% as described by Pereira-Caro *et al*., 2016.

Ethyl acetate modified with acetic acid 1% and ascorbic acid at 1% was chosen as extraction solvent, since it provided satisfactory recoveries and matrix effects compared to the other tested solvents and it offered a higher extraction efficiency, cleaner final sample, a lower background noise in the LC-ESI-MS/MS analysis, and a rapid extraction process due to the low boiling temperature of ethyl acetate that allows a fast evaporation to dryness at 45 °C. All these characteristics lead to a robust extraction method.

The liquid chromatography tandem mass spectrometry LC-ESI-MS/MS conditions were based on those previously established in our group for the analysis of phenolic compounds in table olives (Moreno-González *et al*., 2020a). The LC-ESI-MS/MS were adapted on the one side, from the API3000 to the QTRAP, and on the other, from the determination of phenolic compounds in table olives to plasma samples. In the process of optimization, a sample of rat plasma obtained after the oral administration of Arbequina table olives was injected into LC-ESI-MS/MS system to check the performance of the method. In the beginning, the volume of injection was set at 10 µL. The chromatogram conditions allowed the determination of hydroxytyrosol in MRM mode at the *m/z* 153.2/122.8 Da at retention time of 5.73 min. In the same chromatogram, another peak appeared at 5.37 min, which was a metabolite of hydroxytyrosol because it was absent in blank plasma. The form of the peak indicated the presence of two isomers that could not be separated due to the lack of chromatographic resolution that had to be resolved before all the samples were injected. Since the column and the pre-column were brand new prior to the experiment, the strategy to improve the separation of the peak shape of the compounds was the modification of the volume of the injection. According to Ferrer *et al*. (2011), injecting a smaller amount of sample could be a solution to lessen the matrix effect and to improve the separation of the peaks. Thus, the injection volume was reduced into 2 µL. This approach was feasible thank to the high sensitivity of the method. Since the volume of 2 µL allowed the correct separation of the peaks of metabolites, it was chosen as the injection volume.

After the polyphenols were extracted, they were analyzed by LC-ESI-MS/MS with a short chromatographic run. Method was subsequently validated following the Guidelines on Bioanalytical Method Validation of the European Medicines Agency (2011) and the generally

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accepted recommendations described by Matuszewski *et al.* (2003). The adequacy of the developed experimental conditions was confirmed, since the results obtained in terms of matrix effect, recovery, accuracy, precision, linearity, and sensitivity were satisfactory.

In comparison with other techniques described up to date for the analysis of phenolic compounds in plasma, in the present method, the isolation of analytes is characterized by a fast liquid-liquid extraction that avoids the use of solid-phase extraction as suggested for the analysis of phenolic compounds contained in olive oil in plasma (de la Torre-Carbot *et al.*, 2007, Pastor *et al.*, 2016). In our process, phenolic compounds were injected directly into LC-MS avoiding the step of derivatization of catechols with benzylamine described by Pastor *et al.* (2016), by LC-MS or gas chromatography, where there is a requirement to detect these compounds (Miro-Casas *et al.*, 2003, Kountouri *et al.*, 2007). One of the main features during the validation of the method was sensitivity since it had to be trustworthy adequate for the detection of phenolic compounds in plasma after the oral administration of table olives. Besides, the developed method provides low LOQ, especially for hydroxytyrosol (0.19 nmol/L) that is inferior to the ones found in the work of other authors (de la Torre-Carbot *et al.*, 2007, Pastor *et al.*, 2016). Suárez *et al.* (2011) described a method allowing the determination of 10 olive oil polyphenols belonging to different groups including luteolin, apigenin, and pinoresinol in plasma. In his method, the LOQ of luteolin, hydroxytyrosol, and tyrosol was 0.1, 0.5, and 4.8 μM, respectively, whereas we found LOQ values of 0.12, 0.19, and 1.95 nM for the same compounds, meaning that our method is more sensitive, and it allows the detection even of a small amount of the mentioned compounds (Suarez *et al.*, 2011).

Concerning analytical methods that measure multiple polyphenols, there is only one method described in the literature that allows the determination of 38 phenolic compounds belonging to different classes in urine samples (Achaintre *et al.* 2016). Although many phenolic compounds can be analyzed with the use of the mentioned method, this technique has several limitations identified by the same authors. One of the limitations is the need of costly labeled standards and the partial degradation of flavonols during the dansylation reaction. In addition, the use of a multilayer methodology is limited, and the required equipment is not easily available to many laboratories (Achaintre *et al.*, 2016).

The developed and validated analytical conditions were subsequently applied to the third objective of the present thesis which consisted in the pharmacokinetic assessment of phenolic compounds in plasma after the oral administration of Arbequina table olives to Sprague-Dawley rats. For the development of this pre-clinical study, plasma samples of Sprague-Dawley rats were obtained at different times (0, 30, 60, 90, 120, 180, 240, 360, and 480 min) after the oral administration of the homogenous olive suspensions prepared from table olives of Arbequina variety (harvest 2015/2016). Homogenous suspensions were prepared at doses of 3.85 and 7.70 g/kg that are doses equivalent to the human ingestion of 30 and 60 olives of the same variety.

Considering the concentrations of polyphenols reported for the Arbequina table olives of the season 2015/2016, when the experimental animals were administered with the dose of 3.85 g/kg, each rat received 1.01 mg of polyphenols. Noteworthy the fact that the amount of hydroxytyrosol was 0.81 mg, which corresponded to an 80% of all the polyphenols. Luteolin with 0.086 mg was the ensuing compound and accounted for an 8.5%. The following phenolic compounds were tyrosol verbascoside, vanillic acid and salidroside that were given at 30.4, 28.2, 12.9 and 9.86 μg. These 6 compounds represented in total 96.6% of total phenolic composition of table olives harvested during the season 2015/2016. The other 10 phenolic compounds accounted only for 3.4%. When the compounds were measured in blood, the phenolic profile differed from the one found in table olives and in total, 7 compounds out of 16 were found at concentrations above LOQ. Apigenin, vanillic acid, oleuropein, pinoresinol, and quercetin were identified but not quantified in rat plasma, since they were found at the concentrations that were below the LOQ.

Analysis of the plasma samples showed that hydroxytyrosol was present in plasma along with metabolites that were searched throughout a targeted metabolomic approach. There are several published studies in the literature about metabolism of hydroxytyrosol in rats (Tuck *et al* 2001, Tuck *et al*., 2002, Serra *et al*. 2012, Lopez de las Hazas *et al*., 2015, Rubio *et al*., 2014) that were together with the previous experience in our research group with oral bioavailability of hydroxytyrosol (Ruiz-Gutierrez *et al*., 2000) taken into consideration in the search of metabolites. Hydroxytyrosol has been reported to undergo mainly phase II reactions, yielding two sulfate (M1-a and M1-b) and two glucuronide (M2-a and M2-b) derivatives. This might suggest that hydroxytyrosol follows two different pathways, one mediated by the action of the sulphotransferase enzymes and the other by the glucurotransferases (Suarez *et al*., 2011).

Subsequently, from the obtained plasmatic concentrations of the 7 polyphenols that were found in rat plasma at different times after single oral administration of the homogenous olive suspensions, the main pharmacokinetic parameters were calculated using a non-compartmental approach.

With the intake of the suspension at 3.85 g/kg, *p*-coumaric acid was the compound that reached the maximum plasma concentration (C_{max}) of 4.7 nmol/L. It was followed by hydroxytyrosol (26.3 nmol/L), salidroside (22.8 nmol/L), tyrosol (4.14 nmol/L), verbascoside (2.4 nmol/L), luteolin (1.9 nmol/L), and luteolin-7-*O*-glucoside (0.5 nmol/L). When the dose of 7.70 g/kg was administered, the phenolic compound with the highest C_{max} was salidroside (157.1 nmol/L) that was followed by *p*-coumaric acid (84.1 nmol/L), hydroxytyrosol (45.0 nmol/L), verbascoside (10.0 nmol/L), tyrosol (11.8 nmol/L), luteolin (5.1 nmol/L), and luteolin-7-*O*-glucoside (1.6 nmol/L). All the polyphenols were detected in all sampling times except for salidroside. Salidroside was detected up to 360 when the dose 7.70 g/kg was given, whereas when the dose of 3.85 g/kg was administered, this compound was detected up to 240 min.

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The C_{max} of hydroxytyrosol of 26.3 and 45.0 nmol/L were reached at 30 min after oral intake of table olives at 3.85 and 7.70 g/kg. Hydroxytyrosol was extensively metabolised already at 30 min after oral administration when the maximum plasma concentration of sulfates M1-a reached 53.1 and 104.8 nmol/L and M1-b gave 340.0 and 581.2 nmol/L when table olives at 3.85 and 7.70 g/kg were administered. Glucuronides considered as minor metabolites were found at concentrations lower than 15 nmol/L for both doses. The metabolism observed for this polyphenol was also consistent with data reported in other studies (López de las Hazas *et al.*, 2015, Dominguez-Perles *et al.*, 2017).

T_{max} showed that the phenolic compounds had a relatively fast absorption rate. T_{max} of salidroside, and verbascoside was around 30 min, thus meaning that these compounds were absorbed rapidly in rat plasma after the oral administration of table olives. Luteolin-7-*O*-glucoside had a T_{max} of 45 min and tyrosol, *p*-coumaric acid, luteolin and hydroxytyrosol hold values of approximately 1 hour. Dong *et al.* (2017) reported in their work that luteolin reached T_{max} at 0.42 h when the rats were administered with the extracts of *M. chamomilla* that contained luteolin at 56.49mg/kg (Dong *et al.*, 2017).

The elimination half-life that is the time needed for the plasma concentration of phenolic compounds to decrease by half of the initial value ($t_{1/2z}$) was inferior to 1 hour for salidroside. Hydroxytyrosol and verbascoside had half-life of 2 hours, while luteolin and *p*-coumaric acid of 3 hours. Luteolin-7-*O*-glucoside and tyrosol were the phenolic compounds with the longest half-life of 4 and 5 hours, suggesting that the elimination of them may be slower in comparison with the rest of the compounds. Lin *et al.* (2015) stated the half-life of luteolin to be around 2 h after oral administration of luteolin at 100 mg/kg, whereas luteolin-7-*O*-glucoside had half-life of more than 11 h after oral administration of luteolin-7-*O*-glucoside at 1g/kg (Lin *et al.*, 2015).

Considering the results obtained for the unaltered phenolic compounds, the area under the plasma concentration time curve (AUC) showed that at both doses the highest exposure was achieved by *p*-coumaric acid (50%). It was followed by hydroxytyrosol (23%) salidroside (15%) and tyrosol (7%). Verbascoside and luteolin, compounds with the lowest plasmatic concentrations achieved the AUC that accounted for 2%. Luteolin 7-*O*-glucoside achieved less than 1%. However, hydroxytyrosol that was the most abundant compound in Arbequina olives, although being found unaltered at relatively low amounts, it is the main compound in plasma considering the concentrations achieved by the two sulfate metabolites. The analysis of the AUC of hydroxytyrosol and its derivatives indicated that the two sulfates were the most abundant (~86%), followed by hydroxytyrosol (~10%) and minor amounts of the two glucuronides (~4%).

The fourth aim of the present thesis consisted of the pharmacokinetic analysis of phenolic compounds in plasma after the consumption of Arbequina table olives by healthy human volunteers. Consequently a single centre, randomized, open-label, two-way crossover clinical trial was performed with healthy male volunteers that consume 60 and 120 table olives. For

the establishment of the doses, it was considered that this variety is characterized by its small size, with an average weight of 1.73 ± 0.06 g, which guarantees uniformity in intake. Therefore, it can be indicated that the average weight of 60 olives corresponds to approximately 100 g, so that after discarding the stone giving the value 75 g. This dose corresponds to the one employed by Kountouri *et al*. (2007), when the participants ingested 20 Kalamata olives with the approximate weight of 100 g. This dose provided a sufficient amount of bioactive compounds that allowed its adequate determination in the blood at the various time points required for evaluation of pharmacokinetic parameters. This study was also performed at a dose of 120 olives, in order to evaluate the effect of the dose.

Bearing in mind the concentrations found for the Arbequina table olives harvested at 2016/2017, the total amount of phenolic compounds that the human volunteers received after the consumption of 60 Arbequinas was 69.1 mg of polyphenols. Of this amount, hydroxytyrosol supplied 31.4 mg and verbascoside 22.1 mg, accounting both for approximately 77.5% of the total. Luteolin contributed with 5.9 mg. The ensuing phenolic compounds were hydroxytyrosol acetate, rutin tyrosol and salidroside with values of 1.8, 1.7, 1.5 and 1.1 mg, respectively. Oleuropein, luteolin-7-*O*-glucoside, quercetin, *p*-coumaric acid, caffeic acid, apigenin, vanillic acid and pinoresinol supplied values lower than 1 mg.

In the present clinical trial, the blood was obtained after the oral administration of table olives up to 24 h at 8 different times (0, 30, 60, 90, 120, 240, 360, 480, 1440 min). The sampling period is wider to the ones in the literature that analyze phenolic compounds after the intake of table olives (Kountouri *et al*., 2007; Goldstein *et al*., 2018). The study by Kountouri *et al*. (2007) that assessed polyphenols in plasma after the consumption of 20 Kalamata table olives (approximately 100 g) carried out the pharmacokinetics with only 5 blood withdrawals (0, 60, 120, 180 and 240 min). A short sampling time was also performed by Goldstein and coworkers (2018). After the oral ingestion of 10 Kalamata olives by healthy human volunteers, blood was withdrawn at 15, 30, 45 and 60 min.

Prior to the analysis of plasma samples, the analytical method developed and validated in rat plasma was verified in blank human plasma obtaining appropriate recovery, matrix effect, linearity, precision, accuracy, and sensitivity. In contrast to the 16 polyphenols analyzed in rat plasma samples, due to an unexpected problem with the instrument, only 9 phenolic compounds were evaluated. Of them, hydroxytyrosol, hydroxytyrosol acetate, luteolin, salidroside vanillic acid and verbascoside were found while oleuropein, quercetin and tyrosol were not detected. Vanillic acid, hydroxytyrosol and luteolin were detected in all sampling times, whereas salidroside, verbascoside and hydroxytyrosol acetate were detected up to 8 h. In addition, hydroxytyrosol acetate was only found after the consumption of 120 Arbequina olives.

A targeted metabolomic approach was carried out to search for the metabolites of hydroxytyrosol, hydroxytyrosol acetate, luteolin and tyrosol. For these compounds, derivatives of phase II metabolism, namely glucuronides and sulfates were investigated. Only

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metabolites of hydroxytyrosol were found, with a similar distribution as had been observed in rats. Hydroxytyrosol underwent extensive metabolism yielding two sulfate (M1-a and M1-b) and two glucuronide (M2-a, M2-b) metabolites that were searched throughout a targeted metabolomic approach. There are several published studies in the literature about metabolism of hydroxytyrosol in humans after oral intake of table olives (Kountouri *et al*., 2007, Goldstein *et al*., 2018), olive oil (Miro-Casas *et al*., 2003, Visioli *et al*., 2003, de la Torre-Carbot *et al*., 2007, Suarez *et al*., 2009, Suarez *et al*., 2011, Orozco-Solano *et al*., 2012) or after intake of capsulated olive leaf extract (Del Bock *et al*., 2013, Del Garcia-Villalba *et al*., 2013).

From the obtained plasmatic concentrations, the main pharmacokinetic parameters were evaluated. The value of T_{max} achieved through non-compartmental analysis showed that vanillic acid and hydroxytyrosol were the compounds with the faster absorption of 30 min, followed by luteolin (60 min). Verbascoside had a T_{max} of 75 min, salidroside and hydroxytyrosol acetate had T_{max} of 90 min.

After the oral ingestion of 60 and 120 table olives, the phenolic compound found in human plasma with the highest C_{max} was vanillic acid that reached the values of 31.4 and 57.0 nmol/L. It was followed by hydroxytyrosol (3.2 and 7.5 nmol/L), and salidroside (2.9 nmol/L and 5.3 nmol/L). Luteolin and verbascoside were found below 5 and 1 nmol/L after the administration of both doses. Hydroxytyrosol acetate that was only found after the consumption of 120 Arbequina table olives, yielded a C_{max} of 15.3 nmol/L.

Concerning the metabolites of hydroxytyrosol, in the results obtained in the clinical trial, sulfate M1-b was the most abundant compound that reached C_{max} of 56.5 and 75.2 nmol/L, for the doses of 60 and 120 olives at 30 min post ingestion. Glucuronides were found in amounts below 5 nmol/L, moreover only in few samples, thus they were considered as minor derivatives.

Time needed for the plasma concentration of phenolic compounds to decrease by half of the initial value was 4 hours for luteolin, salidroside and hydroxytyrosol acetate, 5 hours for vanillic acid, and approximately 7 hours for hydroxytyrosol and verbascoside, meaning they eliminate slower when compared with the rest of the compounds.

The compound with the highest AUC of 53% was vanillic acid, followed by hydroxytyrosol acetate (17%), hydroxytyrosol (12%), luteolin (9%), salidroside (7%) and verbascoside (2%). The analysis of the AUC of hydroxytyrosol and its metabolites showed that they followed the same pattern as in rat. Two sulfates represented ~85%, the parent compound hydroxytyrosol accounted for ~10% whereas the two glucuronides were ~5%.

Finally, the fifth objective of the present thesis dealt with the plasmatic concentrations obtained after the repeated consumption of 60 Arbequina table olives by healthy male and female volunteers. The study was part of a single center, randomized, open-label, two-way crossover clinical trial performed with healthy male and female volunteers. Plasma samples

were analyzed following the same analytical method developed, but different from the fourth objective that measured only 9 polyphenols, here the 16 compounds were evaluated. Hence, vanillic acid, catechol, *p*-coumaric acid, quercetin, hydroxytyrosol, hydroxytyrosol acetate, salidroside, apigenin and luteolin were found at in plasma of the volunteers. Of them, *p*-coumaric acid, hydroxytyrosol with its two sulfate derivatives, luteolin and hydroxytyrosol acetate significantly increased at days 15 and 30 after the repeated intake of 60 Arbequina table olives with respect to the values found at day 0.

Given that the main compounds of table olives in both, in rats and human healthy volunteers were not those with the highest plasma concentration, these results reflect the complexity of the processes involving bioavailability. With the consumption of the debittered table olives they face gastrointestinal digestion releasing polyphenols, that have been simulated in vitro for table olives with bioavailability of 100%, 86%, 56%, and 7% for tyrosol, hydroxytyrosol, verbascoside, and luteolin, while apigenin and hydroxytyrosol acetate were not detected (D'Antuono *et al*., 2016). In addition, various polyphenols like oleuropein, ligstroside, comselogoside, verbascoside, salidroside, or different glucosides could be hydrolyzed under experimental conditions like the ones occurring during the transit through the stomach, leading to an increase of *p*-coumaric acid, hydroxytyrosol, and tyrosol, among others (Corona *et al*., 2006, D'Antuono *et al*., 2016, Malapert *et al*., 2018). Once ingested, the absorption of polyphenols is accomplished by various mechanisms, which may involve active transport, as described for *p*-coumaric acid, which provides high bioavailability (Konishi *et al*., 2004), or by simple diffusion, as described for salidroside, hydroxytyrosol, tyrosol, and verbascoside which is a less efficient process (Manna *et al*., 2000, Yasuda *et al*., 2015, Zhou *et al*., 2018). Flavonoid glucosides are hydrolyzed by lactase phlorizin hydrolase to the corresponding aglycone that is followed by pasive diffusion of the aglycone into the cell. Several glycosides, for example luteolin-7-*O*-glucoside, are absorbed into enterocytes via sodium glucose cotransporter 1 (SGLT1) (Yasuda *et al*., 2015). Extensive metabolism occurs within enterocytes, after cellular accumulation of aglycone. Thus, the possible metabolism suffered in the enterocytes and the efflux to the intestinal lumen through ABC protein transporters which affect the bioavailability of different polyphenols cannot be underestimated (Corona *et al*., 2006, Yasuda *et al*., 2015, Zhou *et al*., 2018). Although hydroxytyrosol is the major polyphenol in the Arbequina table olives, concentrations of the free compounds found in plasma are low, due to its extensive metabolism. This was previously described by Kountouri *et al*. (2007) and Goldstein *et al*. (2018) after the consumption of Kalamata table olives by human healthy volunteers that is attributed to extensive presystemic metabolism (Kountouri *et al*., 2007, Goldstein *et al*., 2018).

Various *in vitro* experiments were performed, where the mentioned substances showed low stability during the digestion process (D'Antuono *et al*., 2016, Soler *et al*., 2010), together with a low oral bioavailability (Corona *et al*., 2006, Wang *et al*., 2014). These results were also confirmed by Kountouri *et al*. (2007) who did not detect quercetin, vanillic acid or caffeic acid after the oral intake of Kalamata olives (Kountouri *et al*., 2007).

V. Discussion

The results obtained in the present study show that the plasmatic concentrations of phenolic compounds from table olives are influenced by various factors, like the release of phenolic compounds from the food matrix after ingestion or their bioaccessibility in the gastrointestinal tract. Once in the intestine, the phenolic compounds face uptake process in the enterocytes, where different mechanisms of absorption may occur depending on the hydrophilic or lipophilic nature of the analytes, a process in which intestinal transporters as well as metabolic enzymes may be involved (Rein *et al*., 2012). All these processes have led to results, in the present thesis such as the finding that hydroxytyrosol, the major phenolic compound in table olives is found unaltered in plasma in relatively low concentrations mainly due to its extensive metabolism to sulfate, which is the compound with the highest AUC. Noteworthy, the fact that the *p*-coumaric acid and vanillic acid which were found at relatively low amounts in table olives exhibited the highest AUC in rats and humans, being unexpectedly highly bioavailable. Hence, those results corroborate that the knowledge of the content of polyphenols in a food does not guarantee a prediction of their bioavailability *in vivo*. However, the overall results found in rats and humans after the single and repeated consumption of table olives indicate that this food is an important source of bioactive compounds important for the prevention of chronic diseases.

VI. CONCLUSIONS

The conclusions from the present thesis are:

1. Simultaneous analysis of phenolic compounds in Arbequina table olives by LC-ESI-MS/MS.

1.1. The analysis of Arbequina table olives from the seasons 2015/2016 and 2016/2017 showed the presence of 16 phenolic compounds that accounted for a total of 1.0 g/kg, in both crops. Hydroxytyrosol, luteolin, and verbascoside represented about 90% of the total determined in this variety, whereas the other 13 constituted around 10%.

1.2. The results indicate a high content of polyphenols present in the Arbequina fruit, thus the daily intake of 6-9 small-sized table olives would supply the 5 mg of hydroxytyrosol and derivatives stated by the EFSA (Reg. EU n° 432/2012) to exert a cardioprotective activity.

2. Simultaneous determination of phenolic compounds in rat plasma by LC-ESI-MS/MS.

2.1. A novel analytical method based on a liquid-liquid extraction of polyphenols from rat plasma followed by a highly sensitive LC-ESI-MS/MS analysis was developed.

2.2. With a minimal sample pre-treatment and a short chromatographic run, the method enabled the determination of 16 polyphenols from different classes, namely phenolic alcohols, phenolic acids, flavonoids, secoiridoids and lignans.

2.3. The validation results showed a recovery superior to 80%, no matrix effect, linearity (R>0.99), good precision (RSD<15%) and accuracy (RSD<15%), as well as adequate sensitivity since the LOQ ranged from 0.04 nmol/L for luteolin-7-*O*-glucoside to 2.51 nmol/L for catechol. It was also selective for the different phenolic compounds and no carry-over was observed.

3. Pre-clinical studies: Pharmacokinetics of phenolic compounds in plasma after oral administration of Arbequina table olives to Sprague-Dawley rats.

3.1. Table olives were orally administered to male Sprague-Dawley rats at 3.85 and 7.70 g/kg, which are doses equivalent to the human consumption of 30 and 60 Arbequinas. Analysis of plasma concentrations allowed the determination of *p*-coumaric acid, hydroxytyrosol, luteolin, luteolin-7-*O*-glucoside, salidroside, tyrosol, and verbascoside. Apigenin, oleuropein, pinoresinol, quercetin, and vanillic acid were found at values below the LOQ.

3.2. The pharmacokinetic parameters estimated using a non-compartmental approach indicated a relatively fast absorption of phenolic compounds. Salidroside and verbascoside showed a Tmax of 30 min, luteolin-7-*O*-glucoside was 45 min while tyrosol, *p*-coumaric acid, luteolin and hydroxytyrosol hold values of approximately 1 hour.

3.3. The area under the plasma concentration time curve (AUC) revealed that at both doses

VI. Conclusions

the highest exposure was achieved by *p*-coumaric acid (50%), followed by hydroxytyrosol (23%) salidroside (15%) and tyrosol (7%). The phenolic compounds with the lowest plasmatic concentrations estimated as AUC were verbascoside and luteolin accounting both of them for a 2% while luteolin 7-*O*-glucoside achieved less than 1%.

3.4. Hydroxytyrosol was extensively metabolised yielding two sulfate and two glucuronide metabolites. The analysis of the AUC of hydroxytyrosol and its derivatives, indicated that the two sulfates were the most abundant (~86%), followed by hydroxytyrosol (~10%) and minor amounts of the two glucuronides (~4%).

3.5. The time needed for the plasma concentration of phenolic compounds to decrease by half of the initial value ($t_{1/2z}$) was inferior to 1 hour for salidroside. Hydroxytyrosol and verbascoside hold half-life of 2 hours while luteolin and *p*-coumaric acid were of 3 hours. The phenolic compounds with the longest half-life were luteolin-7-*O*-glucoside and tyrosol with values of 4 and 5 hours, respectively.

4. Clinical trial: Pharmacokinetics of phenolic compounds in plasma after the consumption of Arbequina table olives by healthy human volunteers.

4.1. The developed method was verified in blank human plasma obtaining appropriate recovery and matrix effect (80%−120%), linearity (R>0.99), precision (RSD<15%), accuracy (RSD<15%), and sensitivity, with LOQ ranging from 0.04 nmol/L for luteolin, oleuropein and verbascoside up to 1.58 nmol/L for hydroxytyrosol acetate.

4.2. The application of the validated method to plasma samples obtained after the oral ingestion of 60 and 120 Arbequina table olives enabled the determination of vanillic acid, hydroxytyrosol, salidroside, luteolin, verbascoside, and hydroxytyrosol acetate. Hydroxytyrosol acetate was only found at the dose of 120 olives.

4.3. The Tmax estimated by a non-compartmental analysis showed that vanillic acid and hydroxytyrosol were the compounds with the faster absorption of 30 min, followed by luteolin (60 min). Verbascoside had a Tmax of 75 min whereas for salidroside and hydroxytyrosol acetate it was of 90 min.

4.4. Vanillic acid was the compound with the highest AUC (53%), followed by hydroxytyrosol acetate (17%), hydroxytyrosol (12%), luteolin (9%), salidroside (7%) and verbascoside (2%).

4.5. Hydroxytyrosol in humans had a similar metabolism than in rats, since the percentages obtained by the analysis of the AUC showed that the two sulfates represented ~85%, the parent compound accounted for ~10% whereas the two glucuronides were ~5%. These results evidenced the extensive metabolism underwent by hydroxytyrosol in the humans.

4.6. The analysis of the plasma concentrations by a non-compartmental approach indicated that luteolin, salidroside and hydroxytyrosol acetate had a half-life of 4 hours, vanillic acid of 5 and hydroxytyrosol and verbascoside of approximately 7 hours.

5. Clinical trial: Plasmatic concentrations of phenolic compounds in plasma after the repeated consumption of Arbequina table olives by healthy human volunteers.

5.1. Plasmatic concentrations of phenolic compounds after the daily consumption of 60 Arbequina table olives for 30 days by healthy male and female volunteers were assessed at days 0, 15 and 30. Vanillic acid, catechol, *p*-coumaric acid, quercetin, hydroxytyrosol, hydroxytyrosol acetate, salidroside, apigenin and luteolin were found at concentrations above LOQ. Luteolin-7-*O*-glucoside, pinoresinol, caffeic acid, verbascoside and tyrosol were detected at concentrations below the LOQ.

5.2. No significant differences were observed within the plasmatic concentrations of vanillic acid, catechol, quercetin, salidroside, apigenin. However, plasmatic concentrations of *p*-coumaric acid, hydroxytyrosol with its two sulfate derivatives, luteolin and hydroxytyrosol acetate significantly increased at days 15 and 30 after the repeated intake of 60 Arbequina table olives with respect to the values found at day 0.

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VIII. ANNEX

Article derived from the thesis:

Kundisová, I.; Juan, M.E.; Planas, J.M. Simultaneous determination of phenolic compounds in plasma by LC-ESI-MS/MS and their bioavailability after the ingestion of table olives. *J. Agric. Food Chem.* 2020, *68*(37), 10213–10222. http://doi.org/10.1021/acs.jafc.0c04036.

Simultaneous Determination of Phenolic Compounds in Plasma by LC-ESI-MS/MS and Their Bioavailability after the Ingestion of Table Olives

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ABSTRACT: The role attributed to polyphenols on human health needs to be correlated with their plasmatic concentrations after food consumption. Then, a method based on liquid−liquid extraction followed by highly sensitive LC-ESI-MS/MS analysis was developed to determinate 16 phenolic compounds in plasma. Validation gave appropriate recovery, matrix effect (80%−120%), linear correlation ($R^2 > 0.995$), precision (<15%), LOQ (0.04−2.51 nM), and short chromatographic run. The method was verified after the administration of Arbequina table olives to rats. A single dose of destoned olives was given by gavage, and plasmatic concentrations of polyphenols were analyzed at 30 min. Interestingly, the profile found in plasma greatly differed from that of the olives. Plasmatic concentrations, from highest to lowest, were salidroside, *p*-coumaric acid, hydroxytyrosol, verbascoside, tyrosol, luteolin, and luteolin-7-*O*-glucoside. In conclusion, a simple and robust method was developed, enabling the identification and quantification of unaltered polyphenols in plasma after olives consumption, thus demonstrating its suitability for pharmacokinetics studies.

KEYWORDS: *table olives, polyphenols, LC-ESI-MS/MS, bioavailability, plasma*

INTRODUCTION

Polyphenols constitute a large family of compounds grouped in different classes and are widespread in foods of plant origin, where they are synthesized as secondary metabolites.[1] These phytochemicals elicit health protecting activities such as antioxidant, anti-inflammatory, cardioprotective, anti-tumoral, and neuroprotective, among others.[2] Notwithstanding their potential beneficial effects on health, the studies on the absorption, distribution, and metabolism of polyphenols are mainly focused on certain ones, but few determine the plasmatic concentration of all the phenolic compounds that come from the same food after its ingestion. Additionally, these studies have revealed that the knowledge of the content of polyphenols in foods, although providing valuable nutritional information, does not guarantee a prediction of their bioavailability *in vivo*. Consequently, only when the plasmatic concentrations of each compound are known, it could be possible to establish the relationship between dose and effect and then recommend a daily intake of a determined food. Here, we centered our study on the fruit of *Olea europaea* L., due to its abundant and unique phenolic profile,[3,4] and for being together with olive oil, a source of hydroxytyrosol, an important bioactive compound whose intake at values of 5 mg per day confers protection against cardiovascular disease according to the EFSA health claim.[5]

Several analytical approaches have been established for the determination of polyphenols in plasma after the intake of olive oil; however, they were mainly intended for the study of individual compounds[6,7] or one class of polyphenols.[8,9] Moreover, the results obtained in olive oil cannot be extrapolated to the consumption of table olives, due to the

bioaccessibility from the food matrix, a fact that could imply a different oral bioavailability. There are two studies in the literature describing the plasmatic profile after the intake of Kalamata olives in healthy volunteers.[10,11] Given that hydroxytyrosol is a dopamine metabolite, Goldstein et al.[11] focused their research on the increase of catechols in plasma after the consumption of table olives. This aspect was also considered by Kountouri et al.[10] that extended the research to other polyphenols in olives, mainly phenolic alcohols and acids. However, Kountouri et al.[10] did not include other relevant compounds from table olives, such as luteolin, verbascoside, salidroside, or oleuropein, among others. Consequently, the purpose of the present study was to establish a method that allows the concurrent extraction of 16 polyphenols from different classes in plasma followed by a rapid and sensitive analysis by LC-ESI-MS/MS. The developed procedure was validated following the Guidelines from the European Medicines Agency (EMA).[12] The method was further verified by measuring the plasmatic concentrations of polyphenols after the oral administration of table olives to male Sprague−Dawley rats.

Table 1. MRM Parameters of Each Polyphenol and the Internal Standard Set or Obtained by LC-ESI-MS/MS[a]

compound	retention time (min)	parent ion (m/z)	fragment (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)	fragment function
apigenin	10.58	269.0	117.1	−120	−10	−55	−10	Q
			151.1	−100	−10	−35	−10	I
caffeic acid	7.02	179.1	135.1	−75	−10	−23	−10	Q
			107.1	−75	−10	−30	−15	I
catechol	7.24	109.0	91.2	−80	−10	−28	−10	Q
			65.0	−82	−10	−40	−10	I
p-coumaric acid	8.00	163.2	119.2	−80	−10	−22	−15	Q
			93.2	−80	−10	−45	−15	I
hydroxytyrosol	5.68	153.2	122.8	−78	−10	−20	−10	Q
			94.8	−78	−10	−30	−15	I
hydroxytyrosol acetate	8.78	195.0	59.0	−85	−10	−17	−10	Q
			134.7	−85	−10	−20	−10	I
luteolin	9.73	285.2	133.2	−100	−10	−50	−10	Q
			150.9	−110	−10	−75	−10	I
luteolin-7-O-glucoside	7.47	447.3	285.2	−130	−10	−40	−15	Q
			327.1	−130	−10	−36	−15	I
oleuropein	8.34	539.5	275.0	−109	−10	−30	−10	Q
			307.3	−100	−10	−30	−10	I
(+)-pinoresinol	10.03	357.3	151.1	−97	−10	−27	−10	Q
			136.1	−97	−10	−25	−10	I
quercetin	9.85	301.2	151.1	−110	−10	−30	−10	Q
			179.1	−100	−10	−35	−10	I
rutin	7.18	609.5	300.1	−300	−10	−50	−10	Q
			271.0	−300	−10	−75	−15	I
salidroside	5.70	299.2	119.2	−74	−10	−22	−15	Q
			89.3	−74	−10	−20	−15	I
tyrosol	6.66	137.1	106.2	−70	−10	−20	−15	Q
			118.8	−70	−10	−20	−15	I
vanillic acid	7.11	167.0	152.0	−70	−10	−20	−10	Q
			157.9	−70	−10	−28	−10	I
verbascoside	7.31	623.5	161.3	−140	−10	−50	−10	Q
			461.3	−150	−10	−48	−10	I
2-(3-hydroxyphenyl) ethanol (IS)	7.13	137.0	107.0	−70	−10	−18	−15	Q

[a]DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, collision cell exit potential; Q, quantifier transition; I, identifier transition.

MATERIALS AND METHODS

Chemicals and Reagents. Hydroxytyrosol and hydroxytyrosol acetate were obtained from Seprox BIOTECH (Madrid, Spain). Caffeic acid, catechol, p-coumaric acid, 2-(3-hydroxyphenyl) ethanol (internal standard, IS), oleuropein, pinoresinol, quercetin, rutin, salidroside, and vanillic acid were supplied from Sigma-Aldrich (Tres Cantos, Spain). Apigenin, luteolin, luteolin-7-O-glucoside, tyrosol, and verbascoside were purchased from Extrasynthèse (Genay, France). Acetone, acetonitrile, isopropanol, methanol, and tetrahydrofuran were from Panreac Química SLU (Castellar del Vallés, Spain). Ethyl acetate was from J.T. Baker (Deventer, Netherlands), and glacial acetic acid was obtained from Merck (Darmstadt, Germany). All chemicals were of analytical grade, and the solvents were of LC−MS grade. Ultrapure water was employed in all experiments (Millipore, Milan, Italy).

Animals. Adult male Sprague−Dawley rats weighting 250−300 g (n = 7) were obtained from the breeding colonies from the Animal House Facility at the Facultat de Farmàcia i Ciències de l'Alimentació of the UB. Animals were housed in groups of two per cage under controlled conditions of temperature (22 ± 2 °C), humidity (50 ± 10%), and lighting (12:12 h light−dark cycle). A standard solid diet (2014 Teklad Global 14%, Envigo Rms Spain SLU, Sant Feliu de Codines, Spain) and water were consumed ad libitum. The animal protocol followed in this study was in full accordance with the guidelines established by the European Community for the care and management of laboratory animals. The studies were approved by the Ethic Committee of Animal Experimentation of the Generalitat de Catalunya with reference number 9468.

Working Standards and Calibration Standards. Stock solutions of polyphenols and IS were prepared at 250 μM and stored at −20 °C. Methanol 80% was used in the elaboration of both stock solutions and working standards. Calibration standards were constructed employing plasma withdrawn from overnight fasted animals that did not receive table olives. Aliquots of pooled plasma were kept at −20 °C until analysis. Then, 190 μL of thawed blank plasma was spiked with 10 μL of working standards at 0, 200, 500, 1000, 2000, 3000, and 5000 nM, yielding the final concentrations of 0, 10, 25, 50, 100, 150, and 250 nM. Working and calibration standards were freshly prepared before each experiment.

Extraction Protocol in Plasma Samples. Calibration standards and plasma from rats that were administered table olives (200 μL) were mixed with 10 μL of freshly prepared ascorbic acid (10%, w/v) to a final percentage of 0.5% and 10 μL of acetic acid (1%, v/v) to the final percentage of 0.05%. Moreover, 10 μL of 2-(3-hydroxyphenyl) ethanol (10 μM; IS) was added to a final concentration of 0.50 μM. After vortex-mixing for 2 s, 2 mL of ethyl acetate was added, submitted to vigorous shaking in a vortex for 5 min, placed into an ultrasonic bath for 10 min, and centrifuged at 1500g for 10 min at 2 °C (Megafuge 1.0R, Heraeus, Boadilla, Spain). The supernatant was separated, and a second extraction of the pellet with 2 mL of ethyl acetate was performed. Finally, the ethyl acetate extracts were pooled and 10 μL of 10% ascorbic acid was added prior to the evaporation to dryness at 45 °C using a Concentrator 5301 (Eppendorf Ibérica S.L., San Sebastián de

los Reyes, Spain). The residue was reconstituted by the addition of 100 μL of methanol 80%, (v/v), vortex-mixing for 5 min, an ultrasonic bath for 2 min, and centrifugation at 25 000g for 30 min at 4 °C (Centrifuge 5417R, Eppendorf Ibérica S.L.). The clear supernatant was placed into an amber vial for immediate LC-ESI-MS/MS analysis.

Determination of Polyphenols by Liquid Chromatography Tandem Mass Spectrometry. Polyphenols were analyzed in an Agilent 1260 liquid chromatograph (Agilent Technologies, Santa Clara, CA) coupled to a QTRAP 4000 mass spectrometer (AB Sciex, Toronto, Canada) equipped with a Turbo V electrospray ionization (ESI) source. The instrumentation is located at the Scientific and Technological Centers (CCiTUB). Instrument, data acquisition, and processing were performed with the use of Analyst software version 1.6.2 (AB Sciex).

Vials were kept at 10 °C in a thermostated autosampler until a 2 μL aliquot was injected into a Zorbax Eclipse XDB-C18 (Agilent Technologies) reversed-phase column (150 mm × 4.6 mm, 5 μm) operating at 30 °C. A precolumn of the same material was employed (12.5 mm × 4.6 mm, 5 μm). The mobile phase, delivered at a flow rate of 0.8 mL/min, consisted of solvent A, containing Milli-Q water with 0.025% acetic acid, and solvent B, comprising acetonitrile with 5% acetone. Separation of polyphenols was carried out with the following elution program: 0 min, 95% A and 5% B; 1 min, 90% A and 10% B; 10 min, 35% A and 65% B; 10.5 min, 0% A and 100% B. A period of 5 min of 100% B followed to prevent carry-over before returning to initial conditions. A 6 min delay prior to the next injection was programmed to ensure equilibration of the system. Further carry-over was averted by washing the injector needle with isopropanol, tetrahydrofuran, and Milli-Q water (1:1:1; v/v).

Detection of polyphenols was performed in multiple reaction monitoring (MRM) mode using negative polarity and the following settings: source temperature, 600 °C; curtain gas (N_2), 25 arbitrary units (au); ion source gas 1 (source heating gas, N_2); 50 au; ion source gas 2 (drying gas, N_2); 50 au; and ionization spray voltage, − 3500 V. Table 1 displays the specific parameters of the precursor and the product ions of each analyte obtained by direct infusion of the individual compounds at a concentration of 250 μM dissolved in methanol 80% at a constant flow of 30 μL/min using a Model 11 syringe (Harvard Apparatus, Holliston, MA). The dwell time for the quantifier transition was 60 ms, whereas 10 ms was set for the qualifier transition.

Polyphenols were identified by comparing the retention times of each analyte with those of a standard and considering the mass/charge ratio (m/z) of the precursor ion and the m/z of product ions obtained with the MRM mode (Table 1). Quantification of polyphenols was performed by interpolation of the peak area ratio of polyphenols versus IS on a calibration curve prepared with calibration standards. Within each analytical run, a full set of calibration standards, which included reagent blank and blank plasma, were injected.

Method Validation. The developed analytical method was validated following the guidelines on bioanalytical method validation established by EMA.[12] Validation was performed in three consecutive days using three different batches of blank plasma used to prepare calibration standards in triplicate for each concentration.

Matrix Effect and Recovery. Matrix effect and recovery were evaluated with calibration standards prepared at 25, 100, and 250 nM. The matrix effect (%) was determined by comparing the peak areas of individual polyphenols and IS spiked in extracted blank rat plasma at the expected concentrations at the final volume with those prepared in methanol 80%. Recoveries (%) of polyphenols and IS were calculated by comparing the peak areas of analytes spiked in blank plasma before extraction to those that were spiked after extraction.

Limit of Quantification. The limit of quantification (LOQ) was the concentration of analyte that yielded a signal five times the signal of a blank sample.[12] The LOQ was validated by carrying out the analysis of six independent blank plasma samples spiked with standards of polyphenols at concentrations proximal to the theoretical ones, and their precision and accuracy were below the 20% recommended by the EMA.[12]

Linearity. Linearity was evaluated in the range of application of the analytical method at 0, 10, 25, 50, 100, and 150 nM. The calibration curves were constructed by plotting the peak area ratio of the

polyphenols to the internal standard against the concentration of analytes. The reproducibility of the method was evaluated by comparing the linear regressions of three standard plots prepared during three different days.

Precision and Accuracy. Precision and accuracy were determined by replicate analysis ($n = 5$) of samples at six concentrations levels of 10, 25, 50, 100, 150, and 250 nM. The precision was calculated as CV (%) within a single run (intra-assay) or in three different days (interassay). The accuracy was assessed as the bias or percentage deviation between the nominal and measured concentrations. Precision and accuracy should not exceed 15%.[12]

Selectivity. Selectivity was assessed to evaluate if the extraction procedure was able to differentiate individual polyphenols and IS from endogenous compounds in plasma. Thus, six independent double blank plasma samples containing neither analyte nor IS were compared with the ones spiked with polyphenols at 150 nM and IS at 500 nM.

Carry-Over. The carry-over was routinely assessed six times in each analytical run by sequentially injecting the highest calibration standard followed by blank reagents at regular intervals on the basis of the total number of samples per batch. In addition to this, two independent blank plasma samples were also programmed before the analysis of the first sample.

Oral Administration of Arbequina Table Olives to Rats. Table olives of the Arbequina variety (Cooperativa del Camp, Maials, Lleida, Spain) harvested during the season 2015/2016 and debittered following a natural fermentation in brine were used. The dose administered to Sprague−Dawley rats was 7.70 g of destoned olive/kilogram of body weight, which is equivalent to the consumption of 60 Arbequina table olives by a 60 kg person, calculated by following the body surface area normalization method suggested by Reagan-Shaw et al.[13] Arbequina table olives were administered as a homogeneous suspension. Hence, olives were destoned and mixed with Milli-Q water prior to being carefully grinded using a Polytron homogenizer (Kinematica AG, Lucerne, Switzerland) to which a 20 TS arm had been coupled. The process consisted of six cycles of 30 s at a speed set at 5 with breaks of 1 min between each cycle.

The freshly prepared homogeneous suspension of table olives was orally administered to overnight fasted Sprague−Dawley rats by gavage (18-gauge × 76 mm, ref FFSS-185-76, Instech Laboratories, Inc., Plymouth Meeting, PA) at a volume of administration of 10 mL/kg. Blood was collected from the saphenous vein at times 0 and 30 min and placed in 0.3 mL Microvette CB 300 tubes coated with EDTA-K_2 (Sarstedt). Plasma was immediately obtained by centrifugation at 1500g for 15 min at 4 °C (Megafuge 1.0R) and stored at −20 °C until analysis.

Determination of Polyphenols in Arbequina Table Olives by LC-ESI-MS/MS. Polyphenols in Arbequina olives were analyzed following the method previously described by Moreno-González et al.[3] Briefly, 1 g of the olive suspension was mixed with 6 mL of methanol−ethanol (1:1; v/v) containing 2-(3-hydroxyphenyl) ethanol as IS and vigorously stirred in the vortex for 5 min, followed by centrifugation at 3345g for 30 min at 4 °C (Megafuge 1.0R). The supernatant was removed, and two additional extractions of the pellet were carried out with 3 mL of methanol−ethanol (1:1; v/v). The three supernatants were pooled and centrifuged at 25 000g for 30 min at 2 °C (Centrifuge 5417R) prior to determination by LC-ESI-MS/MS, performed as described for plasma with the only difference being the injection volume set at 10 μL.

Statistical Analysis. Data were expressed as mean ± standard error of the mean (SEM). The concentration of polyphenols was expressed as nanomolar. Chauvenet's criterion was applied to discard outliers. Data evaluation, statistical analysis, and elaboration of the graph were carried out in Prism version 6 (GraphPad Software Inc., San Diego, CA).

■ RESULTS

Setting-up the Method for the Extraction of Polyphenols in Plasma. The development of the analytical method was carried out using blank plasma spiked with pure compounds at 250 nM, and for every examined condition, at least three independent calibration standards were tested for recovery and

Table 2. Matrix Effect and Recovery in Blank Rat Plasma Samples Spiked at Three Concentrations of Polyphenols and Analyzed by LC-ESI-MS/MS

compound	25 nM		100 nM		250 nM	
	matrix effect (%)	recovery (%)	matrix effect (%)	recovery (%)	matrix effect (%)	recovery (%)
apigenin	95.7 ± 10.5	77.7 ± 5.8	86.7 ± 9.9	78.9 ± 2.1	80.7 ± 3.7	75.7 ± 1.9
caffeic acid	112.3 ± 7.9	66.6 ± 1.9	116.2 ± 5.5	64.3 ± 3.2	110.8 ± 0.4	59.9 ± 2.3
catechol	109.1 ± 4.1	81.2 ± 1.7	104.7 ± 5.0	83.4 ± 2.5	90.6 ± 2.2	81.8 ± 0.7
p-coumaric acid	118.8 ± 2.9	78.2 ± 3.3	95.4 ± 8.2	84.4 ± 2.3	85.3 ± 2.2	78.2 ± 1.7
hydroxytyrosol	98.7 ± 2.1	91.3 ± 3.2	100.5 ± 3.5	100.5 ± 2.5	96.0 ± 1.3	91.0 ± 2.3
hydroxytyrosol acetate	92.7 ± 6.0	78.3 ± 1.9	87.7 ± 3.9	77.5 ± 3.7	82.1 ± 1.6	77.4 ± 3.0
luteolin	114.1 ± 4.5	80.0 ± 2.0	107.7 ± 3.1	80.1 ± 1.8	92.7 ± 4.3	80.6 ± 1.3
luteolin-7-*O*-glucoside	118.4 ± 6.2	85.6 ± 2.6	114.4 ± 4.0	85.1 ± 3.2	100.6 ± 1.7	87.5 ± 2.0
oleuropein	103.7 ± 3.8	87.1 ± 3.5	105.4 ± 2.5	89.3 ± 1.4	96.2 ± 0.1	84.5 ± 2.0
(+)-pinoresinol	83.5 ± 10.7	83.4 ± 2.3	99.9 ± 3.3	82.5 ± 3.1	80.8 ± 1.0	80.3 ± 1.4
quercetin	114.0 ± 9.9	79.4 ± 2.6	104.1 ± 7.5	72.4 ± 2.5	84.8 ± 4.1	70.6 ± 0.7
rutin	118.1 ± 7.4	56.4 ± 1.9	112.2 ± 6.9	62.9 ± 5.8	94.1 ± 1.9	64.4 ± 1.7
salidroside	99.9 ± 7.8	48.6 ± 0.7	92.9 ± 2.3	54.7 ± 2.7	90.4 ± 0.3	53.6 ± 1.4
tyrosol	99.5 ± 2.9	89.3 ± 2.8	88.5 ± 2.7	84.0 ± 1.6	88.8 ± 1.0	91.0 ± 3.9
vanillic acid	114.3 ± 7.1	84.3 ± 2.5	113.6 ± 4.7	91.8 ± 2.8	100.5 ± 1.3	80.7 ± 3.1
verbascoside	119.5 ± 7.9	85.8 ± 11.0	119.4 ± 3.1	73.3 ± 1.2	93.7 ± 2.5	72.5 ± 2.8
2-(3-hydroxyphenyl) ethanol (IS)	108.6 ± 5.1	81.5 ± 1.9	99.6 ± 3.3	93.2 ± 7.4	91.7 ± 7.3	80.1 ± 1.2

matrix effect. In the first place, ethanol−methanol (1:1; v/v), acetonitrile 100%, and methanol 100% were assayed as protein precipitants, whereas ethyl acetate 100% was investigated as a liquid−liquid extraction solvent to achieve the maximum recoveries and the lowest endogenous interferences. Although methanol 100% and ethanol−methanol (1:1; v/v) yielded good recovery for most of polyphenols, they were both discarded due to a matrix effect that indicated a poor ionization. Acetonitrile 100% did not exert such a strong suppression of the ionization, but recoveries dropped and this solvent was also dismissed. An improvement of matrix effect and recovery was observed for all the polyphenols when ethyl acetate was used as a solvent, with values ranging from 50% to 80% for both variables. Therefore, liquid−liquid extraction with ethyl acetate was selected for the following steps of the method development owing to its good recovery and matrix effect.

Second, the impact of acidification in the disruption of the bonding of polyphenols to plasma proteins was assessed with formic acid at 0.05% and 0.5% as well as acetic acid at 0.05%, 0.5%, 1%, 2.5%, and 10%. Formic acid at 0.05% gave good recovery of 88.7 ± 7.7% only for tyrosol, decreasing to 40%−70% for apigenin, *p*-coumaric acid, hydroxytyrosol, luteolin, luteolin-7-*O*-glucoside, oleuropein, pinoresinol, and vanillic acid, and dropping to 5.4 ± 0.5% for caffeic acid. No matrix effect was observed for apigenin, *p*-coumaric, hydroxytyrosol, hydroxytyrosol acetate, luteolin-7-*O*-glucoside, pinoresinol, and tyrosol, but the rest of the polyphenols yielded an increase of ionization of around 140%. The use of higher concentrations of formic acid did not enhance either recovery or matrix effect and were discarded. On the contrary, acetic acid improved the recoveries for hydroxytyrosol, but in an inverse proportion to the amount of acid added, the lowest concentration of 0.05% yielded a recovery of 91.2% that dropped to 78.8% when 10% was used. When acetic acid was applied at 1%, the recovery for hydroxytyrosol was higher than 90% and those of the other polyphenols were between 75% and 90%, except for caffeic acid, rutin, and salidroside, which gave values of 64.0%, 58.1%, and 49.8%, respectively. Better recoveries could be achieved for rutin (77.6%) and caffeic acid (75.7%) using acetic acid at 10%, whereas salidroside reached a value of 66.9% when 2.5% acetic

acid was used. However, the use of these amounts of acetic acid was ruled out since it was detrimental to the recovery of most polyphenols, and 1% was the selected amount. These conditions were also adequate to avoid matrix effect, since all the polyphenols had values ranging from 80% to 110%.

The impact of acidification was evaluated concurrently with the assessment of the application of antioxidants to protect polyphenols or sonication to enhance the extraction efficiency. The use of ascorbic acid as an antioxidant was evaluated at 1% and 10%, and the best results were obtained when the highest concentration was added. Also, recoveries improved when ascorbic acid was placed twice: 10 μL to the plasma sample prior to the extraction and 10 μL to the solvent to prevent degradation of polyphenols during the evaporation to dryness. Moreover, the use of an ultrasonic bath for 10 min after vortex-mixing was incorporated to the process since it improved the extraction efficiency. Finally, the influence of the volume of organic solvent was also investigated, being evaluated at the addition of 1.5, 4, and 6 mL to 200 μL of rat plasma. The use of 1.5 mL of solvent decreased the recovery of hydroxytyrosol by a 30% and matrix effect was superior to 140% in comparison with the results obtained when the volume of 6 mL was employed, which gave the best results for all the polyphenols.

Optimization of the Liquid Chromatography Tandem Mass Spectrometry Conditions. The influence of the temperature of the ESI source in the LOQ of polyphenols in plasma samples was investigated. Hence, a standard of polyphenols at 1 μM dissolved in methanol 80% was injected at 350, 400, 450, 500, and 600 °C, and the best signal intensity was achieved at 600 °C. Moreover, different ionization spray voltages (−3000, −3500, −4000, and −4500 V) as well as several ion source gas 2 conditions (25, 50, and 70 arbitrary units) were evaluated, with ion source gas 1 fixed at 50 arbitrary units. The best intensity of the peaks was obtained when the ionization spray voltage was set at −3500 V and the ion source gas 2 was established at 50 arbitrary units. In addition to the optimization of the mass spectrometer, the liquid chromatography conditions were also tuned. Different elution programs were evaluated, and the best selectivity was obtained when the initial percentage of aqueous phase was 95%. Also, injection

volumes of 2, 5, and 10 μL were assessed, and an enhanced peak performance was achieved when 2 μL was used.

Method Validation. *Matrix Effect and Recovery.* No matrix effect was found since the results were within 80%−120% (Table 2). More importantly, the variability of all concentrations evaluated as coefficient of variation (CV), expressed as a percentage, was below 15%, meeting the requirements established by the EMA.[12] Table 2 shows the recoveries (%) for individual polyphenols. Hydroxytyrosol was the analyte with the highest value, with an average of 93.6 ± 2.0% for the three concentrations evaluated. The method also yielded good recoveries for tyrosol (87.9 ± 1.7%), oleuropein (87.1 ± 1.6%), luteolin-7-*O*-glucoside (86.0 ± 1.5%), and vanillic acid (85.9 ± 1.9%). Then, pinoresinol, catechol, *p*-coumaric acid, and luteolin showed recoveries of approximately 81%, whereas apigenin, hydroxytyrosol acetate, verbascoside, and quercetin displayed an acceptable 77%. The lowest recoveries were obtained for caffeic acid (64.0 ± 1.5%), rutin (58.1 ± 2.0%), and salidroside (49.8 ± 1.3%). 2-(3-Hydroxyphenyl)-ethanol gave an average recovery of 85.0 ± 2.9%, which is a value similar to those of most of the polyphenols, thus confirming its adequacy as IS.

Limit of Quantification. The limit of quantification ranged from 0.04 to 2.51 nM (Table 3) for all the analyzed polyphenols.

Linearity. The results obtained indicated that the analytical method was linear with correlation coefficients (R^2) greater than 0.9954 for all the compounds (Table 3).

Table 3. Linearity and Sensitivity of Polyphenols in Blank Rat Plasma Samples Analyzed by LC-ESI-MS/MS

compound	linearity equations	R^2	sensitivity LOQ (nM)
apigenin	$y = (0.00259 \pm 0.00014)x + (-1.12 \times 10^{-7} \pm 1.35 \times 10^{-7})$	0.9985	0.15
caffeic acid	$y = (0.00341 \pm 0.00092)x + (3.31 \times 10^{-4} \pm 8.50 \times 10^{-3})$	0.9993	2.01
catechol	$y = (0.00027 \pm 0.00008)x + (5.59 \times 10^{-4} \pm 5.59 \times 10^{-4})$	0.9997	2.51
p-coumaric acid	$y = (0.00081 \pm 0.00015)x + (-5.05 \times 10^{-7} \pm 4.07 \times 10^{-7})$	0.9979	1.75
hydroxytyrosol	$y = (0.00287 \pm 0.00022)x + (2.21 \times 10^{-6} \pm 2.43 \times 10^{-6})$	0.9981	0.19
hydroxytyrosol acetate	$y = (0.00138 \pm 0.00125)x + (3.67 \times 10^{-3} \pm 3.62 \times 10^{-3})$	0.9975	0.12
luteolin	$y = (0.01245 \pm 0.00579)x + (1.25 \times 10^{-3} \pm 1.25 \times 10^{-3})$	0.9990	0.12
luteolin-7-*O*-glucoside	$y = (0.01616 \pm 0.00735)x + (1.34 \times 10^{-6} \pm 1.38 \times 10^{-6})$	0.9992	0.04
oleuropein	$y = (0.00466 \pm 0.00164)x + (6.61 \times 10^{-3} \pm 6.62 \times 10^{-3})$	0.9997	0.06
(+)-pinoresinol	$y = (0.00195 \pm 0.00041)x + (2.00 \times 10^{-3} \pm 1.45 \times 10^{-3})$	0.9994	0.32
quercetin	$y = (0.00401 \pm 0.00199)x + (5.08 \times 10^{-4} \pm 3.85 \times 10^{-3})$	0.9978	0.40
rutin	$y = (0.00204 \pm 0.00059)x + (4.08 \times 10^{-3} \pm 4.07 \times 10^{-3})$	0.9991	0.11
salidroside	$y = (0.00060 \pm 0.00019)x + (9.44 \times 10^{-4} \pm 9.44 \times 10^{-4})$	0.9979	0.63
tyrosol	$y = (0.00012 \pm 0.00002)x + (4.06 \times 10^{-4} \pm 2.36 \times 10^{-4})$	0.9954	1.95
vanillic acid	$y = (0.00074 \pm 0.00001)x + (-1.09 \times 10^{-6} \pm 3.30 \times 10^{-7})$	0.9990	1.08
verbascoside	$y = (0.00328 \pm 0.00176)x + (2.74 \times 10^{-7} \pm 2.01 \times 10^{-7})$	0.9987	0.06

Precision and Accuracy. The performance of the data on the assay is shown in Table 4. The results obtained for intraday and interday precision as well as accuracy were lower to 15% for all the studied polyphenols.

Selectivity. Figure 1 shows the absence of peaks in blank plasma at the retention times of the analytes, as displayed in the superposed chromatograms depicting blank plasma spiked with standards. Moreover, the developed method enables the selective separation of 16 polyphenols in a short chromatographic run. Under our experimental conditions, hydroxytyrosol at 5.68 min (*m/z* 153.2 → 122.8) and salidroside at 5.70 min (*m/z* 299.2 → 119.2) were the polyphenols that eluted in first place, whereas the last was apigenin (*m/z* 117.1 → 151.1) at 10.58 min. Tyrosol holds precursor and product ions of 137.10 and 106.0, which are similar to the transitions of the IS (*m/z* 137.00 → 107.00). However, both compounds hold different retention times of 6.66 min for tyrosol and 7.13 min for 2-(3-hydroxyphenyl)-ethanol, ensuring their adequate determination.

Carry-Over. No peaks of the analytes or the IS were found in the blank reagents analyzed immediately after the highest calibration standard.

Content of Polyphenols in Arbequina Table Olives. LC-ESI-MS/MS analysis of olive samples showed the presence of 16 compounds from five different classes, namely, phenolic alcohols, phenolic acids, flavonoids, secoiridoids, and lignans, which accounted for a total of 954 mg of polyphenols per kilogram of destoned olives (Table 5). Hydroxytyrosol was the most abundant compound in Arbequina table olives, with concentrations of 764 ± 9 mg/kg that represented 80.1% of all the polyphenols evaluated. The following compounds were luteolin, tyrosol, verbascoside, and vanillic acid at 81.4 ± 3.2, 28.6 ± 1.8, 26.6 ± 2.7, and 12.3 ± 0.55 mg/kg, respectively, which accounted for 8.53%, 3.00%, 2.79%, and 1.29%. Salidroside, apigenin, hydroxytyrosol acetate, quercetin, and catechol were found at concentrations ranging from 9.29 ± 0.13 to 4.53 ± 0.28 mg/kg, with percentages ranging from 1% to 0.5%. Luteolin-7-*O*-glucoside, pinoresinol, oleuropein, rutin, caffeic acid, and *p*-coumaric acid yielded values lower than 0.2%.

Concentrations of Polyphenols in Plasma after the Administration of Arbequina Table Olives. Figure 1 depicts the chromatograms obtained after the oral administration of Arbequina table olives in comparison to the ones from blank and spiked plasmas. Blank plasma samples were checked for the presence of analytes, and apigenin, *p*-coumaric acid, luteolin, luteolin-7-*O*-glucoside, salidroside, vanillic acid, and verbascoside were found in low concentrations. The values attained in blank plasma were subtracted from the ones obtained in plasma withdrawn 30 min after the administration of table olives. Hence, from the 16 polyphenols detected in olives, only 7 compounds were found above the LOQ. Chromatograms showed peaks below the LOQ for apigenin, oleuropein, pinoresinol, quercetin, and vanillic acid, whereas caffeic acid, catechol, hydroxytyrosol acetate, and rutin were not detected.

Plasmatic concentrations of polyphenols at 30 min postadministration are represented in Figure 2. The main polyphenol was salidroside with concentrations of 165.9 ± 28.5 nM at 30 min after the intake of olives. This compound was followed by *p*-coumaric acid (49.2 ± 6.0 nM) and hydroxytyrosol (45.0 ± 6.7 nM). Verbascoside and tyrosol were found at approximately 10 nM, whereas luteolin and luteolin-7-*O*-glucoside gave concentrations lower than 5 nM.

Table 4. Intraday and Interday Precision and Accuracy of Polyphenols in Spiked Rat Blank Plasma Samples Analyzed by LC-ESI-MS/MS

compound	intraday precision (% RSD) concentration (nM)						interday precision (% RSD) concentration (nM)						accuracy (%) concentration (nM)					
	10	25	50	100	150	250	10	25	50	100	150	250	10	25	50	100	150	250
apigenin	6.91	9.70	4.82	4.12	2.91	8.18	6.71	8.19	4.27	5.37	3.05	9.91	−2.23	0.64	−1.99	−2.20	3.52	0.00
caffeic acid	5.56	6.38	6.35	5.69	3.43	7.31	7.33	2.25	2.18	2.73	2.64	5.38	−8.82	2.23	0.71	−0.11	0.69	1.90
catechol	8.23	6.65	7.06	7.19	1.12	6.60	7.78	5.20	6.26	6.39	1.08	5.00	−13.79	−4.50	−2.21	−0.57	7.81	0.00
p-coumaric acid	8.06	9.39	6.42	8.10	1.14	6.40	5.97	8.52	2.28	6.34	1.00	6.01	3.06	−1.06	−2.67	−0.50	1.22	0.00
hydroxytyrosol	9.36	9.92	9.03	4.22	1.79	3.46	12.18	7.64	4.01	7.77	1.67	3.78	−3.12	1.40	2.79	−4.19	1.10	0.00
hydroxytyrosol acetate	6.68	7.33	4.12	9.56	2.38	7.69	9.80	9.60	1.98	8.85	1.62	8.55	−0.88	−1.31	0.94	3.17	−0.32	0.00
luteolin	6.01	8.45	6.38	9.27	7.42	5.50	3.80	8.58	5.12	8.02	7.11	6.43	−0.99	1.99	1.57	−1.23	5.73	0.00
luteolin-7-*O*-glucoside	7.93	6.46	2.57	6.73	3.35	3.25	8.26	5.38	2.14	8.24	3.51	3.49	−0.42	7.83	−2.92	−1.25	3.32	0.00
oleuropein	7.96	8.29	3.16	8.63	6.92	4.29	9.59	7.74	2.58	6.37	5.13	4.06	−3.37	0.11	0.58	−1.71	2.91	0.88
(+)-pinoresinol	8.62	8.45	2.86	5.95	4.61	9.23	6.47	5.56	2.12	4.63	3.61	8.40	−8.34	8.67	−0.91	−1.59	4.65	0.00
quercetin	8.44	5.66	8.32	6.74	5.95	7.76	4.62	4.30	6.53	3.35	7.23	8.43	−5.92	−0.11	5.70	0.38	−1.30	−3.94
rutin	9.63	8.78	4.31	7.20	3.03	7.70	9.46	9.41	3.84	8.83	3.06	7.12	4.38	3.14	−6.79	−0.44	2.00	2.12
salidroside	14.91	7.67	7.10	9.90	8.58	4.82	14.08	9.12	8.43	6.47	9.49	3.41	3.48	2.82	−3.25	−5.66	−2.18	0.00
tyrosol	9.03	9.94	9.25	3.03	0.65	6.94	11.23	9.59	9.72	7.06	4.82	6.04	−1.28	−0.21	−6.42	−2.72	4.83	0.00
vanillic acid	6.79	6.80	3.27	6.39	2.51	0.62	4.44	5.80	3.06	5.48	2.63	0.58	12.31	0.39	−1.69	−1.90	2.12	0.00
verbascoside	6.39	9.75	5.26	9.63	1.34	5.69	7.17	4.92	5.30	7.69	1.01	4.43	−1.34	0.51	4.34	4.95	0.24	0.00

■ DISCUSSION

The determination of polyphenols in plasma involves several critical processes, one of them being the extraction before LC-ESI-MS/MS analysis. Concerning this phase, the selection of the solvent is essential. Here, we have assayed different solvents commonly used, such as methanol 100%, acetonitrile 100%, methanol–ethanol (1:1; v/v), and ethyl acetate.[14−17] Among them, we selected ethyl acetate modified with 1% acetic acid, which offers a higher extraction efficiency, much cleaner final sample, a lower background noise in the LC-ESI-MS/MS analysis, and a fast extraction process due to the low boiling temperature of ethyl acetate that allows for a rapid evaporation to dryness at 45 °C. In addition, the incorporation of ascorbic acid twice, at the beginning of the extraction process and before the evaporation to dryness, as previously indicated,[6,17] ensured the stability of polyphenols. All these characteristics lead to a robust extraction method. Once the polyphenols were extracted, they were analyzed by LC-ESI-MS/MS with a short chromatographic run and the method was validated following the EMA.[12] The results confirmed the adequacy of the developed experimental conditions given the satisfactory results obtained in terms of matrix effect, recovery, precision, accuracy, linearity, and sensitivity.

Compared to the other techniques reported up to now in the literature for the analysis of polyphenols in plasma, in the present method, the isolation of analytes is characterized by a rapid liquid–liquid extraction, avoiding the use of solid-phase extraction, as was proposed for the analysis of olive oil phenolics in plasma.[8,9] Moreover, our process consists of the direct injection of polyphenols into the LC–MS instrument, avoiding the derivatization step applied by Pastor et al.[9] using LC–MS or by gas chromatography where it is a requirement to detect these compounds.[7,10] Besides, the technique proposed here provides a low LOQ, especially for hydroxytyrosol, which is 0.19 nM, an order of magnitude below the ones found using other methods.[8,9] Suárez et al.[18] described a method that allows the determination of 10 olive oil polyphenols from different groups including luteolin, apigenin, and pinoresinol in plasma. However, the method exhibits LOQ values of 0.1, 0.5, and 4.8

μM for luteolin, hydroxytyrosol, and tyrosol, respectively, while we found LOQ values of 0.12, 0.19, and 1.95 nM for the same compounds. Until now, only one procedure that allows the determination of a higher number of polyphenols from different classes has been described by Achaintre et al.[19] Although 38 dietary polyphenols can be analyzed, the technique holds several limitations that have been exposed by the same authors, such as the requirement for costly labeled reagents and the partial degradation of flavonols during the dansylation reaction.[19] Then, the use of multiclass methodology is limited, and the instrumentation required is not easily accessible to all the laboratories.

The developed analytical conditions were further verified *in vivo* by determining the plasmatic concentrations of polyphenols after the oral administration of table olives to Sprague–Dawley rats. Olives were used to substantiate the method due to the interesting polyphenol content of this food, which has largely been overlooked even though it is an important source of nutrients and non-nutrients.[20] The Arbequina variety was selected due to its high content of polyphenols.[4] Considering the content of polyphenols in the Arbequina table olives (954 mg/kg) and the administered amount, the animals received a dose of 7.36 mg of polyphenols/kg of rat body weight. The doses calculated for the more abundant polyphenols were 5.89, 0.63, 0.22, 0.20, 0.09, and 0.07 mg/kg of rat body weight for hydroxytyrosol, luteolin, tyrosol, verbascoside, vanillic acid, and salidroside, respectively. These 6 polyphenols represented 96.6% of the total present in table olives, while the other 10 constituted only 3.4%. Interestingly, the phenolic profile found in plasma 30 min after the administration of table olives differed from the original content, and only 7 compounds from the 16 in the food were above the limit of quantification. The plasmatic concentrations from highest to lowest were salidroside (165.9 nM), *p*-coumaric acid (49.2 nM), hydroxytyrosol (45.0 nM), verbascoside (9.95 nM), tyrosol (8.07 nM), luteolin (4.41 nM), and luteolin-7-*O*-glucoside (1.48 nM). These values represent 2.80%, 8.65%, 0.0045%, 0.11%, 0.021%, 0.006%, and 0.15% from the ingested amount, respectively. These percentages reflect the complexity of the processes involved in bioavailability since the main compounds in table olives were not the ones with the
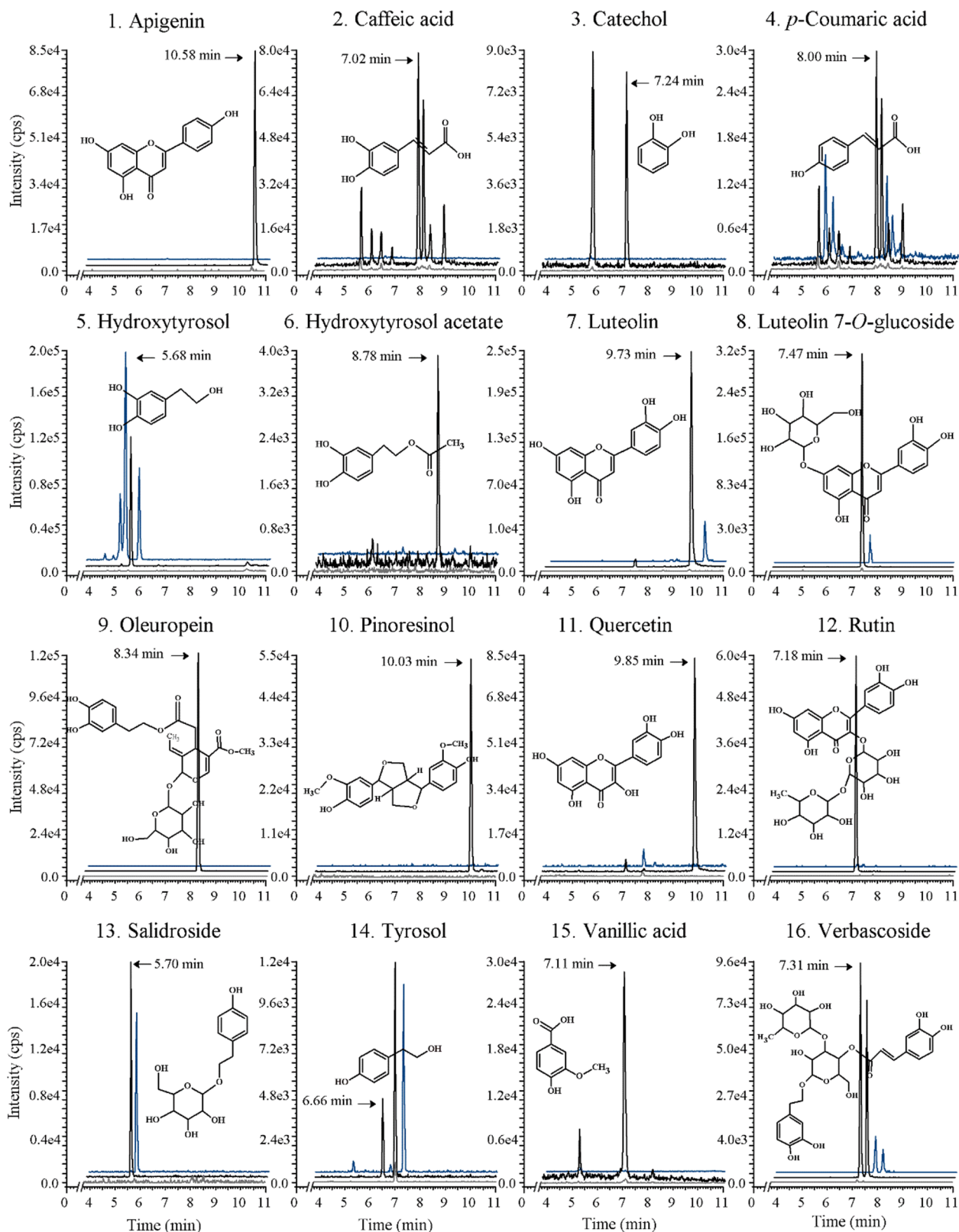
Figure 1. Representative LC-ESI-MS/MS chromatograms obtained in multiple reaction monitoring mode (MRM) of Sprague−Dawley rats blank plasma (gray trace), blank plasma spiked with standards of polyphenols at 150 nM (black trace), and plasma obtained 30 min after the oral administration of a dose equivalent to the human intake of 60 Arbequina table olives (blue trace).

highest plasmatic concentrations. Upon consumption, the fruit of *Olea europaea* L. faces gastrointestinal digestion that releases polyphenols, which has been simulated *in vitro* for table olives with bioaccessibilities of 100%, 86%, 56%, and 7% for tyrosol,

hydroxytyrosol, verbascoside, and luteolin, whereas apigenin and hydroxytyrosol acetate were not detected.[21] It has been also proposed that different polyphenols like oleuropein, ligstroside, comselogoside, verbascoside, salidroside, or different glucosides

Table 5. Determination of Polyphenols by LC-ESI-MS/MS: Concentrations of Polyphenols in Arbequina Table Olives, Dose Administered to Rats and Amount Found in Plasma at 30 min after the Oral Administration

compound	polyphenols in Arbequina table olives			polyphenols in rat plasma	
	mg/kg destoned olive[a]	mg/kg rat body weight[b]	μg in olives[c]	μg in plasma	% in plasma
apigenin	6.77 ± 0.50	0.052	15.3 ± 0.48	<LOQ	
caffeic acid	0.48 ± 0.01	0.004	1.08 ± 0.03		
catechol	4.53 ± 0.28	0.035	10.2 ± 0.32		
p-coumaric acid	0.53 ± 0.03	0.004	1.20 ± 0.04	0.10 ± 0.01	8.65 ± 0.84
hydroxytyrosol	764 ± 9.47	5.891	1725 ± 54.49	0.08 ± 0.01	0.0045 ± 0.0008
hydroxytyrosol acetate	6.67 ± 0.22	0.051	15.1 ± 0.48		
luteolin	81.4 ± 3.17	0.628	183 ± 5.81	0.01 ± 0.003	0.006 ± 0.002
luteolin-7-O-glucoside	2.28 ± 0.19	0.018	5.15 ± 0.16	0.008 ± 0.003	0.15 ± 0.05
oleuropein	1.96 ± 0.21	0.015	4.43 ± 0.14	<LOQ	
pinoresinol	2.33 ± 0.12	0.018	5.26 ± 0.17	<LOQ	
quercetin	5.05 ± 0.33	0.039	11.4 ± 0.36	<LOQ	
rutin	1.47 ± 0.06	0.011	3.32 ± 0.10		
salidroside	9.29 ± 0.13	0.072	21.0 ± 0.66	0.59 ± 0.11	2.80 ± 0.48
tyrosol	28.6 ± 1.77	0.221	64.7 ± 2.04	0.01 ± 0.002	0.02 ± 0.003
vanillic acid	12.3 ± 0.55	0.095	27.8 ± 0.88	<LOQ	
verbascoside	26.6 ± 2.74	0.205	60.0 ± 1.89	0.07 ± 0.01	0.11 ± 0.02

[a]Results are expressed as mean ± SEM of three independent samples analyzed by triplicate. [b]Dose of polyphenols administered to Sprague–Dawley rats. [c]Amount of each polyphenol administered to Sprague–Dawley rats.
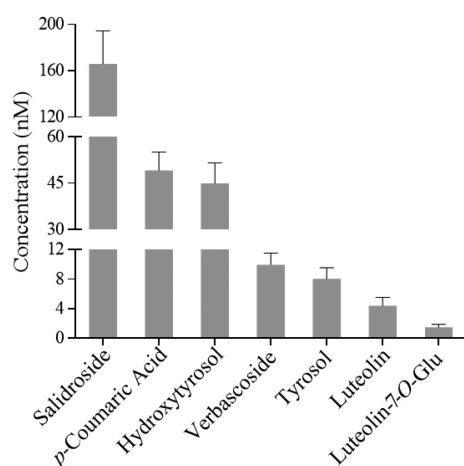

Figure 2. Concentrations of polyphenols in plasma at 30 min after oral administration to Sprague–Dawley rats of a dose equivalent to the human intake of 60 Arbequina table olives. Values are presented as mean ± SEM.

could be hydrolyzed under experimental conditions like the ones occurring during the transit through the stomach, leading to an increase of p-coumaric acid, hydroxytyrosol, and tyrosol, among others.[21−23] Once in the intestine, the uptake of polyphenols takes place through different mechanisms, which may involve active transport, as indicated for p-coumaric acid, ensuring a high bioavailability,[24] or by simple diffusion, as described for salidroside, hydroxytyrosol, tyrosol, and verbascoside,[22,25−27] which is a less efficient process. Flavonoid glucosides have been thought to be deglycosylated by the luminal lactase phlorizin hydrolase and the aglycon absorbed by passive diffusion. Instead, luteolin-7-O-glucoside is found in a low concentration in plasma, and different authors suggested the possible implication of the intestinal sodium/glucose cotransporter.[26,28] Moreover, we cannot underestimate the possible metabolism suffered in the enterocytes[22,26] and the efflux to the intestinal lumen though ABC protein transporters,[27] which affect the bioavailability of different polyphenols. In this regard, despite

hydroxytyrosol being the main polyphenol in the Arbequina variety, the plasmatic concentrations of the free compounds are low, as described previously after the intake of Kalamata table olives by healthy volunteers, a fact attributed to extensive presystemic metabolism.[10,11]

Concerning the polyphenols that we have not detected in plasma, it is noteworthy that they only represented 3.4% of the dose administered, and in this case, apigenin, vanillic acid, oleuropein, pinoresinol, and quercetin were identified but were not quantified since the concentrations found were below the LOQ. These results can be explained, aside from their small values in table olives, different experiments performed in vitro showed a low stability during the digestion process[21,29] along with a low oral bioavailability.[22,30] These results were also corroborated by Kountouri et al.,[10] who could not detect quercetin, vanillic acid, or caffeic acid after the consumption of Kalamata olives.

The assessment of the results obtained in the present study demonstrates that the oral bioavailability of polyphenols from table olives are subdued to different factors, such as the release of polyphenols from the food matrix postingestion and their bioaccessibility in the gastrointestinal track where different mechanisms of absorption may occur depending on the hydrophilic or lipophilic nature of the analytes, a process in which intestinal transporters may be involved as well as metabolizing enzymes.[31] All these processes have driven the results such as the finding that hydroxytyrosol, which is the main polyphenol in table olives, did not achieve the highest plasmatic concentrations and conversely p-coumaric acid was unexpectedly found highly bioavailable. Overall, the fact that the polyphenols accounting for a 96.6% of the content in olives were found unaltered in plasma revealed this food as an important source of bioactive compounds relevant to the prevention of chronic diseases.

In conclusion, we have developed a fast, sensitive, and reliable method that enables the identification and quantification of phenolic alcohols, phenolic acids, flavonoids, secoiridoids, and lignans in plasma, which should contribute to improving our understanding of the role of these phytochemicals from table

olives in human health. Furthermore, the results indicate that the developed and validated method could be applied for the evaluation of pharmacokinetics in preclinical and clinical studies, not only after the administration of table olives but also for other foods containing polyphenols from different classes.

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