



UNIVERSITAT_{DE}
BARCELONA

Health-promoting phenolic compounds in extra virgin olive oil: an integrated study of their concentration and bioavailability

Anallely López Yerena



Aquesta tesi doctoral està subjecta a la llicència **Reconeixement 4.0. Espanya de Creative Commons.**

Esta tesis doctoral está sujeta a la licencia **Reconocimiento 4.0. España de Creative Commons.**

This doctoral thesis is licensed under the **Creative Commons Attribution 4.0. Spain License.**

UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA I CIÈNCIES DE L'ALIMENTACIÓ

HEALTH-PROMOTING PHENOLIC COMPOUNDS IN EXTRA VIRGIN OLIVE OIL: AN INTEGRATED
STUDY OF THEIR CONCENTRATION AND BIOAVAILABILITY

ANALLELY LÓPEZ YERENA

2021

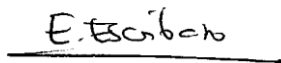
UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA I CIÈNCIES DE L'ALIMENTACIÓ

DOCTORAT EN ALIMENTACIÓ I NUTRICIÓ

HEALTH-PROMOTING PHENOLIC COMPOUNDS IN EXTRA VIRGIN OLIVE OIL: AN INTEGRATED
STUDY OF THEIR CONCENTRATION AND BIOAVAILABILITY

Memòria presentada per Anallely López Yerena per optar al títol de doctor per la universitat de
Barcelona



Dra. Elvira Escribano Ferrer
Directora



Dra. Anna Vallverdú Queralt
Co-directora



Anallely López Yerena
Doctoranda



Dra. Rosa M. Lamuela Raventós
Tutora

ANALLELY LÓPEZ YERENA

2021

FUNDING



Consejo Nacional de Ciencia y
Tecnología
Doctoral scholarship



Ministerio de Ciencia, Innovación y
Universidades
AGL2016-75329-R

ciberobn isciiii

Consorcio Centro de Investigación
Biomédica en Red



Generalitat de Catalunya
2017SGR 196



Universitat de Barcelona, Facultat de
Farmàcia i Ciències de l'Alimentació



Campus de l'Alimentació de Torribera



Institut de Recerca en Nutrició i
Seguretat Alimentària



Fundació Bosch i Gimpera

INDEX

1. ABSTRACT	3
2. INTRODUCTION	7
2.1. Extra virgin olive oil	7
2.1.1. Phenolic content in olive oil	8
2.1.1.1. Chemistry of phenolic compounds	8
2.1.1.2. Factors affecting phenolic composition in EVOO	8
2.1.1.3. Health-promoting properties of secoiridoids	9
2.2. Bioavailability	10
2.2.1. Liberation	10
2.2.2. Absorption	11
2.2.1.1. Physiology of the small intestine	11
2.2.1.2. Absorption mechanisms	12
2.2.1.3. Approaches used to study intestinal absorption	13
2.2.1.3.1. In situ methods to study the absorption	14
2.2.1.4. Absorption of olive oil secoiridoids in the small intestine	15
2.2.3. Distribution	15
2.2.4. Metabolism	16
2.2.1.5. Phase I metabolism	16
2.2.1.6. Phase II metabolism	16
2.2.1.7. Microbial metabolites	18
2.2.7. Excretion	18
2.3. Identification and quantification of phenolic compounds in biological samples	18
3. HYPOTHESIS AND OBJECTIVES	66
3.1. Hypothesis	66
3.2. Objectives	66
4. RESULTS	70
4.1. Phenolic compounds: towards obtaining an olive oil with higher nutritional value	71
4.1.1. Article 3: Conservation of native wild ivory-white olives from the Medes islands natural reserve to maintain virgin olive oil diversity	71
4.1.2. Article 4: Effects of organic and conventional growing systems on the phenolic profile of extra-virgin olive oil	85
4.1.3. Article 5: Influence of the ripening stage and extraction conditions on the phenolic fingerprint of 'Corbella' extra-virgin olive oil	100
4.2. Absorption and metabolism of phenolic compounds assessed by intestinal perfusion method	115
4.2.1. Article 6: Absorption and intestinal metabolic profile of oleocanthal in rats	115
4.2.2. Article 7: Oleacein intestinal permeation and metabolism in rats using an <i>in-situ</i> perfusion technique	130
4.3. Evidence of tissue distribution of olive oil phenols in rats	145
4.3.1. Article 8: Tissue distribution of oleocanthal and its metabolites after oral ingestion in rats	146
4.3.1. Article 9: LC-ESI-LTQ-Orbitrap-MS for profiling the distribution of oleacein and its metabolites in rat tissues	161
5. GLOBAL DISCUSSION	177

5.1. Phenolic compounds: towards obtaining olive oil with higher nutritional value	177
5.2. Absorption and metabolism of phenolic compounds assessed by intestinal perfusion method	177
5.3. Evidence of tissue distribution of olive oil phenols in rats	179
6. CONCLUSIONS	184
7. REFERENCES	188
8. ANEXES	194
8.1. Other publications	194
8.2. Conference communications	195
8.3. Participation in projects	195

Abbreviations

ACET: Acetyltransferase enzymes

AKR: Aldo-keto reductases

ALDH: Aldehyde dehydrogenase

CE: Nonlipolytic carboxylic ester hydrolases

COMT: Catechol-*O*-methyltransferase

CYP450: Cytochrome P450 monooxygenase

EVOO: Extra virgin olive oil

IOC: International Olive Council

IPVS: intestinal perfusion with venous sampling

OLC: Oleocanthal

OLEA: Oleacein

PC: Phenolic compound

PCM: phenolic compound metabolites

PCs: Phenolic compounds

SEC: Secoiridoid

SECs: Secoiridoids

SPIP: intestinal single-pass perfusion.

SULT: Sulfotransferases

UGTs: Glucuronosyltransferases

INDEX OF TABLES

Table 1 Main factors affecting phenolic composition in EVOO _____	9
Table 2. Metabolic enzymes responsible of first pass metabolism of PCs _____	17
Table 3. Challenges and strategies in the analysis of phenolic compounds metabolites _____	19

INDEX OF FIGURES

Figure 1. Molecular structure of secoiridoids, the major constituents of EVOO _____	8
Figure 2. Structure of the small intestine _____	12
Figure 3. Schematic drawing of in situ intestinal perfusion with venous sampling. _____	14
Figure 4. Factors affecting rate of distribution of PCs to target tissues. _____	16

1. ABSTRACT

The health promoting attributes associated with following a traditional Mediterranean diet have been reviewed extensively. The principal source of dietary fat in the Mediterranean diet is Extra virgin olive oil (EVOO) that contribute significantly to the superior health profile observed in Mediterranean populations. Between its minors compounds, EVOO contains at least 30 phenolic compounds (PCs) and has been classified according to their chemical structure into the following main classes: phenolic acids, lignans, flavonoids, phenolic alcohols, secoiridoids (SEC). Regarding this last group, these compounds are responsible for the sensory features of EVOO, mainly the bitter and pungent mouthfeel. Oleocanthal (OLC) and oleacein (OLEA) appear the main compounds responsible for the mouthfeel perceptions, respectively. Moreover, they are the most studied and best-known components in terms of health protecting activities. In this sense, the potential protective role exerted by SECs from EVOO in cancer, cardiovascular, neurodegenerative, aging-related, and immunoinflammatory diseases has been studied. The data obtained so far has clearly shown that SECs has proven biological activity, including antioxidant and antimicrobial properties, anti-inflammatory effects, anticancer properties and neuro-protective effects, among others.

The phenolic concentration and composition of EVOO together with the degree to which these components are absorbed and metabolized are essential in determining the health effects associated with such compounds. Several factors have been shown to affect the concentration of PCs in EVOO and these include olive fruit cultivar and variety, region of growth, agricultural cultivation techniques, maturity at harvest, processing, and storage. In this thesis the study of the main factors affecting the PCs concentration is covered.

Regarding its bioavailability after EVOO dietary intake has been a topic of increasing research in recent years. Bioavailability is a complex process involving several different stages that include liberation, absorption, distribution, metabolism and elimination phases. For polyphenols, this principally involves the following digestive processes: i) release of polyphenols from the food matrix; ii) changes in PCs during gastric/small-intestinal digestion; iii) cellular uptake of aglycons and some conjugated polyphenols by enterocytes; iv) microbiological fermentation of nonabsorbed polyphenols or those re-excreted via bile or the pancreas to yield additional metabolites, v) phase I/II enzyme modifications that occur upon uptake in the small intestine and/or colon; vi) transport in the bloodstream and subsequent tissue redistribution; and vii) excretion via the kidney or re-excretion into the gut via bile and pancreatic juices.

Since many *in vitro*, *in situ*, *in vivo* and clinical studies, have demonstrated the protective effect of SECs, in this thesis we set out to evaluate the intestinal absorption and metabolism of OLC and OLEA for a better understanding of the biological effects attributed to these compounds.

In addition, a tissue distribution of OLC and OLEA and its metabolites also was study after the oral administration of enriched refined olive oil. Target tissues likes as brain, hearth, lungs, among other were evaluated to determine the biological concentrations usually achieved when following a Mediterranean diet.

INTRODUCTION

2. INTRODUCTION

2.1. Extra virgin olive oil

Olive oil, a traditional food product with thousands of years of history, is one of the essential components of the Mediterranean diet and is highly appreciated and consumed in the world. The historical scenario of olive oil begins during the Copper Age (sixth millennium B.C.) when humans began to hunt, fish and perform agricultural activities they realized that from a thorny bush with small fruits, large grain and a little pulp, a thick-oily-beneficial-tasty liquid could be obtained. In the ancient world, olive oil was not only a sacred and precious food, but it was also used as medicine and with cosmetic purposes. Hippocrates called it “the great healer” and Homer “liquid gold”, Galen praised it for its positive effects on health. The olive tree has long been the symbol of peace dating back to 5000 B.C. Olive oil was considered a luxury in ancient Greece. Actually, the Mediterranean olive tree (*Olea europaea* L. subsp. *europaea*, Oleaceae) is one of the earliest fruit trees cultivated in the Mediterranean Basin, to produce both oil and table olives and very significant because of its economic value ^[1,2]. Beside their economical contribution to national economy, it is an important food in terms of their nutritional value ^[3].

According to International Olive Council (IOC), the virgin olive oils are the oils obtained from the fruit of the olive tree (*Olea europaea* L.) solely by mechanical or other physical means under conditions, particularly thermal conditions, that do not lead to alterations in the oil, and which have not undergone any treatment other than washing, decantation, centrifugation and filtration ^[4]. In this sense, extra virgin olive oil (EVOO) is defined as virgin olive oil which has a free acidity, expressed as oleic acid, of not more than 0.8 grams per 100 grams, and the other characteristics of which correspond to those fixed for this category in the IOC standard.

EVOO is one of the key foods of the Mediterranean diet and is distinguished by its high content of nutritional and antioxidant compounds compared to other vegetable oils. Olive oil composition is mainly formed by triglycerides and a variety of several compounds in small quantities. Among the glyceride fraction, olive oil shows a high content of fatty acids and particularly, an elevated proportion of monounsaturated fatty acids (55-83%). In general, the unsaturated acids are up to 85% of olive oil composition and the saturated fats entail around 14%. EVOO has a plethora of minor components of undeniable significance that remain in the oil due to the lack of chemical refining. This minor fraction (2–5%) includes phenolic compounds (PCs), triterpenic compounds, tocopherols, sterols, hydrocarbons and pigments (chlorophylls and carotenoids), among others ^[5–8].

2.1.1. Phenolic content in olive oil

2.1.1.1. Chemistry of phenolic compounds

EVOO contains at least 30 PCs and has been classified according to their chemical structure into the following main classes: phenolic acids, lignans, flavonoids, phenolic alcohols, secoiridoids (SEC), and hydroxy-isocromans ^[9,10]. Tyrosol and hydroxytyrosol appear the most relevant phenolic alcohols, while that acetoxypinoresinol and pinoresinol are for lignans, and luteolin and apigenin for flavonoids.

The SEC compose the most complex and abundant typical family in the EVOO polar fraction reaching more than 70 % of total polyphenols ^[11,12]. The major SEC in olive drupes are oleuropein and ligstroside. These compounds are hydrolyzed through crushing and malaxation process by enzymatic reactions catalyzed by endogenous β -glucosidases yielding SEC aglycons (oleuropein aglycone and ligstroside aglycone). Additionally, oleacein (OLEA) and oleocanthal (OLC), are generated through a decarboxylation step ^[7,13,14].

SEC act as the main natural antioxidants of EVOO, mainly OLEA and oleuropein aglycone, which show remarkable resistance to oxidation ^[15]. In addition, this family is the main contributor to EVOO's organoleptic characteristics, particularly OLC and OLEA appear the main compounds responsible for the mouthfeel perceptions, bitter and pungent, respectively. Moreover, they are the most studied and best-known components in terms of health promoting activities ^[16,17].

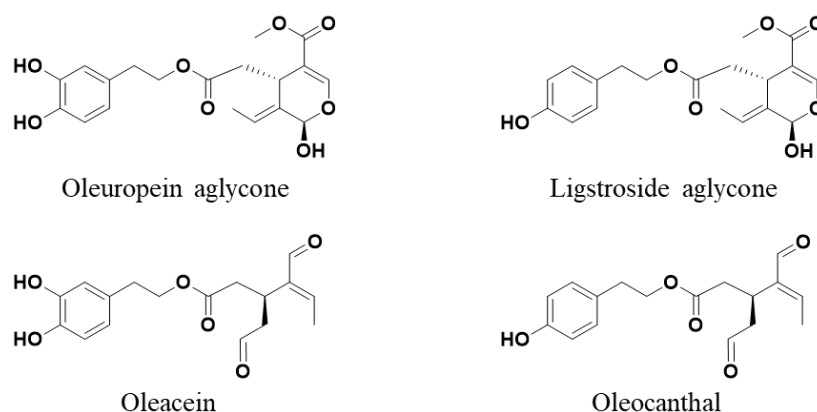


Figure 1. Molecular structure of secoiridoids, the major constituents of EVOO

2.1.1.2. Factors affecting phenolic composition in EVOO

The quality of EVOO is a compromise between multiple factors related to olive cultivar, ripening stage and the technological variables used during the oil mill process and storage. The qualitative and quantitative composition of polyphenols in EVOO is affected by agronomic and technological factors as is showed in the **Table 1**. During olive ripening, chemical reactions and enzymatic activity, such as that of glycosidases, phenol oxidases and phenol polymerases, occur that induce variations in the chemical structure and concentration of some PCs ^[18]. The demand for EVOO is increasing due to growing consumer awareness of the health benefits of the Mediterranean diet,

in which it is the main source of dietary fat. In this sense, consumer preferences point towards high quality EVOO with health-promoting and sensory properties that are associated with a higher content of PCs ^[19]. To meet this demand, several novel and emerging technologies are being under study to be applied in EVOO production ^[19].

Table 1 Main factors affecting phenolic composition in EVOO

Factors		Effect
Agronomic factors		
Genotype	or	Cultivars have a genetic predisposition to provide EVOO with a certain phenolic composition.
Maturity index		Chemical reactions and changes occur in the enzymatic activity that induce variations in the chemical structure and concentration of some PCs of the olive fruit like ligstroside and oleuropein.
Climatic conditions		An increase in water availability involves a reduction in the total phenolic concentration in fresh olive fruit and in the extracted EVOO. Dry summers and autumns seem to increase the phenolic content. Olive fruits in shaded areas of the canopy have higher phenolic contents than those of fruits in well-illuminated zones.
Technological factors		
Crushing		Release and activation of endogenous enzymes (β -glycosidases and oxidoreductases).
Malaxation		Induces the activation of several endogenous fruit enzymes, such as β -glucosidases and esterases, which hydrolyze precursors of oleuropein and ligstroside to produce SEC derivatives
Filtration		The loss of PCs during filtration or natural sedimentation and decantation is related to partitioning of phenols between oil and filter aid or sediment and can be the cause of olive oil stability reduction.
Storage		An improperly storage of EVOO causes unpleasant odor or taste arising due to rancidity process. The SEC derivatives have a more active participation in the oxidative processes, due to hydrolytic reactions, the simple phenols, such as hydroxytyrosol and tyrosol, usually increase and the SEC concentration decreases during storage

EVOO: extra virgin olive oil; PCs: phenolic compounds; SEC: secoiridoids. References: ^[20–24]

2.1.1.3. Health-promoting properties of secoiridoids

The potential protective role exerted by SEC from EVOO in cancer, cardiovascular, neurodegenerative, aging-related, and immunoinflammatory diseases has been summarized in previous works ^[16,25].

The data obtained so far has clearly shown that OLC has proven biological activity, including antioxidant and antimicrobial properties [26,27], anti-inflammatory effects (more potent than ibuprofen) [28], anticancer properties (melanoma, breast, liver and colon cancer and leukemia) [29–31] and neuro-protective effects [32,33].

OLEA may offer far more protection against reactive oxygen/nitrogen species-induced injury of human biomolecules than oleuropein [34]. OLEA also has anti-cancer effects, including antiproliferative and apoptosis-inducing activity in leukemia cells [35] and reduction of viability and migration of non-melanoma skin cancer cells [36]. It offers protection against cardiovascular diseases, decreasing the risk of atherosclerosis [37–39], ischemic stroke [40] and hypertension [41]. An *in vitro* study found that OLEA has significant anti-tumor activity by promoting cell cycle arrest and apoptosis [42]. Moreover, OLEA has been associated with healthy aging in both *in vitro* and *in vivo* studies [26]. Anti-inflammatory properties give OLEA a protective role against several metabolic abnormalities, including dyslipidemia, insulin resistance [36,43] and glucose intolerance.

2.2. Bioavailability

In nutrition science, bioavailability is defined as the fraction of an ingested nutrient or compound that reaches the systemic circulation and the specific sites where it can exert its biological action. Bioavailability depends on proper absorption, release of a dosage form and presystemic elimination. The bioavailability of ingested PCs can be influenced by factors such as phenolic structure, food processing and matrix, diet, host, genomic profile, enzymatic activity and colonic microbiota [44] among others; besides all this factors can interact with each other and influence PCs bioavailability, which makes harder to define the exact mechanisms of action of PCs [45].

Bioavailability is related to other two concepts bioaccessibility and bioactivity. The assessment of bioaccessibility and bioavailability of health associated compounds is important for the understanding of the relationship between food and nutrition [46]. Bioavailability is a complex process involving several different stages: liberation, absorption, distribution, metabolism and elimination phases (LADME).

2.2.1. Liberation

Bioaccessibility has been defined as the fraction of a compound which is released from the food matrix in the gastrointestinal lumen and thereby made available for intestinal absorption. The process begins with mastication in the mouth and then, in the stomach and the rest of the gastrointestinal lumen, various digestive fluids containing different enzymes and pH change continue to break down the food matrix. The phenolic-matrix interaction determines phenolic liberation, however the bioaccessibility is also influenced by the composition of the digested food

matrix, the synergisms and antagonisms of the different components and by physicochemical properties, such as pH, temperature and texture of the matrix ^[46]. It is very important to highlight that only the compounds that are released from the food matrix or absorbed in the small intestine are potentially bioavailable and bioactive. Nonetheless, it is important to note that the fact that a compound is bioaccessible does not always imply it is bioactive ^[45].

In the case of digestion of dietary lipids, the first step in the stomach involves their dispersal into finely divided emulsion particles, generating a lipid-water interface ^[47]. Previous studies have demonstrated that PCs are transferred to the aqueous or oily phase, according to their polarity ^[48]. In an *in vitro* study, it was demonstrated that all the major EVOO PCs (tyrosol, hydroxytyrosol and the related secoiridoid structures, OLEA and OLC showed good stability in the gastric digestion model. The amphiphilic characteristics of the SEC permit their partition between the oily phase and the water phase, and they tended to be more concentrated in the aqueous phase because of their polar functional groups ^[49].

2.2.2. Absorption

Following the ingestion of PCs, some but not all compounds are absorbed into the circulatory system via the small intestine ^[50,51]. Although different factors (physicochemical and physiological, and the matrix effect) can affect the rate and extent of absorption, the absorption of orally administered chemicals is mainly determined by the permeability of the gastrointestinal wall and the solubility/dissolution of the molecule in the gastrointestinal environment ^[52].

2.2.1.1. Physiology of the small intestine

The human gastrointestinal tract is composed of multiple different organs and can be divided into the upper and lower gastrointestinal tract. The upper gastrointestinal tract refers to the mouth, esophagus, stomach, small intestine, while the colon, rectum, and anus make up the lower gastrointestinal tract. The small intestine is a complex tube that can be divided in three different parts that are characterized by having different absorption capacities. The first part, the duodenum, is 25–30 cm long and the passage of the chemicals through this part is relatively quick, resulting in a poor net absorption. The second part is the jejunum and is characterized by a highly active peristalsis, favoring absorption. Finally, the ileum reveals fewer villi than the jejunum but has a similar absorption capability ^[50].

The intestinal epithelial membrane is the main physiological barrier, which must be crossed by chemicals to enter the bloodstream and be bioavailable ^[53]. The intestinal epithelium consists of multiple cell types that are arranged in a columnar layer. Enterocytes are the absorptive cells that facilitate the controlled uptake of a plethora of molecules from the intestinal lumen, including water, various ions, nutrients and drugs. Enterocytes constitute the majority of epithelial cells and can be easily recognized morphologically by their microvilli that form brush borders on the

mucosal side of the enterocyte. PCs move from the intestinal lumen to the cytosol of enterocytes through different paths. Inside the enterocyte cytosol, PCs aglycones are either transferred to the blood stream *via* passive diffusion or transformed to conjugated metabolites *via* phase II pathways. Dietary phase II metabolites are either effluxed into the gut lumen or transported to the blood stream *via* apical and basolateral multi-drug/lipid transporters (phase III transporters: glycoproteins or multidrug resistance associated proteins) respectively ^[54].

The intestinal mucus layer covers the epithelial cell but also acts as a mechanical and physical barrier to potential pathogens, drug absorption and performing the role of a niche for the microbial cells ^[50]. Mucus is an aqueous matrix composed of water (95%), lipids, electrolytes and glycoproteins, with a thickness and composition that is region-dependent ^[55]. The human gut contains trillions of microbes, predominantly bacteria from hundreds of different species, with *Bacteroidetes* and *Firmicutes* being the most prevalent phyla, but also archaea, viruses and fungi. The microbiome exerts a crucial role in a range of physiological functions (e.g., food digestion, synthesis of essential vitamins, contributing to a functioning immune system, regulating intestinal functions) and also produces organic compounds which fulfil an important physiological role ^[50,55]. After oral intake, like drugs, PCs can also be modified by intestinal microorganisms in the small and large intestine. PCs that are not absorbed in the small intestine reach the colon, so there is a substantial structural modification by the colonic microbiota and its microbial metabolites are released back into the circulatory system.

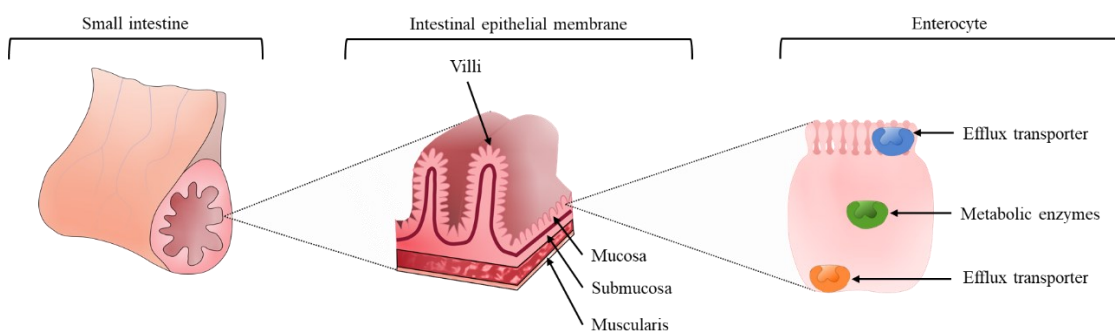


Figure 2. Structure of the small intestine

2.2.1.2. Absorption mechanisms

The two main mechanisms of drug transport across the gastrointestinal epithelium are transcellular (across cells) and paracellular (between cells). The transcellular mechanism is further divided into simple passive diffusion, carrier-mediated transport (active transport and facilitated diffusion), and endocytosis ^[51].

- 1) **Passive Diffusion:** is the main route of absorption and is probably most used by low molecular weight lipophilic chemicals. This mechanism follows Fick's law, whereby the absorption rate is proportional to the chemical concentration and the surface area. It should be noted that

given the different lipid and protein compositions of the apical and basolateral membranes, the rate of passage across these two barriers could be different for the same molecule. Chemical molecules pass the lipid membrane via passive diffusion from a region of higher concentration in the lumen to a region of lower concentration in the blood. The blood flow contributes to maintaining the lower concentration.

- 2) Carrier-mediated transport: In the case of hydrophilic molecules, numerous specialized carrier-mediated transport systems, present especially in the intestine, may be used for their absorption. Normally, these carriers are utilized for the absorption of ions and nutrients and involves specific interaction between the molecule and the transporter or carrier in a saturable mechanism. Two types of transport exist: active and passive carrier-mediated transport.
 - Active carrier-mediated transport: is either Na^+ -or H^+ -linked with bifunctional carriers. This process requires metabolic energy and can work against a concentration gradient of the substrate.
 - Passive carrier-mediated transport: does not require metabolic energy and is driven by the concentration gradient of substrate.

There are two super families of transporters involved in the transport of drugs and xenobiotics across the membranes: ATP-binding cassette (ABC) pumps and solute-carrier (SLC) superfamily. The first ones use energy released by ATP hydrolysis to move substrates across membranes in or out of cells or into cellular vesicles, against their electrochemical gradient ^[56]. Within this family is the so-called p-glycoprotein (or MDR1), which is characterised by its affinity for a large number of lipid substrates, prevents the incorporation of substrates into the cell, and is widely distributed in different organs and tissues of the organism. The SLC superfamily include passive transporters, symporters and antiporters, as well as mitochondrial and vesicular transporters.

- 3) Endocytosis. This is a constitutive process observed in most mammalian cells for the uptake of macromolecules. It requires metabolic energy, and apart from some specialized cell types it is generally a slow uptake mechanism, sometimes resulting in a fusion of endocytocytic vesicles with lysosomes containing high levels of enzymatic activity.

2.2.1.3. Approaches used to study intestinal absorption

Over the recent years, intestinal absorption has been recognized as a critical factor affecting the bioavailability of oral drugs and nutrients. For this reason, many *in silico*, *in vitro*, *in situ* and *in vivo* models are developed and extensively used to investigate the transport mechanisms, to determine the intestinal permeability and to predict the plasma pharmacokinetic profile of PCs ^[57].

2.2.1.3.1 In situ methods to study the absorption

Although that the *in vivo* models are the most clinically relevant to investigate the transport mechanisms, intestinal permeability and plasma pharmacokinetic profile of chemicals include all the physiological factors that can affect absorption and bioavailability, the *in situ* models also have many advantages. In this model, seven different methods can be distinguished: closed-loop method, thiryvella fistula, intestinal single-pass perfusion (SPIP), intestinal recirculating perfusion, intestinal perfusion with venous sampling (IPVS), vascularly perfused intestine-liver (IPIL) and Loc-I-Gut [58].

IPVS is commonly used to investigate trans-membrane chemical transport. The methodology involved perfusion of an isolated intestinal segment with regional mesenteric venous cannulation being used to follow the disappearance kinetics of chemical from the intestinal lumen and its concurrent appearance in blood [58] as it is showed in the **Figure X**. IPVS allows to determine the intestinal effective permeability to be determined based on the disappearance of the compound from the intestinal segment, or its appearance in plasma after a venous sampling procedure [57,59,60], the latter being especially suitable for poorly permeating substances. Among the main advantages of this technique we find [61]:

1. Enables relevant mechanistic investigations of drug absorption and anticipates the effects of various physiological processes.
2. Intact intestinal morphology.
3. Presence of blood flow.
4. Expression of enzymes and transporter.
5. Presence of neural and hormonal feed-back mechanisms.
6. Possibility to control luminal conditions.

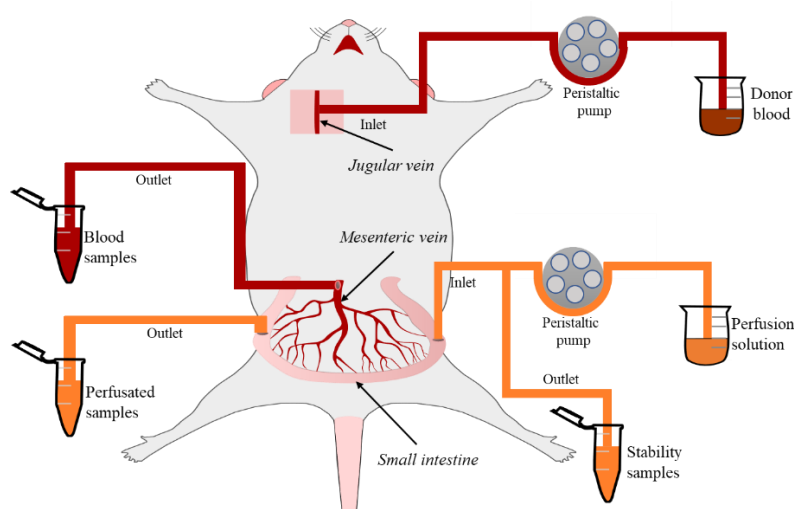


Figure 3. Schematic drawing of *in situ* intestinal perfusion with venous sampling.

2.2.1.4. Absorption of olive oil secoiridoids in the small intestine

Surprisingly, despite the myriad of potential health benefits attributed to EVOO PCs, specifically to SEC, there is currently little information available about its intestinal permeability. In fact, only three studies have focused on evaluating intestinal absorption. In one of them, oleuropein aglycone and OLEA absorption was assessed in two small-intestinal models (Caco-2 cells and SPIP) [62]. In another study, the transepithelial transport hydroxytyrosol, tyrosol and oleuropein was assessed in an *in vitro* study [63]. Finally, only in one study IPVS technique has been used to investigate the intestinal absorption of oleuropein [64].

2.2.3. Distribution

Once absorbed, PCs are widely distributed throughout the organism. In this sense, the key to the positive relationship between the EVOO consumption and its beneficial effect is probably not to be found in such widely analyzed biological fluids as plasma and urine, but must be sought in those tissues in which both PCs and phenolic compound metabolites (PCM) contribute positively to the normal cell metabolic process [65]. Studies in animal models help to determine the tissue distribution and accumulation of compounds and their metabolites and to identify potential sites of action [66]. In the case of hydroxytyrosol, its ability to reach the brain after crossing the blood-brain barrier demonstrated *in vivo* that it fulfills this essential requirement to be used as a neuroprotective agent [67]. To date, only one study has evaluated the tissue distribution of EVOO PCs, including the SEC family. However, in this study information on delivery to target tissues of OLC and OLEA was not given [65].

The PCs rate of distribution to the various tissues of the body will be influenced by different factors as it is showed in the **Figure X**. Regarding to the formation of PCs-plasma protein complexes, in the **Article 1** is summarized how structural modifications affect the affinity of the main dietary PCs and their metabolites for human serum albumin. The elucidation of the main factors involved in their binding, the binding site and the affinity of drugs towards human serum albumin and competitive binding with the most widespread dietary PCs is also discussed.

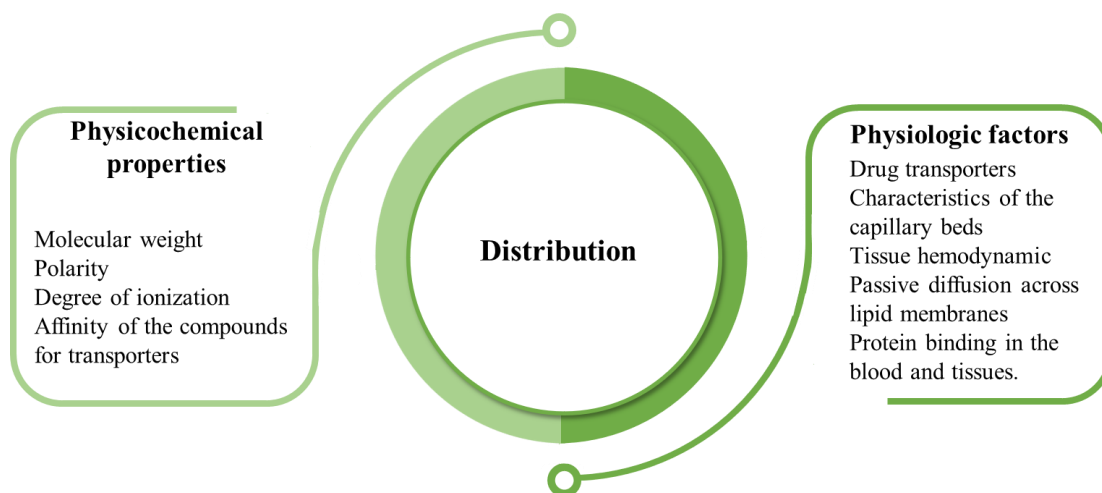


Figure 4. Factors affecting rate of distribution of PCs to target tissues.

2.2.4. Metabolism

PCs are categorized into xenobiotic compounds because humans do not produce them. Therefore, they undergo through biotransformation reactions forming structures called metabolites. Biotransformation is the process of converting lipophilic chemicals into hydrophilic chemicals; thus, making the readily absorbed compounds into readily excreted compounds ^[45]. These metabolites might have either higher or lower activity or no activity at all, as compared to the parent compound. In exceptional cases, some of the metabolites may produce different pharmacological activity than the parent compound and might also exhibit toxicity ^[68].

In the process of crossing enterocytes, EVOO PCs are subjected to an important first pass metabolism through a classic phase I/II biotransformation as well as by gut microbiota.

2.2.1.5. Phase I metabolism

Phase I metabolism broadly involves enzymatic reactions that alter biologic activity, via the addition or transformation of a functional molecular group (hydrolysis, oxidation, and reduction). For lipophilic compounds (e.g. 1.15 and 1.53 partition coefficient (logP) for OLC and OLEA, respectively), these enzymes usually introduces a polar functional group that forms a substrate for subsequent metabolic handling ^[69]. Major phase I enzymes include oxidases (especially monooxygenases), reductases, and hydrolases ^[70]. More detailed information is presented in **Table 2**.

2.2.1.6. Phase II metabolism

Phase II metabolism involves the addition of a cofactor to a compound (glucuronidation, methylation, sulfation). This typically results in a molecule that is highly water-soluble and amenable to excretion within the urinary or biliary tract ^[69,71]. The enzymes involved in phase II usually require specific functional groups on their substrates for conjugation to occur. In this sense, the products of phase I metabolism are often good candidates for subsequent phase II

reactions ^[69]. The main metabolic enzymes responsible for conjugation reactions as well as their location are also presented in **Table 2**.

Table 2. Metabolic enzymes responsible of first pass metabolism of PCs

Reactions	Tipo	Enzymes	Location
Phase I			
Oxidation	Aldehydes → carboxylic acids.	CYP450	Liver, gastrointestinal tract, lung, and kidney,
		ALDH	Small intestine
Reduction	Aldehydes → primary alcohols.	AKR	Stomach, small intestine
Hydrolysis	Hydrolysis of esters, leaving carboxylic acids and alcohols as the hydrolysis products.	CE	Stomach
Phase II			
<i>O</i> -methylation	Transfer of a methyl moiety from the S-adenosyl-L-methionine (SAM) cofactor to one of the hydroxyl groups present in xenobiotics.	COMT	Liver, kidney, intestine, and brain.
Glucuronidation	Transfer of a glucuronyl moiety from the ubiquitous co-substrate UDP-glucuronic acid to hydrophobic molecules possessing one or more electrophilic groups.	UGTs	Stomach, small intestine and liver.
Sulfation	Transfer of a sulfate moiety from 3'-phosphoadenosine-5'-phosphosulfate to a hydroxyl group on various substrates.	SULT	Small intestine, liver
Acetylation	Transfer of acetyl groups from acetyl-CoA to hydroxyl group.	ACET	Small intestine

CYP450: Cytochrome P450 monooxygenase; ALDH: Aldehyde dehydrogenase; AKR: Aldo-keto reductases; CE: Nonlipolytic carboxylic ester hydrolases; COMT: Catechol-O-methyltransferase; UGTs: Glucuronosyltransferases; SULT: Sulfotransferases; ACET: Acetyltransferase enzymes. References: [16,68,72–74].

Regarding to metabolism of EVOO PCs, numerous studies have been focused to elucidate the effect of first pass metabolism. For this purpose, human plasma and/or urine have been analyzed after EVOO intake ^[75–80]. On the other hand, rats also have been used as model to understand the biotransformation that suffer the PCs ^[81–84]. Although, there are numerous reports on the fate on the major phenolic constituents in EVOO, there are still numerous key issues that need to be answered and this will require further research. The metabolism of SEC during its absorption and distribution is a topic that needs to be studied in depth to try to understand the beneficial effects of regular EVOO consumption.

2.2.1.7. Microbial metabolites

PCs that cannot be absorbed in the small intestine or after being secreted in bile after following the enterohepatic cycle can be metabolized by gut microbiota. In the gut, the microbiota can hydrolyze the glycosides and transform the aglycones into new molecules. The PCs transformation, into simpler components, is mediated by different reactions, such as ring fission, hydrolysis, demethylation, reduction, decarboxylation, dehydroxylation and isomerization ^[85]. Regarding to SEC, the microbial metabolism was study by López de las Hazas *et al.* ^[81]. In this study, rats were used as model to study differences in the absorption and metabolism of hydroxytyrosol either in its free form or through its natural occurring esterified precursors: oleuropein and its aglycone forms OLEA.

2.2.7. Excretion

PCs and PCM (mainly phase II metabolites) and bacterial derivatives are eliminated from the consumer through urine and feces (bile). As discussed above, PCs are extensively transformed to more water soluble metabolites by the phase II enzymes and the majority of these metabolites undergo the first-pass metabolism and transfer to the lower gut for further bacterial metabolism. While the phase II transformations minimize the potential harm that may arise from PCs consumption, such transformations give them different biological activities, depending on whether they were methylated, sulfated or glycosylated.^[54]

Regarding to the excretion of EVOO PCs, numerous studies have been focused in evaluated its excretion in human urine after EVOO consumption ^[75,86–89]. Respect to faeces, microbial metabolites were detected in faeces from rats after the diet supplementation (21 days) with 5 mg/kg weight rat/day of hydroxytyrosol, SEC and oleuropein ^[81].

2.3. Identification and quantification of phenolic compounds in biological samples

Metabolomics is an important component of post-genomic omics science that deals with the comprehensive chemical analysis of the entire metabolites, so-called the metabolome, in a particular biological system (tissues, cells, fluids, or organisms) for at a specific developmental stage or under an induced circumstance (disease, drug treatment, dietary intervention, or environmental modulation) ^[90]. In fact, the identification of the PC and its PCM in biological samples produced after foods rich in PC intake, is a first step to understand the overall effect on human health ^[91]. The usual biofluids analyzed in metabolomics are plasma, urine and fecal extracts, however cell cultures also offer the possibility of studying food ingredients or bioactive compounds (as well as new drugs or xenobiotics) in human biological materials more economically ^[92]. However, the analysis is affected by several factors as is summarized in the **Table X**.

Table 3. Challenges and strategies in the analysis of phenolic compounds metabolites

Problems	Strategies
• Enormous structural variety of PCs (i.e., phenolic acids, flavan-3-ols, flavanones, flavones, flavonoids, lignans, among others). • Lack of commercially available standards. • Structural variability of PCM. • Metabolite degradation due to tedious multi-step sample preparation workflows. • Instability of specific compounds. • Low analyte concentration. • Frequent sample contamination.	• Development of sample preparation methods that allow the detection of PCs and PCM with differentiated physicochemical properties. • High analytical power provided by high-resolution mass spectrometry. • Collection of specific metabolite information, typically through the use of low-resolution mass spectrometry.

PCs: phenolic compounds; PCM: phenolic compound metabolites

Given the above, the **Article 2.** aims to provide an overview of the state-of-the-art in the analysis of PCM with a special emphasis on preanalytical separation of plasma and urine samples and the technologies applied in the quantitative and qualitative analysis of PCM.



Review

Insights into the Binding of Dietary Phenolic Compounds to Human Serum Albumin and Food-Drug Interactions

Anallely López-Yerena ¹, Maria Perez ^{1,2}, Anna Vallverdú-Queralt ^{1,3} and Elvira Escribano-Ferrer ^{3,4,*}

- ¹ Department of Nutrition, Food Science and Gastronomy XaRTA, Faculty of Pharmacy and Food Sciences, Institute of Nutrition and Food Safety (INSA-UB), University of Barcelona, 08028 Barcelona, Spain; naye.yerena@gmail.com (A.L.-Y.); mariaperez@ub.edu (M.P.); avallverdu@ub.edu (A.V.-Q.)
- ² Laboratory of Organic Chemistry, Faculty of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain
- ³ CIBER Physiopathology of Obesity and Nutrition (CIBEROBN), Institute of Health Carlos III, 28029 Madrid, Spain
- ⁴ Pharmaceutical Nanotechnology Group I+D+I Associated Unit to CSIC, Biopharmaceutics and Pharmacokinetics Unit, Department of Pharmacy and Pharmaceutical Technology and Physical Chemistry, Institute of Nanoscience and Nanotechnology (IN2UB), Pharmacy and Food Sciences School, University of Barcelona, 08028 Barcelona, Spain
- * Correspondence: eescribano@ub.edu; Fax: +34-9340-35937
- † Current Address: Pharmacy and Pharmaceutical Technology and Physical Chemistry Department, Faculty of Pharmacy and Food Sciences, University of Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain.

Received: 5 November 2020; Accepted: 20 November 2020; Published: 21 November 2020



Abstract: The distribution of drugs and dietary phenolic compounds in the systemic circulation depends on, among other factors, unspecific/specific reversible binding to plasma proteins such as human serum albumin (HSA). Phenolic substances, present in plant-derived feeds, foods, beverages, herbal medicines, and dietary supplements, are of great interest due to their biological activity. Recently, considerable research has been directed at the formation of phenol–HSA complexes, focusing above all on structure–affinity relationships. The nucleophilicity and planarity of molecules can be altered by the number and position of hydroxyl groups on the aromatic ring and by hydrogenation. Binding affinities towards HSA may also differ between phenolic compounds in their native form and conjugates derived from phase II reactions. On the other hand, food–drug interactions may increase the concentration of free drugs in the blood, affecting their transport and/or disposition and in some cases provoking adverse or toxic effects. This is caused mainly by a decrease in drug binding affinities for HSA in the presence of flavonoids. Accordingly, to avoid the side effects arising from changes in plasma protein binding, the intake of flavonoid-rich food and beverages should be taken into consideration when treating certain pathologies.

Keywords: plasma protein binding; distribution; bioavailability; molecular property; noncovalent interaction; pharmacokinetics

1. Introduction

The biological activities of dietary phenolic substances, present in plant-derived feeds, foods, beverages, herbal medicines, and dietary supplements, are of great interest. Polyphenols, which can be classified as flavonoids and non-flavonoids, contain, in addition to other substituents, at least one aromatic ring with one or more hydroxyl groups [1,2]. Flavonoids are a group of natural substances

with variable phenolic structures as flavonols, flavan-3-ols (monomeric and polymeric structures), flavones, isoflavones, flavanones, and anthocyanidins. On the other hand, stilbenes, hydrolyzable tannins, lignans, and phenolic acids can be classified as non-flavonoids [3].

The absorption, distribution and elimination of dietary phytochemicals depend on their intestinal permeability and the influence of pre-systemic enzymes and/or transporters [4]. As systemic exposure can reflect tissue exposure, greater bioavailability should result in higher levels in tissues. Bioavailability is defined as the rate and the extent to which the active ingredient/phytochemical or moiety is absorbed from the ingested matrix and becomes available at the site of action [5]. It is well known that phenolic compounds have a low oral bioavailability, and undergo an extensive biotransformation mediated by phase I and phase II reactions in enterocytes and the liver, as well as by gut microbiota [6]. Polyphenol metabolites are also attracting research interest as their biological effects can be similar to or greater than those of the parent compounds [6,7]. Paradoxically, despite low oral bioavailability, most of the phenolic compounds have proven to have significant biological effects [6].

Once a xenobiotic has entered the systemic circulation, its rate of distribution to the various tissues of the body will be influenced by tissue hemodynamics (blood flow) and the ease with which it can cross the lipoidal cell membranes, either by passive diffusion or by passive/active facilitated transport (carrier-mediated) [8]. Nevertheless, the extent of distribution depends on partitioning into fat and other tissues and on unspecific/specific reversible binding to plasma proteins [9]. Plasma proteins, also called serum proteins, constitute important organic components consisting of simple as well as conjugated proteins [10]. Drugs are transported in the circulation either in a free form, dissolved in the aqueous phase of plasma, or in complex bonds with plasma proteins [11] in varying degrees [12]. Following the principle of reversible equilibrium and the law of mass action [13], an equilibrium exists between bound and free (unbound) molecular forms—additionally because binding is generally reversible [12]. Only the free form is capable of diffusing through membranes and from the vascular space into tissues, being eliminated by metabolism or excretion [14], and therefore pharmacological activity is exerted by the free drug concentration [15,16]. The fraction of a xenobiotic bound to a plasma protein depends on protein affinity towards the compound, protein and compound molar concentration, as well as on the possible competition with other endogenous and exogenous compounds for binding sites [17]. Generally, acidic compounds tend to bind to albumin, basic compounds to α_1 -acid glycoprotein (AAG), neutral compounds can be bound to both human serum albumin (HSA) and AAG, and neutral lipophilic compounds to lipoproteins. Other proteins, such as α -globulin, transcortin, fibrinogen, sex-hormone-binding globulin, and thyroid-binding globulin, bind specific compounds [18,19].

The free drug/xenobiotic concentration depends on the unbound drug clearance and dose, and is not usually changed by plasma protein binding (PPB) [16]. At a steady state, the free drug concentration remains balanced on both sides of any biomembrane [16]. As drug clearance occurs, a new equilibrium between bound and unbound forms is reached, which acts to maintain the free drug fraction [14]. Drug–protein complexes in plasma also serve as a drug reservoir, replacing what is removed by various distribution and elimination processes [8,15,17,20].

The free drug fraction is the ratio between the free drug concentration and total drug plasma concentration, which has values between zero (totally bound) and one (totally free), and remains constant in normal physiological conditions and at low molar drug concentrations. Only if the free fraction remains constant can the total plasma concentration be considered a good measure of changes in the unbound drug/xenobiotic concentration. This concept is important because the total plasma concentration is what is usually measured and not the unbound concentration, which is only occasionally determined. Although at the therapeutic concentration of most drugs, the molar concentration of unbound drugs is usually low in comparison with the molar concentration of the protein binding sites [13,21], in some pathophysiological conditions, the free drug fraction can be reduced/increased with ensuing changes in the distribution process, either by an altered protein–drug affinity or by a change in plasma protein levels [16].

Although it is traditionally considered that only the free form is capable of diffusing through membranes, recent studies have hypothesized that HSA facilitates Dp44mT delivery to the lysosomes by internalization through a carrier mediated transport mechanism enhancing its anti-cancer activity [22]. In addition, an *in vitro* study has shown for proteins with a net negative charge such as albumin, and for drugs highly bound to albumin and in the physiological albumin concentration range, a protein-mediated uptake mechanism [23]. It was observed in hepatocytes and cardiac myocytes. Certainly, this requires further intense investigation since PPB is an important process that determines the pharmacological activity and pharmacokinetics of drugs and other xenobiotics [12,24], and the impact of PPB on the efficacy and safety of a treatment needs to be better understood [25].

As with other xenobiotics, the distribution of phenolic compounds to the various tissues of the body is influenced by unspecific, reversible binding events to plasma protein [9]. PPB and phenolics have been the subject of numerous recent studies, which have focused above all on structure–affinity relationships. The aim of this review is to summarize how structural modifications affect the affinity of the main dietary polyphenols and their metabolites for HSA and to elucidate the main factors involved in their binding and the binding site. Drug binding properties of HSA and competitive binding with the most widespread dietary phenolic compounds are also covered.

2. Plasma Proteins

Plasma contains various proteins with different functions including the transport throughout the circulatory system of endogenous and exogenous molecules. The binding of small molecular weight drug molecules with plasma proteins is mostly associated with HSA and AAG and, to a lesser extent, globulins and lipoproteins [26].

HSA is a 66 kDa non-glycosylated monomeric protein of 585 amino acids present in the human body at a concentration of 0.53–0.75 mM [20,27]. HSA constitutes ~4.5% of the weight of human blood and helps to maintain osmotic pressure and pH in the blood stream [26,27]. Its principal functions are to transport fatty acids, hormones, drugs, nutrients and inorganic ions and to buffer pH [28,29]. Due to a large content of ionic residues, HSA is highly soluble in water and its flexibility allows specific binding to a wide array of molecules [26]. The polypeptide chain of HSA forms a heart-shaped conformation with approximate dimensions of $80 \times 80 \times 30$ Å, about 67% consisting of α -helices [11,28]. It contains three homologous α -helical domains (I–III), each further divided into two subdomains (A and B) [30]. Among them, subdomains IIA and IIIA are two important binding sites. They are delimited by a hydrophobic surface on one side and a positively charged surface on the other side, displaying well determined cavities to specifically bind neutral and negatively charged compounds.

The globulins (α -globulins, β -globulins, and γ -globulin) are a group of globular water-insoluble proteins [31]. AAG, also known as orosomucoid, is acidic, heavily glycosylated (38 to 48 kDa protein, concentration ~12–31 μ M), and comprises a single amino acid chain of 204 residues. An acute phase plasma protein, it is the principal extracellular lipocalin present in blood [32]. Multiple drug-binding sites have been reported for AAG, but one appears to be most important, particularly for basic and neutral drugs [8,27]. It should also be considered that if a compound is available as a racemic mixture in blood/plasma, both HSA and AAG are able to bind preferentially to one stereoisomer [25].

To date, two different approaches to assess drug–protein binding can be distinguished. On one hand, separative methods used to determine directly either the unbound drug or the bound drug concentration by separation of the free ligand from the bound species can be classified as conventional methods (equilibrium dialysis, ultrafiltration and ultracentrifugation), high-performance affinity chromatography and capillary electrophoresis–frontal analysis. On the other hand, non-separative methods have been developed to characterize drug–protein interactions. In this sense, spectroscopic methods (UV–visible, fluorescence, infrared, nuclear magnetic resonance, optical rotatory dispersion, and circular dichroism) based on the perturbation of electronic and spectroscopic energy levels of the ligand or the protein and calorimetric techniques (isothermal titration calorimetry and differential

scanning calorimetry) have been extensively used. In the last year, computational measurements have also been developed to characterize the polyphenol–protein interactions [1,24].

3. Phenolic Compounds and Plasma Protein Binding

PPB and phenolic compounds have been the subject of numerous recent studies, which have focused primarily on structure–affinity relationships. The main observations are summarized in Table 1.

Table 1. Main effect of first pass metabolism of dietary phenolic compounds in human serum albumin (HSA) binding.

Class	Subclass	Reaction	Effect	References
Flavonoids	Flavonols	Hydroxylation	The binding affinity of flavonols for HSA * is structure-dependent, increasing with the number of OH groups in the A, B ring.	[33–35]
		Glucuronidation	Decrease the binding constants.	[36,37]
		Sulfation	Decrease the binding constants.	[38]
	Flavones	Hydroxylation	The HSA affinity of flavones was influenced by the location and number of the OH group.	[33,35,39,40]
		Hydrogenation	Hydrogenation of the unsaturated C2=C3 double bond can reduce binding affinities for CHPP **.	
		Methoxylation	This reaction enhanced hydrophobicity and hydrophobic interactions increasing affinity for HSA.	[33,35,40]
	Isoflavones	Hydroxylation	The hydroxylation at positions 5 and 7 on the A ring increased HSA binding affinity rates. A weakening of isoflavones binding affinities for CHPP after of hydroxylation in C-5 (A ring) and C-3' (B ring).	[33,35]
		Hydrogenation	The C2=C3 double bond conjugated with the oxo group at C-4 plays an important role in flavanone affinity for plasma proteins.	[40]
	Flavanones	Hydroxylation	Affinity increased by the addition of a hydroxyl group on the A ring (C-5 and C-7) and the B ring (C-2').	[35]
		Methoxylation	Slightly increased the protein binding rate.	[40]
		Glucuronidation	Glucuronidation in the B-ring weakly destabilizes the flavanone-HSA complex.	[41]
	Flavan-3-ols	Hydroxylation	The number of hydroxyl groups on the B ring and the presence of a galloyl (3,4,5-trihydroxybenzoyl) moiety increase binding affinities for HSA.	[42–44]
	Anthocyanidins	Hydroxylation	The binding affinities increase with the number of hydroxyl groups on the B ring.	[45–47]
		Methoxylation	The methoxylation could either strengthen or reduce the anthocyanin affinity for HSA.	[46,47]
Phenolic Acids	Hydroxybenzoic acids	Hydroxylation	In the case of benzoic acid, the introduction of (1) an OH group at C-2 on the benzene ring exerted a positive effect and (2) a hydroxy substituent at C-4 had a negative influence.	[48]
		Methoxylation	Both methylation of the hydroxy groups and substituting the hydroxy groups with methyl groups at C-3 and C-4 on the benzene ring resulted in an increase of binding affinity.	
	Hydroxycinnamic acids		Minimal modifications of the chemical structure led to significant changes in binding.	[49–52]
Stilbenes		Hydroxylation	The stilbenoid–HSA affinity was increased.	[35,53]
		Methylation	The stilbenoid–HSA affinity was reduced.	[54]
		Methoxylation		[53]
Hydrolysable Tannins		Hydroxylation	The intensity of the interaction depends not only on the number of OH groups, but also on the bulk, flexibility and hydrophobicity of the chemical structure.	[55,56]

* HSA: human serum albumin; ** CHPP: common human plasma protein.

3.1. Flavonoids

Flavonoids are the major polyphenols in a wide variety of plant sources. Associated with antiaging, antifungal, anti-inflammatory, and anticancer activities, they exhibit high reactivity with reactive oxygen species such as hydroxyl, alkoxy, or peroxy radicals and efficiently inhibit lipid peroxidation in micelle systems [57]. They have a tricyclic structure in which a phenyl (B ring) is attached at position two or three of a benzopyrone or benzopyran system (AC rings), [1] as shown in Figure 1. The most common sub-classes of flavonoids are flavonols, flavones, isoflavones, flavanones, flavan-3-ols, and anthocyanidins, which are differentiated by the structural features of the C ring [2]. The interactions between flavonoids and plasma proteins have been extensively studied. In 2018, it was found that the position of hydroxyl groups and the presence/absence of an unsaturation between C2–C3 in flavonoids could influence binding with HSA [57].

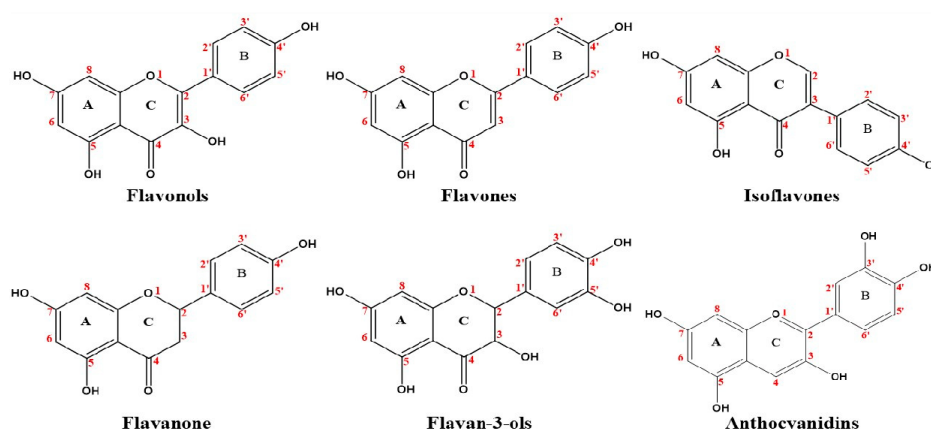


Figure 1. Chemical structure of flavonoids.

3.1.1. Flavonols

Flavonols (3-hydroxyflavones) are compounds with medium nucleophilicity and a partial positive charge at the C-3 position [39]. Given that flavonoids are present in the blood circulation mainly as conjugates resulting from phase II metabolic reactions, it is important to know the affinity of flavonoid conjugates for albumin rather than that of their native forms. In this sense, the study of Dufour and Dangles estimated that the majority of flavonol conjugates (more than 80%) circulate bound to albumin rather than in their free form [38]. This transport through the blood circulation is essential for its further delivery at the cell surface, and consequently, membrane uptake to finally produce its biological effect. In this sense, the binding would facilitate the uptake of circulating flavonol conjugates into tissues.

Structurally, the binding affinity of flavonols for HSA is structure-dependent, increasing with the number of OH groups: the addition of a hydroxyl group at C-5 (A ring) increased the binding rate of fisetin with common human plasma proteins (CHPP) by 11.48% [33]. The same occurs for the B ring where the binding constants were in the following order: myricetin > quercetin > kaempferol [34]. Additionally, hydroxylation at C-4' (B ring) slightly increased the binding affinity of 3,4'-dihydroxyflavone ($88.77 \pm 0.07\%$) compared to 3-hydroxyflavone ($87.78 \pm 0.03\%$) [35] and reduced that of galangin [33].

Quercetin, one of the most abundant flavonoids in our diet, mainly binds to HSA (subdomain IIA), followed by AAG and with no substantial interaction with very-low-density lipoproteins (99.4 ± 0.1 , 39.2 ± 0.5 and $<0.5\%$, respectively) [58]. The binding constants determined for the glucuronide form of quercetin were lower compared to the parent compound, but the stability of quercetin-3-glucuronide and isorhamnetin-3-glucuronide complexes was still high enough to significantly interact with HSA [36]. The main reasons why the glucuronidated form of quercetin had less affinity for HSA are: (1) formation

of an intermolecular hydrogen bond or an ionic interaction between the hydroxyl group of quercetin (C-3) and the nitrogen atom of Lys195 [37]; (2) for steric reasons, the presence of the bulky polar substituent (position three) may reduce the PPB affinity of this metabolite; (3) lower complex stabilities due to the absence of interaction of the 3-hydroxyl moiety with Lys195 [36]. On the other hand, the binding experiments reveal that quercetin sulfate lowers its affinity to albumin by one order of magnitude [38]. However, contradictory results were obtained in the study in which the sulfate form of quercetin (quercetin-3'-sulfate) bound to HSA slightly more readily than quercetin [36].

3.1.2. Flavones

Flavones are compounds with medium to high nucleophilicity and a medium partial negative charge at C-3 [39]. Similar to flavonols, these hydrophobic compounds display stronger binding affinities for HSA (higher binding constants) than the other flavonoids [39,59].

Changes in flavone affinity for HSA and CHPP after oxidation or conjugation reactions have been studied. The introduction of a hydroxyl group on the A ring enhanced the HSA binding rate [35]. Another study has found that the optimal number of hydroxyl groups on the A ring of flavone 7-hydroxyflavone, 5,7-dihydroxyflavone (chrysin), and baicalein was one, and the affinities for HSA decreased with the addition of more hydroxyl groups at positions C-5, C-6, and/or C-7 [60]. In 2016, Rimac and coworkers demonstrated that the HSA affinity of flavones was influenced by the location of the OH group, which had an enhancing effect when attached at C-4 on the A ring [39]. Regarding the B ring, the same authors found that after hydroxylation, apigenin had less affinity than chrysin ($1.32 \pm 0.05 \times 10^5$ and $1.95 \pm 0.08 \times 10^5 \text{ M}^{-1}$, respectively) [39]. Hydroxylation at C-3' slightly enhanced flavone binding affinity for HSA, which was about 1.38-fold higher in luteolin compared to apigenin [40], a difference that increased to 1.74-fold for CHPP [33]. Hydroxylation of the C ring resulted in a 12.5% higher protein binding rate in 3-hydroxyflavone compared to flavone [35]; hydroxylation at C-3 also increased the affinity of luteolin for CHPP, but little effect was observed in chrysin and apigenin [33].

Hydrogenation of the unsaturated C2=C3 double bond of flavone, 6-hydroxyflavone, and 6-methoxyflavone reduced binding affinities for CHPP by 10.02- to 17.82-fold, but had hardly effect in apigenin and 7-hydroxyflavone [33]. This structural modification had a similar outcome on the affinity of flavone, 6-hydroxyflavone and 7-hydroxyflavone for HSA [35]. The explanation is that the reaction modified the flavonoid C ring from a planar to a twisted structure [33], also reducing polarity, and molecules with a non-planar structure were unable to enter the hydrophobic pockets in HSA [35]. Planarity is therefore an important prerequisite for flavonoid binding in the hydrophobic cavity [39].

Methoxylation increased tangeretin affinity for HSA 100-fold, mainly attributed to enhanced hydrophobicity and hydrophobic interactions [40]. Compared to flavone, the affinity of 6-methoxyflavone was slightly higher, whereas a slightly lower affinity was observed when a hydroxyl group was substituted by a methoxy group (methylation) (6-methoxyflavone < 6-hydroxyflavone) [33]. The same behavior was observed with 5-methoxyflavone and 6-methoxyflavone, whose affinity was reduced by 20% and 4%, respectively. The more methoxy groups were added, the more the protein binding rate decreased (tangeretin > nobiletin) [35].

3.1.3. Isoflavones

Soybean and derived food products are the main sources of dietary isoflavones [1], above all genistein, daidzein and glycitein [61]. Isoflavones have low nucleophilicity and a low partial negative charge [39]. Studies on genistein have indicated it binds to HSA via polypeptide polar groups with overall binding constants of $2.4 \pm 0.40 \times 10^4 \text{ M}^{-1}$ [62] and $1.5 \pm 0.2 \times 10^5 \text{ M}^{-1}$ [61]. In the same study, the genistein-HSA complex was found to preclude the attachment of daidzein due to competitive binding. The hydroxylation of isoflavones at positions five and seven on the A ring increased HSA binding affinity rates [35]. This is in contrast with Xiao and coworkers [33], who reported a weakening of isoflavone binding affinities for CHPP after C-5 hydroxylation of the A ring; similar behavior was observed for HSA after C-3' hydroxylation of the B ring (formononetin > calycosin) [35].

3.1.4. Flavanones

Flavanones have very low nucleophilicity and a high partial negative charge at C-3 on the C ring [39]. In 2011, Cao and coworkers demonstrated that the C2=C3 double bond conjugated with the oxo group at C-4 played an important role in flavanone affinity for plasma proteins; hence, the affinity of apigenin for HSA is about 10,000-fold higher than that of naringenin [40]. Moreover, affinity increased by the addition of a hydroxyl group on the A ring (at C-5 and C-7) and the B ring (at C-2') [35]. Regarding methoxylation, the addition of a methyl group at position six on the A ring of flavanone slightly increased the protein binding rate [35] and increased the affinity of tangeretin for HSA 100-fold [40] by polarity reduction and capacity improvement to penetrate the tryptophan-rich hydrophobic regions of HSA [40]. Finally, and considering glucuronidation, it showed weakly destabilized flavanone–HSA complexes and the effect was slightly stronger than if it had occurred in the B ring [41].

3.1.5. Flavan-3-Ols

The subclass of the flavonoid family known as flavan-3-ols includes catechins and plant phenols widely distributed in various fruits, red wine, juices, and cocoa, with green tea being the richest dietary source [63]. The number of hydroxyl groups on the B ring and the presence/absence of a galloyl (3,4,5-trihydroxybenzoyl) moiety are responsible for the functional differences in catechins such as (–)-epigallocatechin, (–)-epicatechin, (–)-epicatechin gallate and (–)-epigallocatechin gallate [42,43]. Thus, those with the galloyl moiety showed enhanced hydrophobic properties and consequently higher binding affinities for HSA than catechins lacking this substituent [43,44]. Differences in protein affinity have also been found among stereoisomeric flavan-3-ols [64]. The binding constant (K_a) obtained for (+)-catechin–HSA lies in the intermediate range, not being so low that it leads to an inefficient distribution or so high that it lowers the plasma concentration [42]. The number of binding sites found for the catechin–HSA systems range from 0.87 to 1.10, suggesting that one HSA molecule associates with one catechin molecule [63].

3.1.6. Anthocyanidins

Anthocyanins are water-soluble pigments found throughout plants but are most obvious in fruits and flowers, presenting a spectrum range from pink, red and violet to dark blue [45,46]. The basic chemical structure of anthocyanins is 3,5,7-trihydroxy-2-phenylbenzopyran and they can be classified according to whether they are glycosylated or not, as well as by the number and location of hydroxyl or methoxyl groups and their substituents on the rings [45]. Thus, under physiological pH conditions, the number and position of the hydroxyl groups on the B ring affected anthocyanin affinities for HSA, which increased with the number of groups [45–47]. This effect can be explained considering that at pH 7.4 quinoidal and anionic quinoidal bases are major species, which favor hydrogen bonding and other types of electrostatic forces [47]. On the other side, the influence of methoxylation and glycosylation could either strengthen or reduce the anthocyanin affinity for HSA [46,47].

3.2. Phenolic Acids

Phenolic acids, including benzoic and cinnamic acids, are the predominant type of polyphenols in fruits, vegetables, and most plant-derived beverages [49]. Structurally, they derive from benzoic or cinnamic acid, as shown in Figure 2.

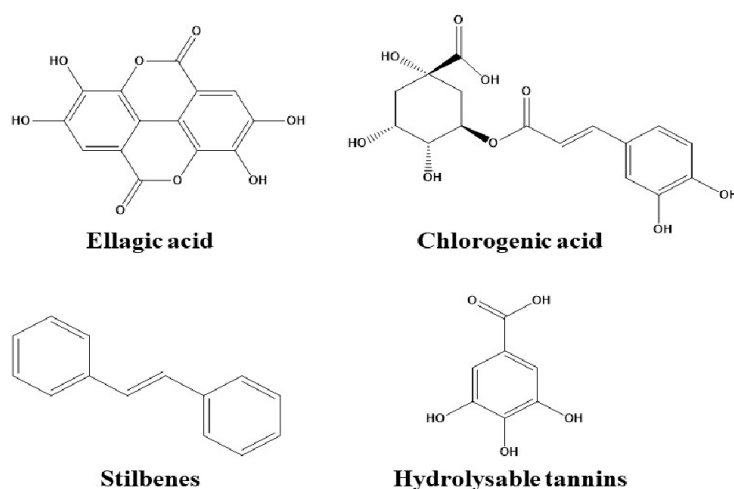


Figure 2. Chemical structure of representative phenolic acids. General chemical structure of stilbenes and hydrolysable tannins.

3.2.1. Hydroxybenzoic Acids

Ellagic acid, a pharmacologically beneficial polyphenol found in fruits (grapes, strawberries, blackcurrants and raspberries) and nuts [65–67], formed a complex with HSA with a binding affinity constant of $15.5 \times 10^4 \text{ M}^{-1}$ [65]. In 2014, He and coworkers demonstrated that ellagic acid binds to HSA more strongly than oleuropein [68]. In the case of benzoic acid, the introduction of different numbers and arrangements of hydroxy groups and other substituents on the aromatic ring altered the binding affinities as follows: (1) a hydroxy group at C-2 on the benzene ring exerted a positive effect; (2) a hydroxy substituent at C-4 had a negative influence; and (3) both methylation of the hydroxy groups and substitution of the hydroxy groups with methyl groups at C-3 and C-4 on the benzene ring resulted in an increase [48].

3.2.2. Hydroxycinnamic Acids

The most representative hydroxycinnamic acids are caffeic acid, chlorogenic acid, and caffeoyl quinic acids [1]. The association constants (K) for the HSA–chlorogenic acid interaction at 10, 25 and 40 °C were 6.0×10^4 , 9.0×10^3 and $2 \times 10^4 \text{ M}^{-1}$, respectively [69], indicating the involvement of two major forces in the binding process. Similar results were reported by Kang and coworkers ($4.37 \times 10^4 \text{ M}^{-1}$) [50]. The formation of a biomolecular complex between chlorogenic acid and its derivatives with HSA was also studied by Sinisi and colleagues [70]; the reported dissociation constants indicated a very high affinity for this family of compounds, and minimal modifications of the chemical structure led to significant changes in binding. In another study, the binding affinity of chlorogenic acid and its isomers for HSA ranked in the decreasing order: cryptochlorogenic acid > neochlorogenic acid > chlorogenic acid; the 4-esteryl structure was associated with a higher binding affinity and larger conformational changes than the 3- or 5-esteryl structures [51]. The binding constant of HSA complexes was calculated to be $4.29 \times 10^4 \text{ M}^{-1}$ at 298 K for rosmarinic acid [49] and $2.23 \times 10^4 \text{ M}^{-1}$ for ferulic acid [50]. Cinnamic acid and its hydroxyl derivatives interacted with HSA in the decreasing order of caffeic acid > p-coumaric acid > cinnamic acid [52].

3.3. Stilbenes

Stilbenoids are an important group of phenolic compounds with a C6–C2–C6 carbon skeleton structure [1], as shown in Figure 2. Among them, resveratrol, found largely in the skin of red grapes and wine, binds to HSA with an association constant of $2.56 \times 10^5 \text{ M}^{-1}$ [71]. The effect of

hydroxylation of stilbenes has been studied in the context of the structure–affinity relationship of stilbenoid–HSA systems. It has been recently demonstrated that the addition of a hydroxyl group to resveratrol (piceatannol and oxyresveratrol) on the B ring increases the binding rates [35]. In addition, hydroxylation increased the binding affinities of stilbenoids for HSA (pterostilbene > pinostilbene > oxyresveratrol > piceatannol > resveratrol > isorhapontigenin > piceid) [53]. The stilbenoid–HSA affinity was reduced by methylation (resveratrol > pterostilbene) [54] and enhanced by methoxylation at C-3 or C-5 of resveratrol [53].

3.4. Hydrolysable Tannins

Little is known about the structural and functional relationship between plasma proteins and tannins. Sekowski and coworkers demonstrated that hydrolysable tannins interact very strongly with HSA, with an intensity of the interaction that depends not only on the number of hydroxyl groups, but also on the bulk, flexibility and hydrophobicity of the chemical structure [55,56]. Tannins with higher molecular weight bind with stronger interaction to proteins, but also tannin flexibility has to be considered: the more flexible the structure, the more rotational capacity of the molecule and easier access to protein binding pockets [55]. Thus, larger and more flexible molecules are capable of changing the secondary structure of albumin through surface interactions, but the presence of valoneoyl structures limit the capacity of tannins to penetrate the hydrophobic tryptophan pocket [55,56].

4. Interactions between Phenolic Compounds and Plasma Proteins

4.1. Non-Covalent Bonds

Phenolic compounds, under non-oxidative conditions, form reversible complexes with plasma proteins [72,73] involving hydrogen bonds, electrostatic interactions, hydrophobic effects and van der Waals forces [45,72,73]. The interactions between polyphenols and proteins are determined by the thermodynamic parameters of enthalpy (ΔH°) and entropy (ΔS°). Positive values of both parameters indicate a hydrophobic interaction; negative values point to van der Waals forces and hydrogen bonding; and $\Delta S^\circ > 0$ and $\Delta H^\circ \approx 0$ indicate that an electrostatic force plays a vital role in aqueous solutions [74].

The attraction between the aromatic ring of the polyphenol and hydrophobic regions of other compounds (aliphatic and aromatic side chains of amino acids) leads to the formation of hydrophobic bonds [75]. These bonds arise from changes in entropy rather than enthalpy and are reversible and independent of pH [75]. Hydrogen bonds involve the sharing of a phenolic proton with numerous amide carbonyl moieties of HSA and occur only in the presence of phenoxyl hydrogen. On the other hand, ionic bonds are only generated when polyphenols are ionized to highly reactive phenolate ions. Electrostatic attraction between the phenolate ion and cationic portions of other compounds leads to the formation of this bond [75].

The nature of phenol–protein complexes depends heavily on individual phenolic structures, and the spatial arrangements of substituents seem to be key factors in phenol–HSA affinities [57,72]. In genistein–HSA complexes, hydrophobic and electrostatic interactions play a central role in the binding [76], although the involvement of hydrogen bonds and ionic interactions has also been suggested [61]. The binding between naringin and HSA strongly involves hydrophobic interactions, according to the positive values of ΔH° and ΔS° , but the involvement of electrostatic interactions cannot be ruled out [77]. The nitrogen atom of Lys195 is suitably positioned to establish an intermolecular ionic interaction with the hydroxyl at C-3 in quercetin [37].

To date, numerous studies on flavanone–HSA complex formation have suggested the involvement of: (1) hydrogen bonds between the phenolic hydroxyl groups and the polypeptide chain or polar amino acid residues; (2) hydrophobic interactions between the aromatic ring and hydrophobic amino acid residues; (3) electrostatic forces generated after the deprotonation of the acidic phenolic hydroxyl

group in the A ring (hesperetin) by the basic amino acid residues; (4) perpendicular and parallel π - π interactions and (5) van der Waals forces [78–81].

There is considerable evidence that catechins bind to HSA via hydrophobic and hydrogen bonding [63,64,82]. It has been demonstrated that hydrogen bonding and van der Waals forces are predominant in (+)-catechin–HSA complexes [42]. However, thermodynamic parameters indicate that tea catechins bind serum proteins via ionic interactions ($+\Delta S^\circ$ 18 to 9 J mol^{−1} K^{−1} and $-\Delta H^\circ$ −13 to −8 kJ mol^{−1}) [82].

As anthocyanins are transported in the circulation system, small molecules bound to plasma proteins are exchanged with their free form in order to maintain an equilibrium [29]. Various studies have focused on the structure–molecular affinity relationship of the anthocyanin–HSA interaction. The van der Waals forces, hydrogen bonds, hydrophobic effect and electrostatic forces are the main drivers of the anthocyanin–protein complex formation [29,47]. The strength of anthocyanin binding to HSA is affected by factors such as pH, temperature, and chemical structure. Thus, the hydrophobic effect plays a major role at pH 4 but is less important at pH 7.4. Moreover, at pH 7.4, the association constant of HSA with aglycones bearing an increasing number of hydroxyl groups indicates the importance of attractive electrostatic interactions such as hydrogen bonding between polar groups [47].

Hydrophobic forces and hydrogen bonds are predominant in the HSA binding interactions with phenolic acids and their methyl esters, respectively [48], as well as with ellagic acid [65,66,68]; the involvement of van der Waals forces has also been proposed [67]. Gallic acid is reported to bind to HSA mainly by hydrophobic and electrostatic interaction in microemulsions [83].

Little is known about the structural and functional relationships in tannin–HSA and stilbenoid–HSA complexes. Tannin interaction with plasma proteins was recently described as complex and opportunistic, occurring without specific binding sites and determined mainly by hydrophobic forces and hydrogen bonds [55,56]. On the other hand, the hydrophobic interaction is the main force binding stilbenoids with HSA [53]. Regarding resveratrol, structural analysis showed that it binds non-specifically (H-bonding) via polypeptide polar groups [71].

4.2. Hydrolysable Tannins

Highly reactive with oxygen species, many polyphenols can be oxidized to their corresponding semiquinones and quinones, which may then undergo covalent reactions with an enormous number of nucleophiles [73], such as amino acids or thiol groups [72]. In this context, covalent bonds can be formed between a phenolic or quinone carbon ring and plasma proteins. The irreversible polyphenol–protein covalent bindings influence the chemical properties of both species, are resistant to disruption by denaturing agents [73,84,85], and can be formed enzymatically and non-enzymatically [75]. Regarding phenolics, covalent bonds are the result of their electrophilic substitution at the ortho and para positions. On the other hand, conjugate addition reactions to the unsaturated carbonyl moiety lead to the formation of covalent bonds with quinones. The differences in the location and type of substituents are a result of differences in electron densities and distribution both in phenols and quinones. The phenolic polymerization through covalent bonds by oxidative processes (generating phenoxyl radicals), or by condensation reactions between polyfunctional nucleophiles (including the anions derived from polyphenols) and quinones can also be formed [75]. Covalent reactions can change the protein structure with a corresponding modification of hydrophobic–hydrophilic properties of the protein derivatives, accompanied by a change in solubility [73].

In 2005, Kaldas and coworkers demonstrated in an *in vitro* study that quercetin oxidized by peroxidase/hydrogen peroxide covalently links to proteins, mainly with a high affinity for HSA [84]. However, in 2010, Cahyana and Gordon reported that non-covalent binding occurred between HSA and anthocyanins with a quite weak negative value of Gibbs free energy (ΔG° , −28.2 to −36.3 kJ mol^{−1}), which is much lower than the value for covalent bond formation (approximately 400 kJ mol^{−1}) [47].

5. Binding Sites

HSA contains three homologous α -helical domains (I–III), each further divided into two subdomains (A and B) [30]. Subdomains IIA and IIIA (Sudlow's sites I and II, respectively) are primary binding sites at which many drugs can bind, with IIA being the most prominent. In this sense, bulky heterocyclic anions with the charge located in a central position of the molecules (such as warfarin) are generally attached at the I site of HSA, while aromatic carboxylic acids with an extended conformation and the negative charge located at one end of the molecule (such as ibuprofen) bind at site II [11,38,79,85].

The IIA subdomain appears to be spacious and is comprised of several individual binding sites that can accommodate ligands with a great variety of chemical structures. In contrast, the IIIA site is smaller and less flexible and can only accept structurally similar ligands [39]. The protein microenvironment of site I of HSA is rich in polar (basic) amino acid residues, which are able to stabilize the negatively charged ligand bound in non-planar conformation [37].

In 2005, Dufour and Dangles proposed that the binding of flavonols mainly takes place in subdomain IIA. Computational mapping of possible binding sites of quercetin revealed binding in the large hydrophobic cavity of subdomain IIA [37], similar to that reported for kaempferol [86], hesperetin [79] and apigenin [87].

The higher affinity of flavones for HSA possibly relies on the benzopyrone moiety (AC rings), which protrudes from the hydrophobic cavity and points towards the entrance of site I, as the hydrophilic phenolic hydroxyl groups can interact with nearby polar residues [88]. However, methoxylation decreased flavone polarity and improved the ability to penetrate the tryptophan-rich hydrophobic regions of proteins, which are often buried in the interior of the folded proteins [40]. To date, it is known that the HSA conformation is altered by binding to trimethoxy flavone, with a decrease in α -helix content and an increase in β -sheets and random coils, suggestive of the partial unfolding of the secondary structure of the protein [89].

The identification of a binding pocket in HSA for isoflavones suggests daidzein and genistein are bound to the subdomain IIA [61]. Structural differences between curcumin and genistein did not affect the hydrophobic binding affinity for HSA and therefore both are mainly bound in the hydrophobic pockets of Trp 214 in site I [62]. Moreover, structural analysis demonstrated that curcumin and genistein bind to HSA via polypeptide polar residues and that the HSA conformation is altered by their complexation, with a reduction in α -helix content and an increase in random coil and turn structures, again indicating a partial protein unfolding [62].

Similar to the other flavonoids, the primary binding site for flavanones is in the subdomain IIA, but it is located closer to the binding site in IIIA [40,77,78,80,81]. The alteration of the protein secondary structure has also been studied [77]. When the flavanone concentration was increased, the percentage of protein α -helix structure gradually decreased [77,79], which implied a rearrangement of the carbonyl hydrogen bonding network of the main polypeptide chain of the protein [78,79]. The flavanones entered the hydrophobic binding cavity located in subdomain IIA by hydrophobic interactions, and the phenolic hydroxyl groups interacted through strong hydrogen bonds with C=O and N–H of the main polypeptide chain, resulting in the aforementioned rearrangement of the polypeptide carbonyl hydrogen bonding network, and a consequent reduction in the protein α -helix structure [79].

A site marker competitive experiment has demonstrated that (+)-catechin is mainly located within site I of HSA [42] and ($\pm$), (+), and (–) forms of catechin bind in the proximity of Trp-214 of HSA [64]. Additionally, studies on conformational changes in plasma protein after binding with flavan-3-ols have reported that galloylated catechins increased the electrophoretic mobility of HSA, which reflects a modification of its molecular charge [44,63]. Changes in the α -helicity of the ($\pm$), (+), and (–) forms of catechin were found to be marginal [64].

Protein conformational changes can also be attributed to the presence of anthocyanins. Interaction of HSA with keracyanin causes changes in the polarity of the hydrophobic microenvironment. The binding site of keracyanin might be in the hydrophobic cavity, the location of Trp-214 and at the

center of the three domains of HSA, which induces slight unfolding or adaptive rearrangement of the polypeptide backbone of the protein [29].

Structural differences affect the binding site of hydroxybenzoic acids. In 2009, a study revealed that protocatechuic acid and its partially methylated products bind to HSA in sites I and II. In contrast, the addition of a hydroxyl group from protocatechuic acid, yielding gallic acid, did not result in competitive binding in sites I or II [90]. An evaluation of the bioactive interaction indicates that the HSA residues for ellagic acid binding are located in subdomain IIA [66] and that the mode of binding reaction is spontaneous [67], which would suggest an alteration of the protein secondary structure. According to circular dichroism spectroscopic data, the fraction of alpha helicity was reduced from 52% to 40% upon binding to ellagic acid [65]. Both ellagic acid and oleuropein interact with the binding pocket of HSA in subdomain IIA [68]. In another study, it has also been demonstrated that gallic acid could bind to the site I of HSA [83].

Differences between hydroxycinnamic acid binding sites have also been studied. Binding with chlorogenic acid induces conformational change in HSA, involving the one tryptophan residue in the binding region [51,69]. The binding of chlorogenic acid and rosmarinic acid most likely takes place in site I and ferulic acid in site II [49,50]. Chlorogenic acids and their derivatives, which are abundant in coffee, form a bimolecular complex within Sudlow's site I and interact with Trp-214 [70]. Multispectroscopic and docking studies on the binding of chlorogenic acid isomers with HSA show that three isomers bound to HSA at Sudlow's site I, affecting the protein secondary structure [51]. Chlorogenic acid is thought to bind in subdomain IIA and ferulic acid in IIIA [50]. In another study, it has been demonstrated that after the interaction between HSA with caffeic acid, p-coumaric acid and cinnamic acid, the α -helix structure was reduced by 9, 5 and 3%, respectively [52]. Finally, in 2015, Nair demonstrated through experimental and theoretical studies that both resveratrol and pterostilbene bind to the hydrophobic cavity at site IA in the subdomain II of HSA [54].

6. Phenolic Compounds–Drug Interaction

Data on food–drug interactions are generally scarce, despite some well documented exceptions (e.g., grapefruit juice and statins), as food consumption and herbal teas/beverages are not usually monitored in patients. Interactions occur after the concomitant intake of food and drugs, with impacts on the absorption and/or metabolism of the active substance. In some cases, the effects of the interactions may benefit the patients, but they frequently undermine the efficacy of the drug or induce adverse reactions [91].

In the case of PPB, a hormone, drug or even a toxin can be displaced by competing phenolic compounds and then circulates in the blood in a free form. The pharmacodynamics and pharmacokinetics of a drug may subsequently be modified, potentially leading to stronger pharmacological activity, adverse effects and faster elimination [39]. It should be noted that such effects are rarely caused by the formation of phenol–HSA complexes, as the low phenol and high HSA concentration in plasma renders saturation at the binding site unlikely. Moreover, phenolic compounds are often subject to high first-pass metabolism, and thus it is the conjugated-HSA complex that should be taken into account in a potential food–drug interaction. Nevertheless, such an outcome should be kept in mind for drugs with high PPB, a low extraction ratio and narrow therapeutic index, and for other plasma proteins more specific than HSA.

To date, most of the research on food–drug interactions has been focused on flavonoids and HSA. Rutin and baicalin have been extensively used to determine the effect of flavonoids on the binding properties of cleviprex, theophylline, nifedipine, promethazine and ticagrelor [91–95]. The results show that (1) both hydrogen bonds and hydrophobic interactions play a central role in the binding process, which is spontaneous; (2) flavonoids can reduce the association constant and increase the distance of drugs binding to HSA due to competitive binding at site I; (3) the synergistic effect of drugs with rutin and baicalin can further change the HSA conformation, and (4) reduced affinities of drugs binding to HSA in the presence of flavonoids may lead to an increase in free drugs in the blood, which would

affect their transportation and/or disposition and may provoke adverse or toxic effects, as shown in Figure 3.

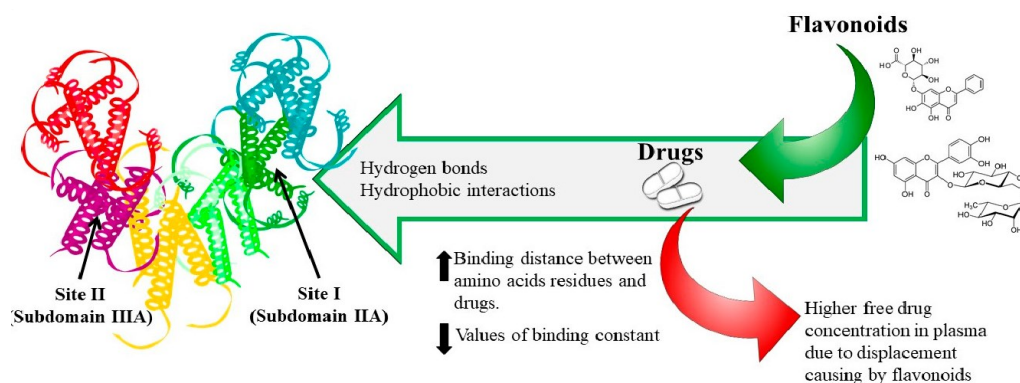


Figure 3. Dietary flavonoid–drug interaction mechanism.

Quercetin had the same effect as rutin and baicalin on ticagrelor and propranolol binding to HSA [95,96]. In 2012, Maciazek-Jurczyk and collaborators reported that competition from curcumin for the binding site of tamoxifen in HSA reduced the binding affinity of this chemopreventive agent, which increased its unbound fraction in the blood with potentially toxic effects [97]. In 2017, Rimac and colleagues showed that warfarin–flavonoid interactions should be regarded as negligible, as they do not share the same binding region in HSA [98]. Conversely, in the same year, it was demonstrated that quercetin metabolites strongly displace warfarin when binding to HSA, suggesting that high quercetin levels can negatively interfere with warfarin therapy [36].

Consequently, the intake of flavonoid-rich foods and beverages should be reduced during treatment with the aforementioned drugs to avoid food–drug interactions and the incidence of toxic symptoms. Alternatively, drugs that do not share the same binding region as flavonoids can be used.

7. Conclusions

PPB is a key process that determines the pharmacokinetics (distribution, metabolism, and elimination) and effects of many drugs and dietary phenolics in the body. This review has gathered valuable information concerning the binding of dietary polyphenols to HSA, highlighting how the properties of these compounds can be substantially modified after the formation of a protein complex. The structural differences among flavonoids, phenolic acids, stilbenes, and hydrolysable tannins strongly affect the binding process with plasma proteins. Thus, the formation of a phenolic–HSA complex is affected by phase I reactions. The number and position of substituted hydroxyls on the aromatic ring of the compounds and hydrogenation can alter the nucleophilicity and planarity of molecules. The binding affinities of phenolic compounds towards HSA are also affected by phase II reactions (glucuronidation, methylation, methoxylation and sulfation). Accordingly, in order to avoid potential therapeutic failures caused by food–drug interactions, the intake of flavonoid-rich food and beverages should be monitored when treating certain pathologies.

Funding: A.L.-Y. wishes to thank the Consejo Nacional de Ciencia y Tecnología (CONACYT) of Mexico for the doctoral scholarship. A.V.-Q. thanks the Ministry of Science Innovation and Universities for the Ramon y Cajal contract (RYC-2016-19355). Financial support from the MINECO/FEDER, Spain (Project RTI2018-09974-B-I00) is gratefully acknowledged. Thanks to the University of Barcelona funding for Open access publishing.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Xiao, J.; Kai, G. A review of dietary polyphenol-plasma protein interactions: Characterization, influence on the bioactivity, and structure-affinity relationship. *Crit. Rev. Food Sci. Nutr.* **2012**, *52*, 85–101. [CrossRef] [PubMed]
2. Ribas-Agustí, A.; Martín-Belloso, O.; Soliva-Fortuny, R.; Elez-Martínez, P. Food processing strategies to enhance phenolic compounds bioaccessibility and bioavailability in plant-based foods. *Crit. Rev. Food Sci. Nutr.* **2018**, *58*, 2531–2548. [CrossRef] [PubMed]
3. Poloni, D.M.; Dangles, O.; Vinson, J.A. Binding of plant polyphenols to serum albumin and LDL: Healthy implications for heart disease. *J. Agric. Food Chem.* **2019**, *67*, 9139–9147. [CrossRef] [PubMed]
4. FDA, G. Bioavailability and Bioequivalence Studies for Orally Administered Drug Products—General; Food and Drug Administration: Rockville, MD, USA, 2002.
5. Neilson, A.P.; Goodrich, K.M.; Ferruzzi, M.G. Bioavailability and Metabolism of Bioactive Compounds from Foods. In *Nutrition in the Prevention and Treatment of Disease*; Elsevier: Amsterdam, The Netherlands, 2017; pp. 301–319. ISBN 978-0-12-802928-2.
6. Luca, S.V.; Macovei, I.; Bujor, A.; Miron, A.; Skalicka-Woźniak, K.; Aprotosoaie, A.C.; Trifan, A. Bioactivity of dietary polyphenols: The role of metabolites. *Crit. Rev. Food Sci. Nutr.* **2020**, *60*, 626–659. [CrossRef]
7. Serreli, G.; Deiana, M. Biological relevance of extra virgin olive oil polyphenols metabolites. *Antioxidants* **2018**, *7*, 170. [CrossRef]
8. Caldwell, J.; Gardner, I.; Swales, N. An introduction to drug disposition: The basic principles of absorption, distribution, metabolism, and excretion. *Toxicol. Pathol.* **1995**, *23*, 102–114. [CrossRef]
9. Talevi, A.; Quiroga, P.A.M. (Eds.) *ADME Processes in Pharmaceutical Sciences: Dosage, Design, and Pharmacotherapy Success*; Springer: Cham, Switzerland, 2018; ISBN 978-3-319-99592-2.
10. Gupta, A. Plasma Proteins. In *Comprehensive Biochemistry for Dentistry: Textbook for Dental Students*; Gupta, A., Ed.; Springer: Singapore, 2019; pp. 67–75. ISBN 978-981-13-1035-5.
11. Otagiri, M. A molecular functional study on the interactions of drugs with plasma proteins. *Drug Metab. Pharmacokinet.* **2005**, *20*, 309–323. [CrossRef]
12. Kamble, S.; Loadman, P.; Abraham, M.H.; Liu, X. Structural properties governing drug-plasma protein binding determined by high-performance liquid chromatography method. *J. Pharm. Biomed. Anal.* **2018**, *149*, 16–21. [CrossRef]
13. Dasgupta, A. Monitoring Free Drug Concentration. In *Clinical Challenges in Therapeutic Drug Monitoring*; Elsevier: Amsterdam, The Netherlands, 2016; pp. 71–100. ISBN 978-0-12-802025-8.
14. Roberts, J.A.; Pea, F.; Lipman, J. The clinical relevance of plasma protein binding changes. *Clin. Pharmacokinet.* **2013**, *52*, 1–8. [CrossRef]
15. Vallner, J.J. Binding of Drugs by Albumin Plasma Protein. *J. Pharm. Sci.* **1977**, *66*, 447–465. [CrossRef]
16. Smith, D.A.; Di, L.; Kerns, E.H. The effect of plasma protein binding on in vivo efficacy: Misconceptions in drug discovery. *Nat. Rev. Drug Discov.* **2010**, *9*, 929–939. [CrossRef] [PubMed]
17. Beasley, V. Absorption, Distribution, Metabolism, and Elimination: Differences among Species. In *Veterinary Toxicology*; International Veterinary Information Service: Ithaca, NY, USA, 1999; pp. 1–19.
18. Hartmut, D.; Schmidt, S. *Clinical Pharmacokinetics and Pharmacodynamics: Concepts and Applications*, 5th ed.; Wolters Kluwer Health/Lippincott William & Wilkins: Philadelphia, PA, USA, 2020.
19. Rowland, M.; Tozer, T.N. *Clinical Pharmacokinetics and Pharmacodynamics: Concepts and Applications*, 4th ed.; Wolters Kluwer Health/Lippincott William & Wilkins: Philadelphia, PA, USA, 2011; ISBN 978-0-7817-5009-7.
20. Kratochwil, N.A.; Huber, W.; Müller, F.; Kansy, M.; Gerber, P.R. Predicting plasma protein binding of drugs: A new approach. *Biochem. Pharmacol.* **2002**, *64*, 1355–1374. [CrossRef]
21. Nation, R.L.; Theuretzbacher, U.; Tsuji, B.T. Concentration-dependent plasma protein binding: Expect the unexpected. *Eur. J. Pharm. Sci.* **2018**, *122*, 341–346. [CrossRef] [PubMed]
22. Merlot, A.M.; Kalinowski, D.S.; Richardson, D.R. Unraveling the mysteries of serum albumin—More than just a serum protein. *Front. Physiol.* **2014**, *5*, 299. [CrossRef] [PubMed]
23. Poulin, P.; Haddad, S. Albumin and Uptake of Drugs in Cells: Additional Validation Exercises of a Recently Published Equation that Quantifies the Albumin-Facilitated Uptake Mechanism(s) in Physiologically Based Pharmacokinetic and Pharmacodynamic Modeling Research. *J. Pharm. Sci.* **2015**, *104*, 4448–4458. [CrossRef]

24. Vuignier, K.; Schappler, J.; Veuthey, J.-L.; Carrupt, P.-A.; Martel, S. Drug–protein binding: A critical review of analytical tools. *Anal. Bioanal. Chem.* **2010**, *398*, 53–66. [CrossRef]
25. Schmidt, S.; Gonzalez, D.; Derendorf, H. Significance of Protein Binding in Pharmacokinetics and Pharmacodynamics. *J. Pharm. Sci.* **2010**, *99*, 1107–1122. [CrossRef]
26. Howard, M.; Hill, J.; Galluppi, G.; McLean, M. Plasma Protein Binding in Drug Discovery and Development. *Comb. Chem. High Throughput Screen.* **2010**, *13*, 170–187. [CrossRef]
27. Trainor, G.L. The importance of plasma protein binding in drug discovery. *Expert Opin. Drug Discov.* **2007**, *2*, 51–64. [CrossRef]
28. Curry, S.; Mandelkow, H.; Brick, P.; Franks, N. Crystal structure of human serum albumin complexed with fatty acid reveals an asymmetric distribution of binding sites. *Nat. Struct. Biol.* **1998**, *5*, 827–835. [CrossRef]
29. Sheng, F.; Wang, Y.; Zhao, X.; Tian, N.; Hu, H.; Li, P. Separation and Identification of Anthocyanin Extracted from Mulberry Fruit and the Pigment Binding Properties toward Human Serum Albumin. *J. Agric. Food Chem.* **2014**, *62*, 6813–6819. [CrossRef] [PubMed]
30. Varshney, A.; Sen, P.; Ahmad, E.; Rehan, M.; Subbarao, N.; Khan, R.H. Ligand binding strategies of human serum albumin: How can the cargo be utilized? *Chirality* **2010**, *22*, 77–87. [CrossRef] [PubMed]
31. Gupta, A. *Comprehensive Biochemistry for Dentistry: Textbook for Dental Students*; Springer: Singapore, 2018; ISBN 9789811310348.
32. Yeggoni, D.P.; Rachamalla, A.; Subramanyam, R. A comparative binding mechanism between human serum albumin and α -1-acid glycoprotein with corilagin: Biophysical and computational approach. *RSC Adv.* **2016**, *6*, 40225–40237. [CrossRef]
33. Xiao, J.; Zhao, Y.; Wang, H.; Yuan, Y.; Yang, F.; Zhang, C.; Yamamoto, K. Noncovalent Interaction of Dietary Polyphenols with Common Human Plasma Proteins. *J. Agric. Food Chem.* **2011**, *59*, 10747–10754. [CrossRef]
34. Das, P.; Chaudhari, S.K.; Das, A.; Kundu, S.; Saha, C. Interaction of flavonols with human serum albumin: A biophysical study showing structure–activity relationship and enhancement when coated on silver nanoparticles. *J. Biomol. Struct. Dyn.* **2019**, *37*, 1414–1426. [CrossRef]
35. Cao, H.; Liu, X.; Ulrih, N.P.; Sengupta, P.K.; Xiao, J. Plasma protein binding of dietary polyphenols to human serum albumin: A high performance affinity chromatography approach. *Food Chem.* **2019**, *270*, 257–263. [CrossRef]
36. Poór, M.; Boda, G.; Needs, P.W.; Kroon, P.A.; Lemli, B.; Bencsik, T. Interaction of quercetin and its metabolites with warfarin: Displacement of warfarin from serum albumin and inhibition of CYP2C9 enzyme. *Biomed. Pharmacother.* **2017**, *88*, 574–581. [CrossRef]
37. Zsila, F.; Bikádi, Z.; Simonyi, M. Probing the binding of the flavonoid, quercetin to human serum albumin by circular dichroism, electronic absorption spectroscopy and molecular modelling methods. *Biochem. Pharmacol.* **2003**, *65*, 447–456. [CrossRef]
38. Dufour, C.; Dangles, O. Flavonoid–serum albumin complexation: Determination of binding constants and binding sites by fluorescence spectroscopy. *Biochim. Biophys. Acta Gen. Subj.* **2005**, *1721*, 164–173. [CrossRef]
39. Rimac, H.; Debeljak, Ž.; Šakić, D.; Weitner, T.; Gabričević, M.; Vrček, V.; Zorc, B.; Bojić, M. Structural and electronic determinants of flavonoid binding to human serum albumin: An extensive ligand-based study. *RSC Adv.* **2016**, *6*, 75014–75022. [CrossRef]
40. Cao, H.; Chen, L.; Xiao, J. Binding Citrus flavanones to human serum albumin: Effect of structure on affinity. *Mol. Biol. Rep.* **2011**, *38*, 2257–2262. [CrossRef] [PubMed]
41. Khan, M.K.; Rakotomanomana, N.; Dufour, C.; Dangles, O. Binding of citrus flavanones and their glucuronides and chalcones to human serum albumin. *Food Funct.* **2011**, *2*, 617. [CrossRef] [PubMed]
42. Li, X.; Wang, S. Study on the interaction of (+)-catechin with human serum albumin using isothermal titration calorimetry and spectroscopic techniques. *New J. Chem.* **2015**, *39*, 386–395. [CrossRef]
43. Ishii, T.; Ichikawa, T.; Minoda, K.; Kusaka, K.; Ito, S.; Suzuki, Y.; Akagawa, M.; Mochizuki, K.; Goda, T.; Nakayama, T. Human Serum Albumin as an Antioxidant in the Oxidation of (–)-Epigallocatechin Gallate: Participation of Reversible Covalent Binding for Interaction and Stabilization. *Biosci. Biotechnol. Biochem.* **2011**, *75*, 100–106. [CrossRef]
44. Minoda, K.; Ichikawa, T.; Katsumata, T.; Onobori, K.; Mori, T.; Suzuki, Y.; Ishii, T.; Nakayama, T. Influence of the Galloyl Moiety in Tea Catechins on Binding Affinity for Human Serum Albumin. *J. Nutr. Sci. Vitaminol.* **2010**, *56*, 331–334. [CrossRef]

45. Tang, L.; Zuo, H.; Shu, L. Comparison of the interaction between three anthocyanins and human serum albumins by spectroscopy. *J. Lumin.* **2014**, *153*, 54–63. [CrossRef]
46. Zhang, J.; Zuo, B.; Ulrih, N.P.; Sengupta, P.K.; Zheng, X.; Xiao, J. Structure-affinity relationship of dietary anthocyanin-HSA interaction. *J. Berry Res.* **2018**, *8*, 1–9. [CrossRef]
47. Cahyana, Y.; Gordon, M.H. Interaction of anthocyanins with human serum albumin: Influence of pH and chemical structure on binding. *Food Chem.* **2013**, *141*, 2278–2285. [CrossRef]
48. Zhang, Y.; Wu, S.; Qin, Y.; Liu, J.; Liu, J.; Wang, Q.; Ren, F.; Zhang, H. Interaction of phenolic acids and their derivatives with human serum albumin: Structure-affinity relationships and effects on antioxidant activity. *Food Chem.* **2018**, *240*, 1072–1080. [CrossRef]
49. Peng, X.; Wang, X.; Qi, W.; Su, R.; He, Z. Affinity of rosmarinic acid to human serum albumin and its effect on protein conformation stability. *Food Chem.* **2016**, *192*, 178–187. [CrossRef]
50. Kang, J.; Liu, Y.; Xie, M.; Li, S.; Jiang, M.; Wang, Y. Interactions of human serum albumin with chlorogenic acid and ferulic acid. *Biochim. Biophys. Acta Gen. Subj.* **2004**, *1674*, 205–214. [CrossRef] [PubMed]
51. Tang, B.; Huang, Y.; Ma, X.; Liao, X.; Wang, Q.; Xiong, X.; Li, H. Multispectroscopic and docking studies on the binding of chlorogenic acid isomers to human serum albumin: Effects of esteryl position on affinity. *Food Chem.* **2016**, *212*, 434–442. [CrossRef] [PubMed]
52. Min, J.; Meng-Xia, X.; Dong, Z.; Yuan, L.; Xiao-Yu, L.; Xing, C. Spectroscopic studies on the interaction of cinnamic acid and its hydroxyl derivatives with human serum albumin. *J. Mol. Struct.* **2004**, *692*, 71–80. [CrossRef]
53. Cao, H.; Jia, X.; Shi, J.; Xiao, J.; Chen, X. Non-covalent interaction between dietary stilbenoids and human serum albumin: Structure-affinity relationship, and its influence on the stability, free radical scavenging activity and cell uptake of stilbenoids. *Food Chem.* **2016**, *202*, 383–388. [CrossRef] [PubMed]
54. Nair, M.S. Spectroscopic study on the interaction of resveratrol and pterostilbene with human serum albumin. *J. Photochem. Photobiol. B Biol.* **2015**, *149*, 58–67. [CrossRef] [PubMed]
55. Sekowski, S.; Bitiucki, M.; Ionov, M.; Zdeb, M.; Abdulladjanova, N.; Rakhimov, R.; Mavlyanov, S.; Bryszewska, M.; Zamaraeva, M. Influence of valoneoyl groups on the interactions between Euphorbia tannins and human serum albumin. *J. Lumin.* **2018**, *194*, 170–178. [CrossRef]
56. Sekowski, S.; Ionov, M.; Kaszuba, M.; Mavlyanov, S.; Bryszewska, M.; Zamaraeva, M. Biophysical studies of interaction between hydrolysable tannins isolated from *Oenothera gigas* and *Geranium sanguineum* with human serum albumin. *Colloids Surf. B Biointerfaces* **2014**, *123*, 623–628. [CrossRef]
57. Wang, B.; Qin, Q.; Chang, M.; Li, S.; Shi, X.; Xu, G. Molecular interaction study of flavonoids with human serum albumin using native mass spectrometry and molecular modeling. *Anal. Bioanal. Chem.* **2018**, *410*, 827–837. [CrossRef]
58. Boulton, D.W.; Walle, U.K.; Walle, T. Extensive Binding of the Bioflavonoid Quercetin to Human Plasma Proteins. *J. Pharm. Pharmacol.* **1998**, *50*, 243–249. [CrossRef]
59. Dangles, O.; Dufour, C.; Bret, S. Flavonol-serum albumin complexation. Two-electron oxidation of flavonols and their complexes with serum albumin. *J. Chem. Soc. Perkin Trans.* **1999**, *2*, 737–744. [CrossRef]
60. Xiao, J.; Cao, H.; Wang, Y.; Yamamoto, K.; Wei, X. Structure-affinity relationship of flavones on binding to serum albumins: Effect of hydroxyl groups on ring A. *Mol. Nutr. Food Res.* **2010**, *54*, S253–S260. [CrossRef] [PubMed]
61. Mahesha, H.G.; Singh, S.A.; Srinivasan, N.; Rao, A.G.A. A spectroscopic study of the interaction of isoflavones with human serum albumin. *FEBS J.* **2006**, *273*, 451–467. [CrossRef]
62. Mandeville, J.-S.; Froehlich, E.; Tajmir-Riahi, H.A. Study of curcumin and genistein interactions with human serum albumin. *J. Pharm. Biomed. Anal.* **2009**, *49*, 468–474. [CrossRef] [PubMed]
63. Trnková, L.; Boušová, I.; Staňková, V.; Dršata, J. Study on the interaction of catechins with human serum albumin using spectroscopic and electrophoretic techniques. *J. Mol. Struct.* **2011**, *985*, 243–250. [CrossRef]
64. Roy, D.; Dutta, S.; Maity, S.S.; Ghosh, S.; Roy, A.S.; Ghosh, K.S.; Dasgupta, S. Spectroscopic and docking studies of the binding of two stereoisomeric antioxidant catechins to serum albumins. *J. Lumin.* **2012**, *132*, 1364–1375. [CrossRef]
65. Pattanayak, R.; Basak, P.; Sen, S.; Bhattacharyya, M. An insight to the binding of ellagic acid with human serum albumin using spectroscopic and isothermal calorimetry studies. *Biochem. Biophys. Rep.* **2017**, *10*, 88–93. [CrossRef]

66. Tang, J.H.; Liang, G.B.; Zheng, C.Z.; Lian, N. Investigation on the Binding Behavior of Ellagic Acid to Human Serum Albumin in Aqueous Solution. *J. Solut. Chem.* **2013**, *42*, 226–238. [CrossRef]
67. Nanda, R.K.; Sarkar, N.; Banerjee, R. Probing the interaction of ellagic acid with human serum albumin: A fluorescence spectroscopic study. *J. Photochem. Photobiol. Chem.* **2007**, *192*, 152–158. [CrossRef]
68. He, J.; Wang, Q.; Zhang, L.; Lin, X.; Li, H. Docking simulations and spectroscopy of the interactions of ellagic acid and oleuropein with human serum albumin. *J. Lumin.* **2014**, *154*, 578–583. [CrossRef]
69. Muralidhara, B.K.; Prakash, V. Interaction of 3⁰-O-cafeoyl d-quinic acid with human serum albumin. *Int. J. Pept. Protein Res.* **2009**, *46*, 1–8. [CrossRef]
70. Sinisi, V.; Forzato, C.; Cefarin, N.; Navarini, L.; Berti, F. Interaction of chlorogenic acids and quinides from coffee with human serum albumin. *Food Chem.* **2015**, *168*, 332–340. [CrossRef] [PubMed]
71. Soukpoé-Kossi, C.N.N.; St-Louis, C.; Beauregard, M.; Subirade, M.; Carpentier, R.; Hotchandani, S.; Tajmir-Riahi, H.A. Resveratrol Binding to Human Serum Albumin. *J. Biomol. Struct. Dyn.* **2006**, *24*, 277–283. [CrossRef] [PubMed]
72. Diniz, A.; Escuder-Gilabert, L.; Lopes, N.P.; Villanueva-Camañas, R.M.; Sagrado, S.; Medina-Hernández, M.J. Characterization of interactions between polyphenolic compounds and human serum proteins by capillary electrophoresis. *Anal. Bioanal. Chem.* **2008**, *391*, 625–632. [CrossRef] [PubMed]
73. Rohn, S. Possibilities and limitations in the analysis of covalent interactions between phenolic compounds and proteins. *Food Res. Int.* **2014**, *65*, 13–19. [CrossRef]
74. Ross, P.D.; Subramanian, S. Thermodynamics of protein association reactions: Forces contributing to stability. *Biochemistry* **1981**, *20*, 3096–3102. [CrossRef]
75. Appel, H.M. Phenolics in ecological interactions: The importance of oxidation. *J. Chem. Ecol.* **1993**, *19*, 1521–1552. [CrossRef]
76. Bian, Q.; Liu, J.; Tian, J.; Hu, Z. Binding of genistein to human serum albumin demonstrated using tryptophan fluorescence quenching. *Int. J. Biol. Macromol.* **2004**, *34*, 275–279. [CrossRef]
77. Zhang, Y.; Li, Y.; Dong, L.; Li, J.; He, W.; Chen, X.; Hu, Z. Investigation of the interaction between naringin and human serum albumin. *J. Mol. Struct.* **2008**, *875*, 1–8. [CrossRef]
78. Ding, F.; Diao, J.-X.; Sun, Y.; Sun, Y. Bioevaluation of Human Serum Albumin–Hesperidin Bioconjugate: Insight into Protein Vector Function and Conformation. *J. Agric. Food Chem.* **2012**, *60*, 7218–7228. [CrossRef]
79. Xie, M.-X.; Xu, X.-Y.; Wang, Y.-D. Interaction between hesperetin and human serum albumin revealed by spectroscopic methods. *Biochim. Biophys. Acta Gen. Subj.* **2005**, *1724*, 215–224. [CrossRef]
80. Feroz, S.R.; Mohamad, S.B.; Bakri, Z.S.D.; Malek, S.N.A.; Tayyab, S. Probing the Interaction of a Therapeutic Flavonoid, Pinostrobin with Human Serum Albumin: Multiple Spectroscopic and Molecular Modeling Investigations. *PLoS ONE* **2013**, *8*, e76067. [CrossRef] [PubMed]
81. Tu, B.; Wang, Y.; Mi, R.; Ouyang, Y.; Hu, Y.-J. Evaluation of the interaction between naringenin and human serum albumin: Insights from fluorescence spectroscopy, electrochemical measurement and molecular docking. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **2015**, *149*, 536–543. [CrossRef] [PubMed]
82. Chanphai, P.; Tajmir-Riahi, H.A. Tea polyphenols bind serum albumins: A potential application for polyphenol delivery. *Food Hydrocoll.* **2019**, *89*, 461–467. [CrossRef]
83. Zhang, Y.; Dong, L.; Li, J.; Chen, X. Studies on the interaction of gallic acid with human serum albumin in membrane mimetic environments. *Talanta* **2008**, *76*, 246–253. [CrossRef]
84. Kaldas, M.I.; Walle, U.K.; van der Woude, H.; McMillan, J.M.; Walle, T. Covalent Binding of the Flavonoid Quercetin to Human Serum Albumin. *J. Agric. Food Chem.* **2005**, *53*, 4194–4197. [CrossRef]
85. Lee, P.; Wu, X. Modifications of human serum albumin and their binding effect. *Curr. Pharm. Des.* **2015**, *21*, 1862–1865. [CrossRef]
86. Matei, I.; Hillebrand, M. Interaction of kaempferol with human serum albumin: A fluorescence and circular dichroism study. *J. Pharm. Biomed. Anal.* **2010**, *51*, 768–773. [CrossRef]
87. Yuan, J.-L.; Lv, Z.; Liu, Z.-G.; Hu, Z.; Zou, G.-L. Study on interaction between apigenin and human serum albumin by spectroscopy and molecular modeling. *J. Photochem. Photobiol. Chem.* **2007**, *191*, 104–113. [CrossRef]
88. Liu, H.; Bao, W.; Ding, H.; Jang, J.; Zou, G. Binding Modes of Flavones to Human Serum Albumin: Insights from Experimental and Computational Studies. *J. Phys. Chem. B* **2010**, *114*, 12938–12947. [CrossRef]
89. Gokara, M.; Sudhamalla, B.; Amooru, D.G.; Subramanyam, R. Molecular Interaction Studies of Trimethoxy Flavone with Human Serum Albumin. *PLoS ONE* **2010**, *5*, e8834. [CrossRef]

90. Nozaki, A.; Kimura, T.; Ito, H.; Hatano, T. Interaction of Polyphenolic Metabolites with Human Serum Albumin: A Circular Dichroism Study. *Chem. Pharm. Bull.* **2009**, *57*, 1019–1023. [CrossRef] [PubMed]
91. Wang, X.; Guo, X.-Y.; Xu, L.; Liu, B.; Zhou, L.-L.; Wang, X.-F.; Wang, D.; Sun, T. Studies on the competitive binding of cleviprex and flavonoids to plasma protein by multi-spectroscopic methods: A prediction of food–drug interaction. *J. Photochem. Photobiol. B Biol.* **2017**, *175*, 192–199. [CrossRef] [PubMed]
92. Wang, X.; He, L.-L.; Liu, B.; Xu, L.; Wang, X.-F.; Sun, T. Decrease of the affinity of theophylline bind to serum proteins induced by flavonoids and their synergies on protein conformation. *Int. J. Biol. Macromol.* **2018**, *107*, 1066–1073. [CrossRef] [PubMed]
93. Wang, X.; Liu, Y.; He, L.-L.; Liu, B.; Zhang, S.-Y.; Ye, X.; Jing, J.-J.; Zhang, J.-F.; Gao, M. Spectroscopic investigation on the food components–drug interaction: The influence of flavonoids on the affinity of nifedipine to human serum albumin. *Food Chem. Toxicol.* **2015**, *78*, 42–51. [CrossRef] [PubMed]
94. He, L.-L.; Wang, Z.-X.; Wang, Y.-X.; Liu, X.-P.; Yang, Y.-J.; Gao, Y.-P.; Wang, X.; Liu, B. Studies on the interaction between promethazine and human serum albumin in the presence of flavonoids by spectroscopic and molecular modeling techniques. *Colloids Surf. B Biointerfaces* **2016**, *145*, 820–829. [CrossRef] [PubMed]
95. Liu, B.-M.; Zhang, J.; Bai, C.-L.; Wang, X.; Qiu, X.-Z.; Wang, X.-L.; Ji, H.; Liu, B. Spectroscopic study on flavonoid–drug interactions: Competitive binding for human serum albumin between three flavonoid compounds and ticagrelor, a new antiplatelet drug. *J. Lumin.* **2015**, *168*, 69–76. [CrossRef]
96. Mohseni-Shahri, F.S.; Housaindokht, M.R.; Bozorgmehr, M.R.; Moosavi-Movahedi, A.A. The influence of the flavonoid quercetin on the interaction of propranolol with human serum albumin: Experimental and theoretical approaches. *J. Lumin.* **2014**, *154*, 229–240. [CrossRef]
97. Maciążek-Jurczyk, M.; Maliszewska, M.; Pożycka, J.; Równicka-Zubik, J.; Góra, A.; Sułkowska, A. Tamoxifen and curcumin binding to serum albumin. Spectroscopic study. *J. Mol. Struct.* **2013**, *1044*, 194–200. [CrossRef]
98. Rimac, H.; Dufour, C.; Debeljak, Ž.; Zorc, B.; Bojić, M. Warfarin and Flavonoids Do Not Share the Same Binding Region in Binding to the IIA Subdomain of Human Serum Albumin. *Molecules* **2017**, *22*, 1153. [CrossRef]

Publisher’s Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).



Review

Metabolomics Technologies for the Identification and Quantification of Dietary Phenolic Compound Metabolites: An Overview

Anallely López-Yerena ^{1,†}, Inés Domínguez-López ^{1,†}, Anna Vallverdú-Queralt ^{1,2}, Maria Pérez ^{1,3}, Olga Jáuregui ^{4,5}, Elvira Escribano-Ferrer ^{2,6,7,*} and Rosa M. Lamuela-Raventós ^{1,2,*}

- ¹ Department of Nutrition, Food Science and Gastronomy XaRTA, Institute of Nutrition and Food Safety (INSA-UB), Faculty of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain; naye.yerena@gmail.com (A.L.-Y.); idominguez@ub.edu (I.D.-L.); avallverdu@ub.edu (A.V.-Q.); mariaperez@ub.edu (M.P.)
 - ² CIBER Physiopathology of Obesity and Nutrition (CIBEROBN), Institute of Health Carlos III, 28029 Madrid, Spain
 - ³ Laboratory of Organic Chemistry, Faculty of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain
 - ⁴ Scientific and Technological Center (CCiTUB), University of Barcelona, 08028 Barcelona, Spain; ojauregui@ccit.ub.edu
 - ⁵ CIBER Fragilidad y Envejecimiento Saludable (CIBERfes), Instituto de Salud Carlos III, 28029 Madrid, Spain
 - ⁶ Biopharmaceutics and Pharmacokinetics Unit, Department of Pharmacy and Pharmaceutical Technology and Physical Chemistry, Institute of Nanoscience and Nanotechnology (IN2UB), Faculty of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain
 - ⁷ Pharmaceutical Nanotechnology Group I+D+I Associated Unit to CSIC, University of Barcelona, 08028 Barcelona, Spain
- * Correspondence: eescribano@ub.edu (E.E.-F.); lamuela@ub.edu (R.M.L.-R.); Tel.: +34-646-421-580 (R.M.L.-R.)
 † Equally Contributing Authors.



Citation: López-Yerena, A.; Domínguez-López, I.; Vallverdú-Queralt, A.; Pérez, M.; Jáuregui, O.; Escribano-Ferrer, E.; Lamuela-Raventós, R.M.

Metabolomics Technologies for the Identification and Quantification of Dietary Phenolic Compound Metabolites: An Overview. *Antioxidants* **2021**, *10*, 846. <https://doi.org/10.3390/antiox10060846>

Academic Editor: Mario Allegra

Received: 22 April 2021

Accepted: 19 May 2021

Published: 25 May 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Abstract: In the search for natural products with properties that may protect against or slow down chronic and degenerative diseases (e.g., cancer, and cardiovascular and neurodegenerative conditions), phenolic compounds (PC) with benefits for human health have been identified. The biological effects of PC in vivo depend on their bioavailability, intestinal absorption, metabolism, and interaction with target tissues. The identification of phenolic compounds metabolites (PCM), in biological samples, after food ingestion rich in PC is a first step to understand the overall effect on human health. However, their wide range of physicochemical properties, levels of abundance, and lack of reference standards, renders its identification and quantification a challenging task for existing analytical platforms. The most frequent approaches to metabolomics analysis combine mass spectrometry and NMR, parallel technologies that provide an overview of the metabolome and high-power compound elucidation. In this scenario, the aim of this review is to summarize the pre-analytical separation processes for plasma and urine samples and the technologies applied in quantitative and qualitative analysis of PCM. Additionally, a comparison of targeted and non-targeted approaches is presented, not available in previous reviews, which may be useful for future metabolomics studies of PCM.

Keywords: phenolic compounds; metabolites; sample treatment; NMR; LC/MS; urine; plasma

1. Introduction

For several years, the benefits of phenolic compound (PC) intake have been described, especially as a protection from cardiovascular diseases [1,2]. The biological effects of PC in vivo depend on their bioavailability, intestinal absorption, metabolism, and interaction with target tissues [3]. It is well-known that PC have a low oral bioavailability, and undergo an extensive biotransformation mediated by phase I reactions (e.g., oxidation, reduction, or hydrolysis), phase II reactions (e.g., glucuronidation, methylation, sulfation), as well as by

gut microbiota (e.g., ring fission, hydrolysis, demethylation, reduction, decarboxylation, dihydroxylation, and isomerization) [4,5]. In fact, the identification, in biological samples, of the phenolic compounds' metabolites (PCM) produced after foods rich in PC intake, is a first step to understand the overall effect on human health [6]. Unfortunately, the (i) enormous structural variety of PC (i.e., phenolic acids, flavan-3-ols, flavanones, flavones, flavonoids, lignans, among others), (ii) the lack of commercially available standards, (iii) the structural variability of PCM, (iv) metabolite degradation due to tedious multi-step sample preparation workflows, (v) instability of specific compounds, (vi) low analyte concentration, and (vii) frequent sample contamination [7–10], render their identification a complex task. In the last decades, pre-analytical separation processes and more powerful separation systems for their analysis have been applied.

Accurate identification and quantification of analytes greatly depends on the extraction step [7–11]. Sample preparation, also known as sample pretreatment or clean-up, is a mandatory step to ensure adequate sensitivity, selectivity, and reproducibility of the analytical process in metabolomics analyses. The objectives of the pre-analytical separation process include: (i) minimization or elimination of interferences or undesired endogenous compounds in the extracted samples, (ii) enhanced selectivity for targeted analytes, (iii) sample preconcentration to improve assay sensitivity, and (iv) sample stabilization by reconstituting the mixture in an inert solvent. The development of analytical methods for biological samples has become increasingly challenging due to growing demands for method reliability and sensitivity, and speed of analysis and sample throughput. However, the extraction approach used is often dictated by the characteristics of the target analyte (hydrophilicity, lipophilicity, and protolytic properties) and the matrix. In liquid chromatography-mass spectrometry (LC-MS)-based metabolite analyses, sample preparation depends on whether a targeted or non-targeted strategy is employed [11]. In non-targeted analysis, the main methods of sample clean-up are the “dilute and shoot” approach, protein precipitation (PP), and ultrafiltration [11]. In targeted analysis, liquid-liquid extraction (LLE), solid-phase extraction (SPE), solid-phase microextraction (μ SPE), solid-liquid extraction (SLE), and PP are the most commonly used [11].

In the SPE technique, analytes from the sample solution or extract are concentrated and purified by sorption on a solid sorbent [12]. SPE efficiency depends on the nature of the stationary phase, solvent and volume sample, solvent pH, and modifier content [13]. An alternative to conventional SPE, μ SPE allows analyte extraction from a small amount of sample using a low elution volume. Its advantage is that sample extraction and preconcentration can be performed in one step, avoiding evaporation and reconstitution, and therefore reducing the time of analysis [14]. LLE is a technique used to isolate target compounds that consists of adding an immiscible organic solvent, commonly ethyl acetate, to the biological sample, which is subjected to vortex-mixing and centrifugation to achieve the transfer of the analytes to the organic phase [7]. This technique stands out for being simple to assemble and providing pure extracts, resulting in improved sensitivity in the chromatographic column [15]. However, if the number of samples is large, LLE is a slow procedure and requires a high consumption of solvents [16]. Finally, PP involves treating the samples with a protein precipitant (e.g., water-miscible organic solvents such as acetonitrile (ACN), acetone, ethanol, and methanol), its efficiency depending on the volume of organic solvent added and the presence of acid [7].

The analytical techniques commonly used for compound identification can measure a wide range of molecule classes, but each has its limitations regarding detection, sensitivity, dynamic range, and quantification. In metabolomics, the analytical tools of choice for small-molecule analysis are mass spectrometry (MS) and nuclear magnetic resonance (NMR) [17]. Chromatographic techniques for the separation of target compounds, typically coupled with MS, are liquid chromatography (LC), gas chromatography (GC), or capillary electrophoresis (CE) [7,17,18].

MS is a highly sensitive method that can detect, quantitate, and structurally elucidate several hundred metabolites in a single measurement [17,18]. The sensitivity and

accuracy of detection by MS depend on the experimental conditions and instrumental settings, especially the methods of metabolite extraction, separation, and ionization (and possibly ion suppression) [17]. The most commonly applied ionization techniques, which are key for compound detection and quantification [17], are electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and matrix-assisted laser desorption/ionization (MALDI) [11,18,19]. The ionization charges neutral molecules so they can be manipulated by electrical and/or magnetic and/or radio frequency energies according to their m/z ratio [19]. The most conventional method of ion-fragmentation is collision-induced dissociation (CID), although surface-induced dissociation (SID) and infrared multiphoton dissociation (IRMPD) have also been employed [18]. Target compound detection with a high degree of resolution and sensitivity generally cannot be achieved using a single MS detection mode, as higher sensitivity leads to lower resolution, and vice versa [17]. Single configuration mass analyzers include quadrupole (Q), linear ion trap (LIT), quadrupole ion trap (QIT), time of flight (TOF), Fourier transform ion cyclotron resonance (FTICR), and Orbitrap. Hybrid configuration can further aid metabolite identification by providing highly resolved and accurate MS/MS spectra [20]. Tandem (MS/MS) mass analyzers for quantitative and qualitative metabolomics studies include triple-quadrupole ion trap (QTrap), triple quadrupole (QqQ), quadrupole-time-of-flight (Q-TOF), and linear-quadrupole ion trap-Orbitrap (LTQ-Orbitrap) [17,18,20].

The aim of this review is to highlight recent advances in PCM identification and quantification in plasma and urine from humans and animals after the consumption of dietary PC. Sample preparation methods that enhance analytical sensitivity, identification, confidence, or the range of PCM that can be identified in these biological samples are described. Finally, the limitations and advantages of analytical methods in metabolomics studies are outlined.

2. Biological Sample Preparation Methods

There has been considerable progress in the development of sample preparation methods that allow the detection of PC and PCM with differentiated physicochemical properties. In LC-MS-based metabolite analyses, the main sample preparation methods used in non-targeted strategies are “dilute and shoot”, PP, and ultrafiltration, whereas LLE, SPE, μ SPE, SLE, and PP are the most common techniques in targeted analysis [11]. Table 1, drawing on work of the last two decades, includes the dietary source of each analyzed PC, the precursor compounds, the PCM type, and the biological sample analyzed. Additionally, detailed information about PCM of secoiridoids, phenolic alcohols, flavonoids, phenolic acids, enterolignans, stilbenes, ellagitannins, and others is presented in the Supporting Information (Supporting Information (Tables S1–S6, respectively)). Figure 1 shows the general clean-up steps for human or animal plasma and urine.

Table 1. Food source of phenolic compounds and the predominant phenolic compound metabolites identified in plasma and urine samples.

Food or Supplement	Compounds	Metabolites	Sample	Ref
VOO/ EVOO	Phenolic acids and tyrosols	PI	Plasma	[21]
	Oleuropein			[22]
	Hydroxytyrosol	PII	Urine	[23,24]
	Tyrosols			[25]
	Phenolic acids and tyrosols		Plasma	[14]
	Hydroxytyrosol			[26]
	Flavones, lignans, and tyrosols	PI/ PII	Urine	[27]
	Tyrosols			[28]
	Secoiridoids		Plasma/ urine	[29]
	Hydroxytyrosol and derivatives	PII/ MM		[30,31]

Table 1. Cont.

Food or Supplement	Compounds	Metabolites	Sample	Ref
Coffee	Chlorogenic acids	PII/ MM	Plasma/ urine	[32,33]
	Phenolic acids	PI		[34]
		PII	Plasma	[35]
		MM		[36]
Green tea	Flavan-3-ols	PII	Urine	[37]
Black tea	Hydroxybenzoic acids	PII/ MM		[38]
	Flavonoids and phenolic acids		Plasma/ urine	[39]
Yerba mate	Flavonols and phenolic acids			[40]
Orange juice	Flavonoids and phenolic acids	PII	Plasma/ urine	[41]
	Flavanones and flavonols			[42]
	Flavanoids	PII/ MM	Urine	[43]
	Flavanones		Plasma/ urine	[44]
				[45]
Red wine	Flavonoids, phenolic acids, lignans, stilbenes	PII/ MM	Urine	[46]
	Stilbenes			[47]
	Proanthocyanidin		Plasma/ urine	[48]
Bilberry	Anthocyanins	PII	Plasma/ urine	[49]
Black raspberry	Anthocyanins	PII	Urine	[50]
Red raspberry	Flavan-3-ols and phenolic acids	PII/ MM	Plasma/ urine	[51]
Chokeberry extract	Anthocyanins	PI/ PII	Urine	[52]
Strawberry drink	Anthocyanins	PII	Plasma	[53]
	Anthocyanins and phenolic acids	PII/ MM		[54]
Cranberry juice	Flavonols, flavan-3-ols, and phenolic acids	PII/ MM	Plasma/ urine	[55]
	Flavan-3-ols			[56]
Cranberry extract	Procyanidins	PII/ MM	Plasma	[57]
Cranberry-syrup	Flavonoids and phenolic acids	PI/ PII	Urine	[58]
Grape pomace drink	Flavan-3-ols and phenolic acids	PII/ MM	Plasma/ urine	[59]
Concord grape juice	Flavonoids and hydroxycinnamic acids	PII	Plasma/ urine	[60]
	Phenolic acids	MM	Urine	[61]
Grape extract	Flavonoids, phenolic acids, and stilbenes	PII/ MM	Urine	[62]
PRJD	Flavonoids and phenolic acids	PII/ MM	Plasma/ urine	[63]
PRBF	Flavonoids and phenolic acids	PII	Urine	[64]
Apple	Flavonoids and phenolic acids	PII/ MM	Plasma	[65]
	Flavan-3-ol		Urine	[66]
AAPO	Quercetin	PII	Plasma	[67]
Cocoa	Flavan-3-ols and phenolic acids	MM	Urine	[68]
	Flavonoids and procyanidin	PII/ MM	Plasma/ urine	[69]
	Flavan-3-ols			[70]

Table 1. Cont.

Food or Supplement	Compounds	Metabolites	Sample	Ref
Cocoa cream			Plasma	[73]
	Flavan-3-ols	PII		[74]
Cocoa drink			Plasma/ urine	[75]
	(-)-Epicatechin		Urine	[76]
	Flavan-3-ols	PII	Plasma/ urine	[77]
Dark chocolate/ chocolate	Isoflavones		Urine	[78]
	Flavan-3-ols	PII/ MM		[79]
Tomato sauce	Flavonoids and phenolic acids	PII		[80]
Tomato sauce with oil	Flavanones and hydroxycinnamic acids		Plasma/ urine	[3]
Tomato/ tomato sauce	Flavonoids and phenolic acids	PII/ MM		[81]
Almonds	Flavonoids	PII/ MM	Plasma/ urine	[82]
Beans	Flavonoids and phenolic acids	PII/ MM	Plasma/ urine	[83]
Black soybean extract	Isoflavones	PII	Plasma/ urine	[84]
	Catechin	PII/ MM	Urine	[85]
	Quercetin			[86]
	(-)-Epicatechin	PI/ PII/ MM	Plasma/ urine	[87]
	Daidzein			[88]
	Gallic acid	PII		[89]
	Trans-resveratrol		Urine	[90]
	Caffeic, ferulic, and isoferulic acid		Plasma/ urine	[91]
	Hesperetin and hesperidin	PI/ PII		[92]
	Oleocanthal		Plasma	[93,94]
	Oleacein			[95]
Capsule	(-)-Epicatechin and procyanidins	PII/ MM	Plasma/ urine	[96]
	Luteolin	PII	Plasma	[97]
Pill	Trans-resveratrol	PII/ MM	Plasma	[98]

PI: Phase I metabolites; PII: Phase II metabolites; MM: microbial metabolites; VOO: Virgin olive oil; EVOO: Extra virgin olive oil. PRJD: Polyphenol-rich juice drink, PRBF: Puree of (poly)phenol-rich berry fruits; AAPO: Applesauce with apple peel and onion.

2.1. Plasma

Plasma is an aqueous solution that contains a great variety of substances, including proteins, glucose, mineral ions, hormones, carbon dioxide, and blood cells [8], some of which may interfere with detection of the target analyte. As PCM may have a higher binding affinity to plasma proteins than the parent compounds [99], proteins and other cellular components such as phospholipids need to be removed prior to analysis to reduce potential matrix effects and ion suppression phenomena [100]. An appropriate sample treatment is therefore a crucial step that influences the reliability and accuracy of the analysis [16].

2.1.1. Solid-Phase Extraction

PC and PCM extraction from plasma samples is typically performed by C18-bonded SPE, as it produces results with greater repeatability than techniques such as PP [101]. Other advantages over methods such as LLE include higher recoveries, shorter analysis time, and lower solvent requirements [12].

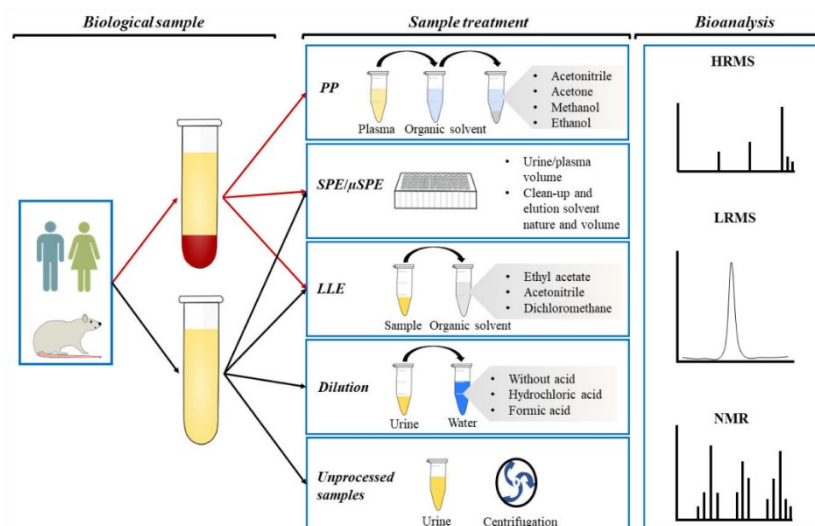


Figure 1. Graphical representation of plasma and urine clean-up and the bioanalysis for the identification and quantification of phenolic metabolites.

A range of solvents have been used to extract anthocyanins and their metabolites from plasma by SPE. PCM elution is frequently carried out with methanol, which efficiently precipitates proteins and deactivates enzymes, and can be combined with water to improve the extraction of polar phenolic acids. Some studies have used methanol acidified with different percentages of formic acid to elute anthocyanins and their metabolites in C18 cartridges [55]. The isolation of anthocyanins and their glucuronide metabolites was also achieved using hydrophilic-lipophilic-balance (HLB) cartridges and eluting the analytes with acetone/ formic acid (9:1), obtaining a recovery rate of 71% for cyanidine-3-glucose [51]. Anthocyanin and phenolic acid metabolites were also eluted with acetone/ formic acid (9:1) [56]. Acetone has an advantage over methanol in that its lower boiling point allows a faster evaporation.

The extraction of different classes of flavonoids and their metabolites was performed using methanol as the elution solvent in mixed-mode cation-exchange (MCX) plates [82]. The samples were previously hydrolyzed or nonhydrolyzed to analyze the metabolites in both their free and conjugated forms. With this procedure, phase-II metabolites of flavonoids and their microbial derivatives were identified and quantified, with recovery values ranging from 87% to 109%. Epicatechin and its metabolites were extracted with different solvents using Strata X cartridges. Actis-Goretta et al., employed methanol/ pyridine (1:1 v/v) to elute the metabolites, mainly glucuronides, sulfates, and O-methyl sulfates, achieving recoveries from 96% to 104% [77]. These results constitute an improvement on previous studies that used HLB cartridges with methanol containing formic acid (0.1%) [76] or N,N-dimethyl formamide/ methanol (7:3).

The first validated methodology for the quantification of oleuropein and its metabolites in plasma was based on sample pretreatment with SPE, using HLB cartridges and acetone as the eluent. This approach allowed the simultaneous determination of metabolites previously undetected by other techniques such as LLE, namely 2-(3,4-dihydroxyphenyl)acetic acid, homovanillyl alcohol, homovanillic acid, and elenolic acid [21].

In a study on procyanidin metabolites, rat plasma samples after ingestion of procyanidin-enriched cocoa cream were pretreated in off-line SPE HLB cartridges. The retained compounds were eluted with acetone/ water/ acetic acid (70:29.5:0.5, v/v/v), which produced better results than methanol/ acetone, with the recoveries being over 84%. The iso-

lated procyanidin metabolites were mainly phase-II-conjugated catechin-glucuronide and epicatechin-glucuronide [73].

A method based on high-throughput μ SPE using HLB μ Elution plates was developed by Feliciano et al. for the identification and quantification of different classes of PCM, including flavonols, benzoic acids, catechols, and cinnamic acids. Recovery rates of 89% were obtained when the μ -elution was performed with methanol followed by 70% aqueous acidified acetone [57].

Suárez et al. obtained the highest recovery of hydroxytyrosol using 50% aqueous ACN, although this method was less efficient for other PCM, whose recoveries were better with methanol [14]. The μ SPE method has been used for the analysis of hydroxytyrosol and derivatives in plasma samples [26,31]. In another study evaluating methanol as the elution solvent, recoveries of over 75% were achieved for all the studied PCM, including catechol sulfate and valerolactone glucuronide [67].

2.1.2. Liquid-Liquid Extraction

In a study on the pharmacokinetic processes of flavan-3-ols [96], LLE was carried out to extract phase-II-conjugated compounds and gut microbiota metabolites. The spiked samples, previously acidified with formic acid, were extracted 3 times with ethyl acetate, yielding recoveries of 70–100%. However, the values for cinnamic acids varied considerably, suggesting that the solvent was not suitable for its extraction. Ethyl acetate was also employed in an LLE of gallic acid metabolites in human plasma, with the recoveries ranging from 67% to 96% [89], and in the clean-up of rat plasma containing caffeic, ferulic, and isoferulic acid metabolites [91].

2.1.3. Protein Precipitation

Plasma contains a large amount of proteins that should be removed to avoid interference in the final analysis [99]. Samples are treated with a protein precipitant, commonly water-miscible organic solvents such as ACN, acetone, perchloric acid, ethanol, and methanol. Both composition and volume of the organic precipitant can affect PP efficiency, which may be improved by adding an acid (e.g., formic, acetic, trichloroacetic, and phosphoric) to the sample or PP solution to alter the pH of the sample matrix [7]. Although PP is a simple and fast method, it does not provide a completely clean extract. Protein removal can also be achieved by the addition of acids such as trichloroacetic or perchloric acids, which have a denaturation and aggregation effect.

Other components of plasma samples that could interfere with the analysis are phospholipid species, which may impair both the ionization process and chromatographic separation, thereby reducing extraction efficiency, recovery, and reproducibility, and increasing inter-sample variability. The removal of phospholipids (highly ionic) has been successfully accomplished with recently developed commercial plates [102].

ACN and formic acid (2%) were used to extract red raspberry anthocyanins and ellagitannins and their phase-II metabolites from human plasma [53]. The same solvents were employed in an analysis of phase-II metabolites of flavonoids and phenolic acids and phase-II and microbial metabolites of flavanone in plasma after the consumption of orange juice.

Ethanol has been utilized in different studies as a protein precipitant, including in an evaluation of chlorogenic and phenolic acids and their metabolites in human plasma after coffee consumption, and the recoveries ranged from 98.1% to 108.6% for all the glucuronide, sulfate, and lactone metabolites of the studied phenolic acids [36]. A similar procedure was performed to isolate chlorogenic acids and their metabolites, although the plasma samples were resuspended in sodium acetate buffer and acidified with hydrochloric acid in aqueous methanol (40%, v/v), achieving an 88% recovery of chlorogenic acid [35]. In another study, coffee phenolic metabolites thought to be formed by the gut microbiota were identified after ethanol-induced PP and sample acidification with perchloric acid [37].

2.2. Urine

Urine contains high concentrations of urea, inorganic salts (chloride, sodium, and potassium), creatinine, ammonia, organic acids, various water-soluble toxins, and pigmented products of hemoglobin breakdown [103]. Although urine is one of the easiest biological fluids to collect, its high salt concentration can easily interfere with ESI. Thus, the electrolytes need to be removed prior to the analysis [27], which is usually partially achieved by SPE, LLE, and sample dilution. Nevertheless, direct analysis of urine (unprocessed urine) has also been carried out.

2.2.1. Solid-Phase Extraction

One of the most widely used techniques for the preconcentration and clean-up of urine samples is SPE, in which the nature and volume of the elution solvent are important factors [73]. This technique has been used for metabolite extraction of PC from the main dietary sources such as tomato [3], tomato sauce [80], extra virgin olive oil [22,25,30], red wine [49,104], black raspberry [52], chokeberry [54], cocoa [70,71,79], chocolate [77], and anthocyanins [105]. An SPE method for the quantification of 350 dietary markers, including (poly)phenolic aglycones, phase-II metabolites, microbial-transformed compounds, and other dietary components, was also developed [106].

Methanol and water are by far the most extensively used solvents for PCM extraction [3,22,25,30,52,54,70,71,77,79,80,105]. Methanol and ethyl acetate have also been used to isolate PCM, specific, and resveratrol metabolites [49,104]. On the other hand, the methanol and ammonium formate have been less used [106].

Another strategy to optimize the PCM extractions is the use of different additives during its washing and elution. In this sense, formic acid [3,30,52,70,71,77,80,105,106], acetic acid [22,49,104], and trifluoroacetic acid [54] are the most common additives used. In other studies, both the methanol and water were employed without the addition of any additive [25,79].

In some cases, urine dilution, before SPE extraction, may be necessary to reduce the ionic strength prior to ion-exchange SPE due to a high concentration of salts [7]. Water [3], water with trifluoroacetic acid [22,54], water with phosphoric acid [25,49,104], water with formic acid [106], and methanol [30] have been extensively used prior to SPE extraction. In other studies, the urine dilution was not employed [52,70,71,79]. Finally, in another study, it was demonstrated that a complete evaporation of the eluent (to dryness) significantly reduced the recovery of certain compounds [105].

μ SPE constitutes an excellent option for PCM analysis as it requires small sample volumes. Feliciano and coworkers developed a rapid and high-throughput μ SPE method for the analysis of the most representative polyphenol classes (flavan-3-ols, benzoic acids, phenylacetic acids, propionic acids), achieving recoveries of up to 88% when using methanol followed by 70% aqueous-acidified acetone as the elution solvent [57]. In 2013, Serra demonstrated that a clean-up step was not necessary in the μ SPE method for hydroxytyrosol metabolites, which was attributed to their polarity. Thus, these compounds were eluted with water without affecting the matrix effect (lower than 18%) [24]. μ SPE was used with the aim to detect phenol metabolites in urine samples to identify the most appropriate compliance olive oil markers and eventually relate them to the expected biological effects [31].

2.2.2. Liquid-Liquid Extraction

This technique has been used for the extraction of PCM from the main dietary sources, such as extra virgin olive oil [27,107], orange juice [44], tea [38], yerba mate [42], puree of five (poly)phenol-rich berry fruits [66], cocoa products [72], and specific compounds, such as resveratrol [90] and daidzein and genistein [78].

ACN has been extensively employed for LLE of PCM [44,46,48,66]. In other studies, ethyl acetate [27,107] and dichloromethane have also been employed for the extraction of PCM [38]. Although LLE can be carried out with a single solvent, the use of solvent

mixtures can improve extraction [7]. An ACN/ methanol mixture (8:2, v/v) was used to extract major phase-II metabolites of resveratrol [90], adding an acetate buffer to increase the density of the aqueous layer.

LLE constitutes an excellent option for PCM analysis as it requires small urine volumes (<500 µL). Another advantage of LLE is that sample extraction can be performed in one step, avoiding evaporation and reconstitution, and therefore reducing the time of analysis. In this sense, after centrifugation (and/ or filtration), the samples can be directly injected in MS.

2.2.3. Urine Dilution

The preparation of samples using the “dilute and shoot” approach reduces the time required to obtain results [11]. Different volumes of water (1:1, 1:3, 1:4, 1:5, or 1:10 v/v, urine:water), with or without formic acid (0.1%) or acetic acid [23], have been used to analyze a wide variety of PCM. After dilution, the samples are usually mixed, centrifuged, and filtered prior to the bioanalysis. In 2019, González-Domínguez and colleagues demonstrated that a 10-fold dilution factor was optimum for a minimal matrix effect provoked by the high salt content of urine [106]. The same authors found that this strategy yielded LOQs above $10 \mu\text{g L}^{-1}$ for almost all compounds monitored, which can hinder the detection of food-derived metabolites in real urine samples. Hydrochloric acid (0.1 M) has been employed in urine treatment for the analysis of microbial metabolites, glucuronides, and sulfate derivatives [64]. The same acid was used to retard bacterial growth and ensure metabolite stability in human urine after the consumption of black tea [64].

2.2.4. Unprocessed Urine

The direct injection of urine samples is known to be difficult due to problems such as column clogging and MS signal alterations caused by endogenous compounds. Nevertheless, unprocessed urine samples have been analyzed, in nontargeted approaches, to determine the profile of excretable PC. Unprocessed urine containing metabolites of flavonoids and hydroxycinnamic acids after the acute consumption of grape juice [62], hydroxycinnamate derivatives after the acute consumption of coffee [34], and PCM after polyphenol-rich juice drink [65] and moderate wine [48] consumption have been analyzed. The urine samples were centrifuged, to eliminate cells and noncellular suspended particles, before being directly analyzed by UHPLC–TOF-MS [48] or HPLC-PDA-LIT [34,62,65].

3. Identification and Quantitation of Metabolites

3.1. Nontargeted Approaches

Nontargeted approaches to PC and PCM characterization are becoming more feasible with the high analytical power provided by high-resolution mass spectrometry (HRMS) instruments (MS and MS/MS mode) [108,109]. In Table 2, the main chromatographic and MS conditions employed in nontargeted studies of PCM in urine and plasma samples are summarized.

Q-TOF-MS is a hybrid analytical technique widely used in metabolomics. This combination of analyzers takes advantage of the higher MS/MS efficiency demonstrated by QqQ instruments and the speed and sensitivity of a TOF analyzer [69,110,111]. A Q-TOF-based metabolomics approach is a suitable strategy to understand the effect of nutritional interventions, reveal metabolomic changes, and obtain data on bioaccessibility and bioavailability [111]. A comprehensive list of the main conditions used to analyze dietary PCM in urine and plasma is provided in Table 2.

Table 2. Methods for untargeted analysis of PCM in urine and plasma samples.

Phenolic Compounds	Sample; Sample Clean-Up; Internal Standard	Chromatographic Conditions	MS Conditions
Phase-I and Phase-II Metabolites			
Quercetin [86]	Urine and plasma; PP	Acquity BEH C ₁₈ (2.1 × 50 mm, 1.7 μm); A: Water (0.1% FA). B: ACN.	QTOF; ESI-[M-H] ⁻
Secoiridoids [29]	Urine and plasma; UU and PP	Hypersil gold C ₁₈ (2.1 × 100 mm, 1.9 μm); A: Water (0.01% AA), B: ACN.	Q-Exactive; ESI-[M-H] ⁻
Epicatechin [87]	Urine and plasma; SPE	Acquity BEH C ₁₈ (2.1 × 100 mm, 1.7 μm); A: Water (0.1% FA), A: ACN:MeOH (3:1 v/v).	LTQ-Orbitrap; ESI-[M-H] ⁻
Daidzein [88], hesperetin, and hesperidin [92]		Acquity BEH C ₁₈ (2.1 × 100 mm, 1.7 μm); A: Water (0.1% FA). B: ACN.	LTQ-Orbitrap; ESI-[M-H] ⁻ / [M+H] ⁺
Oleocanthal [93]	Plasma; SPE	Acquity BEH C ₁₈ (2.1 × 50 mm, 1.7 μm); A: Water, B: MeOH, both with 0.1% FA.	LTQ-Orbitrap; ESI-[M-H] ⁻
Phenolic acids and f avonoids [60]	Urine; SPE	Zorbax Eclipse C ₁₈ (4.6 × 150 mm, 1.8 μm); A: 1% FA in water/ ACN (90:10, v/v), B: ACN	QTOF; ESI-[M-H] ⁻
		Zorbax Eclipse C ₁₈ (4.6 × 150 mm, 1.8 μm); A: Water (10% FA), B: ACN.	QTOF; ESI-[M+H] ⁺
Phase-II Metabolites			
Procyanidins [59]	Plasma; PP; mixture solution **	ACE Excel 2 C ₁₈ -PFP (2.1 × 100 mm, 2 μm); A: Water (0.1% FA). B: ACN.	Q-Exactive; HESI-[M-H] ⁻
Resveratrol [98]	Plasma; PP; mixture solution *	Nucleodur C ₁₈ Isis (2 × 150 mm,1.8 μm); A: Water, B: MeOH, both with 0.1% FA.	
Quercetin [69]	Plasma; PP; Quercetin 4 ⁰ O-glucoside	Poroshell C ₁₈ (2.1 × 100 mm, 2.7 mm); A: Water, B: ACN, both with 0.1% FA.	QTOF; ESI-[M-H] ⁻ / [M+H] ⁺
Flavonoids [44]	Urine; UD; Naringin-d4	Kinetex C ₁₈ (3.0 × 150 mm, 2.6 μm); A: Water, B: MeOH, both with 0.1% FA.	QTOF; ESI-[M-H] ⁻
Phenolic acids and f avonoids [66]	Urine; LLE; Rutin and taxifolin	Synergi Hydro RP18 C ₁₈ (2 × 150 mm, 4 μm); A: Water, B: ACN (0.1% FA).	LTQ-Orbitrap; ESI-[M-H] ⁻
Isof avones [84]	Urine and plasma; SPE	Kinetex C ₁₈ (2.1 × 100 mm, 1.7 μm); A: Buffer (0.1% FA). B: ACN.	
Phase-II and Microbial Metabolites			
Quercetin [86]	Urine and plasma; PP	Acquity BEH C ₁₈ (2.1 × 50 mm, 1.7 μm); A: Water (0.1% FA). B: ACN,	QTOF; ESI-[M-H] ⁻
Phenolic acids and f avonoids [57,83]	Urine and plasma; μSPE; Taxifolin	Zorbax Eclipse Plus RRHD (2.1 × 50 mm, 1.8 μm); A: Water, B: ACN, both with 0.1% FA.	
Phenolic acids and f avonols [42,72]	Urine and plasma; LLE, PP and UD	Ascentis Express C ₁₈ (3 × 150 mm, 2.7 μm);	
Phenolic acids [32]		A: Water, B: ACN, both with 0.1% FA.	
Flavan-3-ol [50]	Urine and plasma; PP	Kinetex Phenyl-Hexyl (4.6 × 150 mm, 2.6 μm); 0.1% FA methanol in 0.1% aqueous FA	Q-Exactive; HESI-[M-H] ⁻
Phenolic acids and f avonoids [41]	Plasma; PP; Baicalin and genistein 7-β-D-O-glucuronide	Acquity BEH C ₁₈ (2.1 × 150 mm, 1.7 μm); ACN (0.1% FA).	LTQ-Orbitrap; ESI-[M-H] ⁻

Table 2. Cont.

Phenolic Compounds	Sample; Sample Clean-Up; Internal Standard	Chromatographic Conditions	MS Conditions
Flavanone [47]	Plasma; PP	Kinetex C ₁₈ (4.6 × 150 mm, 5 µm); 0.1% FA methanol in 0.1% aqueous FA	Q-Exactive; HESI-[M-H] [−]
Phenolic acids and flavonoids [48]	Urine; UU	Zorbax Eclipse Plus C ₈ (2.1 mm × 100 mm, 1.8 µm); A: Water, B: ACN, both with 0.01% FA.	QTOF; ESI-[M-H] [−]
Procyanidins, phenolic acids, and flavonoids [64]	Urine; UD	Luna C ₁₈ (2.0 × 50 mm, 5 µm); A: Water, B: ACN, both with 0.1% FA	LTQ-Orbitrap; ESI-[M-H] [−]
Flavan-3-ol [79]	Urine; SPE: Hesperetin	Poroshell C ₁₈ (0.5 × 250 mm, 2.7 µm); A: Water, B: ACN, both with 0.1% FA	QTOF; ESI-[M-H] [−] / [M+H] ⁺

AA: acetic acid; ACN: acetonitrile; FA: formic acid; UD: urine dilution; UU: unprocessed urine; SPE: solid-phase extraction; PP: protein precipitation; µSPE: micro-elution solid-phase extraction; * L-tryptophan-D3, L-leucine-D10, creatine-D3, and caffeine-D3; ** trans-resveratrol-13C6, trans-resveratrol-3-O-sulfate-D4, trans-resveratrol-3-O-β-D-glucuronide-D4, and trans-resveratrol-4-O-β-D-glucuronide-D4.

Although Q-TOF-MS has been applied to detect phase-I metabolites in vivo [60,86], conjugated metabolites have received more attention. Thus, glucuronide and sulfate forms of quercetin were the main metabolites identified in human plasma post-consumption of applesauce enriched with apple peel and onion [69]. In another study, phase-II metabolites were identified in rat plasma and urine after a single oral administration of quercetin [86]. Human urinary metabolites, mainly sulfated forms of caffeic and ferulic/ isoferulic acids, were detected after consumption of yerba mate [42]. Other studies have focused on the identification of PCM after ingestion of cranberry syrup [60], cranberry juice [57], orange juice [44], and chocolate [79].

The key role played by phenolic microbial metabolites in the health effects of dietary PC has prompted experiments on Q-TOF-based metabolomic fingerprints in biological fluids. Gut microbial metabolites were analyzed in plasma and urine samples following the consumption of cranberry juice [57], cocoa products [72], beans [83], and tomato products [81]. Hydroxycinnamate metabolites derived from microbiota were detected in both plasma and urine after coffee intake [32], and microbial metabolites in urine were found to be strongly affected by moderate red wine consumption [48]. In summary, negative ESI (Table 2) is the most frequently used mode in analysis of PCM, regardless of whether they are phase I, phase II, or microbial metabolites. Regarding chromatographic separation, the most employed mobile phases are water and ACN with formic acid (0.1% or 0.01%).

The prediction, screening, and identification of PCM in foods is rarely straightforward. The Orbitrap mass analyzer, the first high-performance mass analyzer able to trap ions in electrostatic fields, provides the high-resolution, mass accuracy, and sensitivity required for metabolomic analyses [112,113]. Two major families of instruments are used to study PCM: the hybrid linear ion-trap Orbitrap MS (LTQ-Orbitrap) and Q Exactive. Table 2 summarizes the chromatographic and MS conditions used in the analysis of PCM.

In the most frequently employed operation mode, the Orbitrap mass analyzer acquires FTMS data and the LTQ provides data-dependent MS/MS scans after trapping. The advantages are the high trapping capacity and MSⁿ scanning function of the LTQ along with accurate mass measurements, resulting in greater throughput and identification of major or trace metabolites. To date, the LTQ-Orbitrap mass analyzer is the most popular platform used in dietary PC metabolomics research, applied to study the metabolism of compounds such as (-)-epicatechin [87], oleocanthal [93,94], oleacein [95], daidzein [88], hesperetin, and hesperidin [92], and to identify PCM in biological samples after the intake of foods or beverages [41,64,66,81,84]. As can be seen in Table 2, negative ESI is the most routinely used mode in PCM analysis. Although high mass resolution is necessary to separate or resolve two peaks with a small mass difference, in most of the studies, the resolution was set at 30,000 in FTMS mode. A resolution of 60,000 was used in only

one study and 70,000 in another. Regarding the chromatographic conditions, the most employed mobile phases are water and ACN or methanol with formic acid (0.1% or 0.01%).

Another newly developed method is Q Exactive hybrid quadrupole-orbitrap mass spectrometry (Q Exactive MS), which combines high-performance quadrupole precursor selection with high-resolution and accurate-mass orbitrap detection. In the field of PC analysis, from 2015 until the present, this hybrid technology has been used in only a few studies, to identify proanthocyanidin [50], procyanidins [59], resveratrol [106], flavanone, and secoiridoids [29]. Most (80%) of these studies used heated electrospray ionization (HESI), which generally gives improved signals compared to the more traditional unheated ESI (Table 2). Chromatographic separation was carried out as in the LTQ-Orbitrap system, using water and ACN or methanol with formic acid (0.1% or 0.01%).

3.2. Targeted Approaches

Targeted analyses require the collection of specific metabolite information, typically through the use of low-resolution mass spectrometry (LRMS) instrumentation. There are several types of mass analyzers that can generate mass measurements, but the most frequently used are QqQ or QTrap [114]. Table 3 summarizes the main chromatographic and MS conditions employed in targeted studies of PCM in urine and plasma samples. It is well-known that due to the lack of bioanalytical standards for some PCM, various strategies have been used trying to solve this problem: (i) quantification of PCM using the calibration curves corresponding to their phenolic precursors or with the most structurally similar compound [42,43], (ii) expressing the concentration as equivalents of parent compounds [71,94], (iii) expressing the concentration as ratios [29,93,95,115], and (iv) the synthesis of the target metabolites [39]. In the case of the expression of the concentration as ratios, the peak abundance ratio (analyte/ internal standard) and the concentration ratio (analyte/ internal standard) are used [115]. In other cases, the peak area ratios of each analyte vs. that of the internal standard (analyte/ internal standard) are employed [21,63].

3.2.1. Quadrupole Ion Trap (QIT)

QIT, despite being one of the first technologies employed to analyze dietary PC, has been applied in only a few studies to date. Its principal limitation is a relatively low limiting value of the mass/ charge ratio. As can be seen in Table 3, the quantification of PCM with this technology has focused on flavonoids [38,62,65,116] and phenolic acids [62], both in plasma and urine samples, using ESI and fragmentation by CID. A single mobile phase was employed in two studies [62,65], whereas others used a combination of two [116] or three [38]. In all cases, formic or acetic acids (0.05%, 0.1%, or 0.5%) were the major additives used to improve compound ionization.

3.2.2. Triple Quadrupole (QqQ)

Due to its high sensitivity and selectivity, QqQ mass spectrometry has been primarily applied in targeted analysis of dietary PCM. Table 3 summarizes the chromatographic and MS conditions routinely used in the study of PCM.

Phase-I metabolites of oleuropein in plasma samples were analyzed with QqQ technology, employing a C8 column with water (pH 5, adjusted with acetic acid) and ACN for chromatographic separation [21]. To quantify phase-I and phase-II metabolites of oleocanthal, a C18 column and water:MeOH, both with 0.1% formic acid, was used [93]. In addition, phase-II metabolites of phenolic acids [14,36], phenolic alcohols [14,25], flavonoids [14,77], phenyl- γ -valerolactones [39], procyanidins [117], and resveratrol [90] were studied, employing a C18 column and water:ACN for plasma samples and ammonium acetate:ACN for urine.

Water and ACN containing formic acid (0.1%, 0.2%, and 0.5%) in combination with C18 columns have been extensively used in the analysis of phase-II metabolites and microbial metabolites of flavonoids [56,61,66,68,71,80] and phenolic acids [61,66,80] in plasma or urine samples. The same mobile phases have been used in the analysis of epicatechin and

procyanidin microbial metabolites [70] in urine samples. In another study, a PFP column was used to separate polar compounds, including phenolic acid metabolites [53].

3.2.3. Triple-Quadrupole Ion Trap (QTrap)

QTrap is the one of the most widely used instruments for analyzing specific metabolites of interest [108]. Recently, targeted metabolomic profiling of the urinary [106] and plasma [118] food metabolome has resulted in the simultaneous quantitation of PCM, including (poly)phenolic aglycones, conjugated metabolites, and colonic microbial metabolites. QTrap technology has been extensively utilized to develop a targeted metabolomic approach for specific PC groups, being applied to plasma and human urine containing phenolic acid metabolites [119] or specific compounds such as chlorogenic acid [33]. Regarding flavonoids, phase-I, phase-II, or microbial metabolites were detected in plasma [97] and urine [46,78,105]. As shown in Table 3, acetic and formic acids are the major additives used to improve compound ionization. The most common mobile phases for urine are both ammonium acetate and water (with acid), together with ACN/ methanol, and for plasma, water, and ACN.

Table 3. Chromatographic and MS conditions for the quantification of PCM in plasma and urine samples.

Phenolic Compounds	Sample; Sample Clean-Up; Internal Standard	Chromatographic Conditions	MS Conditions	LOQ
Phase I Metabolites				
Oleuropein [21]	Plasma; SPE; 2-hydroxyphenylethanol	RP-C8 (150 × 2.1 mm, 3.5 µm); A: Water pH 5, adjusted with AA. B: ACN	QqQ; MRM; ESI-[M-H] ⁻	5–50 ng L ⁻¹
Phase-II Metabolites				
Flavonoids and phenolic acids [65]	Plasma and urine; PP and UU; Ethyl gallate	Synergi RP-Max (4.6 × 250 mm, 4 µm); MeOH (0.5% AA or 1% FA).	QIT; FS, CRM, and SIM; ESI- [M-H] ⁻ / [M+H] ⁺	
Flavonoids [116]	Plasma and urine; LLE, SPE, and PP	Symmetry C18 (2.1 × 50 mm, 3.5 µm); A: Water, B: ACN, both with 0.05% FA.	QIT; FS; ESI- [M-H] ⁻ / [M+H] ⁺	
Catechins [38]	Urine; LLE	Supelcosil LC18 (3.0 × 100 mm, 5 µm); A: MeOH-water-AA (1:98:1), B: MeOH- water-AA (50:49:1), C: MeOH-water (4:1).	QIT; FS; ESI- [M-H] ⁻	
Phenolic acids [36]	Plasma; PP; mixture solution *	Acquity BEH C18 (2.1 × 150 mm, 1.7 µm); A: Water, B: ACN, both with 1% AA.	QqQ; MRM; ESI- [M-H] ⁻	5 nM
Procyanidins [117]	Plasma; SPE; Catechol	Acquity HSS T3 (2.1 × 100 mm, 1.8 µm); A: Water (0.2% AA), B: ACN.	QqQ; SRM; ESI- [M-H] ⁻	0.01–0.98 µM
Phenolic acids and alcohols and flavonoids [14]	Plasma; µSPE; Catechol and caffeic acid	Acquity BEH C18 (2.1 × 100 mm, 1.7 µm); A: Water (0.2% AA), B: ACN.	QqQ; SRM; ESI- [M-H] ⁻	0.02–8.2 µM
Phenolic alcohols [25]	Urine; SPE; 4'-O-hydroxy phenylpropanol and 3-(4-hydroxyphenyl) propanol	Acquity BEH C18 (2.1 × 100 mm, 1.7 µm); A: 1 mM ammonium acetate (pH 5), B: ACN	QqQ; MRM; ESI- [M-H] ⁻	1.87–20 ng L ⁻¹

Table 3. Cont.

Phenolic Compounds	Sample; Sample Clean-Up; Internal Standard	Chromatographic Conditions	MS Conditions	LOQ
Resveratrol [90]	Urine; UD; Wogonin	ODS-3 (5 × 150 mm, 2.1 μm); A: 5 mM ammonium acetate, B: ACN	QqQ; SRM; ESI- [M−H] [−]	4–20 ng L ^{−1}
(−)-Epicatechin [77]	Plasma and urine; SPE; Umbelliferone sulfate and glucuronide	HSS C18 (1.8 × 100 mm, 2.1 μm); A: Water, B: ACN, both with 0.1% AA.	QqQ; MRM; ESI- [M−H] [−]	12–30 nM;
Phenyl-γ- valerolactone [39]	Plasma and urine; PP and UD	Kinetex EVO C18 (2.1 × 100 mm, 2.6 μm); A: Water, B: ACN, both with 0.2% FA.	QqQ; MRM; HESI- [M−H] [−]	0.6–2.2 nM
Flavonoids and phenolic acids [43]				
Daidzein and genistein [78]	Urine; LLE; Taxifolin	Phenomenex C18 (3 × 150 mm, 3 μm); A: ammonium acetate (13 mM, pH 4 with 0.1% AA), B: MeOH (0.1% AA).	QTRAP; MRM; ESI- [M−H] [−]	3 ng mL ^{−1}
Luteolin [97]	Plasma; PP	Capcell pak C18 MGII (4.6 × 150 mm, 3 μm); A: water (0.1% TFA), B: ACN.	QTRAP; MRM; ESI- [M+H] ⁺	
Phase I and II Metabolites				
Chlorogenic acid [33]	Urine and plasma; PP; mixture solution *	Synergi Polar RP C18 (4.6 × 250 mm, 4 μm); A: Water, B: ACN, both with 0.1% AA.	QTRAP; MRM; ESI- [M−H] [−]	5 nM
Flavonoids and phenolic acids [62]	Plasma and urine; SPE and UU; Cyanidin-3-O- sambubioside-5-O- glucoside	Synergi Polar RP C18 (4.6 × 250 mm, 4 μm); MeOH (0.5% AA or 1% FA).	QIT; FS, SRM, SIM, and CRM; ESI-[M−H] [−] / [M+H] ⁺	
Oleocanthal [93]	Plasma; SPE	AcQuity BEH C18 (2.1 × 50 mm, 1.7 μm); A: Water, B: MeOH, both with 0.1% FA.	QqQ; MRM; ESI- [M−H] [−]	
Anthocyanins [120]	Plasma and urine; SPE; Scopoletin	Kinetex PFP (4.6 × 100 mm, 2.6 μm); A: Water, B: ACN, both with 0.1% FA.	QTRAP; MRM; ESI- [M+H] ⁺	
Microbial Metabolites				
Epicatechin, procyanidins [70]	Urine; SPE; Ethyl gallate	Luna C18 (2.0 × 50 mm, 5 μm); A: Water/ ACN (94.9:5, v/v), B (ACN), both with 0.1% FA.	QqQ; MRM; ESI- [M−H] [−]	0.03–44.4 μg L ^{−1}
Flavanones [46]	Urine; LLE	ACE 3 C18-AR (4.6 × 75 mm, 3 μm); A: Water, B: ACN, both with 0.1% FA.		
Phase-II and Microbial Metabolites				
Flavonoids and phenolic acids [66]	Urine; LLE; Rutin and taxifolin	Atlantis T3 (2.1 × 100 mm, 3 μm); A: Water/ ACN (95:5, v/v), B: ACN/ water (95:5, v/v), both with 0.5% FA.	QqQ; MRM; ESI- [M−H] [−]	

Table 3. Cont.

Phenolic Compounds	Sample; Sample Clean-Up; Internal Standard	Chromatographic Conditions	MS Conditions	LOQ
(-)-Epicatechin [71]	Plasma and urine; SPE; Ethyl gallate	Luna C18 (2.0 × 50 mm, 5 µm); A: Water, B: ACN, both with 0.1% FA.	QqQ; MRM; TIS-[M-H] ⁻	28.4–75.8 ng L ⁻¹
Flavonoids and phenolic acids [80]			QqQ; MRM; ESI-[M-H] ⁻	
Flavonoids and phenolic acids [61]	Plasma and urine; SPE and UD	Kinetex EVO C18 (2.1 × 100 mm, 2.6 µm); A: Water, B: ACN, both with 0.2% FA.	QqQ; SRM; HESI-[M-H] ⁻	
Flavan-3-ols [68]	Urine; UD		QqQ; MRM; ESI-[M-H] ⁻	
Phenolic acids [56]	Plasma; SPE; Syringic acid	Pursuit 3 PFP (150 × 2.0 mm); A: Water, B: ACN, both with 0.1% FA.	QqQ; MRM; ESI-[M-H] ⁻ / M+H] ⁺	
Anthocyanins [56]	Plasma; SPE; Malvidin-3-O-glucoside	Poroshell 120 C18 (2.1 mm × 150 mm, 2.7 µm); A: Water (1% FA), B: ACN.		
Flavonoids, phenolic acids, stilbenes, lignans, and tyrosol derivatives [106,118]	Urine; SPE; Taxifolin	Luna Omega Polar C18 (100 × 2.1 mm, 1.6 µm); A: 10 mM ammonium formate (0.1% FA), B: ACN.	QTRAP; MRM; ESI-[M-H] ⁻	10 µg L ⁻¹
	Plasma; PP and SPE; Ferulic acid-1,2,3-13C ₃ , L-phenylalanine-15N	Luna Omega Polar C18 (100 × 2.1 mm, 1.6 µm); A: Water, B: ACN, both with 0.5% FA.	QTRAP; MRM; ESI-[M+H] ⁺	0.5–5 µmol L ⁻¹
Anthocyanins [105]	Urine; SPE; Phloridzin, scopoletin, taxifolin, and 7,8-dihydroxycoumarin	Kinetex PFP (4.6 × 100 mm, 2.6 µm); A: Water, B: ACN, both with 0.1% FA.	QTRAP; MRM; ESI-[M-H] ⁻	

AA: acetic acid; FA: formic acid; TFA: trifluoroacetic acid; SRM: selective reaction monitoring; SIM: selected ion monitoring; CRM: consecutive reaction monitoring; TIS: turbo ion spray; UD: urine dilution; UU: unprocessed urine; HESI: heated-electrospray ionization source. * d¹³C₂-caffeic acid, d₃-dihydroisoferrulic acid, and d₃-dihydroisoferrulic-3⁰O-glucuronide.

3.3. GC-MS

GC is generally recognized to be more reproducible, cheaper, and producing higher resolution than LC [112,121]. However, in PC analysis, its limitations have tipped the scales in favor of LC. Since the development of LC, there has been little evolution in GC methodologies, which in recent publications are clearly surpassed by LC [121]. The main disadvantage of GC is that the analytes need to be volatile (or transformed into volatile or semi-volatile compounds) and thermally stable, so their boiling temperature should be lower than 350–400 °C [122]. Secondly, it is necessary to add a derivatization step prior to the injection to generate phenolic derivatives that can be analyzed by GC. This procedure is performed with methylating or silylating reagents, most commonly N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS), N-methyl-N-(trimethylsilyl) (MSTFA), 1,1,1,3,3,3-hexamethyldisilazane (HMDS), N-methyl-N-(tert-butyltrimethylsilyl)trifluoroacetamide (MTBSTFA), and tetramethylammonium hydroxide (TMAH) [112].

The combination of GC with MS provides a sensitive technique for the identification and quantification of PCM [121]. Flavonoids possess physicochemical properties that make them especially suitable for GC-MS analysis [123]. Isoflavones and flavonoids and their metabolites were studied in urine samples previously derivatized with MSTFA [124]. Another study identified phase I metabolites of isoflavones after performing the derivatization with pyridine/ HMDS/ TMCS, 9:3:1 [125].

In 2018, Ordóñez et al. compared the use of GC-MS and HPLC-MS for the analysis of microbial-derived phenolic acids in urine. Interestingly, they observed that GC-MS is not suitable for the analysis of phase-II glucuronide and sulfate metabolites, which are insufficiently volatile. However, other microbial urinary phenolic metabolites, such as caffeic acid, ferulic acid, or isoferulic acid, were detected after using MSTFA as a derivatization agent [126]. Most studies employing GC-MS to analyze PCM have focused on colonic metabolites after the consumption of polyphenol-rich foods. It was used by Draijer et al. to obtain a fingerprint of phenolic acids in urine, employing BSTFA with 10% TMCS to derivatize the metabolites [127]. Other studies used pyridine and MSTFA (1:4, v/v) or BSTFA/ TMCS (90:10 v/v) to achieve the separation and identification of phenolic acids and aromatic acids produced by the gut microbiota [45,63]. The main metabolites of chlorogenic acid, caffeic, and ferulic acid were analyzed in plasma samples after coffee intake. The derivatization of the analytes was carried out with MTBSTF and 1% tert-butyldimethylsilyl chloride, and the products were redissolved in hexane prior to injection [110].

GC-MS/MS was used to characterize the urinary metabolites of olive oil PC in samples derivatized with MSTFA/ 1-(trimethylsilyl)imidazole (TMSI) 1000/ 2 (v/v), resulting in the identification and quantification of free and conjugated forms of hydroxytyrosol, tyrosol, and elenolic acid [22]. In another study on olive oil, metabolites of hydroxytyrosol, homovanillic alcohol, and homovanillic acid were identified and quantified after their transformation into trimethylsilyl ether derivatives [28].

The application of GC to determine PCM is not limited to its coupling with MS. GC-QqQ/MS was applied by Carry et al. to analyze microbial phenolic acid metabolites of grape epicatechin and catechin [128]. Furthermore, Kay et al., employed GC with a flame ionization detector to identify the glycosylating structures of cyanidin, and the derivatization of sugars was performed with TMSI + pyridine [54].

3.4. NMR

NMR spectroscopy is a potent tool for the qualitative and quantitative analysis of complex mixtures of small molecules and has been used to analyze PC in food [129–131]. NMR-based metabolomics offers a powerful approach to metabolite fingerprinting and profiling [132]. As well as being non-destructive, quantitative, and highly reproducible, this technique requires minimal sample preparation, allows the identification of new compounds, and is information-rich for the characterization of molecular structures, particularly in complex mixture analyses [133]. However, a limiting factor is the complexity of interpreting the NMR spectra of biological samples, due to signal overlap and crowding, so multivariate statistical analysis is essential [129,130,134]. A study in 2005 profiled the flavonol metabolites of black tea in urine samples using ^1H NMR spectroscopy followed by pattern recognition techniques, which converted the NMR spectra into a set of spectroscopic integrals used as descriptors for principal component analysis [40].

An ^1H NMR metabolomic approach was also used to profile the metabolomic changes in plasma arising from the consumption of cranberry procyanidins [59]. Similarly, urinary metabolome changes in female Sprague–Dawley rats were examined after the administration of partially purified cranberry or apple procyanidins [135], and recently, an in-depth analysis of the effects of resveratrol on the urinary and fecal metabolome of female Wistar rats [136] was performed. 2D NMR spectra (heteronuclear single-quantum correlation (HSQC) or heteronuclear multiple-quantum correlation (HMQC) spectroscopy) were used in some examples to assist the metabolite identification and confirm the metabolite assignments.

The complexity of the urine matrix, together with the low concentration of the conjugates, which have many possible isomeric forms, requires the occasional use of an SPE sample preparation procedure to both purify and concentrate the PC metabolites before NMR analysis. In this context, Jacobs et al. developed a method in which three complementary metabolite sub-profiles were generated with different compound classes by SPE fractionation [137]. Separation of phenolic from polar metabolites improved the identification by reducing signal overlap in the ^1H NMR spectra and increasing phenolic

concentrations. This SPE-NMR sub-profiling method was tested on urine samples collected from a crossover human nutritional intervention trial in which healthy volunteers consumed a mixture of wine and grape polyphenol extracts or a placebo.

In another study, van der Hooft reported a procedure for the identification and quantification of PC metabolites in urine after intake of tea using a methodology in which MS-based SPE trapping was coupled to ^1H NMR spectroscopy [138]. A urine clean-up and preconcentration by means of SPE has also been recently employed by de Roo for the spectral assignment of conjugated valerolactone metabolites of catechin-based polyphenols isolated from the urine of black tea consumers [139]. In this study, an SPE-preparative liquid chromatography (prepLC)–MS–LC–MS–NMR workflow allowed the full spectral ^1H and ^{13}C NMR assignments of five conjugated valerolactones, applying 1D and 2D homo- and hetero-nuclear NMR experiments.

Moazzami et al. developed a quantitative NMR analysis to ascertain the pattern of sesamin urinary metabolites in humans. In this study, after collection by HPLC, the major catechol metabolites were characterized by ^1H -NMR, homonuclear correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), heteronuclear multiple-bond correlation (HMBC), HSQC, and two-dimensional nuclear Overhauser effect (NOESY) experiments, and the excretion post-consumption of a specific dose of sesame oil was quantified from urine extracts by ^1H -NMR spectra [140].

While MS/MS and MS^n fragmentation can be very helpful in metabolite characterization and identification [133], it is not useful to establish a substitution position, unless the reference of the compounds are available [18]. Therefore, to elucidate their complete structure, additional information from NMR spectra is required. A noteworthy application was reported by Natsume in 2003 [141], where the elucidation of the chemical structure of several (-)-epicatechin metabolites (methylated and glucuronidated forms) in human and rat urine was performed by ^1H NMR, ^{13}C NMR, and HMBC (and LC-MS) analyses. In another study, Miksits et al. located the sulfate at position 3 and identified the full structure of the main resveratrol monosulfated metabolite by NMR experiments (1D ^1H , 2D double-quantum filtered-COSY, HSQC, and HMBC) [142]. The elucidation of the truly active structures of (+)-catechin and (-)-epicatechin in biological rat fluids was established as (+)-catechin 5-O- β -glucuronide and (-)-epicatechin 5-O- β -glucuronide by both MS analysis and the full assignment of ^1H and ^{13}C NMR, which was achieved by 2D experiments (COSY, HSQC, and HMBC). In this study, the conjugation position of the glucuronide was established by HMBC spectra and was additionally corroborated by NOESY analysis [143].

4. Conclusions

The characterization of PCM in plasma and urine is a strategy to understand the biological effects of PC present in plant-derived feeds, foods, beverages, herbal medicines, and dietary supplements. Due to their beneficial impacts on health, considerable research has been focused on the identification and quantification of PCM in plasma and urine of human and rats after the consumption of dietary PC. During the last two decades, considerable progress has been achieved in sample preparation methods (PCM extraction, cleaning/purification, preconcentration, and derivation), allowing the analysis of PC and PCM with differentiated physicochemical properties. However, problematic issues in sample clean-up include a lack of commercial standards for PCM and internal standards, metabolite degradation due to tedious multi-step sample preparation workflows, instability of specific compounds, and low analyte concentration.

PCM analysis has benefited from recent advances in high-throughput identification by hyphenated chromatographic techniques. The presence of PCM with highly variable physicochemical traits (hydrophilicity, lipophilicity, and protolytic properties), their small structural differences, and low levels of abundance remain a challenge for their identification and quantitation by existing analytical platforms. Currently, there is lack of clear guidelines or strategies for the identification and mainly for the targeted quantification of PCM without standards. Reversed-phase LC is actually the best approach, but the column

specificity (particle size or the use of core shell particles), the mobile phase, and additives included in the mobile phases, and the performance of interface with detection systems, especially with the MS, are all important to ensure and sustain data quality, mainly for isomeric or isobaric compounds that are challenging and require both chromatographic separation and mass spectrometry detection.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/antiox10060846/s1>, Table S1. Secoiridoids metabolites detected in plasma and/or urine from humans and rats; Table S2. Phenolic alcohol metabolites detected in plasma and/or urine from humans and rats; Table S3. Flavonoids metabolites detected in plasma and/or urine from humans and rats; Table S4. Metabolites of phenolic acids detected in plasma and/or urine from humans and rats; Table S5. Metabolites of enterolignans, stilbenes, and ellagitannins detected in plasma and/or urine from humans and rats; Table S6. Metabolites of other phenolic compounds detected in plasma and/or urine from humans and rats.

Author Contributions: All authors have given approval to the final version of the manuscript.

Funding: This research was funded by CICYT (AGL2016-75329-R), CIBEROBN from the Instituto de Salud Carlos III, ISCIII from the Ministerio de Ciencia, Innovación y Universidades (AEI/ FEDER, UE) and Generalitat de Catalunya (GC) (2017SGR 196). A.L.-Y. wishes to thank the Consejo Nacional de Ciencia y Tecnología (CONACYT) of Mexico for the doctoral scholarship. I.D.-L. thanks the FI-AGAUR Research Fellowship Program for the FI contract (FI_B 00256). A.V.-Q. thanks the Ministry of Science Innovation and Universities for the Ramon y Cajal contract (RYC-2016-19355). M.-P. thanks to Ministry of Science Innovation (MICIU/ FEDER) for the project (RTI2018-093974-B-I00).

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

Atmospheric Pressure Chemical Ionization (APCI); Capillary Electrophoresis (CE); Collision-Induced Dissociation (CID); Electrospray Ionization (ESI); Gas Chromatography (GC); Gas Chromatography-Mass Spectrometry (GC-MS); Heteronuclear Single-Quantum Correlation Spectroscopy (HSQC); Heteronuclear Multiple-Bond Correlation Spectroscopy (HMBC); Heteronuclear Multiple-Quantum Correlation Spectroscopy (HMQC); Homonuclear Correlation Spectroscopy (COSY); Liquid Chromatography (LC); Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (LC-ESI-MS); Liquid-liquid Extraction (LLE); Mass Spectrometry (MS); Matrix-Assisted Laser Desorption Ionization (MALDI); Micro-elution Solid-Phase Extraction (μ SPE); Nuclear Magnetic Resonance (NMR); N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA); N-trimethylsilyl-N-methyl trifluoroacetamide (MSTFA); N-methyl-N-(tert-butyltrimethylsilyl) trifluoroacetamide and tetramethylammonium hydroxide (TMAH); Phenolic Compounds (PC); Phenolic Compounds Metabolites (PCM); Protein Precipitation (PP); Solid-Phase Extraction (SPE); Solid-Liquid Extraction (SLE); Surface-Induced Dissociation (SID); Total Correlation Spectroscopy (TOCSY); Trimethylchlorosilane (TMCS); Two-dimensional Nuclear Overhauser Effect (NOESY); Ultra-High-Performance Liquid Chromatography-Mass Spectrometry (UHPLC-MS).

References

1. Tresserra-Rimbau, A.; Rimm, E.B.; Medina-Remón, A.; Martínez-González, M.A.; De la Torre, R.; Corella, D.; Salas-Salvadó, J.; Gómez-Gracia, E.; Lapetra, J.; Arós, F. Inverse Association between Habitual Polyphenol Intake and Incidence of Cardiovascular Events in the PREDIMED Study. *Nutr. Metab. Cardiovasc. Dis.* **2014**, *24*, 639–647. [CrossRef] [PubMed]
2. Tresserra-Rimbau, A.; Rimm, E.B.; Medina-Remón, A.; Martínez-González, M.A.; López-Sabater, M.C.; Covas, M.I.; Corella, D.; Salas-Salvadó, J.; Gómez-Gracia, E.; Lapetra, J. Polyphenol Intake and Mortality Risk: A Re-Analysis of the PREDIMED Trial. *BMC Med.* **2014**, *12*, 1–11. [CrossRef] [PubMed]
3. Tulipani, S.; Martínez Huelamo, M.; Rotches Ribalta, M.; Estruch, R.; Ferrer, E.E.; Andres-Lacueva, C.; Illan, M.; Lamuela-Raventós, R.M. Oil Matrix Effects on Plasma Exposure and Urinary Excretion of Phenolic Compounds from Tomato Sauces: Evidence from a Human Pilot Study. *Food Chem.* **2012**, *130*, 581–590. [CrossRef]
4. Luca, S.V.; Macovei, I.; Bujor, A.; Miron, A.; Skalicka-Woźniak, K.; Aprotosoaie, A.C.; Trifan, A. Bioactivity of Dietary Polyphenols: The Role of Metabolites. *Crit. Rev. Food Sci. Nutr.* **2020**, *60*, 626–659. [CrossRef]

5. Marhuenda-Muñoz, M.; Laveriano-Santos, E.P.; Tresserra-Rimbau, A.; Lamuela-Raventós, R.M.; Martínez-Huélamo, M.; Vallverdú-Queralt, A. Microbial Phenolic Metabolites: Which Molecules Actually Have an Effect on Human Health? *Nutrients* **2019**, *11*, 2725. [CrossRef]
6. Mayorga-Gross, A.L.; Esquivel, P. Impact of Cocoa Products Intake on Plasma and Urine Metabolites: A Review of Targeted and Non-Targeted Studies in Humans. *Nutrients* **2019**, *11*, 1163. [CrossRef]
7. Li, W.; Jian, W.; Fu, Y. Basic Sample Preparation Techniques in LC-MS Bioanalysis: Protein Precipitation, Liquid-Liquid Extraction, and Solid-Phase Extraction. *Sample Prep. LC-MS Bioanal.* **2019**, 1–30. [CrossRef]
8. Niu, Z.; Zhang, W.; Yu, C.; Zhang, J.; Wen, Y. Recent Advances in Biological Sample Preparation Methods Coupled with Chromatography, Spectrometry and Electrochemistry Analysis Techniques. *TrAC Trends Anal. Chem.* **2018**, *102*, 123–146. [CrossRef]
9. Reyes-Garcés, N.; Gionfriddo, E. Recent Developments and Applications of Solid Phase Microextraction as a Sample Preparation Approach for Mass-Spectrometry-Based Metabolomics and Lipidomics. *TrAC Trends Anal. Chem.* **2019**, *113*, 172–181. [CrossRef]
10. Maciel, E.V.S.; de Toffoli, A.L.; Neto, E.S.; Nazario, C.E.D.; Lanças, F.M. New Materials in Sample Preparation: Recent Advances and Future Trends. *TrAC Trends Anal. Chem.* **2019**, *119*, 115633. [CrossRef]
11. Aszyk, J.; Byliński, H.; Namieśnik, J.; Kot-Wasik, A. Main Strategies, Analytical Trends and Challenges in LC-MS and Ambient Mass Spectrometry-Based Metabolomics. *TrAC Trends Anal. Chem.* **2018**, *108*, 278–295. [CrossRef]
12. Ötles, S.; Kartal, C. Solid-Phase Extraction (SPE): Principles and Applications in Food Samples. *Acta Sci. Pol. Technol. Aliment.* **2016**, *15*, 5–15. [CrossRef]
13. Quirantes-Pine, R.; Verardo, V.; Arraez-Roman, D.; Fernandez-Arroyo, S.; Micol, V.; Caboni, M.; Segura-Carretero, A.; Fernandez-Gutierrez, A. Evaluation of Different Extraction Approaches for the Determination of Phenolic Compounds and Their Metabolites in Plasma by NanoLC-ESI-TOF-MS. *Anal. Bioanal. Chem.* **2012**, *404*, 3081–3090. [CrossRef]
14. Suárez, M.; Romero, M.-P.; Macià, A.; Valls, R.M.; Fernández, S.; Solà, R.; Motilva, M.-J. Improved Method for Identifying and Quantifying Olive Oil Phenolic Compounds and Their Metabolites in Human Plasma by Microelution Solid-Phase Extraction Plate and Liquid Chromatography–Tandem Mass Spectrometry. *J. Chromatogr. B* **2009**, *877*, 4097–4106. [CrossRef]
15. Alexović, M.; Dotsikas, Y.; Bober, P.; Sabo, J. Achievements in Robotic Automation of Solvent Extraction and Related Approaches for Bioanalysis of Pharmaceuticals. *J. Chromatogr. B* **2018**, *1092*, 402–421. [CrossRef]
16. Hyötyläinen, T. Critical Evaluation of Sample Pretreatment Techniques. *Anal. Bioanal. Chem.* **2009**, *394*, 743–758. [CrossRef]
17. Gowda, G.N.; Djukovic, D. Overview of Mass Spectrometry-Based Metabolomics: Opportunities and Challenges. In *Mass Spectrometry in Metabolomics*; Human Press: New York, NY, USA; Springer: New York, NY, USA, 2014; Volume 1198, pp. 3–12, ISBN 978-1-4939-1257-5.
18. Moco, S.; Vervoort, J.; Moco, S.; Bino, R.J.; De Vos, R.C.H.; Bino, R. Metabolomics Technologies and Metabolite Identification. *TrAC Trends Anal. Chem.* **2007**, *26*, 855–866. [CrossRef]
19. Nash, W.J.; Dunn, W.B. From Mass to Metabolite in Human Untargeted Metabolomics: Recent Advances in Annotation of Metabolites Applying Liquid Chromatography-Mass Spectrometry Data. *TrAC Trends Anal. Chem.* **2019**, *120*, 115324. [CrossRef]
20. Xiao, J.F.; Zhou, B.; Ressom, H.W. Metabolite Identification and Quantitation in LC-MS/MS-Based Metabolomics. *TrAC Trends Anal. Chem.* **2012**, *32*, 1–14. [CrossRef]
21. Bazoti, F.N.; Gikas, E.; Tsarbopoulos, A. Simultaneous Quantification of Oleuropein and Its Metabolites in Rat Plasma by Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry. *Biomed. Chromatogr.* **2010**, *24*, 506–515. [CrossRef]
22. Bazoti, F.N.; Gikas, E.; Puel, C.; Coxam, V.; Tsarbopoulos, A. Development of a Sensitive and Specific Solid Phase Extraction–Gas Chromatography–Tandem Mass Spectrometry Method for the Determination of Elenolic Acid, Hydroxytyrosol, and Tyrosol in Rat Urine. *J. Agric. Food Chem.* **2005**, *53*, 6213–6221. [CrossRef]
23. Khymenets, O.; Crespo, M.C.; Dangles, O.; Rakotomanomana, N.; Andres-Lacueva, C.; Visioli, F. Human Hydroxytyrosol's Absorption and Excretion from a Nutraceutical. *J. Funct. Foods* **2016**, *23*, 278–282. [CrossRef]
24. Serra, A.; Rubió, L.; Macià, A.; Valls, R.-M.; Catalán, Ú.; de la Torre, R.; Motilva, M.-J. Application of Dried Spot Cards as a Rapid Sample Treatment Method for Determining Hydroxytyrosol Metabolites in Human Urine Samples. Comparison with Microelution Solid-Phase Extraction. *Anal. Bioanal. Chem.* **2013**, *405*, 9179–9192. [CrossRef]
25. Khymenets, O.; Farré, M.; Pujadas, M.; Ortiz, E.; Joglar, J.; Covas, M.I.; de la Torre, R. Direct Analysis of Glucuronidated Metabolites of Main Olive Oil Phenols in Human Urine after Dietary Consumption of Virgin Olive Oil. *Food Chem.* **2011**, *126*, 306–314. [CrossRef]
26. López de las Hazas, M.-C.; Rubió, L.; Kotronoulas, A.; de la Torre, R.; Solà, R.; Motilva, M.-J. Dose Effect on the Uptake and Accumulation of Hydroxytyrosol and Its Metabolites in Target Tissues in Rats. *Mol. Nutr. Food Res.* **2015**, *59*, 1395–1399. [CrossRef]
27. García-Villalba, R.; Carrasco-Pancorbo, A.; Nevedomskaya, E.; Mayboroda, O.A.; Deelder, A.M.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Exploratory Analysis of Human Urine by LC-ESI-TOF MS after High Intake of Olive Oil: Understanding the Metabolism of Polyphenols. *Anal. Bioanal. Chem.* **2010**, *398*, 463–475. [CrossRef]
28. Caruso, D.; Visioli, F.; Patelli, R.; Galli, C.; Galli, G. Urinary Excretion of Olive Oil Phenols and Their Metabolites in Humans. *Metabolism* **2001**, *50*, 1426–1428. [CrossRef]
29. Silva, S.; Garcia-Aloy, M.; Figueira, M.E.; Combet, E.; Mullen, W.; Bronze, M.R. High Resolution Mass Spectrometric Analysis of Secoiridoids and Metabolites as Biomarkers of Acute Olive Oil Intake-An Approach to Study Interindividual Variability in Humans. *Mol. Nutr. Food Res.* **2018**, *62*, 1700065. [CrossRef]

30. Domínguez-Perles, R.; Auñón, D.; Ferreres, F.; Gil-Izquierdo, A. Gender Differences in Plasma and Urine Metabolites from Sprague–Dawley Rats after Oral Administration of Normal and High Doses of Hydroxytyrosol, Hydroxytyrosol Acetate, and DOPAC. *Eur. J. Nutr.* **2017**, *56*, 215–224. [CrossRef]
31. Rubiò, L.; Farràs, M.; de La Torre, R.; Macià, A.; Romero, M.-P.; Valls, R.M.; Solà, R.; Farré, M.; Fitó, M.; Motilva, M.-J. Metabolite Profiling of Olive Oil and Thyme Phenols after a Sustained Intake of Two Phenol-Enriched Olive Oils by Humans: Identification of Compliance Markers. *Food Res. Int.* **2014**, *65*, 59–68. [CrossRef]
32. Gómez-Juaristi, M.; Martínez-López, S.; Sarria, B.; Bravo, L.; Mateos, R. Bioavailability of Hydroxycinnamates in an Instant Green/ Roasted Coffee Blend in Humans. Identification of Novel Colonic Metabolites. *Food Funct.* **2018**, *9*, 331–343. [CrossRef] [PubMed]
33. Scherbl, D.; Renouf, M.; Marmet, C.; Poquet, L.; Cristiani, I.; Dahbane, S.; Emady-Azar, S.; Sauser, J.; Galan, J.; Dionisi, F.; et al. Breakfast Consumption Induces Retarded Release of Chlorogenic Acid Metabolites in Humans. *Eur. Food Res. Technol.* **2017**, *243*, 791–806. [CrossRef]
34. Stalmach, A.; Mullen, W.; Barron, D.; Uchida, K.; Yokota, T.; Cavin, C.; Steiling, H.; Williamson, G.; Crozier, A. Metabolite Profiling of Hydroxycinnamate Derivatives in Plasma and Urine after the Ingestion of Coffee by Humans: Identification of Biomarkers of Coffee Consumption. *Drug Metab. Dispos.* **2009**, *37*, 1749–1758. [CrossRef] [PubMed]
35. Monteiro, M.; Farah, A.; Perrone, D.; Trugo, L.C.; Donangelo, C. Chlorogenic Acid Compounds from Coffee Are Differentially Absorbed and Metabolized in Humans. *J. Nutr.* **2007**, *137*, 2196–2201. [CrossRef]
36. Marmet, C.; Actis-Goretti, L.; Renouf, M.; Giuffrida, F. Quantification of Phenolic Acids and Their Methylates, Glucuronides, Sulfates and Lactones Metabolites in Human Plasma by LC–MS/MS after Oral Ingestion of Soluble Coffee. *J. Pharm. Biomed. Anal.* **2014**, *88*, 617–625. [CrossRef]
37. Renouf, M.; Marmet, C.; Giuffrida, F.; Lepage, M.; Barron, D.; Beaumont, M.; Williamson, G.; Dionisi, F. Dose-Response Plasma Appearance of Coffee Chlorogenic and Phenolic Acids in Adults. *Mol. Nutr. Food Res.* **2014**, *58*, 301–309. [CrossRef]
38. Li, C.; Meng, X.; Winnik, B.; Lee, M.-J.; Lu, H.; Sheng, S.; Buckley, B.; Yang, C.S. Analysis of Urinary Metabolites of Tea Catechins by Liquid Chromatography/ Electrospray Ionization Mass Spectrometry. *Chem. Res. Toxicol.* **2001**, *14*, 702–707. [CrossRef]
39. Brindani, N.; Mena, P.; Calani, L.; Benzie, I.; Choi, S.-W.; Brighenti, F.; Zanardi, F.; Curti, C.; Del Rio, D. Synthetic and Analytical Strategies for the Quantification of Phenyl- γ -Valerolactone Conjugated Metabolites in Human Urine. *Mol. Nutr. Food Res.* **2017**, *61*, 1700077. [CrossRef]
40. Daykin, C.A.; Duynhoven, J.P.M.V.; Groenewegen, A.; Dachtler, M.; Amelsvoort, J.M.M.V.; Mulder, T.P.J. Nuclear Magnetic Resonance Spectroscopic Based Studies of the Metabolism of Black Tea Polyphenols in Humans. *J. Agric. Food Chem.* **2005**, *53*, 1428–1434. [CrossRef]
41. Van Duynhoven, J.; van der Hooft, J.J.J.; van Dorsten, F.A.; Peters, S.; Foltz, M.; Gomez-Roldan, V.; Vervoort, J.; de Vos, R.C.H.; Jacobs, D.M. Rapid and Sustained Systemic Circulation of Conjugated Gut Microbial Catabolites after Single-Dose Black Tea Extract Consumption. *J. Proteome Res.* **2014**, *13*, 2668–2678. [CrossRef]
42. Gómez-Juaristi, M.; Martínez-López, S.; Sarria, B.; Bravo, L.; Mateos, R. Absorption and Metabolism of Yerba Mate Phenolic Compounds in Humans. *Food Chem.* **2018**, *240*, 1028–1038. [CrossRef]
43. Castello, F.; Fernández-Pachón, M.-S.; Cerrillo, I.; Escudero-López, B.; Ortega, Á.; Rosi, A.; Bresciani, L.; Del Rio, D.; Mena, P. Absorption, Metabolism, and Excretion of Orange Juice (Poly)Phenols in Humans: The Effect of a Controlled Alcoholic Fermentation. *Arch. Biochem. Biophys.* **2020**, *695*, 108627. [CrossRef]
44. Zeng, X.; Su, W.; Bai, Y.; Chen, T.; Yan, Z.; Wang, J.; Su, M.; Zheng, Y.; Peng, W.; Yao, H. Urinary Metabolite Profiling of Flavonoids in Chinese Volunteers after Consumption of Orange Juice by UFLC-Q-TOF-MS/MS. *J. Chromatogr. B* **2017**, *1061–1062*, 79–88. [CrossRef]
45. Pereira-Caro, G.; Borges, G.; van der Hooft, J.; Clifford, M.N.; Del Rio, D.; Lean, M.E.; Roberts, S.A.; Kellerhals, M.B.; Crozier, A. Orange Juice (Poly)Phenols Are Highly Bioavailable in Humans. *Am. J. Clin. Nutr.* **2014**, *100*, 1378–1384. [CrossRef]
46. Aschoff, J.K.; Riedl, K.M.; Cooperstone, J.L.; Högel, J.; Bosy-Westphal, A.; Schwartz, S.J.; Carle, R.; Schweiggert, R.M. Urinary Excretion of Citrus Flavanones and Their Major Catabolites after Consumption of Fresh Oranges and Pasteurized Orange Juice: A Randomized Cross-over Study. *Mol. Nutr. Food Res.* **2016**, *60*, 2602–2610. [CrossRef]
47. Pereira-Caro, G.; Clifford, M.N.; Polyviou, T.; Ludwig, I.A.; Alfheaid, H.; Moreno-Rojas, J.M.; Garcia, A.L.; Malkova, D.; Crozier, A. Plasma Pharmacokinetics of (Poly)Phenol Metabolites and Catabolites after Ingestion of Orange Juice by Endurance Trained Men. *Free Radic. Biol. Med.* **2020**, *160*, 784–795. [CrossRef]
48. Esteban-Fernández, A.; Ibañez, C.; Simó, C.; Bartolomé, B.; Moreno-Arribas, M.V. An Ultrahigh-Performance Liquid Chromatography–Time-of-Flight Mass Spectrometry Metabolomic Approach to Studying the Impact of Moderate Red-Wine Consumption on Urinary Metabolome. *J. Proteome Res.* **2018**, *17*, 1624–1635. [CrossRef]
49. Rotches-Ribalta, M.; Andres-Lacueva, C.; Estruch, R.; Escubano, E.; Urpi-Sarda, M. Pharmacokinetics of Resveratrol Metabolic Profile in Healthy Humans after Moderate Consumption of Red Wine and Grape Extract Tablets. *Pharmacol. Res.* **2012**, *66*, 375–382. [CrossRef]
50. Pereira-Caro, G.; Ordóñez, J.L.; Ludwig, I.; Gaillet, S.; Mena, P.; Del Rio, D.; Rouanet, J.-M.; Bindon, K.A.; Moreno-Rojas, J.M.; Crozier, A. Development and Validation of an UHPLC-HRMS Protocol for the Analysis of Flavan-3-Ol Metabolites and Catabolites in Urine, Plasma and Feces of Rats Fed a Red Wine Proanthocyanidin Extract. *Food Chem.* **2018**, *252*, 49–60. [CrossRef]

51. Cooke, D.N.; Thomasset, S.; Boocock, D.J.; Schwarz, M.; Winterhalter, P.; Steward, W.P.; Gescher, A.J.; Marczylo, T.H. Development of Analyses by High-Performance Liquid Chromatography and Liquid Chromatography/ Tandem Mass Spectrometry of Bilberry (*Vaccinium Myrtillus*) Anthocyanins in Human Plasma and Urine. *J. Agric. Food Chem.* **2006**, *54*, 7009–7013. [CrossRef]
52. Tian, Q.; Giusti, M.M.; Stoner, G.D.; Schwartz, S.J. Urinary Excretion of Black Raspberry (*Rubus Occidentalis*) Anthocyanins and Their Metabolites. *J. Agric. Food Chem.* **2006**, *54*, 1467–1472. [CrossRef] [PubMed]
53. Ludwig, I.A.; Mena, P.; Calani, L.; Borges, G.; Pereira-Caro, G.; Bresciani, L.; Del Rio, D.; Lean, M.E.J.; Crozier, A. New Insights into the Bioavailability of Red Raspberry Anthocyanins and Ellagitannins. *Free Radic. Biol. Med.* **2015**, *89*, 758–769. [CrossRef] [PubMed]
54. Kay, C.D.; Mazza, G.; Holub, B.J.; Wang, J. Anthocyanin Metabolites in Human Urine and Serum. *Br. J. Nutr.* **2004**, *91*, 933–942. [CrossRef] [PubMed]
55. Sandhu, A.K.; Huang, Y.; Xiao, D.; Park, E.; Edirisinghe, I.; Burton-Freeman, B. Pharmacokinetic Characterization and Bioavailability of Strawberry Anthocyanins Relative to Meal Intake. *J. Agric. Food Chem.* **2016**, *64*, 4891–4899. [CrossRef]
56. Sandhu, A.K.; Miller, M.G.; Thangthaeng, N.; Scott, T.M.; Shukitt-Hale, B.; Edirisinghe, I.; Burton-Freeman, B. Metabolic Fate of Strawberry Polyphenols after Chronic Intake in Healthy Older Adults. *Food Funct.* **2018**, *9*, 96–106. [CrossRef]
57. Feliciano, R.P.; Mecha, E.; Bronze, M.R.; Rodriguez-Mateos, A. Development and Validation of a High-Throughput Micro Solid-Phase Extraction Method Coupled with Ultra-High-Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry for Rapid Identification and Quantification of Phenolic Metabolites in Human Plasma and Urine. *J. Chromatogr. A* **2016**, *1464*, 21–31. [CrossRef]
58. Favari, C.; Mena, P.; Curti, C.; Istas, G.; Heiss, C.; Del Rio, D.; Rodriguez-Mateos, A. Kinetic Profile and Urinary Excretion of Phenyl-γ-Valerolactones upon Consumption of Cranberry: A Dose–Response Relationship. *Food Funct.* **2020**, *11*, 3975–3985. [CrossRef]
59. Liu, H.; Garrett, T.J.; Tayyari, F.; Gu, L. Profiling the Metabolome Changes Caused by Cranberry Procyanidins in Plasma of Female Rats Using ¹H NMR and UHPLC-Q-Orbitrap-HRMS Global Metabolomics Approaches. *Mol. Nutr. Food Res.* **2015**, *59*, 2107–2118. [CrossRef]
60. Iswaldi, I.; Arráez-Román, D.; Gómez-Caravaca, A.M.; del Mar Contreras, M.; Uberos, J.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Identification of Polyphenols and Their Metabolites in Human Urine after Cranberry-Syrup Consumption. *Food Chem. Toxicol.* **2013**, *55*, 484–492. [CrossRef]
61. Castello, F.; Costabile, G.; Bresciani, L.; Tassotti, M.; Naviglio, D.; Luongo, D.; Ciciola, P.; Vitale, M.; Vetrani, C.; Galaverna, G.; et al. Bioavailability and Pharmacokinetic Profile of Grape Pomace Phenolic Compounds in Humans. *Arch. Biochem. Biophys.* **2018**, *646*, 1–9. [CrossRef]
62. Stalmach, A.; Edwards, C.A.; Wightman, J.D.; Crozier, A. Identification of (Poly)Phenolic Compounds in Concord Grape Juice and Their Metabolites in Human Plasma and Urine after Juice Consumption. *J. Agric. Food Chem.* **2011**, *59*, 9512–9522. [CrossRef]
63. Stalmach, A.; Edwards, C.A.; Wightman, J.D.; Crozier, A. Colonic Catabolism of Dietary Phenolic and Polyphenolic Compounds from Concord Grape Juice. *Food Funct.* **2013**, *4*, 52–62. [CrossRef]
64. Sasot, G.; Martínez-Huélamo, M.; Vallverdú-Queralt, A.; Mercader-Martí, M.; Estruch, R.; Lamuela-Raventós, R.M. Identification of Phenolic Metabolites in Human Urine after the Intake of a Functional Food Made from Grape Extract by a High Resolution LTQ-Orbitrap-MS Approach. *Food Res. Int.* **2017**, *100*, 435–444. [CrossRef]
65. Mullen, W.; Borges, G.; Lean, M.E.J.; Roberts, S.A.; Crozier, A. Identification of Metabolites in Human Plasma and Urine after Consumption of a Polyphenol-Rich Juice Drink. *J. Agric. Food Chem.* **2010**, *58*, 2586–2595. [CrossRef]
66. Pimpão, R.C.; Dew, T.; Figueira, M.E.; McDougall, G.J.; Stewart, D.; Ferreira, R.B.; Santos, C.N.; Williamson, G. Urinary Metabolite Profiling Identifies Novel Colonic Metabolites and Conjugates of Phenolics in Healthy Volunteers. *Mol. Nutr. Food Res.* **2014**, *58*, 1414–1425. [CrossRef]
67. Yuste, S.; Macià, A.; Ludwig, I.A.; Romero, M.-P.; Fernández-Castillejo, S.; Catalán, Ú.; Motilva, M.-J.; Rubió, L. Validation of Dried Blood Spot Cards to Determine Apple Phenolic Metabolites in Human Blood and Plasma After an Acute Intake of Red-Fleshed Apple Snack. *Mol. Nutr. Food Res.* **2018**, *62*, 1800623. [CrossRef]
68. Anesi, A.; Mena, P.; Bub, A.; Ulaszewska, M.; Del Rio, D.; Kulling, S.E.; Mattivi, F. Quantification of Urinary Phenyl-γ-Valerolactones and Related Valeric Acids in Human Urine on Consumption of Apples. *Metabolites* **2019**, *9*, 254. [CrossRef]
69. Lee, J.; Ebeler, S.E.; Zweigenbaum, J.A.; Mitchell, A.E. UHPLC-(ESI)QTOF MS/MS Profiling of Quercetin Metabolites in Human Plasma Postconsumption of Applesauce Enriched with Apple Peel and Onion. *J. Agric. Food Chem.* **2012**, *60*, 8510–8520. [CrossRef]
70. Urpi-Sarda, M.; Monagas, M.; Khan, N.; Lamuela-Raventós, R.M.; Santos-Buelga, C.; Sacanella, E.; Castell, M.; Permanyer, J.; Andrés-Lacueva, C. Epicatechin, Procyanidins, and Phenolic Microbial Metabolites after Cocoa Intake in Humans and Rats. *Anal. Bioanal. Chem.* **2009**, *394*, 1545–1556. [CrossRef]
71. Urpi-Sarda, M.; Monagas, M.; Khan, N.; Llorach, R.; Lamuela-Raventós, R.M.; Jáuregui, O.; Estruch, R.; Izquierdo-Pulido, M.; Andrés-Lacueva, C. Targeted Metabolic Profiling of Phenolics in Urine and Plasma after Regular Consumption of Cocoa by Liquid Chromatography–Tandem Mass Spectrometry. *J. Chromatogr. A* **2009**, *1216*, 7258–7267. [CrossRef]
72. Gómez-Juaristi, M.; Sarria, B.; Martínez-López, S.; Bravo Clemente, L.; Mateos, R. Flavanol Bioavailability in Two Cocoa Products with Different Phenolic Content. A Comparative Study in Humans. *Nutrients* **2019**, *11*, 1441. [CrossRef] [PubMed]

73. Serra, A.; Macià, A.; Rubió, L.; Anglès, N.; Ortega, N.; Morelló, J.R.; Romero, M.-P.; Motilva, M.-J. Distribution of Procyanidins and Their Metabolites in Rat Plasma and Tissues in Relation to Ingestion of Procyanidin-Enriched or Procyanidin-Rich Cocoa Creams. *Eur. J. Nutr.* **2013**, *52*, 1029–1038. [CrossRef] [PubMed]
74. Ottaviani, J.I.; Momma, T.Y.; Kuhnle, G.K.; Keen, C.L.; Schroeter, H. Structurally Related (–)-Epicatechin Metabolites in Humans: Assessment Using de Novo Chemically Synthesized Authentic Standards. *Free Radic. Biol. Med.* **2012**, *52*, 1403–1412. [CrossRef] [PubMed]
75. Roura, E.; Andrés-Lacueva, C.; Jáuregui, O.; Badia, E.; Estruch, R.; Izquierdo-Pulido, M.; Lamuela-Raventós, R.M. Rapid Liquid Chromatography Tandem Mass Spectrometry Assay To Quantify Plasma (–)-Epicatechin Metabolites after Ingestion of a Standard Portion of Cocoa Beverage in Humans. *J. Agric. Food Chem.* **2005**, *53*, 6190–6194. [CrossRef]
76. Roura, E.; Andrés-Lacueva, C.; Estruch, R.; Lourdes Mata Bilbao, M.; Izquierdo-Pulido, M.; Lamuela-Raventós, R.M. The Effects of Milk as a Food Matrix for Polyphenols on the Excretion Profile of Cocoa (–)-Epicatechin Metabolites in Healthy Human Subjects. *Br. J. Nutr.* **2008**, *100*, 846–851. [CrossRef]
77. Actis-Goretti, L.; Lévêque, A.; Giuffrida, F.; Romanov-Michailidis, F.; Viton, F.; Barron, D.; Duenas-Paton, M.; Gonzalez-Manzano, S.; Santos-Buelga, C.; Williamson, G.; et al. Elucidation of (–)-Epicatechin Metabolites after Ingestion of Chocolate by Healthy Humans. *Free Radic. Biol. Med.* **2012**, *53*, 787–795. [CrossRef]
78. Saha, S.; Kroon, P.A. A Simple and Rapid LC-MS/MS Method for Quantification of Total Daidzein, Genistein, and Equol in Human Urine. *J. Anal. Methods Chem.* **2020**, *2020*, 1–9. [CrossRef]
79. Hakeem Said, I.; Truex, J.D.; Heidorn, C.; Retta, M.B.; Petrov, D.D.; Haka, S.; Kuhnert, N. LC-MS/MS Based Molecular Networking Approach for the Identification of Cocoa Phenolic Metabolites in Human Urine. *Food Res. Int.* **2020**, *132*, 109119. [CrossRef]
80. Martínez-Huélamo, M.; Tulipani, S.; Torrado, X.; Estruch, R.; Lamuela-Raventós, R.M. Validation of a New LC-MS/MS Method for the Detection and Quantification of Phenolic Metabolites from Tomato Sauce in Biological Samples. *J. Agric. Food Chem.* **2012**, *60*, 4542–4549. [CrossRef]
81. Martínez-Huélamo, M.; Vallverdú-Queralt, A.; Di Lecce, G.; Valderas-Martínez, P.; Tulipani, S.; Jáuregui, O.; Escribano-Ferrer, E.; Estruch, R.; Illan, M.; Lamuela-Raventós, R.M. Bioavailability of Tomato Polyphenols Is Enhanced by Processing and Fat Addition: Evidence from a Randomized Feeding Trial. *Mol. Nutr. Food Res.* **2016**, *60*, 1578–1589. [CrossRef]
82. Urpi-Sarda, M.; Garrido, I.; Monagas, M.; Gómez-Cordovés, C.; Medina-Remón, A.; Andrés-Lacueva, C.; Bartolomé, B. Profile of Plasma and Urine Metabolites after the Intake of Almond [*Prunus Dulcis* (Mill.) D.A. Webb] Polyphenols in Humans. *J. Agric. Food Chem.* **2009**, *57*, 10134–10142. [CrossRef]
83. Mecha, E.; Feliciano, R.P.; Rodríguez-Mateos, A.; Silva, S.D.; Figueira, M.E.; Vaz Patta, M.C.; Bronze, M.R. Human Bioavailability of Phenolic Compounds Found in Common Beans: The Use of High-Resolution MS to Evaluate Inter-Individual Variability. *Br. J. Nutr.* **2020**, *123*, 273–292. [CrossRef]
84. Zhang, J.; Guo, Q.; Wei, M.; Bai, J.; Huang, J.; Liu, Y.; Su, Z.; Qiu, X. Metabolite Identification and Pharmacokinetic Profiling of Isoflavones from Black Soybean in Rats Using Ultrahigh-Performance Liquid Chromatography with Linear-Ion-Trap–Orbitrap and Triple-Quadrupole Tandem Mass Spectrometry. *J. Agric. Food Chem.* **2018**, *66*, 12941–12952. [CrossRef]
85. Fardet, A.; Llorach, R.; Martin, J.-F.; Besson, C.; Lyan, B.; Pujos-Guillot, E.; Scalbert, A. A Liquid Chromatography–Quadrupole Time-of-Flight (LC–QTOF)-Based Metabolomic Approach Reveals New Metabolic Effects of Catechin in Rats Fed High-Fat Diets. *J. Proteome Res.* **2008**, *7*, 2388–2398. [CrossRef]
86. Wu, M.; Wu, X.; Zhang, D.; Qiu, F.; Ding, L.; Ma, H.; Chen, X. Metabolic Profiling of Quercetin in Rats Using Ultra-Performance Liquid Chromatography/Quadrupole-Time-of-Flight Mass Spectrometry. *Biomed. Chromatogr.* **2017**, *31*, e4016. [CrossRef]
87. Shang, Z.; Wang, F.; Dai, S.; Lu, J.; Wu, X.; Zhang, J. Profiling and Identification of (–)-Epicatechin Metabolites in Rats Using Ultra-High Performance Liquid Chromatography Coupled with Linear Trap–Orbitrap Mass Spectrometer: Study of EC in Vivo Metabolites. *Drug Test. Anal.* **2017**, *9*, 1224–1235. [CrossRef]
88. Zhao, W.; Shang, Z.; Li, Q.; Huang, M.; He, W.; Wang, Z.; Zhang, J. Rapid Screening and Identification of Daidzein Metabolites in Rats Based on UHPLC-LTQ–Orbitrap Mass Spectrometry Coupled with Data-Mining Technologies. *Molecules* **2018**, *23*, 151. [CrossRef]
89. Shahrzad, S.; Bitsch, I. Determination of Gallic Acid and Its Metabolites in Human Plasma and Urine by High-Performance Liquid Chromatography. *J. Chromatogr. B Biomed. Sci. App.* **1998**, *705*, 87–95. [CrossRef]
90. Qiu, Z.; Yu, J.; Dai, Y.; Chen, X.; Huang, F.; Li, N. A Simple LC–MS/MS Method for the Simultaneous Quantification of Resveratrol and Its Major Phase II Metabolites: Assessment of Their Urinary and Biliary Excretions in Rats. *J. Chromatogr. B* **2017**, *1048*, 85–93. [CrossRef]
91. Zhang, Z.; Xu, M.; Sun, S.; Qiao, X.; Wang, B.; Han, J.; Guo, D. Metabolic Analysis of Four Phenolic Acids in Rat by Liquid Chromatography–Tandem Mass Spectrometry. *J. Chromatogr. B* **2008**, *87*, 7–14. [CrossRef]
92. Jiao, Q.; Xu, L.; Jiang, L.; Jiang, Y.; Zhang, J.; Liu, B. Metabolism Study of Hesperetin and Hesperidin in Rats by UHPLC-LTQ–Orbitrap MSⁿ. *Xenobiotica* **2020**, *50*, 1311–1322. [CrossRef]
93. López-Yerena; Vallverdú-Queralt; Mols; Augustijns; Lamuela-Raventós; Escribano-Ferrer Absorption and Intestinal Metabolic Profile of Oleocanthal in Rats. *Pharmaceutics* **2020**, *12*, 134. [CrossRef]
94. López-Yerena, A.; Vallverdú-Queralt, A.; Jáuregui, O.; García-Sala, X.; Lamuela-Raventós, R.M.; Escribano-Ferrer, E. Tissue Distribution of Oleocanthal and Its Metabolites after Oral Ingestion in Rats. *Antioxidants* **2021**, *10*, 688. [CrossRef]

95. López-Yerena, A.; Pérez, M.; Vallverdú-Queralt, A.; Miliarakis, E.; Lamuela-Raventós, R.M.; Escribano-Ferrer, E. Oleacein Intestinal Permeation and Metabolism in Rats Using an In Situ Perfusion Technique. *Pharmaceutics* **2021**, *13*, 719. [CrossRef]
96. Wiese, S.; Esatbeyoglu, T.; Winterhalter, P.; Kruse, H.-P.; Winkler, S.; Bub, A.; Kulling, S.E. Comparative Biokinetics and Metabolism of Pure Monomeric, Dimeric, and Polymeric Flavan-3-Ols: A Randomized Cross-over Study in Humans. *Mol. Nutr. Food Res.* **2015**, *59*, 610–621. [CrossRef]
97. Hayasaka, N.; Shimizu, N.; Komoda, T.; Mohri, S.; Tsushida, T.; Eitsuka, T.; Miyazawa, T.; Nakagawa, K. Absorption and Metabolism of Luteolin in Rats and Humans in Relation to in Vitro Anti-Inflammatory Effects. *J. Agric. Food Chem.* **2018**, *66*, 11320–11329. [CrossRef]
98. Svilar, L.; Martin, J.-C.; Defoort, C.; Paut, C.; Tourniaire, F.; Brochot, A. Quantification of Trans-Resveratrol and Its Metabolites in Human Plasma Using Ultra-High Performance Liquid Chromatography Tandem Quadrupole-Orbitrap Mass Spectrometry. *J. Chromatogr. B* **2019**, *1104*, 119–129. [CrossRef]
99. López-Yerena, A.; Perez, M.; Vallverdú-Queralt, A.; Escribano-Ferrer, E. Insights into the Binding of Dietary Phenolic Compounds to Human Serum Albumin and Food-Drug Interactions. *Pharmaceutics* **2020**, *12*, 1123. [CrossRef]
100. Tulipani, S.; Llorach, S.; Urpi-Sarda, M.; Andres-Lacueva, C. Comparative Analysis of Sample Preparation Methods To Handle the Complexity of the Blood Fluid Metabolome: When Less Is More. *Anal. Chem.* **2013**, *85*, 341–348. [CrossRef]
101. David, A.; Abdul-Sada, A.; Lange, A.; Tyler, C.R.; Hill, E.M. A New Approach for Plasma (Xeno)Metabolomics Based on Solid-Phase Extraction and Nanoflow Liquid Chromatography-Nanoelectrospray Ionisation Mass Spectrometry. *J. Chromatogr. A* **2014**, *1365*, 72–85. [CrossRef]
102. Koller, D.; Zubiaur, P.; Saiz-Rodríguez, M.; Abad-Santos, F.; Wojnicz, A. Simultaneous Determination of Six Antipsychotics, Two of Their Metabolites and Caffeine in Human Plasma by LC-MS/MS Using a Phospholipid-Removal Microelution-Solid Phase Extraction Method for Sample Preparation. *Talanta* **2019**, *198*, 159–168. [CrossRef] [PubMed]
103. Bouatra, S.; Aziat, F.; Mandal, R.; Guo, A.C.; Wilson, M.R.; Knox, C.; Bjorn Dahl, T.C.; Krishnamurthy, R.; Saleem, F.; Liu, P.; et al. The Human Urine Metabolome. *PLoS ONE* **2013**, *8*, e73076. [CrossRef] [PubMed]
104. Urpi-Sarda, M.; Zamora-Ros, R.; Lamuela-Raventós, R.; Cherubini, A.; Jauregui, O.; de la Torre, R.; Covas, M.I.; Estruch, R.; Jaeger, W.; Andres-Lacueva, C. HPLC–Tandem Mass Spectrometric Method to Characterize Resveratrol Metabolism in Humans. *Clin. Chem.* **2007**, *53*, 292–299. [CrossRef] [PubMed]
105. De Ferrars, R.M.; Czank, C.; Saha, S.; Needs, P.W.; Zhang, Q.; Raheem, K.S.; Botting, N.P.; Kroon, P.A.; Kay, C.D. Methods for Isolating, Identifying, and Quantifying Anthocyanin Metabolites in Clinical Samples. *Anal. Chem.* **2014**, *86*, 10052–10058. [CrossRef]
106. González-Domínguez, R.; Urpi-Sarda, M.; Jauregui, O.; Needs, P.W.; Kroon, P.A.; Andrés-Lacueva, C. Quantitative Dietary Fingerprinting (QDF)—A Novel Tool for Comprehensive Dietary Assessment Based on Urinary Nutrimetabolomics. *J. Agric. Food Chem.* **2019**, *68*, 1851–1861. [CrossRef]
107. Del Boccio, P.; Di Deo, A.; De Curtis, A.; Celli, N.; Iacoviello, L.; Rotilio, D. Liquid Chromatography–Tandem Mass Spectrometry Analysis of Oleuropein and Its Metabolite Hydroxytyrosol in Rat Plasma and Urine after Oral Administration. *J. Chromatogr. B* **2003**, *785*, 47–56. [CrossRef]
108. Liu, X.; Zhou, L.; Shi, X.; Xu, G. New Advances in Analytical Methods for Mass Spectrometry-Based Large-Scale Metabolomics Study. *TrAC Trends Anal. Chem.* **2019**, *121*, 115665. [CrossRef]
109. Alvarez-Rivera, G.; Ballesteros-Vivas, D.; Parada-Alfonso, F.; Ibañez, E.; Cifuentes, A. Recent Applications of High Resolution Mass Spectrometry for the Characterization of Plant Natural Products. *TrAC Trends Anal. Chem.* **2019**, *112*, 87–101. [CrossRef]
110. Glish, G.L.; Burinsky, D.J. Hybrid Mass Spectrometers for Tandem Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **2008**, *19*, 161–172. [CrossRef]
111. Jové, M.; Serrano, J.C.E.; Ortega, N.; Ayala, V.; Anglès, N.; Reguant, J.; Morelló, J.R.; Romero, M.P.; Motilva, M.J.; Prat, J.; et al. Multicompartmental LC-Q-TOF-Based Metabonomics as an Exploratory Tool to Identify Novel Pathways Affected by Polyphenol-Rich Diets in Mice. *J. Proteome Res.* **2011**, *10*, 3501–3512. [CrossRef]
112. Hu, Q.; Noll, R.J.; Li, H.; Makarov, A.; Hardman, M.; Graham Cooks, R. The Orbitrap: A New Mass Spectrometer. *J. Mass Spectrom.* **2005**, *40*, 430–443. [CrossRef]
113. Eliuk, S.; Makarov, A. Evolution of Orbitrap Mass Spectrometry Instrumentation. *Annu. Rev. Anal. Chem.* **2015**, *8*, 61–80. [CrossRef]
114. Gorrochategui, E.; Jaumot, J.; Lacorte, S.; Tauler, R. Data Analysis Strategies for Targeted and Untargeted LC-MS Metabolomic Studies: Overview and Workflow. *TrAC Trends Anal. Chem.* **2016**, *82*, 425–442. [CrossRef]
115. López de las Hazas, M.-C.; Piñol, C.; Macià, A.; Romero, M.-P.; Pedret, A.; Solà, R.; Rubió, L.; Motilva, M.-J. Differential Absorption and Metabolism of Hydroxytyrosol and Its Precursors Oleuropein and Secoiridoids. *J. Funct. Foods* **2016**, *22*, 52–63. [CrossRef]
116. Davis, B.D.; Needs, P.W.; Kroon, P.A.; Brodbelt, J.S. Identification of Isomeric Flavonoid Glucuronides in Urine and Plasma by Metal Complexation and LC-ESI-MS/MS. *J. Mass Spectrom.* **2006**, *41*, 911–920. [CrossRef]
117. Serra, A.; Macià, A.; Romero, M.-P.; Salvadó, M.-J.; Bustos, M.; Fernández-Larrea, J.; Motilva, M.-J. Determination of Procyanidins and Their Metabolites in Plasma Samples by Improved Liquid Chromatography–Tandem Mass Spectrometry. *J. Chromatogr. B* **2009**, *877*, 1169–1176. [CrossRef]
118. González-Domínguez, R. Quantifying the Human Diet in the Crosstalk between Nutrition and Health by Multi-Targeted Metabolomics of Food and Microbiota-Derived Metabolites. *Int. J. Obes.* **2020**, *1–10*. [CrossRef]

119. Obrenovich, M.E.; Donskey, C.J.; Schiefer, I.T.; Bongiovanni, R.; Li, L.; Jaskiw, G.E. Quantification of Phenolic Acid Metabolites in Humans by LC–MS: A Structural and Targeted Metabolomics Approach. *Bioanalysis* **2018**, *10*, 1591–1608. [CrossRef]
120. De Ferrars, R.M.; Cassidy, A.; Curtis, P.; Kay, C.D. Phenolic Metabolites of Anthocyanins Following a Dietary Intervention Study in Post-Menopausal Women. *Mol. Nutr. Food Res.* **2014**, *58*, 490–502. [CrossRef]
121. Nolvachai, Y.; Marriott, P.J. GC for Flavonoids Analysis: Past, Current, and Prospective Trends: Gas Chromatography. *J. Sep. Sci.* **2013**, *36*, 20–36. [CrossRef]
122. Juszczak, A.M.; Zovko-Končić, M.; Tomczyk, M. Recent Trends in the Application of Chromatographic Techniques in the Analysis of Luteolin and Its Derivatives. *Biomolecules* **2019**, *9*, 731. [CrossRef]
123. Szultka, M.; Buszewski, B.; Papaj, K.; Szeja, W.; Rusin, A. Determination of Flavonoids and Their Metabolites by Chromatographic Techniques. *TrAC Trends Anal. Chem.* **2013**, *47*, 47–67. [CrossRef]
124. Magiera, S.; Uhlschmied, C.; Rainer, M.; Huck, C.W.; Baranowska, I.; Bonn, G.K. GC–MS Method for the Simultaneous Determination of β -Blockers, Flavonoids, Isoflavones and Their Metabolites in Human Urine. *J. Pharm. Biomed. Anal.* **2011**, *56*, 93–102. [CrossRef]
125. Heinonen, S.-M.; Hoikkala, A.; Wähälä, K.; Adlercreutz, H. Metabolism of the Soy Isoflavones Daidzein, Genistein and Glycitein in Human Subjects. *J. Steroid Biochem. Mol. Biol.* **2003**, *87*, 285–299. [CrossRef]
126. Ordóñez, J.L.; Pereira-Caro, G.; Ludwig, I.; Muñoz-Redondo, J.M.; Ruiz-Moreno, M.J.; Crozier, A.; Moreno-Rojas, J.M. A Critical Evaluation of the Use of Gas Chromatography- and High Performance Liquid Chromatography-Mass Spectrometry Techniques for the Analysis of Microbial Metabolites in Human Urine after Consumption of Orange Juice. *J. Chromatogr. A* **2018**, *1575*, 100–112. [CrossRef]
127. Draijer, R.; van Dorsten, F.; Zebregs, Y.; Hollebrands, B.; Peters, S.; Duchateau, G.; Grün, C. Impact of Proteins on the Uptake, Distribution, and Excretion of Phenolics in the Human Body. *Nutrients* **2016**, *8*, 814. [CrossRef]
128. Carry, E.; Zhao, D.; Mogno, I.; Faith, J.; Ho, L.; Villani, T.; Patel, H.; Pasinetti, G.M.; Simon, J.E.; Wu, Q. Targeted Analysis of Microbial-Generated Phenolic Acid Metabolites Derived from Grape Flavonols by Gas Chromatography-Triple Quadrupole Mass Spectrometry. *J. Pharm. Biomed. Anal.* **2018**, *159*, 374–383. [CrossRef]
129. Son, H.-S.; Kim, K.M.; van den Berg, F.; Hwang, G.-S.; Park, W.-M.; Lee, C.-H.; Hong, Y.-S. H Nuclear Magnetic Resonance-Based Metabolomic Characterization of Wines by Grape Varieties and Production Areas. *J. Agric. Food Chem.* **2008**, *56*, 8007–8016. [CrossRef]
130. Falasca, A.; Melck, D.; Paris, D.; Saviano, G.; Motta, A.; Iorizzi, M. Seasonal Changes in the Metabolic Fingerprint of Juniperus Communis L. Berry Extracts by ¹H NMR-Based Metabolomics. *Metabolomics* **2014**, *10*, 165–174. [CrossRef]
131. Olmo-Cunillera, A.; López-Yerena, A.; Lozano-Castellón, J.; Tresserra-Rimbau, A.; Vallverdú-Queralt, A.; Pérez, M. NMR Spectroscopy: A Powerful Tool for the Analysis of Polyphenols in Extra Virgin Olive Oil. *J. Sci. Food Agric.* **2020**, *100*, 1842–1851. [CrossRef]
132. D'Urso, G.; Piacente, S.; Pizza, C.; Montoro, P. Metabolomics of Healthy Berry Fruits. *Curr. Med. Chem.* **2019**, *25*, 4888–4902. [CrossRef] [PubMed]
133. Wishart, D.S. Advances in Metabolite Identification. *Bioanalysis* **2011**, *3*, 1769–1782. [CrossRef] [PubMed]
134. Li, W.; Ruan, C.-J.; Teixeira da Silva, J.A.; Guo, H.; Zhao, C.-E. NMR Metabolomics of Berry Quality in Sea Buckthorn (*Hippophae L.*). *Mol. Breed.* **2013**, *31*, 57–67. [CrossRef]
135. Liu, H.; Tayyari, F.; Edison, A.S.; Su, Z.; Gu, L. NMR-Based Metabolomics Reveals Urinary Metabolome Modifications in Female Sprague–Dawley Rats by Cranberry Procyanidins. *J. Nutr. Biochem.* **2016**, *34*, 136–145. [CrossRef] [PubMed]
136. Torres Santiago, G.; Serrano Contreras, J.I.; Meléndez Camargo, M.E.; Zepeda Vallejo, L.G. NMR-Based Metabolomic Approach Reveals Changes in the Urinary and Fecal Metabolome Caused by Resveratrol. *J. Pharm. Biomed. Anal.* **2019**, *162*, 234–241. [CrossRef]
137. Jacobs, D.M.; Spiesser, L.; Garnier, M.; de Roo, N.; van Dorsten, F.; Hollebrands, B.; van Velzen, E.; Draijer, R.; van Duynhoven, J. SPE–NMR Metabolite Sub-Profiling of Urine. *Anal. Bioanal. Chem.* **2012**, *404*, 2349–2361. [CrossRef]
138. Van der Hooft, J.J.J.; de Vos, R.C.H.; Mihaleva, V.; Bino, R.J.; Ridder, L.; de Roo, N.; Jacobs, D.M.; van Duynhoven, J.P.M.; Vervoort, J. Structural Elucidation and Quantification of Phenolic Conjugates Present in Human Urine after Tea Intake. *Anal. Chem.* **2012**, *84*, 7263–7271. [CrossRef] [PubMed]
139. De Roo, N.; Wilmsen, S.M.J.; Mihaleva, V.V.; Jacobs, D.M.; van Duynhoven, J.P.M. Full ¹H and ¹³C NMR Spectral Assignment of Conjugated Valerolactone Metabolites Isolated from Urine of Black Tea Consumers by Means of SPE–PrepLC–MS–LC–MS–NMR. *Magn. Reson. Chem.* **2019**, *57*, 548–557. [CrossRef] [PubMed]
140. Moazzami, A.A.; Andersson, R.E.; Kamal-Eldin, A. Quantitative NMR Analysis of a Sesamin Catechol Metabolite in Human Urine. *J. Nutr.* **2007**, *137*, 940–944. [CrossRef]
141. Natsume, M.; Osakabe, N.; Oyama, M.; Sasaki, M.; Baba, S.; Nakamura, Y.; Osawa, T.; Terao, J. Structures of (–)-Epicatechin Glucuronide Identified from Plasma and Urine after Oral Ingestion of (–)-Epicatechin: Differences between Human and Rat. *Free Radic. Biol. Med.* **2003**, *34*, 840–849. [CrossRef]

142. Miksits, M.; Maier-Salamon, A.; Aust, S.; Thalhammer, T.; Reznicek, G.; Kunert, O.; Haslinger, E.; Szekeres, T.; Jaeger, W. Sulfation of Resveratrol in Human Liver: Evidence of a Major Role for the Sulfotransferases SULT1A1 and SULT1E1. *Xenobiotica* **2005**, *35*, 1101–1119. [CrossRef]
143. Harada, M.; Kan, Y.; Naoki, H.; Fukui, Y.; Kageyama, N.; Nakai, M.; Miki, W.; Kiso, Y. Identification of the Major Antioxidative Metabolites in Biological Fluids of the Rat with Ingested (+)-Catechin and (-)-Epicatechin. *Biosci. Biotechnol. Biochem.* **1999**, *63*, 973–977. [CrossRef]

HYPOTHESIS AND OBJECTIVES

3. HYPOTHESIS AND OBJECTIVES

3.1. Hypothesis

The content of PCs in EVOO is affected by the unique characteristics of the olive fruits, by agronomic and processing factors.

Evidence on the health-promoting properties of SEC could suggest that OLC and OLE have high bioavailability.

3.2. Objectives

To evaluate how the unique conditions of the olive fruits as well as the agronomic and processing factors affect the phenolic composition of the EVOO.

1. To study the natural habitat and unique characteristics of olive fruits and its phenolic composition to contribute to maintain the biodiversity of olive oil in the Mediterranean basin.
2. To evaluate the effect of organic and conventional production systems, under the same environmental, on the content of PCs in Hojiblanca EVOO.
3. To optimize the agronomic (ripening stage) and technological variables (crushing temperature and size and malaxation temperature and time) to obtain an EVOO with a high content of PCs.

To study the intestinal absorption and metabolism of OLC and OLEA using an IPVS technique in rats involving simultaneous sampling from the luminal perfusate and mesenteric blood.

1. To evaluate the stability of OLC and OLEA in the perfusion solution, at 37°C, during IPVS experiment.
2. To determine the intestinal permeability considering the disappearance of the test product in the lumen (P_{eff}) and appearance in the mesenteric circulation (P_{app}),
3. To compare the intestinal permeability of OLC and OLEA with the highly permeable drugs levofloxacin and naproxen, respectively.
4. To evaluate the intestinal metabolic profile of OLC and OLEA monitored in the intestinal lumen and mesenteric blood plasma.

To evaluate the distribution of OLC and OLEA and its metabolites in multiple tissues and at different times post-administration.

1. To determine the qualitative and quantitative distribution of OLC or OLEA in rat tissues after the administration of refined olive oil containing the compounds.

2. To study the OLC and OLEA metabolism and its metabolites distribution in organs of the digestive system (stomach, small intestine, and liver), excretory tissue (kidney), and several target tissues (brain, lungs, heart, spleen, thyroids, and skin).

RESULTS

4. RESULTS

This section presents the results obtained during the doctoral thesis. These results have been classified into three sections as it is showed:

1. Phenolic compounds: towards obtaining an olive oil with higher nutritional value.
2. Absorption and metabolism of phenolic compounds assessed by intestinal perfusion method.
3. Evidence of tissue distribution of olive oil phenols in rats.

All results are explained in seven articles. Five of the manuscripts have been published in indexed scientific journals of the first-quartile, one in the second-quartile and the last one is under review in a first-quartile journal.

4.1. Phenolic compounds: towards obtaining an olive oil with higher nutritional value

4.1.1. Article 3: Conservation of native wild ivory-white olives from the Medes islands natural reserve to maintain virgin olive oil diversity

Anallely López-Yerena, Antònia Ninot, Julián Lozano-Castellón, Elvira Escribano-Ferrer, Agustí J. Romero-Aroca, Angjelina Belaj, Anna Vallverdú-Queralt and Rosa M. Lamuela-Raventós

Antioxidants, 9(10), 1009, 2020

Abstract

Background: Food diversity, and in particular genetic diversity, is being lost at an alarming rate and the protection of natural areas is crucial to safeguard the world's threatened species. The Medes Islands (MI), located in the northwest Mediterranean Sea, are a protected natural reserve. Wild olive trees also known as oleasters make up part of the vegetation of the Meda Gran island. Among them, in 2012, a wild albino ivory-white olive tree with fruit was identified.

Scope: Our objective was to explore the natural habitat of the MI for the development of native oleaster olive fruit as a resource to maintain olive oil biodiversity in the Mediterranean Basin. The quality of this oil was extensively analyzed using sensory, physicochemical, and stability parameters.

Methods and results: Fruits were collected from this tree and their seeds were first sown in a greenhouse and then planted in an orchard in order to preserve the natural habitat. Seven out of the 78 seedling trees obtained (12%) produced ivory-white fruits. In autumn 2018, fruits from these trees were sampled. Although the fruits had low oil content, virgin olive oil with unique sensory, physicochemical, and stability characteristics was produced. Respect to the polyphenols content, OLEA was the main compound identified (373.29 ± 72.02 mg/kg) and the OLC was the second most abundant PC (204.84 ± 52.58 mg/kg). Such very high contents of these compounds, responsible for the bitter and pungent EVOO test, are really interesting from the health point of view, due to its anti-inflammatory activity that can be relevant in many pathologies. Regarding pigments, samples were characterized by an intense yellow color, with 12.5 ± 4.6 mg/kg of chlorophyll and 9.2 ± 3.3 mg/kg of carotenoids.

Conclusions: This study explored the resources of the natural habitat of the MI as a means of enrichment of EVOO diversity and authenticity of this traditional Mediterranean food. Physicochemical characterization of these fruits and olive oil revealed unique sensory physicochemical and stability characteristics, highlighting the need to maintain the biodiversity of olive oil and food authenticity of this traditional Mediterranean food.



Article

Conservation of Native Wild Ivory-White Olives from the MEDES Islands Natural Reserve to Maintain Virgin Olive Oil Diversity

Anallely López-Yerena ^{1,†} , Antònia Ninot ^{2,†} , Julián Lozano-Castellón ^{1,3} ,
Elvira Escribano-Ferrer ^{3,4}, Agustí J. Romero-Aroca ² , Angjelina Belaj ⁵,
Anna Vallverdú-Queralt ^{1,3} and Rosa M. Lamuela-Raventós ^{1,3,*}

¹ Nutrition, Food Science and Gastronomy Department, XaRTA, Institute of Nutrition and Food Safety (INSA-UB), Pharmacy and Food Sciences School, University of Barcelona, 08028 Barcelona, Spain; naye.yerena@gmail.com (A.L.-Y.); jullozcas@gmail.com (J.L.-C.); avallverdu@ub.edu (A.V.-Q.)

² Institute of Agrifood Research and Technology (IRTA), Fruit Science Program, Olive Growing and Oil Technology research team, 43120 Constantí, Spain; Antonia.Ninot@irta.cat (A.N.); Agusti.Romero@irta.cat (A.J.R.-A.)

³ CIBER Physiopathology of Obesity and Nutrition (CIBEROBN), Institute of Health Carlos III, 28029 Madrid, Spain; eescribano@ub.edu

⁴ Department of Pharmacy and Pharmaceutical Technology and Physical Chemistry, Biopharmaceutics and Pharmacokinetics Unit, Institute of Nanoscience and Nanotechnology (IN2UB), Pharmacy and Food Sciences School, University of Barcelona, 08028 Barcelona, Spain

⁵ Instituto de Investigación y Formación Agraria y Pesquera (IFAPA)—Centro “Alameda del Obispo”, Avda. Menéndez Pidal s/n, E-14004 Córdoba, Spain; angjelina.belaj@juntadeandalucia.es

* Correspondence: lamuela@ub.edu; Tel.: +34-64-642-1580; Fax: +34-93-403-5931

† These authors contributed equally to the work.

Received: 23 September 2020; Accepted: 15 October 2020; Published: 17 October 2020



Abstract: Food diversity, and in particular genetic diversity, is being lost at an alarming rate. Protection of natural areas is crucial to safeguard the world’s threatened species. The Medes Islands (MI), located in the northwest Mediterranean Sea, are a protected natural reserve. Wild olive trees also known as oleasters make up part of the vegetation of the Meda Gran island. Among them, in 2012, a wild albino ivory-white olive tree with fruit was identified. Fruits were collected from this tree and their seeds were first sown in a greenhouse and then planted in an orchard for purposes of ex situ preservation. Seven out of the 78 seedling trees obtained (12%) produced ivory-white fruits. In autumn 2018, fruits from these trees were sampled. Although the fruits had low oil content, virgin olive oil with unique sensory, physicochemical, and stability characteristics was produced. With respect to the polyphenols content, oleacein was the main compound identified (373.29 ± 72.02 mg/kg) and the oleocanthal was the second most abundant phenolic compound (204.84 ± 52.58 mg/kg). Regarding pigments, samples were characterized by an intense yellow color, with 12.5 ± 4.6 mg/kg of chlorophyll and 9.2 ± 3.3 mg/kg of carotenoids. Finally, oleic acid was the main fatty acid identified. This study explored the resources of the natural habitat of the MI as a means of enrichment of olive oil diversity and authenticity of this traditional Mediterranean food

Keywords: conservation; food; oleaster; plant diversity; polyphenol; fatty acid profile; sensorial analysis

1. Introduction

Despite increasing recognition of the importance of biodiversity of natural and human communities and the tremendous conservation strategies aimed at detaining the decline in wild habitats at regional

and national levels, biodiversity is rapidly diminishing worldwide [1,2]. The protection of natural areas is crucial to safeguard threatened species and represents the fundamental building blocks of most national and international conservation strategies [3]. Until 2018, 15 national parks, 152 natural parks, 291 nature reserves, 342 monuments natural, 57 protected landscapes, and more than 800 spaces have been declared as natural protected areas in Spain. In this sense, the Natura 2000 network protects 27% of the land area and about 13% of the waters in Spain [4]. The Medes Islands (MI) located in the northwest Mediterranean Sea were declared in 2010 a National Protected Natural Park for the great biodiversity of marine fauna and flora of the islands. The MI vegetation has been adapted to a series of abiotic (climate, wind, salinity, geology) and biotic factors. Wild olive-trees are included among the vegetation of the Meda Gran island. Nonetheless, the number of trees is very limited, since the plant life on the islands is influenced by the materials brought mainly by birds, especially seagulls [5].

The Mediterranean olive tree (*Olea europaea* L. subsp. *europaea*, Oleaceae) is one of the earliest fruit trees cultivated in the Mediterranean Basin. The natural distribution of the Mediterranean olive encompasses the var. *Sylvestris* (also named oleaster) and the cultivated tree var. *europaea* [6,7], some of which produce ivory-white fruit. Oleasters are usually located in isolated areas far from cultivated zones and are characterized by small fruits with a thin mesocarp [7,8]. For this reason, the biological resource monitoring provides vital information to guide conservation management and improve its effectiveness [9]. Actually, true wild forms are becoming increasingly scarce, and a few genuine wild populations have been localized in isolated areas from olive tree cultivation in Spain [10]. Wild olive-trees forests in the south of Spain can still be found, and scattered genuine wild forms in other parts such as Catalonia. [11,12]. The presence of a few wild olive trees which produce ivory-white fruits has been observed in prospecting surveys in Spain (Belaj A, unpublished results) but up to now no further attention has been paid to their study. In this sense, to the best of our knowledge, the only cultivated genotypes with ivory-white fruits are *Leucocarpa* in Italy [13,14], *Marf I* in Spain [15], and *Bajda* in the Maltese islands [16].

Olive oil contains bioactive components, such as monounsaturated fatty acids (55–83%), unsaponifiable compounds (1–2%), and soluble or hydrophilic compounds, including α -tocopherol, phenolic compounds, and other compounds with antioxidant properties [17]. However, most studies on wild olive genotypes deal with their diversity and only a few of them with their agronomic and oil quality evaluation [18,19]. In this sense, genotype is one of the main agronomic factors responsible for the chemical and organoleptic footprint of the olive oil strongly contributing in its qualitative and nutritional characteristics [20,21] and productivity as well [22]. On the other hand, virgin olive oil plays a crucial role in the eating habits of several dietary regimes, mainly of the Mediterranean diet [23]. The present study is part of an ongoing project related with the valorization of local cultivated and wild olive genetic resources in Catalonia (Northeastern Spain). In this sense, we aimed to explore the natural habitat of the MI for the development of native oleaster olive fruit as a resource to maintain olive oil biodiversity in the Mediterranean Basin. The quality of this oil was extensively analyzed using sensory, physicochemical, and stability parameters.

2. Materials and Methods

2.1. Chemicals

Oleocanthal and oleacein were purchased from PhytoLab GmbH (Vestenbergsgreuth, Germany); oleuropein aglycone from the Toronto Research Chemical Inc. (Ontario, Canada); luteolin and nonadecanoic acid from Sigma-Aldrich (Madrid, Spain); ferulic acid and apigenin from Fluka (Buchs, Switzerland) and hydroxytyrosol from Extrasynthese (Genay, France). Hexane, methanol, acetonitrile were purchased from Sigma-Aldrich, and cyclohexane from Panreac.

2.2. Plant Material

The study was undertaken using plant material obtained from targeted monitoring performed in 2012 with permission from the Catalan Government. The Institute of Agrifood Research and Technology (IRTA, Constantí, Spain) and the Andalusian Institute for Research and Training in Agriculture, Fisheries, Food and Ecological Production (IFAPA, Cordoba, Spain) surveyed the wild olive population in the MI. Two oleasters with ivory-white fruit were localized. In order to maintain *ex situ* and monitor the natural process under controlled conditions seeds from one ivory olive tree were collected and sown in a greenhouse at the IRTA facilities. In the spring of 2014, the surviving plantlets were transplanted in the field. Cultural practices at the orchard are the usual in the producing area, and no irrigation is supplied. The orchard is sited at latitude 41.172° N and longitude 1.169° E with 100 m altitude.

2.3. Olive Oil Extraction

Seven out of 78 trees (12%) produced ivory-white fruits. In autumn 2018, healthy olive fruits (3–5 kg) from these trees were picked by hand and carefully stored in ventilated boxes and processed within 8 h. The physical characteristics of the fruit were measured including the ripening index (adapted to the whitish characteristic of these fruits), fruit and pit weight, moisture and oil content [24]. Shortly, fruits were classified depending on their skin and flesh color into eight categories (0—green, 1—yellow, 2—whitish areas covering less than half of the surface, 3—white color more than half of the surface, 4—white color total surface with green flesh). Average fruit and pit weight were measured using a weighing scale and flesh to pit ratio was scored. One hundred fruits were grounded in order to measure moisture content (in oven at 105 °C till constant weight) and oil content was measured by nuclear magnetic resonance (Maran-S60; Oxford Instruments, Abingdon, UK).

The washed fruits were pressed using an Abencor oil extraction system MC2 (Ingeniería y Sistemas, Sevilla, Spain), which reproduces the industrial process at a laboratory scale. The olive paste was fed into a thermomixer at 28 °C for 30 min and subsequently centrifuged at 3000 rpm (7402203.301 N) for 3 min without the addition of either warm water or talc as coadjuvant. The oil was separated by decanting, transferred into glass bottles, and then stored in the dark at −20 °C until analysis.

2.4. Olive Oil Characterization

2.4.1. Fatty Acid Composition

Fatty acids were transmethyated with boron trifluoride (BF₃) and methanolic potassium hydroxide [25]. Then, fatty acids were determined by gas chromatography (Agilent 6890N, Boston, USA), fitted with a flame ionization detector (FID) and a capillary column (0.25 mm × 0.25 µm × 30 m; DB23, Agilent, Bellefonte, USA). Both the detector temperatures and injector were kept at 250 °C; and the oven temperature at 170 °C. The carrier gas was helium with a flux of 55.8 mL/min, and a pressure of 10.99 psi at the column head. The injection volume was set 1 µL. The internal standard (nonadecanoic acid) was analyzed in the same experimental conditions.

2.4.2. Pigments and Color

The oil color was measured according to the CIELab system under 2 degrees and D65 illumination conditions, using a spectrophotometer (Konica-Minolta CM-3500d, Osaka, Japan). Palette colors were described using three parameters: lightness (L*, from black to white), and the chromatic components a* (from green to red), and b* (from blue to yellow). The other parameter used was chroma [$C^* = (2a^* + 2b^*)/2$].

Chlorophyll and carotenoid pigments were measured by using a spectrophotometer (Shimadzu UV-160A; Kyoto, Japan). The maximum absorption at 670 and 470 nm is related to the chlorophyll and carotenoid fractions, respectively. They were used for calculation of the total content of these pigments in olive oil according to the method described by Minguez-Mosquera et al. [26]. The values

of the coefficients of specific extinction applied were $E_0 = 613$ for pheophytin as a major component in the chlorophyll fraction and $E_0 = 2000$ for lutein as a major component in the carotenoid fraction. The pigment contents were calculated as follows:

$$\text{Chlorophyll (mg/kg)} = \frac{A_{670} \times \text{DF}}{613 \times W_s} \quad (1)$$

$$\text{Carotenoid (mg/kg)} = \frac{A_{470} \times \text{DF}}{2000 \times W_s} \quad (2)$$

where A is the absorbance, DF is dilution factor, and W_s is the sample weight.

2.4.3. Phenolic Compounds

Then of liquid–liquid extraction, the quantification and identification of individual polyphenols were carried out by liquid chromatography coupled to mass spectrometry in tandem mode (LC-MS/MS) following procedures described previously [27]. For the liquid–liquid extraction, 0.5 g of olive oil was dissolved in hexane (oil/hexane 1:2, w/v) in a 10 mL centrifuge tube and shaken during 1 min. Later, 2 mL of MeOH:H₂O (8:2 v/v) was added to phenolic compound extraction and the samples were homogenized during 1 min. The two phases were separated by centrifugation at 3000 rpm, 4 °C during 4 min. The methanolic fraction was separated and was subjected to a second cleaning with hexane, while the remaining phenolic content in the hexane fraction was extracted adding one more time MeOH:H₂O (8:2 v/v). Before all, extracts were shaken and centrifuged in using the same conditions previously detailed. Both methanolic extracts were recovered and evaporated with N₂. Finally, the extracts were reconstituted with 800 µL of ACN and stored at −80 °C until chromatographic analysis.

An AcquityTM UPLC (Waters; Milford, MA, EUA) coupled to an API 3000 triple-quadrupole mass spectrometer (PE Sciex, Concord, Ontario, Canada) with a turbo ion spray source was used. The column was an Acquity UPLC[®] BEH C18 column (2.1 × 50 mm, i.d., 1.7 µm particle size) and Acquity UPLC[®] BEH C18 Pre-Column (2.1 × 5 mm, i.d., 1.7 µm particle size) (Waters Corporation[®], Wexford, Ireland). The separation of oleocanthal, oleacein, ligstroside, and oleuropein aglycone was performed MeOH (A) and H₂O (B), both with 0.1% of formic acid as mobile phases. An increasing linear gradient (v/v) of B was used (t (min), %B), as follows: (0, 100); (2, 100); (4.75, 46.4); (4.9, 100); (5.9, 0); (6, 100); (6.5, 100), at a constant flow rate of 0.6 mL/min, the injection volume was 5 µL, and the column temperature was 50 °C. The separation of other phenolic compounds was achieved using H₂O with 0.2% acetic acid (A) and ACN (B) as mobile phases. An increasing linear gradient (v/v) of B was used (t (min), %B), as follows: (0, 5); (2.5, 5); (12.5, 40); (12.6, 100); (13.5, 100); (13.6, 5); (15, 5), at a constant flow rate of 0.4 mL/min, the injection volume was 5 µL and the column temperature was 40 °C.

The ionization was achieved using electrospray interface operating in negative mode [M−H] and the compounds were monitored in the multiple monitoring mode (MRM) with the following settings: −3500 V to capillary voltage and arbitrary units for nebulizer gas and curtain gas (N₂) (10 and 12, respectively); drying gas (N₂) heated to 450 °C. The mass spectrometry potentials are presented in the supporting information (Table S1). The system was controlled by Analyst version 1.4.2 software supplied by Applied Biosystems. The calibration curves were prepared in refined olive oil and were linear over the concentration ranges 0–20 mg/mL using hydroxytyrosol, apigenin, luteolin, oleocanthal, oleacein, oleuropein aglycone as standards.

2.4.4. Oxidative Stability

Oxidative stability was evaluated by the Rancimat method [28]. Stability was expressed as the oxidation induction time (h), measured with the Rancimat 743 apparatus (Metrohm Co., Basel, Switzerland), using an oil sample of 3.0 g that was heated to 120 °C and an air flow of 20 L/h. The oxidation induction time is the time needed for an abrupt change in conductivity of an aqueous solution in which the volatile compounds resulting from oxidation of the oil were collected.

2.4.5. Sensorial Analysis

A sensorial analysis was performed by the Catalan Official Olive Oil Tasting Panel according to the UE2568/91 regulations [29]. Briefly, each oil sample was tasted by eight trained tasters in groups of four samples per session (randomly presented within each group). Positive descriptors analyzed were those of the official method (fruity, bitter, pungent), joined with other complementary ones (astringent, sweet, apple, ripe fruits, green, and other secondary undertones). The intensity of each attribute was measured using a continuous scale of 10 cm, anchored at zero and open at 10. The Catalan Panel relies under ISO-17025 certification rules and tasters were previously trained in the identification and intensity measurement of each attribute.

In all experiments, the means of five experimental runs are shown. Data are presented as mean and standard deviation.

3. Results and Discussion

3.1. Physical Characterization

The physicochemical characteristics of the ivory-white olives are shown in Table 1. With ripening, the color of the olive skin changed from green to light green-yellow and finally to white and ivory. The ripening index was 3.2 ± 0.4 . The olive fruits were small (0.78 ± 0.08 g), with flesh accounting for 73% (0.57 ± 0.06 g) and the remainder to the pit (0.21 ± 0.03 g). The average fat content expressed on dry basis (db) was $28.22 \pm 3.91\%$. Oleasters are usually characterized by small fruits with a thin mesocarp [7,8]. The results of our study are similar to that of a wild species of Tunisian trees which produce fruit of only 1 g in weight with 20.6% db of fat [19,30] and in other wild species in the Southern Spain [31]. Indeed, even the Marf I variety of ivory olives has larger fruits (2.2 g) with a higher fat content (40% db) [15]. Though fruit size and fat content can change along the ripening process [32], the very low values observed, at a relative high ripe index, makes unexpected to reach those values typical for traditional cultivated varieties. This means that possibly we are facing a real wild olive population.

Table 1. Physical and chemical characterization of olive oil from ivory-white olives.

Physical Characteristics		Chromatic Parameters		Pigments (mg/kg)	
Fruit weight (g)	0.78 ± 0.08	L*	88.6 ± 6.7	Chlorophylls	12.5 ± 4.6
Flesh weight (g)	0.57 ± 0.06	a*	-9.3 ± 1.4	Carotenoids	9.2 ± 3.3
Pit weight (g)	0.21 ± 0.03	b*	83.7 ± 23.3	Chlorophylls/carotenoids	1.4 ± 1.4
Fat content (%)	28.22 ± 3.91	Chroma	84.2 ± 23.3	Oxidative Stability (h at 120 °C)	
Ripening index	3.18 ± 0.42	a*/b*	-0.1 ± 0.06	Stability	15.8 ± 5.9

3.2. Fatty Acid Profile

In this work, the composition of the following nine fatty acid was determined: palmitic (C16:0), palmitoleic (C16:1), margaric (17:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), linolenic (C18:3), arachidic (C20:0), and eicosenoic (C20:1) acid (Table 2). The fatty acid composition of virgin oil is strongly affected by cultivar and other agronomical factors such as fruit ripeness, crop yield, and growing medium [33]. The fatty acid profile of the wild ivory olive population from the MI showed the typical traits of oleaster, being very rich in palmitic and palmitoleic acids and poor in oleic acid compared to oil from cultivated varieties [18,34]. Moreover, the fatty acid profile was similar to that of most common virgin olive oils; although with lower polyunsaturated fatty acid concentrations (14.9%). In our samples, the monounsaturated fatty acids comprise the largest group in the olive oil (62.5%). The high content of MUFA, particularly the oleic acid, and phenolic compounds can protect the oil to oxidation [35]. Similar ivory-white cultivars (Leucocarpa and Bajda) had been previously studied without reporting the fatty acid profile [13,14,16].

In general, the SFA content in our samples (22.6%) was found in higher concentrations than in Italian olive cultivars (13.1–17.6%) [36] and in Arbequina produced in different regions of Brazil and

Spain [37]. Previous studies have demonstrated that the cultivar is an important factor that affects the composition of fatty acid in olive oils [38].

The PUFA are the substrates of enzymes that generate volatile compounds responsible for oil aroma [39] but have also been found to contribute to the rancidification of oils [40]. The MUFA / PUFA ratio holds great importance because of its effects on the nutritional properties, oxidative stability, and organoleptic characteristics of olive oils [41,42]. In the olive oil analyzed, the MUFA / PUFA ratio could indicate a moderate susceptibility to oxidation attributed to that a high ratio favors the resistance to oxidative deterioration [42]. Indeed, it has been demonstrated that oils with a high content of MUFA and low PUFA and SFA values have the best oxidative stability [37]. Moreover, the C18:1 / C18:2 ratio (oleic acid/linoleic acid ratio) can be useful to characterize olive cultivars and to have a marked relationship with stability [43].

Table 2. Physical and chemical characterization of ivory-white olives.

Fatty Acid Composition (%)			
Palmitic	20.11 ± 2.46	Fatty acid indices	
Palmitoleic	3.51 ± 0.96	MUFA (%)	62.51 ± 6.46
Margaric	0.39 ± 0.37	PUFA (%)	14.90 ± 4.20
Stearic	1.79 ± 0.07	MUFA / PUFA	4.20 ± 2.60
Oleic	58.80 ± 7.05	C18:1 / C18:2	4.36 ± 3.16
Linoleic	13.48 ± 4.09	SFA (%)	22.60 ± 2.48
Linolenic	1.28 ± 0.18	UFA / SFA	3.43 ± 0.53
Arachidic	0.30 ± 0.02		
Eicosenoic	0.20 ± 0.04		
Eicosadienoic	0.14 ± 0.13		

SFA saturated fatty acids, PUFA polyunsaturated fatty acids, MUFA monounsaturated fatty acid, UFA unsaturated fatty acids.

3.3. Pigments and Color

Table 1 shows the pigments and chromatic parameters of the examined oils. The color characteristics of the virgin olive oil obtained from the ivory cultivar showed an intense yellow color (+b* values), with a poor green color (−a* values), bright and vivid (+L*, and chroma, respectively). The color of the oil was yellowish due to a balanced ratio between chlorophyll and carotenoid content (close to 1) [44]. Similar results were previously reported for the oil from ivory-white *Leucocarpa* cultivar [14].

The chlorophylls and carotenoids are very common pigments and their presence in olive oil also depends on olive variety, the stage of fruits ripeness, environmental conditions, the extraction process, and storage conditions [45,46]. Pigment determination showed chlorophyll and carotenoid concentrations of 12.5 ± 4.6 and 9.2 ± 3.3 mg/kg, respectively. Different concentrations of pigments have been reported in virgin olive oil from *Leucocarpa* cultivar. In this sense, higher concentrations of the total chlorophylls and carotenoids were reported (23–104 and 14–30 mg/kg, respectively) [13] and at the same time lowest concentrations were also detected (2.03–5.17 and 2.77–2.79 mg/kg, respectively) [14]. Likewise, lower concentrations of these compounds were reported previously in non-ivory cultivars from Greek islands, with values between 0.08–4.45 mg/kg and 0.87–2.28 mg/kg for chlorophylls and carotenoids, respectively [46]. Similar results have been reported in olive cultivars planted in Tunisia (Arbequina, Koroneiki, Leccino, Oueslati, and Chemchali) in the chlorophylls content (7.51–15.39 mg/kg) and less concentration in carotenoids (3.02–6.32 mg/kg) [47].

3.4. Phenolic Compounds

The minor polar fraction in virgin oil include different subclasses, among these: secoiridoids, phenolic alcohols, phenolic acids, flavonoids, and lignans [48] which act as antioxidant and may contribute to the prevention of several human diseases [49]. Table 3 shows the total phenol and

individual phenol concentrations in olive oil obtained by liquid chromatography coupled to masses. To date, several studies have shown that the phenolic composition of virgin olive oil depends on a very complex multivariate interaction between agronomic, biochemical, and technological factors. Initially, the genetic origin of the olive, the geographical area of cultivation, the climate, and agronomic practices such as fertilization and water availability are determining factors in the composition of the olive [27,50]. Given the biological importance of these compounds, one of the purposes of this research was the phenolic profile characterization, for the first time, in ivory-white wild olive trees of Spain. As shown in Table 3, the total polyphenols were 669.97 ± 119.38 mg/kg and was higher than that obtained in oil from other wild olives (Algerian variety 242 ± 19 to 341 ± 58 mg/kg) [51].

Table 3. Phenolic compound profile of olive oil from ivory-white olives.

Phenolic Compounds (mg/kg)	
Total phenols	669.97 ± 119.38
Oleacein	373.29 ± 72.02
Oleocanthal	204.84 ± 52.58
Oleuropein aglycone	33.82 ± 12.61
Ligstroside aglycone	14.15 ± 3.34
Elenolic acid	17.18 ± 9.47
Hydroxytyrosol	0.77 ± 0.57
Hydroxyelenolic acid	0.27 ± 0.19
Lactone	4.94 ± 1.77
HDCM-OA	0.37 ± 0.18
Luteolin	18.76 ± 10.77
Apigenin	1.56 ± 0.88

HDCM-OA hydroxydecarboxymethyl-oleuropein aglycone.

Secoiridoids were by far the most abundant group of phenolic compounds (93%) in agreement with previous studies [52]. Oleacein (373.29 ± 72.02 mg/kg) was the main compound followed by oleocanthal (204.84 ± 52.58 mg/kg). Both compounds are responsible for the bitter and pungent test, respectively, of olive oil [53]. Such very high secoiridoids contents are really interesting from the health point of view, due to its anti-inflammatory activity that can be relevant in many pathologies [53,54]. To date, there is little information on the phenolic profile of virgin olive of ivory-white olives. In a previous study carried out with Bajda cultivar, oleacein, oleocanthal, oleuropein aglycone, and ligstroside aglycone were only identified but not quantified [16]. Leucocarpa cultivar also was analyzed and low concentrations of oleuropein aglycone were detected in the fruit (6.63 ± 0.22 to 16.8 ± 0.07 mg/g of fresh weight) whereas other compounds were not analyzed [13]. Concerning non-ivory-white cultivars, different concentrations of OLE have been reported previously in monovarietal commercial samples from Greece, reaching a maximum of 291.7 ± 10.2 mg/kg [55]. Moreover, Pendolino and Kalamon cultivars have been reported as very rich in OLE as well, with a maximum of 903 ± 394 and 866 ± 285 mg/kg, respectively [56]. Regarding OLC, high concentrations were found in Alfafara, Kalamon, and Plementa Bjelica (1602 ± 688 to 2931 ± 26 mg/kg) [56]. Similar results to our white progenies were obtained in Koroneiki cultivar (183.9 ± 5.0 to 355.0 ± 12.1 mg/kg) [55].

The hydroxytyrosol, an essential quality biomarker in virgin olive oil, was also identified in olive samples (0.77 ± 0.57 mg/kg). This phenolic alcohol was also identified in olive oil from ivory-whites [16]. The variations in the phenolic alcohols is also attributed to ripening and varietal factors [57]. Olive oil is in between the European Food Safety Authority (EFSA) parameters for claiming that polyphenols from olive oil and olive leaves protect low-density lipoproteins from oxidative damage when consumed at a daily rate of 5 mg of hydroxytyrosol and its derivatives (e.g., oleuropein complex and tyrosol) [58].

Two main flavonoids were quantified for the first time in virgin oil from wild ivory-white olive trees: luteolin (18.76 ± 10.77 mg/kg) and apigenin (1.56 ± 0.88 mg/kg). These flavonoids have been quantified in an Algerian wild olive (not ivory) but with a lower luteolin (0.97 ± 0.17 to 13 ± 3 mg/kg) and apigenin content (0.07 ± 0.01 to 1.1 ± 0.1 mg/kg) [51]. These two compounds were also identified

in Bajda cultivar [16]. The Leucocarpa cultivar also produces ivory-white fruit due to low or null accumulation of flavonoid compounds [13] which is suggestive of purity and has led to the use of these olives to produce holy anointing oil [14] and with potential use as table olives [59]. The Marf I cultivar presents tolerance to olive leaf spot disease and also produces ivory-colored fruits with high oil quality [15]. In fact, the particular color of the ripe fruit of these cultivars is due to a blockage of flavonoid biosynthesis [60], mainly anthocyanins [13]. In previous studies, the content of luteolin found in Picual and Hojiblanca oils (41.3 and 22.69 ± 5.09 mg/kg, respectively) was higher to those found in the present assay. In the same studies, higher concentrations of apigenin were reported reaching concentrations to the 8.72 and 5.51 ± 0.69 mg/kg, respectively [27,61]. Differences in the concentration of both compounds in non-ivory cultivars growing in an identical climate/environment area in Italy also was reported with concentrations between 2.2 ± 0.5 – 16.7 ± 0.6 mg/kg for the luteolin and 0.2 ± 0.1 – 9.2 ± 0.3 mg/kg for the apigenin [62]. The ripening stage is another factor that affects the concentrations of these compounds, increasing the content of luteolin with the maturity index whereas apigenin does not show any definite trend. Moreover, during oil extraction the flavonoids are hardly affected by time and temperature of malaxation [61].

Other compounds were also identified in concentrations lower than 1 mg/kg (hydroxyelenolic acid and hydroxydecarboxymethyl-oleuropein aglycone, HDCM-OA).

3.5. Oxidative Stability

Table 1 also shows the oxidative stability measured by the Rancimat method. Oxidative stability is an important parameter for evaluating the quality of oils and fats, providing a good estimation of susceptibility to oxidative degeneration, the main cause of alterations in olive oil [63]. The oxidative stability for the ivory-white oil genotype measured by the Rancimat method was 15.8 h, which is quite a high value and is higher than that of the Marf I olive oils (12.59 h) [15,64]. High oxidative stability may be due to the equilibrium among the fatty acid profile, and the carotenoid and phenolic compounds (Tables 2 and 3). The chlorophyll/carotenoid ratio is low. In the presence of light, chlorophylls and their derivatives are the most active promoters of photosensitized oxidation in virgin olive oil contributing greatly to a high susceptibility to oxidation [65]. Carotenoids possess conjugated hydrocarbons which are potent protectors against photosensitized oxidation, acting as singlet oxygen quencher [66]. Phenolic compounds have also been correlated with oil shelf life due to the antioxidant properties of mainly oleacein and oleuropein aglycone, which show remarkable resistance to oxidation [67]. Finally, the low content of polyunsaturated fatty acids (14.9%), which are the fatty acids most prone to oxidation, also contributed to oxidative stability [68].

3.6. Sensorial Analysis

Sensorial analysis resulted in medium to high intensity fruitiness (5.0 ± 0.3), clearly pungent (5.5 ± 0.3) and bitter (5.0 ± 0.7). Secondary attributes were also detected such as medium intensity sweetness, cut grass aroma, and slight floral almond and astringent mouthfeel. Neither ripe fruit nor apple attributes were detected. The sensory parameters of the oil of the ivory-white genotype revealed the complexity of olive oil. The taste has fruity, bitter, and pungent characteristics, the latter two sensations being generated in the same gustative papillae [69,70]. Two phenolic compounds, oleocanthal and oleacein, that are exclusively present in virgin olive oil, seem to be the main compounds responsible for these mouthfeel perceptions and were the major phenolic compounds found in the olive oil from the ivory-white cultivar. Volatile compounds are responsible for the aggregate aroma of virgin olive oil. The fruity notes are perceived directly and/or through the back of the nose and are the characteristic set of olfactory sensations of the oil from sound, fresh olives, either ripe or unripe [71]. Fruity notes are mainly given by volatile compounds produced by the lipoxygenase pathway during oil extraction. Green flavor, and sweet, grassy, and almond aromas [72] have been described in olives of ivory-white progeny (Figure 1).

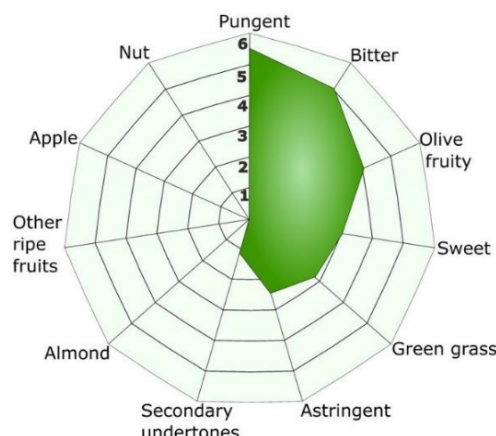


Figure 1. Sensory virgin olive oil characteristics.

4. Conclusions

Actions to promote the conservation of indigenous oleaster genotypes will prevent their decline at regional, national, and global levels. Native wild olive trees are becoming increasingly scarce, and thus, protected reserve zones are fundamental pillars for the preservation of these wild species which have adapted to a series of abiotic and biotic factors. At least, two wild olive-tree were localized in the natural reserve of the MI what produces ivory-white olive fruits. Physicochemical characterization of these fruits and olive oil revealed unique sensory physicochemical and stability characteristics, highlighting the need to maintain the biodiversity of olive oil and food authenticity of this traditional Mediterranean food. However, further research should be performed in this case on sensory characteristics to determine its possible applications as an edible oil for human nutrition. Nevertheless, additional studies during several years and involving more oleasters are crucial to corroborate the results obtained and to better characterize wild olive genetic resources in Catalonia.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2076-3921/9/10/1009/s1>, Table S1: Multiple reaction monitoring conditions for the polyphenols.

Author Contributions: Conceptualization, A.L.-Y., A.N., A.J.R.-A. and R.M.L.-R.; methodology, A.L.-Y., A.N., A.J.R.-A., J.L.-C. and A.V.-Q.; validation, A.L.-Y., A.N., and A.J.R.-A.; formal analysis, A.L.-Y., A.N., A.J.R.-A., and J.L.-C.; investigation, A.L.-Y., A.N., A.J.R.-A., A.B., and J.L.-C.; resources, A.N., A.J.R.-A., and A.B.; writing—original draft preparation, A.L.-Y., and A.N.; writing—review and editing, A.L.-Y., A.N., A.J.R.-A., J.L.-C., E.E.-F., and A.V.-Q.; visualization, A.L.-Y., A.N., A.J.R.-A., J.L.-C., E.E.-F., and A.V.-Q.; supervision, A.N., A.J.R.-A., E.E.-F. and R.M.L.-R.; project administration, R.M.L.-R.; funding acquisition, R.M.L.-R. All authors have read and agreed to the published version of the manuscript.”

Funding: This research was funded by CICYT [AGL2016-75329-R]. A.L.-Y. wishes to thank the Consejo Nacional de Ciencia y Tecnología (CONACYT) of Mexico for the doctoral scholarship. J.L.-C. thanks the Ministry of Science Innovation and Universities for the FPI contract (BES-2017-080017). A.V.-Q. thanks the Ministry of Science Innovation and Universities for the Ramon y Cajal contract (RYC-2016-19355). A.R.-A. and A.N. acknowledge financial support from the CERCA Program of the Generalitat of Catalonia.

Acknowledgments: The authors wish to thank the CCiT-UB for use of the mass spectrometry equipment. A.N. and A.B. acknowledge the government of Catalonia for its support in the surveying of the Medes Islands.

Conflicts of Interest: R.M.L.-R. reports receiving lecture fees from Cerveceros de España and receiving lecture fees and travel support from Adventia. The other authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Williams, D.R.; Balmford, A.; Wilcove, D.S. The past and future role of conservation science in saving biodiversity. *Conserv. Lett.* **2020**, *1*–7. [CrossRef]

2. Kitzes, J.; Berlow, E.; Conlisk, E.; Erb, K.; Iha, K.; Martinez, N.; Newman, E.A.; Plutzar, C.; Smith, A.B.; Harte, J. Consumption-based conservation targeting: Linking biodiversity loss to upstream demand through a global wildlife footprint. *Conserv. Lett.* **2017**, *10*, 531–538. [CrossRef]
3. Dudley, N. Guidelines for Applying Protected Area Management Categories; IUCN: Gland, Switzerland, 2008.
4. De la Guerra, M.M.; Alandi, C.M.; Blázquez, J.P.; Mezquida, J.A.A.; García, J.G.-L.; Ventura, D.G. Anuario 2018 del Estado de Las Áreas Protegidas en España; EUROPARC-España: Madrid, Spain, 2018.
5. Ros, J. The Medes isles, paradise around the corner. *Catalonia* **1992**, *31*, 20–23.
6. Julca, I.; Marcet-Houben, M.; Cruz, F.; Gomez-Garrido, J.; Gaut, B.S.; Diez, C.M.; Gut, I.G.; Alioto, T.S.; Vargas, P.; Gabaldon, T. Genomic evidence for recurrent genetic admixture during domestication mediterranean olive trees (*Olea europaea*). *bioRxiv* **2020**. [CrossRef]
7. Green, P.S. A revision of *Olea* L. (Oleaceae). *KEW Bull.* **2002**, *57*, 91–140. [CrossRef]
8. Lumaret, R.; Ouazzani, N. Ancient wild olives in Mediterranean forests. *Nature* **2001**, *413*, 700. [CrossRef]
9. Nichols, J.D.; Williams, B.K. Monitoring for conservation. *Trends Ecol. Evol.* **2006**, *21*, 668–673. [CrossRef]
10. Belaj, A.; Muñoz-Diez, C.; Baldoni, L.; Satovic, Z.; Barranco, D. Genetic diversity and relationships of wild and cultivated olives at regional level in Spain. *Sci. Hortic.* **2010**, *124*, 323–330. [CrossRef]
11. Belaj, A.; Muñoz-Diez, C.; Baldoni, L.; Porceddu, A.; Barranco, D.; Satovic, Z. Genetic diversity and population structure of wild olives from the north-western Mediterranean assessed by SSR markers. *Ann. Bot.* **2007**, *100*, 449–458. [CrossRef]
12. Besnard, G.; Hernández, P.; Khadari, B.; Dorado, G.; Savolainen, V. Genomic profiling of plastid DNA variation in the Mediterranean olive tree. *BMC Plant Biol.* **2011**, *11*, 80. [CrossRef]
13. Cirilli, M.; Bellincontro, A.; Urbani, S.; Servili, M.; Esposto, S.; Mencarelli, F.; Muleo, R. On-field monitoring of fruit ripening evolution and quality parameters in olive mutants using a portable NIR-AOTF device. *Food Chem.* **2016**, *199*, 96–104. [CrossRef] [PubMed]
14. Pasqualone, A.; Di Rienzo, V.; Blanco, A.; Summo, C.; Caponio, F.; Montemurro, C. Characterization of virgin olive oil from *Leuocarpa* cultivar by chemical and DNA analysis. *Food Res. Int.* **2012**, *47*, 188–193. [CrossRef]
15. Tous, J.; Romero, A. Marf l' olive. *HortScience* **1998**, *33*, 162–163. [CrossRef]
16. Lia, F.; Zammit-Mangion, M.; Farrugia, C. A first description of the phenolic profile of EVOOs from the Maltese islands using SPE and HPLC: Peco-climatic conditions modulate genetic factors. *Agriculture* **2019**, *9*, 107. [CrossRef]
17. Yubero-Serrano, E.M.; Lopez-Moreno, J.; Gomez-Delgado, F.; Lopez-Miranda, J. Extra virgin olive oil: More than a healthy fat. *Eur. J. Clin. Nutr.* **2019**, *72*, 8–17. [CrossRef]
18. León, L.; De La Rosa, R.; Velasco, L.; Belaj, A. Using wild olives in breeding programs: Implications on oil quality composition. *Front. Plant Sci.* **2018**, *9*, 232. [CrossRef]
19. Hannachi, H.; Breton, C.; Msallem, M.; El Hadj, S.B.; El Gazzah, M.; Berville, A. Differences between native and introduced olive cultivars as revealed by morphology of drupes, oil composition and SSR polymorphisms: A case study in Tunisia. *Sci. Hortic.* **2008**, *116*, 280–290. [CrossRef]
20. Di Vaio, C.; Nocerino, S.; Paduano, A.; Sacchi, R. Influence of some environmental factors on drupe maturation and olive oil composition. *J. Sci. Food Agric.* **2013**, *93*, 1134–1139. [CrossRef] [PubMed]
21. Rotondi, A.; Alfei, B.; Magli, M.; Pannelli, G. Influence of genetic matrix and crop year on chemical and sensory profiles of Italian monovarietal extra-virgin olive oils. *J. Sci. Food Agric.* **2010**, *90*, 2641–2648. [CrossRef]
22. Salazar-García, D.C.; Malheiro, R.; Pereira, J.A.; Lopéz-Cortés, I. Unexplored olive cultivars from the Valencian Community (Spain): Some chemical characteristics as a valorization strategy. *Eur. Food Res. Technol.* **2019**, *245*, 325–334. [CrossRef]
23. Fratianni, F.; Cozzolino, R.; Martignetti, A.; Malorni, L.; d'Acierno, A.; De Feo, V.; da Cruz, A.G.; Nazzaro, F. Biochemical composition and antioxidant activity of three extra virgin olive oils from the Irpinia Province, Southern Italy. *Food Sci. Nutr.* **2019**, *7*, 3233–3243. [CrossRef]
24. Uceda, M.; Frías, L. Seasons of harvest. Changes on fruit oil content, oil composition and oil quality. In Proceedings of the II Seminario Oleícola Internacional. IOOC, Cordoba, Spain, 1975; pp. 25–46.
25. Morrison, W.R.; Smith, L.M. Preparation of fatty acid methyl esters and dimethylacetals from lipids. *J. Lipid Res.* **1964**, *5*, 600–608.
26. Minguez-Mosquera, I.M.; Rejano-Navarro, L.; Gandul-Rojas, B.; SanchezGomez, A.H.; Garrido-Fernandez, J. Color-pigment correlation in virgin olive oil. *J. Am. Oil Chem. Soc.* **1991**, *68*, 332–336. [CrossRef]

27. Lopez-Yerena, A.; Lozano-Castellon, J.; Olmo-Cunillera, A.; Tresserra-Rimbau, A.; Quifer-Rada, P.; Jiménez, B.; Perez, M.; Vallverdu-Queralt, A. Effects of organic and conventional growing systems on the phenolic profile of extra-virgin olive oil. *Molecules*. **2019**, *24*, 1986. [CrossRef]
28. Gutiérrez Rosales, F. Determinación de la estabilidad oxidativa de aceites de oliva vírgenes: Comparación entre el método del oxígeno activo (AOM) y el método rancimat. *Grasas y Aceites*. **1989**, *40*, 1–5.
29. Commission Regulation (EEC). On the Characteristics of Olive Oil and Olive-Residue Oil and on the Relevant Methods of Analysis; European Commission: Brussels, Belgium, 1991.
30. Hannachi, H.; Nasri, N.; Elfalleh, W.; Tlili, N.; Ferchichi, A.; Msallem, M. Fatty acids, sterols, polyphenols, and chlorophylls of olive oils obtained from Tunisian wild olive trees (*Olea europaea* L. Var *Sylvestris*). *Int. J. Food Prop.* **2013**, *16*, 1271–1283. [CrossRef]
31. Belaj, A.; Leon, L.; Satovic, Z.; de la Rosa, R. Variability of wild olives (*Olea europaea* Subsp. *europaea* Var. *Sylvestris*) analyzed by agro-morphological traits and SSR markers. *Sci. Hortic.* **2011**, *129*, 561–569. [CrossRef]
32. Conte, P.; Squeo, G.; Difonzo, G.; Caponio, F.; Fadda, C.; Del Caro, A.; Urgeghe, P.P.; Montanari, L.; Montinaro, A.; Piga, A. Change in quality during ripening of olive fruits and related oils extracted from three minor autochthonous Sardinian cultivars. *Emir. J. Food Agric.* **2019**, *31*, 196–205. [CrossRef]
33. Beltran, G.; del Rio, C.; Sanchez, S.; Martinez, L. Influence of harvest date and crop yield on the fatty acid composition of virgin olive oils from Cv. Picual. *J. Agric. Food Chem.* **2004**, *52*, 3434–3440. [CrossRef] [PubMed]
34. Baccouri, B.; Guerfel, M.; Zarrouk, W.; Taamalli, W.; Daoud, D.; Zarrouk, M. Wild olive (*Olea Europaea* L.) selection for quality oil production. *J. Food Biochem.* **2011**, *35*, 161–176. [CrossRef]
35. Ozcan, M.M.; Al Juhaimi, F.; Uslu, N.; Ghafoor, K.; Mohamed Ahmed, I.A.; Babiker, E.E. The effect of olive varieties on fatty acid composition and tocopherol contents of cold pressed virgin olive oils. *J. Oleo Sci.* **2019**, *68*, 307–310. [CrossRef]
36. Aguilera, M.P.; Beltran, G.; Ortega, D.; Fernandez, A.; Jimenez, A.; Uceda, M. Characterisation of virgin olive oil of Italian olive cultivars: “Frantoio” and “Leccino”, grown in Andalusia. *Food Chem.* **2005**, *89*, 387–391. [CrossRef]
37. Borges, T.H.; Pereira, J.A.; Cabrera-Vique, C.; Lara, L.; Oliveira, A.F.; Seiquer, I. Characterization of Arbequina virgin olive oils produced in different regions of Brazil and Spain: Physicochemical properties, oxidative stability and fatty acid profile. *Food Chem.* **2017**, *215*, 454–462. [CrossRef]
38. Yu, L.; Wang, Y.; Wu, G.; Jin, J.; Jin, Q.; Wang, X. Quality and composition of virgin olive oils from indigenous and European cultivars grown in China. *JAOCS J. Am. Oil Chem. Soc.* **2019**, *97*, 341–353. [CrossRef]
39. Salas, J.J.; Sanchez, J.; Ramli, U.S.; Manaf, A.M.; Williams, M.; Harwood, J.L. Biochemistry of lipid metabolism in olive and other oil fruits. *Prog. Lipid Res.* **2000**, *39*, 151–180. [CrossRef]
40. Al-Bachir, M.; Sahloul, H. Fatty acid profile of olive oil extracted from irradiated and non-irradiated olive fruits. *Int. J. Food Prop.* **2017**, *20*, 2550–2558. [CrossRef]
41. Dag, A.; Kerem, Z.; Yogev, N.; Zipori, I.; Lavee, S.; Ben-David, E. Influence of time of harvest and maturity index on olive oil yield and quality. *Sci. Hortic.* **2011**, *127*, 358–366. [CrossRef]
42. Reboredo-Rodriguez, P.; Gonzalez-Barreiro, C.; Cancho-Grande, B.; Fregapane, G.; Salvador, M.D.; Simal-Gandara, J. Characterisation of extra virgin olive oils from Galician autochthonous varieties and their co-crushings with Arbequina and Picual cv. *Food Chem.* **2015**, *176*, 493–503. [CrossRef]
43. Allalout, A.; Krichene, D.; Methenni, K.; Taamalli, A.; Oueslati, I.; Daoud, D.; Zarrouk, M. Characterization of virgin olive oil from super intensive spanish and greek varieties grown in northern Tunisia. *Sci. Hortic.* **2009**, *120*, 77–83. [CrossRef]
44. Criado, M.N.; Romero, M.P.; Casanovas, M.; Motilva, M.J. Pigment profile and colour of monovarietal virgin olive oils from Arbequina cultivar obtained during two consecutive crop seasons. *Food Chem.* **2008**, *110*, 873–880. [CrossRef]
45. Giuffrida, D.; Salvo, F.; Salvo, A.; La Pera, L.; Dugo, G. Pigments composition in monovarietal virgin olive oils from various sicilian olive varieties. *Food Chem.* **2007**, *101*, 833–837. [CrossRef]
46. Karabagias, I.K.; Badeka, A.; Casiello, G.; Longobardi, F.; Kontominas, M.G. Rapid screening of olive oil cultivar differentiation based on selected physicochemical parameters, pigment content and fatty acid composition using advanced chemometrics. *Eur. Food Res. Technol.* **2019**, *245*, 2027–2038. [CrossRef]

47. Dabbou, S.; Brahmi, F.; Taamali, A.; Issaoui, M.; Ouni, Y.; Braham, M.; Zarrouk, M.; Hammami, M. Extra virgin olive oil components and oxidative stability from olives grown in Tunisia. *JAOCS J. Am. Oil Chem. Soc.* **2010**, *87*, 1199–1209. [CrossRef]
48. Romani, A.; Ieri, F.; Urciuoli, S.; Noce, A.; Marrone, G.; Nediani, C.; Bernini, R. Health effects of phenolic compounds found in extra-virgin olive oil, by-products, and leaf of *Olea europaea* L. *Nutrients* **2019**, *11*, 1776. [CrossRef]
49. Juliano, P.; Bainczyk, F.; Swiergon, P.; Supriyatna, M.I.M.; Guillaume, C.; Ravetti, L.; Canamasas, P.; Cravotto, G.; Xu, X.Q. Extraction of olive oil assisted by high-frequency ultrasound standing waves. *Ultrason. Sonochemistry* **2017**, *38*, 104–114. [CrossRef]
50. Taticchi, A.; Selvagini, R.; Esposto, S.; Sordini, B.; Veneziani, G.; Servili, M. Physicochemical characterization of virgin olive oil obtained using an ultrasound-assisted extraction at an industrial scale: Influence of olive maturity index and malaxation time. *Food Chem.* **2019**, *289*, 7–15. [CrossRef]
51. Bouarroudj, K.; Tamendjari, A.; Larbat, R. Quality, composition and antioxidant activity of Algerian wild olive (*Olea Europaea* L. Subsp. *Oleaster*) oil. *Ind. Crop. Prod.* **2016**, *83*, 484–491. [CrossRef]
52. Celano, R.; Piccinelli, A.L.; Pugliese, A.; Carabetta, S.; Di Sanzo, R.; Rastrelli, L.; Russo, M. Insights into the analysis of phenolic secoiridoids in extra virgin olive oil. *J. Agric. Food Chem.* **2018**, *66*, 6053–6063. [CrossRef]
53. Lozano-Castellon, J.; Lopez-Yerena, A.; Rinaldi de Alvarenga, J.F.; Romero del Castillo-Alba, J.; Vallverdu-Queralt, A.; Escribano-Ferrer, E.; Lamuela-Raventos, R.M. Health-promoting properties of oleocanthal and oleacein: Two secoiridoids from extra-virgin olive oil. *Crit. Rev. Food Sci. Nutr.* **2019**, *60*, 2532–2548. [CrossRef]
54. Francisco, V.; Ruiz-Fernandez, C.; Lahera, V.; Lago, F.; Pino, J.; Skaltsounis, L.; Gonzalez-Gay, M.A.; Mobasheri, A.; Gómez, R.; Scotece, M.; et al. Natural molecules for healthy lifestyles: Oleocanthal from extra virgin olive oil. *J. Agric. Food Chem.* **2019**, *67*, 3845–3853. [CrossRef]
55. Karkoula, E.; Skantzari, A.; Melliou, E.; Magiatis, P. Direct measurement of oleocanthal and oleacein levels in olive oil by quantitative ¹H NMR. Establishment of a new index for the characterization of extra virgin olive oils. *J. Agric. Food Chem.* **2012**, *60*, 11696–11703. [CrossRef]
56. Miho, H.; Diez, C.M.; Mena-Bravo, A.; Sanchez de Medina, V.; Moral, J.; Melliou, E.; Magiatis, P.; Rallo, L.; Barranco, D.; Priego-Capote, F. Cultivar influence on variability in olive oil phenolic profiles determined through an extensive germplasm survey. *Food Chem.* **2018**, *266*, 192–199. [CrossRef]
57. Deiana, P.; Santona, M.; Dettori, S.; Culeddu, N.; Dore, A.; Molinu, M.G. Multivariate approach to assess the chemical composition of Italian virgin olive oils as a function of variety and harvest period. *Food Chem.* **2019**, *300*, 125243. [CrossRef]
58. EFSA. Scientific opinion on the substantiation of a health claim related to polyphenols in olive and maintenance of normal blood HDL cholesterol concentrations (ID 1639, further assessment) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. *EFSA J.* **2012**, *10*, 2848. [CrossRef]
59. Lanza, B.; Di Marco, S.; Simone, N.; Di Marco, C.; Gabriele, F. Table olives fermented in iodized sea salt brines: Nutraceutical/sensory properties and microbial biodiversity. *Foods* **2020**, *9*, 301. [CrossRef]
60. Lanza, B.; Amoroso, F.; Di Serio, M.G. Nutritional composition and kinaesthetic properties of white table olives from *Olea europaea* L. *Leucocarpa* Cultivar. *Rivista Italiana delle Sostanze Grasse* **2016**, *93*, 157–164.
61. de Torres, A.; Espinola, F.; Moya, M.; Alcalá, S.; Vidal, A.M.; Castro, E. Assessment of phenolic compounds in virgin olive oil by response surface methodology with particular focus on flavonoids and lignans. *LWT - Food Sci. Technol.* **2018**, *90*, 22–30. [CrossRef]
62. Negro, C.; Aprile, A.; Luvisi, A.; Nicoli, F.; Nutricati, E.; Vergine, M.; Miceli, A.; Blando, F.; Sabella, E.; De Bellis, L. Phenolic profile and antioxidant activity of Italian Monovarietal extra virgin olive oils. *Antioxidants* **2019**, *8*, 161. [CrossRef]
63. Aparicio, R.; Roda, L.; Albi, M.A.; Gutierrez, F. Effect of various compounds on virgin olive oil stability measured by Rancimat. *J. Agric. Food Chem.* **1999**, *47*, 4150–4155. [CrossRef]
64. Tous, J.; Romero, A.; Francisco, J.; Ninot, A. Mediterranean clonal selections evaluated for modern hedgerow olive oil production in Spain. *Calif. Agric.* **2011**, *65*, 34–40. [CrossRef]
65. Velasco, J.; Dobarganes, C. Oxidative stability of virgin olive oil. *Eur. J. Lipid Sci. Technol.* **2002**, *104*, 661–676. [CrossRef]
66. Bradley, D.G.; Min, D.B. Singlet oxygen oxidation of foods. *Crit. Rev. Food Sci. Nutr.* **1992**, *31*, 211–236. [CrossRef]

67. Baldioli, M.; Servili, M.; Perretti, G.; Montedoro, G.F. Antioxidant activity of tocopherols and phenolic compounds of virgin olive oil. *J. Am. Oil Chem. Soc.* **1996**, *73*, 1589–1593. [CrossRef]
68. Franco, M.N.; Galeano-Diaz, T.; Sanchez, J.; De Miguel, C.; Martin-Vertedor, D. Antioxidant capacity of the phenolic fraction and its effect on the oxidative stability of olive oil varieties grown in the southwest of Spain. *J. Oleo Sci.* **2014**, *65*, 004.
69. Bate-Smith, E.C. Haemanalysis of tannins: The concept of relative astringency. *Phytochemistry* **1973**, *12*, 907–912. [CrossRef]
70. Whitehead, M.C.; Beeman, C.S.; Kinsella, B.A. Distribution of taste and general sensory nerve endings in fungiform papillae of the hamster. *Am. J. Anat.* **1985**, *173*, 185–201. [CrossRef]
71. Reboredo-Rodriguez, P.; Gonzalez-Barreiro, C.; Cancho-Grande, B.; Simal-Gaandara, J. Concentrations of aroma compounds and odor activity values of odorant series in different olive cultivars and their oils. *J. Agric. Food Chem.* **2013**, *61*, 5252–5259. [CrossRef]
72. Morales, M.T.; Rios, J.J.; Aparicio, R. Changes in the volatile composition of virgin olive oil during oxidation: Flavors and off-flavors. *J. Agric. Food Chem.* **1997**, *45*, 2666–2673. [CrossRef]

Publisher’s Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).

4.1.2. Article 4: Effects of organic and conventional growing systems on the phenolic profile of extra-virgin olive oil

Anallely López-Yerena, Julián Lozano-Castellón, Alexandra Olmo-Cunillera, Anna Tresserra-Rimbau, Paola Quifer-Rada, Brígida Jiménez, Maria Pérez and Anna Vallverdú-Queralt

Molecules, 24(10), 1986, 2019

Abstract

Background: EVOO is largely appreciated for its proven nutritional properties. The qualitative and quantitative composition of PCs in EVOO is affected by many variables, such as the degree of olive ripeness, the technological production process and storage conditions. Previous studies demonstrate that the organic fruits have higher phenolic content than conventional ones and that the organic foods are perceived as healthier by consumers.

Scope: The objective of our study was to compare the content of PCs (secoiridoids, flavones, phenolic alcohols, phenolic acids and lignans) in 'Hojiblanca' EVOO produced by organic and conventional production systems under the same environmental conditions. Moreover, we applied a quantitative ^1H nuclear magnetic resonance (qNMR) method to corroborate the concentration of OLC in our EVOOs obtained by LC-MS.

Methods and results: The quantification and identification of individual PCs was carried out by liquid chromatography coupled to mass spectrometry in tandem mode (LC-MS/MS). Significantly higher levels ($p < 0.05$) of PCs were found in organic EVOOs. The major PCs in EVOO were SEC, whereas lignans, phenolic acids and flavones were present in low concentrations. The SEC represented 91–92% of the PCs, with higher levels in the organic than conventional EVOO (420.72 ± 59.42 and 306.48 ± 48.09 mg/kg, respectively). The methodology used was able to detect previously unreported differences in bioactive components between organic and conventional EVOOs.

Conclusion: The total polyphenols in EVOO made from 'Hojiblanca' olives were analyzed, comparing organic and conventional growing systems under the same environmental conditions, and levels were significantly higher in organic samples ($p < 0.05$). The concentration of SEC, which are synthesized through the secologanin pathway without the need for nitrogen or phosphorus, was higher in oils from olives cultivated under organic conditions. Among them, OLC- the less studied compound to date- was satisfactorily analyzed by LC-MS/MS, as demonstrated by results obtained by qNMR.

Article

Effects of Organic and Conventional Growing Systems on the Phenolic Profile of Extra-Virgin Olive Oil

Anallely López-Yerena ^{1,†}, Julián Lozano-Castellón ^{1,2,†}, Alexandra Olmo-Cunillera ¹, Anna Tresserra-Rimbau ^{2,3}, Paola Quifer-Rada ⁴ , Brígida Jiménez ⁵, Maria Pérez ^{1,6}  and Anna Vallverdú-Queralt ^{1,2,*} 

¹ Department of Nutrition, Food Science and Gastronomy XaRTA, Institute of Nutrition and Food Safety (INSA-UB), Faculty of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain; naye.yerena@gmail.com (A.L.-Y.); jullozcas@gmail.com (J.L.-C.); alexandra.oc.94@gmail.com (A.O.-C.); mariaperez@ub.edu (M.P.)

² CIBER Physiopathology of Obesity and Nutrition (CIBEROBN), Institute of Health Carlos III, 28029 Madrid, Spain; anna.tresserra@iispv.cat

³ Human Nutrition Unit, University Hospital of Sant Joan de Reus, Department of Biochemistry and Biotechnology, Faculty of Medicine and Health Sciences, Pere Virgili Health Research Center, Universitat Rovira i Virgili, 43002 Reus, Spain

⁴ Department of Endocrinology & Nutrition, CIBER of Diabetes and Associated Metabolic Diseases (CIBERDEM), Biomedical Research Institute Sant Pau, Hospital de la Santa Creu i Sant Pau, 08041 Barcelona, Spain; pquifer@santpau.cat

⁵ Instituto Andaluz de Investigación y Formación Agraria, Pesquera, Alimentaria y de la Producción Ecológica, Centro de Cabra, Antigua Ctra, Cabra-Doña Mencía, Km. 2.5, 14940 Córdoba, Spain; brigida.jimenez@juntadeandalucia.es

⁶ Laboratory of Organic Chemistry, Faculty of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain

* Correspondence: avallverdu@ub.edu; Tel.: +34-93-403-4843

† Equally Contributing Authors.

Academic Editor: Fátima Paiva-Martins

Received: 8 April 2019; Accepted: 18 May 2019; Published: 23 May 2019



Abstract: Extra-virgin olive oil (EVOO) is largely appreciated for its proven nutritional properties. Additionally, organic foods are perceived as healthier by consumers. In this context, the aim of the present study was to compare the phenolic profiles of EVOO from olives of the Hojiblanca variety, cultivated under organic and conventional systems. The quantification and identification of individual polyphenols was carried out by liquid chromatography coupled to mass spectrometry in tandem mode (LC-MS/MS). Significantly higher levels ($p < 0.05$) of phenolic compounds were found in organic EVOOs. The methodology used was able to detect previously unreported differences in bioactive components between organic and conventional EVOOs.

Keywords: phenolic compounds; Hojiblanca; variety; organic; conventional; agriculture; mass spectrometry; oleocanthal; secoiridoids; ripening; NMR

1. Introduction

Extra virgin olive oil (EVOO), a key component of the Mediterranean diet, is highly appreciated for its nutritional and organoleptic attributes. The minor compounds include aliphatic and triterpene alcohols, sterols, hydrocarbons, volatile compounds and antioxidants such as carotenoids and polyphenols, which contribute to the organoleptic characteristics, stability and nutritional value of EVOO [1,2]. The qualitative and quantitative composition of polyphenols in EVOO is affected by

many variables, such as the degree of olive ripeness, the technological production process and storage conditions [3,4]. The most important changes in the polyphenol content occur during the crushing and malaxation of olives [5,6] as well as during the storage and filtration of EVOO [7]. Other influential factors include the use of organic and conventional growing systems [8,9].

The market for organic products, generally perceived as healthier and safer than conventional foods, is growing annually [10], despite the higher costs and lower productivity of organic compared to traditional agriculture. In 2016, on a global level, up to 178 countries practiced organic agriculture, on an extension of 57.8 million hectares, with a market size of 89.7 billion US dollars [11]. A key difference between the two growing systems is soil fertility management, which can affect the nutritive composition of plants, including levels of secondary metabolites [12]. In organic agriculture, which is associated with the promotion of biodiversity and biological cycles, crops obtain nitrogen and nutrients from a diverse soil ecosystem. Contrastingly, conventional farming uses fertilizers containing soluble inorganic nitrogen and other nutrients, which are more directly available to plants [13]. Phenolic biosynthesis in plants is known to be strongly affected by the cultivar, the environmental conditions (especially light), as well as the type of fertilization [14,15]. Previous studies demonstrate that the organic fruits have higher phenolic content than conventional ones [16–18].

Therefore, the objective of our study was to compare the content of polyphenols (secoiridoids, flavones, phenolic alcohols, phenolic acids and lignans) in Hojiblanca EVOO produced by organic and conventional production systems under the same environmental conditions. Moreover, we applied a quantitative ^1H nuclear magnetic resonance (qNMR) method to corroborate the concentration of oleocanthal (OLC) in our EVOOs obtained by LC-MS.

2. Results

2.1. Total Amount of Phenolic Compounds

The average of total phenols (TP) in organic and conventional EVOO, with the p-value for the differences assessed by the Mann–Whitney test, are shown in Table 1. In order to control the ripening factor when assessing the differences in phenolic concentrations between the two types of EVOO, generalized linear models adjusting for ripening index (RI) were used (Table S1). The mean TP content of organic and conventional EVOO was 456.89 ± 56.74 and $338.19 \pm 42.96 \text{ mg}\cdot\text{kg}^{-1}$, respectively, being 26% higher in EVOO produced by the organic system (Figure 1).

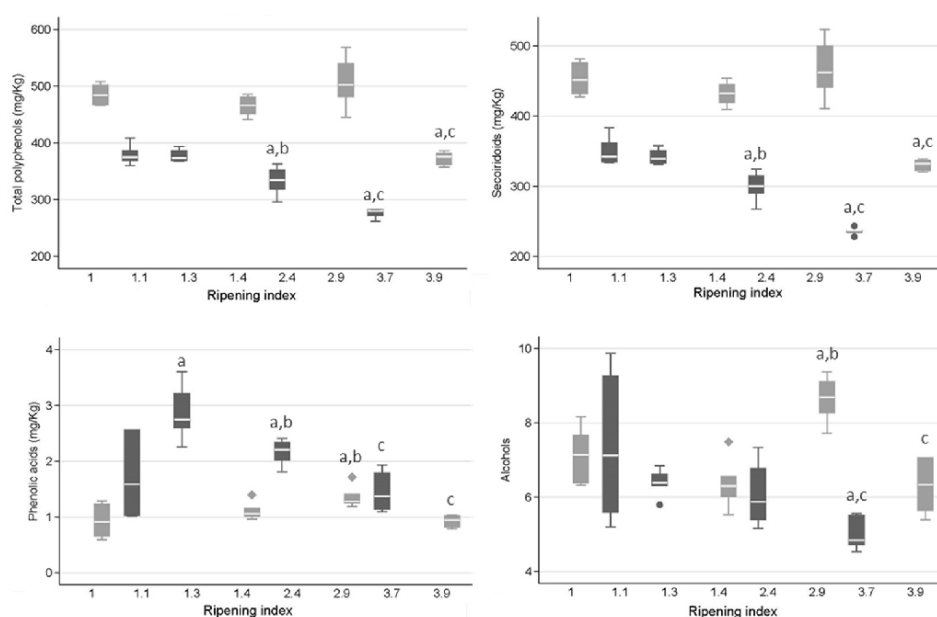


Figure 1. Cont.

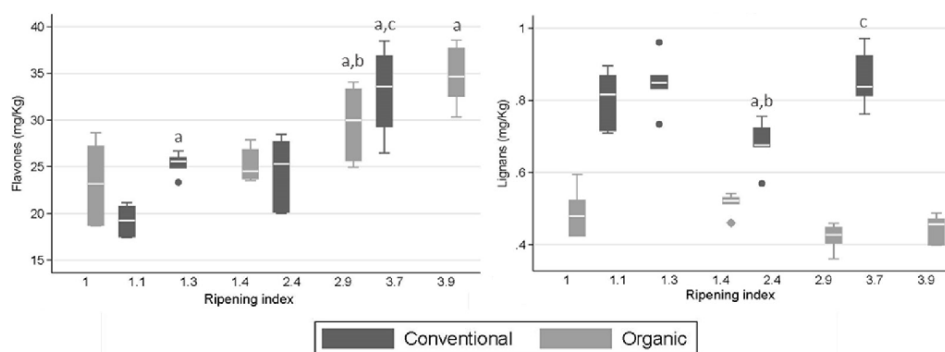


Figure 1. Boxplots for concentrations ($\text{mg}\cdot\text{kg}^{-1}$) of total phenols (TP) and polyphenolic groups in extra-virgin olive oil (EVOO) by ripening index (RI) and growing method. (a means $p < 0.05$ vs. 1st RI, b means $p < 0.05$ vs. 2nd RI, c means $p < 0.05$ vs. 3rd RI within the same growing).

Table 1. Phenolic compound contents ($\text{mg}\cdot\text{kg}^{-1}$) of conventional and organic EVOO made from Hojiblanca olives. * p-values of U Mann–Whitney test.

	Organic	Conventional	p *
Total Phenols	456.89 ± 56.74	338.19 ± 42.96	<0.001
Secoiridoids	420.72 ± 59.42	306.48 ± 48.09	<0.001
Oleuropein	0.82 ± 0.02	0.81 ± 0.02	0.2
Oleuropein derivatives			
Oleuropein der I	22.77 ± 3.01	34.83 ± 4.44	<0.001
Oleuropein der II	3.21 ± 0.54	1.67 ± 0.25	<0.001
Oleuropein der III	3.63 ± 0.58	2.33 ± 0.33	<0.001
me-3,4-DHPEA-EA	1.46 ± 0.25	0.97 ± 0.07	<0.001
Hydroxy oleuropein aglycone I (HOA I)	1.44 ± 0.34	1.17 ± 0.18	0.007
Hydroxy oleuropein aglycone II (HOA II)	2.18 ± 0.77	1.67 ± 0.41	0.02
HDCM OA	9.13 ± 3.53	6.37 ± 1.94	0.01
3,4-DHPEA-EA I	7.18 ± 0.98	4.74 ± 0.42	<0.001
3,4-DHPEA-EA II	5.82 ± 0.94	3.02 ± 0.74	<0.001
Lactone	0.19 ± 0.04	0.33 ± 0.20	<0.001
Ligstroside derivatives			
Ligstroside I	20.45 ± 2.77	12.32 ± 2.47	<0.001
Ligstroside II	41.61 ± 3.68	24.41 ± 4.02	<0.001
Ligstroside III	54.59 ± 10.63	34.75 ± 8.00	<0.001
Oleocanthal	186.72 ± 40.61	132.10 ± 37.02	<0.001
Elenolic acid	55.35 ± 8.10	40.37 ± 7.39	<0.001
Elenolic acid derivatives			
Hydroxyelenolic acid	3.41 ± 1.42	3.19 ± 1.70	0.5
Flavones	28.21 ± 5.55	25.53 ± 5.85	0.09
Luteolin	22.69 ± 5.09	19.35 ± 5.38	0.03
Apigenin	5.51 ± 0.69	6.17 ± 0.78	0.008
Phenolic alcohols	7.11 ± 1.15	6.21 ± 1.37	0.07
Hydroxytyrosol	4.47 ± 1.10	3.65 ± 1.32	0.01
Dihydroxytyrosol	1.73 ± 0.09	1.78 ± 0.10	0.001
3,4-DHPEA-AC	0.91 ± 0.02	0.91 ± 0.03	0.51
Lignans	0.47 ± 0.06	0.79 ± 0.09	<0.001
Pinoresinol	0.47 ± 0.06	0.79 ± 0.09	<0.001
Phenolic acids	1.08 ± 0.26	2.05 ± 0.71	<0.001
Ferulic acid	0.05 ± 0.01	0.07 ± 0.01	0.003
p-coumaric acid	0.67 ± 0.15	1.04 ± 0.36	<0.001
Vanillic acid	0.35 ± 0.12	0.93 ± 0.37	<0.001

2.2. Concentrations of Phenolic Groups and Selected Phenolic Compounds

The major phenolic compounds in EVOO were secoiridoids (SEC), whereas lignans, phenolic acids and flavones were present in low concentrations. The SEC represented 91–92% of the phenolic compounds, with higher levels in the organic than conventional EVOO (420.72 ± 59.42 and $306.48 \pm 48.09 \text{ mg}\cdot\text{kg}^{-1}$, respectively). OLC was the predominant lignostroside derivative found in organic and conventional EVOO samples (186.72 ± 40.61 and $132.10 \pm 37.02 \text{ mg}\cdot\text{kg}^{-1}$, respectively), being 30% higher in the former. Also, the elenolic acid concentration was positively affected by the organic system ($55.35 \pm 8.10 \text{ mg}\cdot\text{kg}^{-1}$), being 27% lower in conventional EVOO ($40.37 \pm 7.39 \text{ mg}\cdot\text{kg}^{-1}$).

Luteolin was the predominant flavonoid in both organic and conventional EVOO (22.69 ± 5.09 and $19.35 \pm 5.38 \text{ mg}\cdot\text{kg}^{-1}$, respectively) and the apigenin represented 20–24% of the total flavonoids and its content was not affected by the agronomic conditions (6.17 ± 0.78 and $5.51 \pm 0.69 \text{ mg}\cdot\text{kg}^{-1}$, conventional and organic, respectively).

The concentration of the total phenolic alcohols was not affected by the organic or conventional growing systems (7.11 ± 1.15 and $6.21 \pm 1.37 \text{ mg}\cdot\text{kg}^{-1}$, respectively). However, the content of hydroxytyrosol was higher under the organic than the conventional system (4.47 ± 1.10 and $3.65 \pm 1.32 \text{ mg}\cdot\text{kg}^{-1}$, respectively).

The content of lignans and phenolic acids, which are important phenolic components of EVOO, were higher under the conventional system (0.79 ± 0.09 and $2.05 \pm 0.71 \text{ mg}\cdot\text{kg}^{-1}$, respectively). The only lignan found was pinoresinol and the phenolic acids were p-coumaric, ferulic and vanillic acid (1.04 ± 0.36 , 0.07 ± 0.01 , and $0.93 \pm 0.37 \text{ mg}\cdot\text{kg}^{-1}$, respectively).

2.3. EVOO Phenolic Profile and Olive Fruit Ripening

The concentration of total SEC, phenolic acids, flavones and lignans in EVOO samples extracted from olives of the Hojiblanca cultivar, grown in conventional and organic conditions and harvested at different RI, are presented in Figure 1. To assess the effect of the RI of the olives on the content of phenolic compounds in conventional and organic EVOO, regression models were fitted (Table 2).

Table 2. Linear regressions (polyphenol content vs. RI) by type of cultivation.

	Organic		Conventional	
	Coefficient	p	Coefficient	p
Total phenols	−27.4	0.004	−40.2	<0.001
Secoiridoids	−31.2	0.001	−44.7	<0.001
Phenolic alcohols	0.05	0.81	−0.76	0.002
Phenolic acids	0.02	0.74	−0.29	0.04
Flavones	3.77	<0.001	4.23	<0.001
Lignans	−0.02	0.01	0.003	0.9

The concentration of TP and SEC decreased during ripening in both conventional and organic EVOO (Table 2). Conversely, an increase in the content of flavones was correlated with ripeness in both organic and conventional EVOO ($p < 0.001$). The total phenolic acids and phenolic alcohols were affected by the olive ripening stage only in the conventional system, showing lower levels with later harvests. Lignans were not affected by the RI in any system.

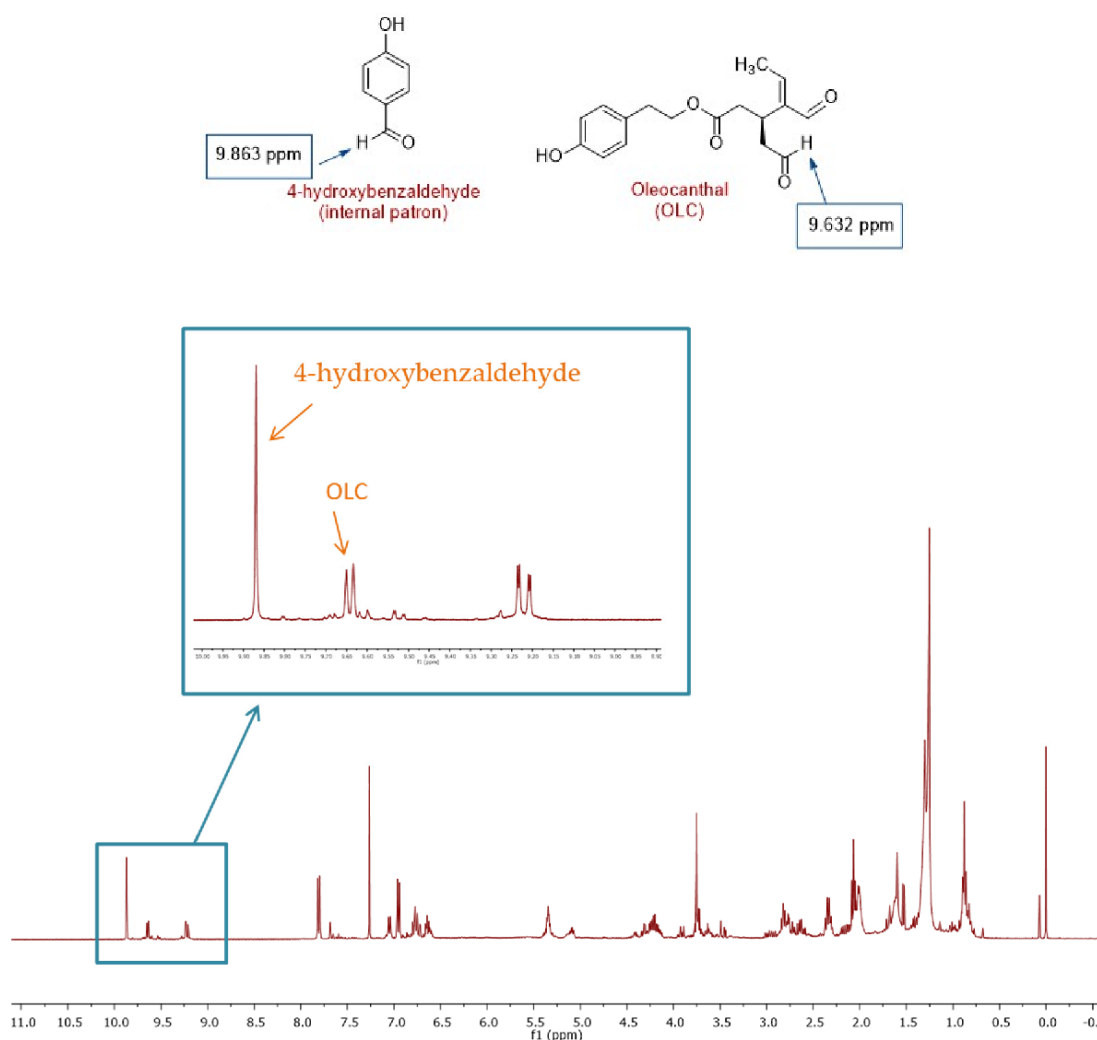
2.4. Analysis of Oleocanthal by NMR

The qNMR showed that the level of OLC was higher ($168.96 \text{ mg}\cdot\text{kg}^{-1}$) in the EVOO made from organic vs. conventionally cultivated olives ($118.21 \text{ mg}\cdot\text{kg}^{-1}$) (Table 3). Thus, the significant variation in OLC concentrations among the samples of the Hojiblanca EVOO is in accordance with previous studies recently reviewed [19] and the results of our OLC analysis by mass spectrometry (MS).

Table 3. Concentration of oleocanthal (OLC) measured by qNMR in EVOOs of Hojiblanca variety.

	Integration	Concentration (mg·kg ⁻¹)
Conventional	0.477	118.21
Organic	0.684	168.96

As 1D ¹H NMR typically provides an excellent linear response to component concentrations, it was envisaged as a simple and reliable methodology to validate the UPLC-MS monitoring of OLC levels. The aldehydic proton region of the target compound in the ¹H NMR spectrum of EVOO acetonitrile extracts, when recorded in CDCl₃, presented a well-resolved set of peaks, making feasible the integration of one of the aldehydic protons and its comparison with the peak of the internal standard. OLC was quantified by integrating the singlet at 9.632 ppm (Figure 2).

**Figure 2.** ¹H NMR spectrum of EVOO acetonitrile extracts recorded in CDCl₃ and using 4-hydroxybenzaldehyde as the internal standard. The signals of the OLC and internal standard are shown in the expanded spectrum.

3. Discussion

3.1. Total Amount of Phenolic Compounds

Recent studies found a higher polyphenol content in organic EVOO [20] and a different acid composition, as well as a higher degree of bitterness (cv. Leccino and Frantoio) and pungency (cv. Frantoio) and less sweetness (cv. Frantoio) [21]. A similar enhancing effect of organic cultivation on TP content was observed in EVOO extracted from olives of the Casaliva variety, both unripe (51% increase) and ripe (40% increases), whereas in a multi-varietal organic EVOO this effect was only observed with unripe olives [22]. However, in other studies, agronomic factors did not play a clear role in the TP content of EVOO made from olives of different cultivars, which instead was mainly affected by the year of the harvest [8], or water availability [23]. The type of farming (organic or conventional) only becomes a major factor in the TP content of tomatoes [24], pepper [25] and fruits [26] when these are grown under similar environmental conditions.

3.2. Concentrations of Phenolic Groups and Selected Phenolic Compounds

Organic agriculture is associated with a natural increase in the amount of defense substances, as the plant is exposed to greater stress in the absence of synthetic pesticides. In addition, without synthetic fertilizers there is less bioavailable nitrogen, with concomitant lower plant growth rates and an enhanced production of secondary metabolites such as phenolic compounds [27,28].

Previous studies have established that SEC (oleuropein and oleuropein, ligstroside and elenolic acid derivatives) are the most complex and abundant family of polyphenols in EVOO polar fractions [29–31] and are the principal contributors to organoleptic traits [32]. SEC are synthesized through the secologanin pathway, which does not depend on nitrogen or phosphorus, so their production is not impeded with the low nitrogen and phosphorus availability of organic soil [33]. A recent study demonstrated that the foliar fertilization with a biofertilizer rich in calcium increased oleuropein aglycone and OLC levels in EVOO, which in contrast, decreased significantly with the use of a biofertilizer rich in nitrogen, phosphorus and potassium [34]. Therefore, conventional practices could explain the lower content of most of the SEC compounds (oleuropein derivatives and ligstrosides derivatives) compared to organic practices. The ester breakdown of SEC leads to the formation of elenolic acid and derivatives [35]. In our study, the elenolic acid concentration was in accordance with previously reported results in different EVOO (16.8–58.6 mg·kg^{−1}) [36,37]. Since the content of SEC was higher in organic EVOO, it is also logical to have higher content of elenolic acid (Table 1), because the more SEC, the more elenolic acid is released from SEC breakdown.

The main flavonoids present in EVOO are luteolin and apigenin [38,39] and a high luteolin content is of great interest, as it has been associated with health-promoting and antioxidant properties of foods [40,41]. The luteolin concentration reported here (Table 1) is higher than in other studies (3.12 and 7.57 mg·kg^{−1}) [36,42], including one in which EVOO was also extracted from Hojiblanca olives (3.69–6.67 mg·kg^{−1}) [43]. These differences among studies are not surprising, since it has been shown that the concentration of luteolin depends considerably on the olive variety, geographical area, season, environmental conditions and cultivation method [36,44]. In another study, the use of a biofertilizer rich in nitrogen, phosphorus and potassium led to a significant increase in the apigenin content of EVOO (1.66 ± 0.32 mg·kg^{−1}), whereas luteolin levels increased when the biofertilizer was supplemented with calcium (2.12 ± 0.39 mg·kg^{−1}) [34].

In our work, the concentration of the total phenolic alcohols is similar to that of another study comparing organic and conventional EVOO made from the same type of olives (8.33–11.0 and 10.5–16.3 mg·kg^{−1}, respectively) [43]. Other authors reported a decrease in hydroxytyrosol concentration in EVOO when olive trees were fertilized with nitrogen, phosphorus and potassium, as occurred with SEC [34]. The phenolic alcohols are derived from the SEC, so their biosynthesis does not depend on the nitrogen or the phosphorus [33].

Both lignans and phenolic acids are synthesized through the phenylpropanoid pathway [45,46] and depend on the shikimic pathway, in which nitrogen and phosphorus take part [47]. The greater availability of nitrogen and phosphorus in conventional farming could thus explain the lower lignan and phenolic acid concentration in the organic EVOO.

3.3. EVOO Phenolic Profile and Olive Fruit Ripening

During ripening, the chemical structure and concentrations of compounds in olives can be modified by chemical reactions and the enzymatic activity of glycosidases, phenol oxidases and phenol polymerases [48,49]. The amount of these enzymes depends on the cultivar and maturation stage [41]. Thus, the degree of olive fruit ripeness is a crucial parameter in EVOO quality [50]. Previous studies have reported a reduction in TP, beginning at a maturation index of 2.5–3 [51], or a significant gradual decrease from the first to the fifth harvest [23]. It has been suggested that the TP content depends more on the olive cultivar than an early or late harvest [52].

The amount of SEC decreases significantly with ripeness, both in organic and conventional systems, as reported in the literature [48,53,54]. The SEC concentration was found to decrease by 31% ($92.1\text{--}63.0\text{ mg}\cdot\text{kg}^{-1}$) between the first and last harvests (maturation index of 2.4–5.6), due to oleuropein degradation during ripening [55]. Also, Gutierrez-Rosales et al. [56] showed that high contents of oleuropein aglycone at the initial stage of ripening were caused by a high activity of β -glucosidase. This indicates that oleuropein biosynthesis combines with enzymatic hydrolysis to produce the aglycone form. Thus, when the olive is in a green stage, the level of β -glucosidase activity increases proportionally with the amount of oleuropein and ligstroside, whereas in the black stage, when the phenolic glycoside concentration is reduced, the glucosidase activity is low [49].

Reports in the literature on the influence of ripening on flavonoid content are contradictory. The content of flavones (luteolin and apigenin) in olives was observed to increase up to a maturation index close to 4, decreasing thereafter [38], whereas elsewhere this tendency was found at an index of 0.76–1.27 [54]. Furthermore, an increase in flavonoid concentration has been reported in EVOO made from olives at an intermediate ripening stage [57,58]. In Hojiblanca EVOO, a higher content of luteolin was obtained by harvesting medium-ripe olives (6.10 and $6.59\text{ mg}\cdot\text{kg}^{-1}$, conventional and organic EVOO, respectively), whereas an early harvest resulted in increased apigenin (3.32 and $3.65\text{ mg}\cdot\text{kg}^{-1}$, conventional and organic EVOO, respectively) [43]. The association of a higher concentration of luteolin with an intermediate ripening stage could be because apigenin is the substrate for a hydroxylase enzyme in the flavonoid pathway, giving rise to luteolin [53].

With respect to phenolic acids, our results are in agreement with those of Jimenez et al., who reported that *p*-coumaric and ferulic acid contents were higher in EVOO extracted from olives at an early RI, decreasing progressively thereafter, and that the concentration of vanillic acid was apparently not affected by the ripening process [59]. Another study found an increase in phenolic acids in EVOO extracted from more mature olives, which may be due to the activity of hydrolytic enzymes on the complex phenols [53].

3.4. Analysis of Oleocanthal by NMR

The various extraction procedures and analytical methods developed for the quantification of EVOO phenolic compounds have generated ambiguous results that are difficult to compare. The most commonly used methods are liquid chromatography (LC), followed by UV-Vis or detection by MS [60,61]. However, OLC can react with different solvents and consequently both liquid–liquid extraction and chromatographic analysis may interfere with its determination by LC-MS, leading in some cases to broader or multiple peaks in MS detection. [62]. A promising new method has been published recently by Sánchez de Medina et al. [63], but further studies are needed to assess its accuracy and reliability. For this reason, the chromatographic results obtained with this method were validated by acetonitrile extraction from random samples of EVOO and directly measured OLC levels by qNMR, as described by Karkoula et al. [64]. The interest of qNMR is due to the repeatability and

reproducibility of measurements, as well as its rapidity compared to more classical methods, and its reliability [65]. Other advantages include simple sample preparation, low sample consumption and non-destructive measurement.

This validation study was performed by NMR using CDCl_3 as the solvent, avoiding both the undesired interactions of other solvents like methanol or water with the target compound, and the overlap of the aldehydic proton peaks. We chose 4-hydroxybenzaldehyde as the internal standard due to its price, stability, and solubility in our deuterated solvent, and the simplicity of the resulting ^1H NMR spectrum. Moreover, the aldehydic proton peaks of this compound and OLC do not overlap.

4. Material and Methods

4.1. Chemicals

OLC was purchased from PhytoLab GmbH (Vestenbergsgreuth, Germany); oleuropein, lutein, m-coumaric acid, pinosresinol, lariciresinol, isolariciresinol, secoisolariciresinol and taxifolin were obtained from Sigma-Aldrich (Madrid, Spain). p-Coumaric acid, vanillic acid, ferulic acid and apigenin were obtained from Fluka (Buchs, Switzerland), hydroxytyrosol from Extrasynthese (Genay, France), and verbascoside from HWI ANALYTIK GmbH (Rülzheim, Germany). Hexane, methanol, acetonitrile, and chloroform-d were purchased from Sigma-Aldrich and cyclohexane from Carlo Erba (Madrid, Spain).

4.2. Olive Fruit Samples

Olive fruits were collected from olive trees of the Hojiblanca cultivar, which were cultivated using organic and conventional agricultural practices without irrigation. The orchard was located on the experimental farm of the Agricultural Research Training Centre in Cabra in the province of Cordoba, at an altitude of approximately 547 m. The soil pH was 8 and its composition was limestone and sand. The climate was continental Mediterranean, with hot summers and cold winters, and the average temperature between October of 2017 and January of 2018 was 12.7°C , with an average relative humidity of 64.4%. Ten trees were selected per cultivation system. The olives were harvested on 4 different days with 2 weeks of difference between every picking.

The RI of each harvest was determined according to the methodology proposed by Uceda and Frías [66], which is based on the color of the skin and the pulp. 100 olives were randomly selected and the following formula was applied: $\text{RI} = (A \times 0 + B \times 1 + C \times 2 + D \times 3 + E \times 4 + F \times 5 + G \times 6 + H \times 7) / 100$. Where A, B, C, D, E, F, G, H are the number of olives with the 8 different ripening stages. Those are: stage 0: intense green skin; stage 1: yellowish green skin; stage 2: green skin with red spots, in less than half of the fruit; stage 3: reddish or purple skin in more than half of the fruit; stage 4: black skin and white pulp; stage 5: black skin and pulp purple; stage 6: black skin and more than half of the pulp purple; stage 7: black skin and totally purple pulp. The fruit RI in organic and conventional system was 1 to 3.945 and 1.06 to 3.68, respectively.

4.3. Oil Samples

Three representative olive samples, each weighing a minimum of one kilogram, were processed and the corresponding EVOOs were obtained using an Abencor milling system (Abengoa S.A., Seville, Spain). This system reproduced the industrial process on a laboratory scale. The apparatus consisted of three elements: a hammer mill, a thermobeater and a pulp centrifuge. The olive fruits (6 kg) were milled using a stainless-steel hammer mill equipped with a 5-mm sieve that was operated at 3000 rpm. The resulting olive paste was immediately kneaded in a mixer at 50 rpm for 30 min at 30°C , with hot water added at 20 min. Centrifugation of the kneaded olive paste was performed in a basket centrifuge at 3500 rpm for 1 min. After centrifugation, the oil was decanted and stored in amber glass bottles at 4°C in darkness and without headspace until analysis.

4.4. Polyphenol Analysis by Liquid Chromatography

The liquid–liquid extraction of phenolic compounds was performed with the method proposed by Capriotti et al. [67]. 1 g of EVOO was dissolved in hexane (oil/hexane 1:1, w/v) in a 10 mL centrifuge tube and shaken for 30 s. The polyphenols were extracted with 2 mL of MeOH and stirred for 30 s; the emulsion was then centrifuged at 3000 rpm and 4 °C for 3 min. The supernatant (methanolic extract) was subjected to a second cleaning with hexane, and the hexane extract was subjected to a second extraction of polyphenols with MeOH. All extracts were shaken for 30 s and centrifuged at 3000 rpm and 4 °C for 3 min. The methanolic extracts were recovered and cleaned up by dispersing 50 mg of C18. The samples were evaporated and reconstituted with 800 µL of MeOH:H₂O (80:20 v/v), filtered with (Polytetrafluoroethylene) PTFE syringe filters (0.2 µm), transferred to an amber glass vial and stored at −80 °C until analysis. The internal standard was added to the EVOO to obtain a final concentration of 5 ppm after the reconstitution. The experiment was done in triplicate.

The identification and quantification of phenolic compounds was performed using an AcquityTM UPLC (Waters; Milford, MA, EUA) coupled to an API 3000 triple-quadrupole mass spectrometer (PE Sciex) with a turbo ion spray source. Separation of compounds was achieved using an Acquity UPLC[®] BEH C₁₈ Column (2.1 × 50 mm, i.d., 1.7 µm particle size) and Acquity UPLC[®] BEH C₁₈ Pre-Column (2.1 × 5 mm, i.d., 1.7 µm particle size) (Waters Corporation[®], Ireland) (See supporting information). The mobile phases were H₂O with 0.2% acetic acid (A) and ACN (B). An increasing linear gradient (v/v) of B was used (t (min), %B), as follows: (0, 5); (2.5, 5); (12.5, 40); (12.6, 100); (13.5, 100); (13.6, 5); (15, 5), at a constant flow rate of 0.4 mL/min. The injection volume was 10 µL and the column temperature 40 °C.

The quantification of OLC was performed using a methodology proposed by Sánchez de Medina et al. with some modifications. Separation was achieved using an Acquity UPLC[®] BEH C₁₈ Column (2.1 × 50 mm, i.d., 1.7 µm particle size) and Acquity UPLC[®] BEH C₁₈ Pre-Column (2.1 × 5 mm, i.d., 1.7 µm particle size) (Waters Corporation[®], Ireland). The mobile phases were MeOH (A) and H₂O (B), both with 0.1% of formic acid. An increasing linear gradient (v/v) of B was used (t (min), %B), as follows: (0, 100); (2, 100); (4.75, 46.4); (4.9, 0); (5.9, 0); (6.100); (6.5, 100), at a constant flow rate of 0.6 mL·min^{−1}. The injection volume was 5 µL and the column temperature 50 °C. The MS potentials were optimized for the compound (Supporting Table S2). Method suitability was evaluated by submitting random samples to a comparative NMR study.

Ionization was achieved using an electrospray interface operating in the negative mode [M−H] and all the compounds were monitored in the multiple monitoring mode (MRM) with the following settings: capillary voltage, −3500 V; nebuliser gas (N₂), 10 (arbitrary units); curtain gas (N₂), 12 (arbitrary units); and drying gas (N₂) heated to 450 °C. The declustering potential, focusing potential, collision energy and entrance potential were optimized to detect phenolic compounds with the highest signals, following the method described by Suárez et al. [39]. The system was controlled by Analyst version 1.4.2 software supplied by Applied Biosystems.

The calibration curves were prepared in refined oil and were linear over the concentration ranges 0–20 mg·mL^{−1} using oleuropein, hydroxytyrosol, p-coumaric acid, m-coumaric acid, vanillic acid, ferulic acid, apigenin, luteolin, pinoresinol, lariciresinol, isolariciresinol, secoisolariciresinol, verbascoside and OLC.

4.5. Analysis of Oleocanthal by NMR

The OLC extraction and sample preparation for NMR analysis were carried out using the methodology proposed by Karkoula et al. [30]. Olive oil (8.0 g) was mixed with cyclohexane (32 mL) and ACN (40 mL). The mixture was homogenized using a vortex mixer for 30 s and centrifuged at 4000 rpm for 5 min. The ACN phase (40 mL) was collected, mixed with 1.6 mL of 4-hydroxybenzaldehyde solution (0.5 mg·mL^{−1}) in ACN, and evaporated under reduced pressure using a rotary evaporator (Buchi, Model R-200 with dry ice and acetone cold-trap condenser, Switzerland).

The residue of the above procedure was dissolved in CDCl₃ (750 µL), and an accurately measured volume of the solution (550 µL) was transferred to a 5 mm NMR tube. ¹H NMR spectra were recorded at 400 MHz using an NMR spectrometer (Varian VNMRs 400 MHz). Typically, 128 scans were collected into 32K data points over a spectral width of 16 ppm (6410 Hz), with a relaxation delay of 1 s and an acquisition time of 2.5 s. The spectra were phase corrected and integrated automatically using MNova. Accurate integration was performed manually for the peaks of interest.

4.6. Statistical Analysis

Significant differences between organic and conventional samples were assessed by the Mann–Whitney test (Table 1). The relationship between categorical exposure variables (organic vs conventional cultivation) and concentration of polyphenols was assessed by Generalized Linear Models adjusting for RI (Table 2). Regression models were also fitted to assess associations between polyphenol concentration as a dependent variable and RI as an independent variable. Statistical analyses were conducted using STATA software (version 14.0; StataCorp, College Station, TX, USA). *p* values < 0.05 were considered statistically significant.

5. Conclusions

The TP in EVOO made from Hojiblanca olives were analyzed, comparing organic and conventional growing systems under the same environmental conditions, and levels were significantly higher in organic samples (*p* < 0.05). The concentration of SEC, which are synthesized through the secologanin pathway without the need for nitrogen or phosphorus, was higher in oils from olives cultivated under organic conditions. These included oleocanthal, which was satisfactorily analyzed by LC-MS/MS, as demonstrated by results obtained by qNMR.

In contrast, the concentrations of lignans and phenolic acids were higher under the conventional system, as their synthesis is through the phenylpropanoid pathway via shikimic acid, which requires nitrogen and phosphorus. In the case of phenolic alcohols and flavones, there were no significant differences associated with the cultivation method.

When the effect of the ripening stage of the olive fruit was assessed, both the TP and SEC concentrations were found to decrease with maturation in both production systems, whereas the flavone content increased. Olive maturation was also associated with a decline in certain compounds: lignans in organic EVOO and phenolic acids and phenolic alcohols in conventional samples.

It should be emphasized that long-term experiments are required to eliminate the effect of seasonality. There is also a need for more randomized, controlled dietary intervention trials to corroborate the potentially greater beneficial effects of organic food on human health compared to those produced conventionally. However, organic food may be recommended, not only for its health benefits, but also because its production has less of an environmental impact.

Supplementary Materials: The following are available online, Table S1: GLM model adjusted for ripeness (conventional vs. organic); Table S2: Multiple reaction Monitoring conditions for the polyphenols.

Author Contributions: Methodology, A.L.-Y., J.L.-C., P.Q.-R.; formal analysis A.L.-Y., J.L.-C., A.O.-C. and A.T.-R.; investigation, A.L.-Y., J.L.-C., M.P. and A.V.-Q.; writing—original draft preparation, A.L.-Y., J.L.-C., M.P., A.T.-R. and A.V.-Q.; writing—review and editing, A.L.-Y., J.L.-C., M.P., A.T.-R. and A.V.-Q.; visualization, M.P. and A.V.-Q.; supervision, M.P. and A.V.-Q.

Funding: This research received no external funding.

Acknowledgments: A.L.-Y. thanks the National Council for Science and Technology (CONACYT) of Mexico for the doctoral scholarship. J.L.-C. thanks the Ministry of Science Innovation and Universities for the FPI contract. P.Q.-R. is grateful for the Sara Borrell postdoctoral program from the Instituto de Salud Carlos III (ISCIII). A.T.-R. thanks the Ministry of Science Innovation and Universities for the Juan de la Cierva-formación contract A.V.-Q. thanks the Ministry of Science Innovation and Universities for the Ramon y Cajal contract. The authors wish to thank the CCIT-UB for the mass spectrometry equipment.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Servili, M.; Sordini, B.; Esposto, S.; Urbani, S.; Veneziani, G.; Di Maio, I.; Selvaggini, R.; Taticchi, A. Biological Activities of Phenolic Compounds of Extra Virgin Olive Oil. *Antioxidants* **2013**, *3*, 1–23. [CrossRef]
2. Servili, M.; Esposto, S.; Fabiani, R.; Urbani, S.; Taticchi, A.; Mariucci, F.; Selvaggini, R.; Montedoro, G.F. Phenolic compounds in olive oil: Antioxidant, health and organoleptic activities according to their chemical structure. *Inf ammopharmacology* **2009**, *17*, 76–84. [CrossRef] [PubMed]
3. Koseoglu, O.; Unal, M.K. The effect of phenolic compounds on the quality and stability of virgin olive oil. In Proceedings of the VI International Symposium on Olive Growing, Evora, Portugal, 9–13 September 2008; Volume 791, pp. 655–663.
4. Di Giovacchino, L.; Costantini, N.; Serraiocco, A.; Surricchio, G.; Basti, C. Natural antioxidants and volatile compounds of virgin olive oils obtained by two or three-phases centrifugal decanters. *Eur. J. Lipid Sci. Technol.* **2001**, *103*, 279–285. [CrossRef]
5. El Riachy, M.; Priego-Capote, F.; León, L.; Rallo, L.; Luque de Castro, M.D. Hydrophilic antioxidants of virgin olive oil. Part 2: Biosynthesis and biotransformation of phenolic compounds in virgin olive oil as affected by agronomic and processing factors. *Eur. J. Lipid Sci. Technol.* **2011**, *113*, 692–707. [CrossRef]
6. Clodoveo, M.L. Malaxation: Influence on virgin olive oil quality. Past, present and future—An overview. *Trends Food Sci. Technol.* **2012**, *25*, 13–23. [CrossRef]
7. Lozano-Sánchez, J.; Cerretani, L.; Bendini, A.; Gallina-Toschi, T.; Segura-Carretero, A.; Fernández-Gutiérrez, A. New filtration systems for extra-virgin olive oil: Effect on antioxidant compounds, oxidative stability, and physicochemical and sensory properties. *J. Agric. Food Chem.* **2012**, *60*, 3754–3762. [CrossRef]
8. Ninfali, P.; Bacchiocca, M.; Biagiotti, E.; Esposto, S.; Servili, M.; Rosati, A.; Montedoro, G. A 3-year study on quality, nutritional and organoleptic evaluation of organic and conventional extra-virgin olive oils. *JAOCS J. Am. Oil Chem. Soc.* **2008**, *85*, 151–158. [CrossRef]
9. Vallverdú-Queralt, A.; Lamuela-Raventós, R.M. Foodomics: A new tool to differentiate between organic and conventional foods. *Electrophoresis* **2016**, *37*, 1784–1794. [CrossRef] [PubMed]
10. Hurtado-Barroso, S.; Tresserra-Rimbau, A.; Vallverdú-Queralt, A.; Lamuela-Raventós, R.M. Organic food and impact on human health. *Int. J. PharmTech Res.* **2016**, *9*, 316–324. [CrossRef]
11. González, N.; Marquès, M.; Nadal, M.; Domingo, J.L. Occurrence of environmental pollutants in foodstuffs: A review of organic vs. conventional food. *Food Chem. Toxicol.* **2019**, *125*, 370–375. [CrossRef] [PubMed]
12. Williams, C.M. Nutritional quality of organic food: Shades of grey or shades of green? *Proc. Nutr. Soc.* **2008**, *61*, 19–24. [CrossRef]
13. Worthington, V. Effect of Agricultural Methods on Nutritional Quality: A Comparison of Organic with Conventional Crops. *Altern. Ther. Health Med.* **1998**, *4*, 58–69.
14. Häkkinen, S.H.; Törrönen, A.R. Content of flavonols and selected phenolic acids in strawberries and Vaccinium species: Influence of cultivar, cultivation site and technique. *Food Res. Int.* **2000**, *33*, 517–524. [CrossRef]
15. Romero-Pérez, A.I.; Lamuela-Raventós, R.M.; Andrés-Lacueva, C.; De La Carmen Torre-Boronat, M. Method for the quantitative extraction of resveratrol and piceid isomers in grape berry skins. Effect of powdery mildew on the stilbene content. *J. Agric. Food Chem.* **2001**, *49*, 210–215. [CrossRef]
16. Vallverdú-Queralt, A.; Medina-Remón, A.; Casals-Ribes, I.; Amat, M.; Lamuela-Raventós, R.M. A metabolomic approach differentiates between conventional and organic ketchups. *J. Agric. Food Chem.* **2011**, *59*, 11703–11710. [CrossRef]
17. Martí, R.; Leiva-Brondo, M.; Lahoz, I.; Campillo, C.; Cebolla-Cornejo, J.; Roselló, S. Polyphenol and L-ascorbic acid content in tomato as influenced by high lycopene genotypes and organic farming at different environments. *Food Chem.* **2018**, *239*, 148–156. [CrossRef]
18. Cuevas, F.J.; Pradas, I.; Ruiz-Moreno, M.J.; Arroyo, F.T.; Perez-Romero, L.F.; Montenegro, J.C.; Moreno-Rojas, J.M. Effect of organic and conventional management on bio-functional quality of thirteen plum cultivars (*Prunus salicina* Lindl.). *PLoS ONE* **2015**, *10*, e0136596. [CrossRef]
19. Cicerale, S.; Lucas, L.J.; Keast, R.S.J. Antimicrobial, antioxidant and anti-inflammatory phenolic activities in extra virgin olive oil. *Curr. Opin. Biotechnol.* **2012**, *23*, 129–135. [CrossRef]

20. Barbieri, S.; Bendini, A.; Valli, E.; Gallina Toschi, T. Do consumers recognize the positive sensorial attributes of extra virgin olive oils related with their composition? A case study on conventional and organic products. *J. Food Compos. Anal.* **2015**, *44*, 186–195. [CrossRef]
21. Rosati, A.; Cafiero, C.; Paoletti, A.; Alfei, B.; Caporali, S.; Casciani, L.; Valentini, M. Effect of agronomical practices on carpology, fruit and oil composition, and oil sensory properties, in olive (*Olea europaea* L.). *Food Chem.* **2014**, *159*, 236–243. [CrossRef]
22. Trombetta, D.; Smeriglio, A.; Marcoccia, D.; Giofrè, S.; Toscano, G.; Mazzotti, F.; Giovanazzi, A.; Lorenzetti, S. Analytical evaluation and antioxidant properties of some secondary metabolites in northern Italian mono- and multi-varietal extra virgin olive oils (EVOOs) from early and late harvested olives. *Int. J. Mol. Sci.* **2017**, *18*, 797. [CrossRef]
23. Alowaish, B.; Singh, Z.; Fang, Z.; Kailis, S.G. Harvest time impacts the fatty acid compositions, phenolic compounds and sensory attributes of Frantoio and Manzanilla olive oil. *Sci. Hortic. (Amsterdam)* **2018**, *234*, 74–80. [CrossRef]
24. Vallverdú-Queralt, A.; Medina-Remón, A.; Casals-Ribes, I.; Lamuela-Raventós, R.M. Is there any difference between the phenolic content of organic and conventional tomato juices? *Food Chem.* **2012**, *130*, 222–227. [CrossRef]
25. Hallmann, E.; Rembialska, E. Characterisation of antioxidant compounds in sweet bell pepper (*Capsicum annuum* L.) under organic and conventional growing systems. *J. Sci. Food Agric.* **2012**, *92*, 2409–2415. [CrossRef]
26. Mditshwa, A.; Magwaza, L.S.; Tesfay, S.Z.; Mbili, N. Postharvest quality and composition of organically and conventionally produced fruits: A review. *Sci. Hortic. (Amsterdam)* **2017**, *216*, 148–159. [CrossRef]
27. Assumpção, C.F.; Nunes, I.L.; Mendonça, T.A.; Bortolin, R.C.; Jablonski, A.; Flôres, S.H.; De Oliveira Rios, A. Bioactive Compounds and Stability of Organic and Conventional *Vitis labrusca* Grape Seed Oils. *JAOCS J. Am. Oil Chem. Soc.* **2016**, *93*, 115–124. [CrossRef]
28. Hunter, D.; Foster, M.; McArthur, J.O.; Ojha, R.; Petocz, P.; Samman, S. Evaluation of the micronutrient composition of plant foods produced by organic and conventional agricultural methods. *Crit. Rev. Food Sci. Nutr.* **2011**, *51*, 571–582. [CrossRef]
29. Miho, H.; Díez, C.M.; Mena-Bravo, A.; Sánchez de Medina, V.; Moral, J.; Melliou, E.; Magiatis, P.; Rallo, L.; Barranco, D.; Priego-Capote, F. Cultivar influence on variability in olive oil phenolic profiles determined through an extensive germplasm survey. *Food Chem.* **2018**, *266*, 192–199. [CrossRef]
30. Karkoula, E.; Skantzari, A.; Melliou, E.; Magiatis, P. Quantitative measurement of major secoiridoid derivatives in olive oil using qNMR. Proof of the artificial formation of aldehydic oleuropein and ligstroside aglycon isomers. *J. Agric. Food Chem.* **2014**, *62*, 600–607. [CrossRef]
31. García-Rodríguez, R.; Belaj, A.; Romero-Segura, C.; Sanz, C.; Pérez, A.G. Exploration of genetic resources to improve the functional quality of virgin olive oil. *J. Funct. Foods* **2017**, *38*, 1–8. [CrossRef]
32. Campestre, C.; Angelini, G.; Gasbarri, C.; Angerosa, F. The compounds responsible for the sensory profile in monovarietal virgin olive oils. *Molecules* **2017**, *22*, 1833. [CrossRef] [PubMed]
33. Jensen, S.R.; Franzyk, H.; Wallander, E. Chemotaxonomy of the oleaceae: Iridoids as taxonomic markers. *Phytochemistry* **2002**, *60*, 213–231. [CrossRef]
34. Dabbaghi, O.; Tekaya, M.; Flamini, G.; Zouari, I.; El-Gharbi, S.; M'barki, N.; Laabidi, F.; Cheheb, H.; Attia, F.; Aïachi Mezghani, M.; et al. Modification of Phenolic Compounds and Volatile Profiles of Chemlali Variety Olive Oil in Response to Foliar Biofertilization. *J. Am. Oil Chem. Soc.* **2019**, *96*, 585–593. [CrossRef]
35. Figueiredo-González, M.; Reboredo-Rodríguez, P.; González-Barreiro, C.; Carrasco-Pancorbo, A.; Cancho-Grande, B.; Simal-Gándara, J. The involvement of phenolic-rich extracts from Galician autochthonous extra-virgin olive oils against the α -glucosidase and α -amylase inhibition. *Food Res. Int.* **2019**, *116*, 447–454. [CrossRef] [PubMed]
36. Kelebek, H.; Kesen, S.; Selli, S. Comparative study of bioactive constituents in Turkish olive oils by LC-ESI/MS/MS. *Int. J. Food Prop.* **2015**, *18*, 2231–2245. [CrossRef]
37. Arslan, D.; Karabekir, Y.; Schreiner, M. Variations of phenolic compounds, fatty acids and some qualitative characteristics of Sariulak olive oil as induced by growing area. *Food Res. Int.* **2013**, *54*, 1897–1906. [CrossRef]
38. de Torres, A.; Espínola, F.; Moya, M.; Alcalá, S.; Vidal, A.M.; Castro, E. Assessment of phenolic compounds in virgin olive oil by response surface methodology with particular focus on flavonoids and lignans. *LWT Food Sci. Technol.* **2018**, *90*, 22–30. [CrossRef]

39. Suárez, M.; Macià, A.; Romero, M.P.; Motilva, M.J. Improved liquid chromatography tandem mass spectrometry method for the determination of phenolic compounds in virgin olive oil. *J. Chromatogr. A* **2008**, *1214*, 90–99. [CrossRef] [PubMed]
40. Murkovic, M.; Lechner, S.; Pietzka, A.; Bratacos, M.; Katzogiannos, E. Analysis of minor components in olive oil. *J. Biochem. Biophys. Methods* **2004**, *61*, 155–160. [CrossRef]
41. Ye, J.H.; Wijesundera, C.; Shi, M. Effects of Agronomic and Oil Processing Conditions on Natural Antioxidative Phenolics in Olive (*Olea europaea* L.). *Austin J. Nutr. Food Sci.* **2014**, *2*, 1050.
42. Collado-González, J.; Grosso, C.; Valentão, P.; Andrade, P.B.; Ferreres, F.; Durand, T.; Guy, A.; Galano, J.M.; Torrecillas, A.; Gil-Izquierdo, Á. Inhibition of α -glucosidase and α -amylase by Spanish extra virgin olive oils: The involvement of bioactive compounds other than oleuropein and hydroxytyrosol. *Food Chem.* **2017**, *235*, 298–307. [CrossRef]
43. Jimenez, B.; Sánchez-Ortiz, A.; Lorenzo, M.L.; Rivas, A. Effect of organic cultivation of Picual and Hojiblanca olive varieties on the quality of virgin olive oil at four ripening stages. *Eur. J. Lipid Sci. Technol.* **2014**, *116*, 1634–1646. [CrossRef]
44. Kalogiouri, N.P.; Aalizadeh, R.; Thomaidis, N.S. Investigating the organic and conventional production type of olive oil with target and suspect screening by LC-QTOF-MS, a novel semi-quantification method using chemical similarity and advanced chemometrics. *Anal. Bioanal. Chem.* **2017**, *409*, 5413–5426. [CrossRef]
45. Dean, J.F.D.; LaFayette, P.R.; Rugh, C.; Tristram, A.H.; Hoopes, J.T.; Eriksson, K.-E.L.; Merkle, S.A. Laccases Associated with Lignifying Vascular Tissues. In *ACS Symposium Series*; American Chemical Society: Washington, DC, USA, 2009; Volume 78, pp. 96–108. ISBN 9780841235663.
46. Ali Ghasemzadeh Flavonoids and phenolic acids: Role and biochemical activity in plants and human. *J. Med. Plants Res.* **2011**, *5*, 6697–6703. [CrossRef]
47. Fraser, C.M.; Chapple, C. The Phenylpropanoid Pathway in Arabidopsis. *Am. Soc. Plant Biol.* **2011**, *9*, e0152. [CrossRef]
48. Ben Brahim, S.; Kelebek, H.; Ammar, S.; Abichou, M.; Bouaziz, M. LC–MS phenolic profiling combined with multivariate analysis as an approach for the characterization of extra virgin olive oils of four rare Tunisian cultivars during ripening. *Food Chem.* **2017**, *229*, 9–19. [CrossRef] [PubMed]
49. Clodoveo, M.L.; Hbaieb, R.H.; Kotti, F.; Mugnozza, G.S.; Gargouri, M. Mechanical strategies to increase nutritional and sensory quality of virgin olive oil by modulating the endogenous enzyme activities. *Compr. Rev. Food Sci. Food Saf.* **2014**, *13*, 135–154. [CrossRef]
50. Amanpour, A.; Kelebek, H.; Selli, S. Characterization of aroma, aroma-active compounds and fatty acids profiles of cv. Nizip Yaglik oils as affected by three maturity periods of olives. *J. Sci. Food Agric.* **2019**, *99*, 726–740. [CrossRef] [PubMed]
51. Peres, F.; Martins, L.L.; Ferreira-Dias, S. Influence of enzymes and technology on virgin olive oil composition. *Crit. Rev. Food Sci. Nutr.* **2017**, *57*, 3104–3126. [CrossRef]
52. Bilušić, T.; Žanetić, M.; Ljubenković, I.; Generalić Mekinić, I.; Štambuk, S.; Bojović, V.; Soldo, B.; Magiatis, P. Molecular characterization of Dalmatian cultivars and the influence of the olive fruit harvest period on chemical profile, sensory characteristics and oil oxidative stability. *Eur. Food Res. Technol.* **2018**, *244*, 281–289. [CrossRef]
53. Amanpour, A.; Kelebek, H.; Selli, S. LC-DAD-ESI-MS/MS-based phenolic profiling and antioxidant activity in Turkish cv. Nizip Yaglik olive oils from different maturity olives. *J. Mass Spectrom.* **2018**, *227*–238. [CrossRef]
54. Lukić, I.; Krapac, M.; Horvat, I.; Godena, S.; Kosić, U.; Brkić Bubola, K. Three-factor approach for balancing the concentrations of phenols and volatiles in virgin olive oil from a late-ripening olive cultivar. *LWT Food Sci. Technol.* **2018**, *87*, 194–202. [CrossRef]
55. Lozano-Sánchez, J.; Amir, Y.; Fernández-Gutiérrez, A.; Bakhouch, A.; Bengana, M.; Segura-Carretero, A.; Youyou, A. Influence of olive ripeness on chemical properties and phenolic composition of Chemlal extra-virgin olive oil. *Food Res. Int.* **2013**, *54*, 1868–1875. [CrossRef]
56. Gutierrez-Rosales, F.; Romero, M.P.; Casanovas, M.; Motilva, M.J.; Mínguez-Mosquera, M.I. Metabolites involved in oleuropein accumulation and degradation in fruits of *Olea europaea* L.: Hojiblanca and Arbequina varieties. *J. Agric. Food Chem.* **2010**, *58*, 12924–12933. [CrossRef]
57. Jiménez, B.; Sánchez-Ortiz, A.; Rivas, A. Influence of the malaxation time and olive ripening stage on oil quality and phenolic compounds of virgin olive oils. *Int. J. Food Sci. Technol.* **2014**, *49*, 2521–2527. [CrossRef]

58. Artajo, L.S.; Romero, M.P.; Motilva, M.J. Transfer of phenolic compounds during olive oil extraction in relation to ripening stage of the fruit. *J. Sci. Food Agric.* **2006**, *86*, 518–527. [CrossRef]
59. Jiménez, B.; Sánchez-Ortiz, A.; Lorenzo, M.L.; Rivas, A. Influence of fruit ripening on agronomic parameters, quality indices, sensory attributes and phenolic compounds of Picudo olive oils. *Food Res. Int.* **2013**, *54*, 1860–1867. [CrossRef]
60. Bendini, A.; Cerretani, L.; Carrasco-Pancorbo, A.; Gómez-Caravaca, A.M.; Segura-Carretero, A.; Fernández-Gutiérrez, A.; Lercker, G. Phenolic molecules in virgin olive oils: A survey of their sensory properties, health effects, antioxidant activity and analytical methods. An overview of the last decade. *Molecules* **2007**, *12*, 1679–1719. [CrossRef] [PubMed]
61. Kanakis, P.; Termentzi, A.; Michel, T.; Gikas, E.; Halabalaki, M.; Skaltsounis, A.L. From olive drupes to olive Oil. An HPLC-orbitrap-based qualitative and quantitative exploration of olive key metabolites. *Planta Med.* **2013**, *79*, 1576–1587. [CrossRef] [PubMed]
62. Di Donna, L.; Benabdelkamel, H.; Mazzotti, F.; Napoli, A.; Nardi, M.; Sindona, G. High-throughput assay of oleopentanedialdehydes in extra virgin olive oil by the UHPLC-ESI-MS/MS and isotope dilution methods. *Anal. Chem.* **2011**, *83*, 1990–1995. [CrossRef] [PubMed]
63. Sánchez de Medina, V.; Miho, H.; Melliou, E.; Magiatis, P.; Priego-Capote, F.; Luque de Castro, M.D. Quantitative method for determination of oleocanthal and oleacein in virgin olive oils by liquid chromatography–tandem mass spectrometry. *Talanta* **2017**, *162*, 24–31. [CrossRef] [PubMed]
64. Karkoula, E.; Skantzari, A.; Melliou, E.; Magiatis, P. Direct measurement of oleocanthal and oleacein levels in olive oil by quantitative ¹H NMR. Establishment of a new index for the characterization of extra virgin olive oils. *J. Agric. Food Chem.* **2012**, *60*, 11696–11703. [CrossRef] [PubMed]
65. Godelmann, R.; Kost, C.; Patz, C.D.; Ristow, R.; Wachter, H. Quantitation of compounds in wine using ¹H NMR spectroscopy: Description of the method and collaborative study. *J. AOAC Int.* **2016**, *99*, 1295–1304. [CrossRef] [PubMed]
66. Uceda, M.; Frías, L. Epocas de recolección. Evolución del fruto y de la composición y calidad del aceite. (Seasons of harvest. Changes on fruit oil content, oil composition and oil quality). In *Proceeding of II Seminario Olícola Internacional*; International Olive-Oil Council: Cordoba, Spain, 1975.
67. Capriotti, A.L.; Cavaliere, C.; Crescenzi, C.; Foglia, P.; Nescatelli, R.; Samperi, R.; Laganà, A. Comparison of extraction methods for the identification and quantification of polyphenols in virgin olive oil by ultra-HPLC-QToF mass spectrometry. *Food Chem.* **2014**, *158*, 392–400. [CrossRef] [PubMed]

Sample Availability: Samples of the compounds are available from the authors.



© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).

4.1.3. Article 5: Influence of the ripening stage and extraction conditions on the phenolic fingerprint of 'Corbella' extra-virgin olive oil

Anallely López-Yerena, Antonia Ninot, Núria Jiménez-Ruiz, Julián Lozano-Castellón, Maria Pérez, Elvira Escribano-Ferrer, Agustí Romero-Aroca, Rosa M. Lamuela-Raventós, Anna Vallverdú-Queralt

Antioxidants, 10(6), 877, 2021

Abstract

Background: The ancient 'Corbella' olive variety, native to Bages in the center-north of Catalonia, is being recovered to obtain quality EVOO with unique organoleptic properties. EVOO produced exclusively with 'Corbella' olives has a powerful, fruity, and balanced taste. In recent years, centenary 'Corbella' olive trees have been recovered to promote the cultivation of this autochthonous variety and offer the market ecological products with added value.

Scope: The aim of this work was to determine the effect of agronomic and technical factors on the phenolic fingerprint of EVOO and to establish the optimum harvesting time and crushing and malaxation conditions for 'Corbella' olives.

Methods and results: Three different ripening indices (0.3, 1.2, and 3.2) and three crushing temperatures (10, 18, and 25 °C) were studied. Additionally, a factorial design to optimize the phenolic concentration of the EVOO was developed, applying a range of sieve diameters (4 and 6 mm), and malaxation time (30 and 60 min) and temperature (27, 32, and 37 °C). The phenolic profile was analyzed by ultra-high performance liquid chromatography coupled to mass spectrometry in a tandem detector (LC-MS/MS). The level of secoiridoids, the major PCs in the oil, was higher when using olives harvested earlier. Oleuropein aglycone and ligstroside aglycone were degraded during crushing at high temperatures, resulting in the formation of OLEA and OLC. The best processing conditions in terms of total phenolic content were found to be 30 min of malaxation at 37 °C, the crushing size not having any affect.

Conclusions: A negative correlation was found between the total phenols and higher crushing temperatures, although individual PCs such as OLC and OLEA increased. In summary, to obtain a 'Corbella' EVOO rich in PCs, an early harvest and low crushing temperature should be employed. On the contrary, if a higher concentration of specific phenols such as OLC or OLEA is desired a moderate crushing temperature should be used. Regarding malaxation conditions, the results suggest that a higher temperature (37 °C) increased the total phenol content and the concentration of the most relevant phenols. A short malaxation time (30 min) enhanced the total phenols and oleuropein and ligstroside aglycone, whereas a longer process (60 min) increased OLC concentration. Finally, a clear trend was not observed with respect to crushing size.



Article

Influence of the Ripening Stage and Extraction Conditions on the Phenolic Fingerprint of ‘Corbella’ Extra-Virgin Olive Oil

Anallely López-Yerena ^{1,†}, Antonia Ninot ^{2,†}, Núria Jiménez-Ruiz ¹, Julián Lozano-Castellón ^{1,3}, Maria Pérez ^{1,4}, Elvira Escribano-Ferrer ^{3,5}, Agustí Romero-Aroca ², Rosa M. Lamuela-Raventós ^{1,3} and Anna Vallverdú-Queralt ^{1,3,*}

¹ Department of Nutrition, Food Science and Gastronomy, XIA, Faculty of Pharmacy and Food Sciences, Institute of Nutrition and Food Safety (INSA-UB), University of Barcelona, 08028 Barcelona, Spain; naye.yerena@gmail.com (A.L.-Y.); nuriajimenru@gmail.com (N.J.-R.); julian.lozano@ub.edu (J.L.-C.); mariaperez@ub.edu (M.P.); lamuela@ub.edu (R.M.L.-R.)

² Institute of Agrifood Research and Technology (IRTA), Fruit Science Program, Olive Growing and Oil Technology Research Team, 43120 Constantí, Spain; Antonia.Ninot@irta.cat (A.N.); agusti.romero@irta.cat (A.R.-A.)

³ CIBER Physiopathology of Obesity and Nutrition (CIBEROBN), Institute of Health Carlos III, 28029 Madrid, Spain; eescribano@ub.edu

⁴ Laboratory of Organic Chemistry, Faculty of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain

⁵ Pharmaceutical Nanotechnology Group I+D+I Associated Unit to CSIC, Biopharmaceutics and Pharmacokinetics Unit, Department of Pharmacy and Pharmaceutical Technology and Physical Chemistry, Institute of Nanoscience and Nanotechnology (IN2UB), Faculty of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain

* Correspondence: avallverdu@ub.edu; Tel.: +34-934-024-508

† These authors equally contributed to the work.



Citation: López-Yerena, A.; Ninot,

A.; Jiménez-Ruiz, N.;

Lozano-Castellón, J.; Pérez, M.;

Escribano-Ferrer, E.; Romero-Aroca,

A.; Lamuela-Raventós, R.M.;

Vallverdú-Queralt, A. Influence of the

Ripening Stage and Extraction

Conditions on the Phenolic

Fingerprint of ‘Corbella’ Extra-Virgin

Olive Oil. *Antioxidants* **2021**, *10*, 877.

[https://doi.org/10.3390/](https://doi.org/10.3390/antiox10060877)

[antiox10060877](https://doi.org/10.3390/antiox10060877)

Academic Editors: Monica

Rosa Loizzo and Rosa Tundis

Received: 6 May 2021

Accepted: 26 May 2021

Published: 30 May 2021

Publisher’s Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors.

Licensee MDPI, Basel, Switzerland.

This article is an open access article

distributed under the terms and

conditions of the Creative Commons

Attribution (CC BY) license ([https://](https://creativecommons.org/licenses/by/4.0/)

[creativecommons.org/licenses/by/](https://creativecommons.org/licenses/by/4.0/)

[4.0/](https://creativecommons.org/licenses/by/4.0/)).

Abstract: The ancient ‘Corbella’ olive variety from the center-north of Catalonia is being recovered to obtain quality extra-virgin olive oil (EVOO) with unique organoleptic properties. The aim of this work was to determine the effect of agronomic and technical factors on the phenolic fingerprint of EVOO and to establish the optimum harvesting time and crushing and malaxation conditions for ‘Corbella’ olives. Therefore, three different ripening indices (0.3, 1.2, and 3.2) and three crushing temperatures (10, 18, and 25 °C) were studied. Additionally, a factorial design to optimize the phenolic concentration of the EVOO was developed, applying a range of sieve diameters (4 and 6 mm), and malaxation time (30 and 60 min) and temperature (27, 32, and 37 °C). The phenolic profile was analyzed by ultra-high performance liquid chromatography coupled to mass spectrometry in a tandem detector. The level of secoiridoids, the major phenolic compounds in the oil, was higher when using olives harvested earlier. Oleuropein aglycone and ligstroside aglycone were degraded during crushing at high temperatures, resulting in the formation of oleacein and oleocanthal. The best processing conditions in terms of total phenolic content were found to be 30 min of malaxation at 37 °C, the crushing size not having any affect.

Keywords: polyphenols; malaxation; crushing size; oleocanthal; oleacein

1. Introduction

The demand for extra virgin olive oil (EVOO) is increasing due to growing consumer awareness of the health benefits of the Mediterranean diet, in which it is the main source of dietary fat [1]. The nutritional and health-promoting properties of EVOO [2,3] are mainly correlated with its highly bioactive components, such as monounsaturated fatty acids (55–83%), unsaponifiable compounds (1–2%), and soluble or hydrophilic compounds, including α -tocopherol, phenolic compounds, and other compounds with antioxidant properties [4]. In 2012, the European Food Safety Authority (EFSA) recognized that a daily

consumption of 5 mg hydroxytyrosol and/ or its derivatives (e.g., the oleuropein complex and tyrosol) per 20 g of olive oil afforded protection of LDL particles from oxidative damage and maintenance of a normal blood HDL-cholesterol concentration [5].

Since this recognition, the scientific community and olive oil industry have focused considerable efforts on optimizing the EVOO extraction process, searching for a balance between yield and quality [6]. To date, many studies have shown that the phenolic composition of EVOO depends on a highly complex multivariate interaction between agronomic, biochemical, and technological factors [7–10]. Firstly, the composition of the olives depends on their genetic origin, the geographical area of cultivation and its climate, and agronomic practices such as fertilization and water availability [11,12]. Then, the parameters associated with EVOO quality can be controlled by regulating the main variables of mechanical extraction (temperature, oxygen, enzymatic activities, and the disruption level of olive cell tissues) [13,14]. In this context, crushing is a determinant factor in the release and activation of endogenous enzymes (β -glycosidases and oxidoreductases), whereas malaxation is crucial for the modulation of the enzymatic activity [14–17] and production of high quality EVOO. During malaxation, the chemical composition of the oil undergoes considerable changes due to partition phenomena between oil and water, as well as reactions catalyzed by the fruit enzymes released during crushing [13].

Given the above, the main novelty of this work lies in the study of both agronomic and technological variables to obtain EVOO with a high content of phenolic compounds. For this purpose, the objectives of this study were (i) to determine the effect of three different ripening indices (RI, 0.3, 1.2, and 3.2) on the phenolic profile of EVOO, (ii) to evaluate changes in polyphenol content of EVOO obtained at three crushing temperatures (10, 18, and 25 °C), and (iii) to optimize the malaxation parameters (time and temperature) and crushing size to obtain an EVOO with higher phenolic compounds content. Regarding this last objective, a factorial design was developed to optimize the time (30 and 60 min) and temperature (27, 32, and 37 °C) of malaxation, applying two sieve diameters (4 and 6 mm). The study was carried out with olives from the ‘Corbella’ cultivar, native to Bages in the center-north of Catalonia, an ancient variety that was abandoned and at risk of extinction. EVOO produced exclusively with ‘Corbella’ olives has a powerful, fruity, and balanced taste. In recent years, centenary ‘Corbella’ olive trees have been recovered to promote the cultivation of this autochthonous variety and offer the market ecological products with added value.

2. Materials and Methods

2.1. Chemicals and Reagents

Regarding the secoiridoids, oleocanthal ($\geq 95\%$ purity) was purchased from Merck (Darmstadt, Germany), while oleacein and oleuropein aglycone (≥ 90 and 95% purity, respectively) from Toronto Research Chemical Inc. (ON, Canada). Luteolin was acquired from Sigma-Aldrich (Madrid, Spain), ferulic acid and apigenin from Fluka (Buchs, Switzerland), and hydroxytyrosol from Extrasynthese (Genay, France). Hexane, methanol, and acetonitrile were purchased from Sigma-Aldrich. Ultrapure water was obtained using a Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2. Plant Material

Olive fruits were hand collected from mature olive trees of the ‘Corbella’ cultivar in the Oil Migjorn farm in Bages, Barcelona Province, Spain. The olives used to study the crushing temperature, crushing size, and malaxation were cultivated under controlled deficit irrigation conditions, whereas those used for the ripening study were obtained from non-irrigated trees. On the day of harvesting, the olives were sent to the IRTA laboratories in Constantí, Tarragona, where, after a health assessment, they were treated as described below.

2.3. Oil Extraction

All the fruit samples were processed using an Abencor analyser (Abengoa S.A., Seville, Spain), which reproduces the industrial process on a laboratory scale. The fruits were milled using a stainless-steel rotatory hammer mill operating at 3000 rpm and equipped with different sieves, depending on the experiment. The resulting olive paste was immediately passed into a malaxation unit with blades spinning at 50 rpm. The time and temperature of this step were set depending on each experiment. A basket centrifuge (3000 rpm for 3 min) was used to centrifuge the kneaded olive paste without addition of hot water or talcum. After centrifugation, the oil was decanted and stored in amber glass bottles at -20°C in darkness and without headspace until analysis.

2.3.1. Ripening Study

The fruits were collected at 3 different stages of maturity and, following the method described by Uceda and Frías [18], the 3 RI were calculated to be 0–1, 1–2, and 2–3. For each RI, the olives were crushed in a 5 mm diameter sieve, and the olive paste was placed in a 28°C malaxator for 30 min without the addition of talcum powder or hot water. The centrifugation and storage conditions were as described above (Section 2.2).

2.3.2. Crushing Temperature Study

Healthy olives were stored for two hours in a fridge (7°C) and then washed using clean water heated to three different temperatures: (10, 18, and 25°C) in a water bath. Once the desired temperature was reached, fruits were crushed in a 5 mm diameter sieve, and the resulting paste was placed in a malaxator at $18\text{--}19^{\circ}\text{C}$ for 30 min without adding either talcum or water. It was then centrifuged at 3000 rpm for 3 min without adding water. The EVOO obtained was separated and stored under the conditions described above (Section 2.2).

2.3.3. Crushing Size and Malaxation Study

The olive oil was extracted following a factorial model with three factors: crushing size (4 and 6 mm), malaxation temperature (27, 32, and 37°C), and malaxation time (30 and 60 min). The trials were performed using a 3.3 kg batch of drupes. Three representative olive samples, each weighing a minimum of one kilogram, were processed, and the corresponding EVOOs were obtained using an Abencor analyser (Abengoa S.A., Seville, Spain). The olive fruits were milled using a stainless-steel hammer mill equipped with a 4- and 6-mm sieve that was operated at 3000 rpm. The resulting olive paste was immediately kneaded in a mixer at 50 rpm for 30 or 60 min at 27, 32, or 37°C . The centrifugation and storage conditions were as described above (Section 2.2).

2.4. Phenolic Compound Composition

After a liquid–liquid extraction, the identification and quantification of phenolic compounds were carried out by liquid chromatography coupled to mass spectrometry in tandem mode (LC-MS/MS), as described in our previous studies [11,19]. Samples were analyzed using an AcquityTM UPLC (Waters; Milford, MA, EUA) system with an API 3000 triple-quadrupole mass spectrometer (PE Sciex, Concord, Ontario, Canada). The system was equipped with a turbo ion spray source. Separation of phenolic compounds was conducted using an Acquity UPLC[®] BEH C₁₈ column (2.1 × 50 mm, i.d., 1.7 μm particle size) and Acquity UPLC[®] BEH C₁₈ Pre-Column (2.1 × 5 mm, i.d., 1.7 μm particle size) (Waters Corporation[®], Wexford, Ireland).

The liquid–liquid extraction was carried out following procedures described previously [19]. For this purpose, 0.5 g of EVOO was dissolved in hexane (oil/hexane 1:2, w/v) in a 10 mL centrifuge tube and shaken for 1 min. For the phenolic compound extraction, 2 mL of MeOH: H₂O (4:1 v/v) was added and the samples were homogenized for 1 min. The two phases were separated by centrifugation (3000 rpm, 4°C , 4 min). The methanolic fraction was separated and subjected to a second cleaning using hexane, while the remain-

ing phenolic compounds in the hexane fraction were extracted by a further addition of MeOH: H₂O (4:1 v/v). The extracts were shaken and centrifuged under the same conditions as previously described. The two methanolic fractions were pooled and reduced to dryness with N₂, redissolved in 800 µL of ACN, and stored at −80 °C prior to analysis.

The separation of oleocanthal, oleacein, ligstroside, and oleuropein aglycone was performed following the procedure proposed by Lozano-Castellon [19]. MeOH (A) and H₂O (B) were used as mobile phases, both with 0.1% of formic acid. An increasing linear gradient (v/v) of B was used (t (min), %B), as follows: (0, 100); (2, 100); (4.75, 46.4); (4.9, 100); (5.9, 0); (6, 100); (6.5, 100), at 0.6 mL/min, 5 µL, and 50 °C of constant flow rate, injection volume, and column temperature, respectively. The separation of other polyphenols was achieved following procedures described previously [11] using H₂O with 0.2% acetic acid (A) and ACN (B) as mobile phases. An increasing linear gradient (v/v) of B was used (t (min), %B), as follows: (0, 5); (2.5, 5); (12.5, 40); (12.6, 100); (13.5, 100); (13.6, 5); (15, 5), at 0.4 mL/min, 5 µL, and 40 °C of constant flow rate, injection volume, and column temperature, respectively.

The ionization was achieved using an electrospray (ESI) interface operating in negative ionization mode (M[−]H), and the compounds were monitored in the multiple monitoring mode (MRM). The system was controlled by Analyst version 1.4.2 software supplied by Applied Biosystems. The calibration curves were prepared in refined olive oil and were linear over the concentration range 0–20 mg/mL using hydroxytyrosol, apigenin, luteolin, oleocanthal, oleacein, and oleuropein aglycone as standards.

2.5. Statistical Analyses

The normality of the data was tested by the Q–Q plot diagnostic graphic. After viewing the results, all subsequent tests were non-parametric. In the ripening and crushing study, to assess differences between groups, a Kruskal–Wallis test was performed, and the post-hoc test was the pairwise Mann–Whitney U-test. To investigate the main effects of the investigated factors, as well as to determine possible interactions between them, phenolic concentration analysis data were subjected to three-way analysis of variance (ANOVA), with 2 crushing size, malaxation temperature, and malaxation duration as factors (3 malaxation temperatures × 2 malaxation durations × 2 crushing size × 3 replicates). Means were compared by least significant difference (LSD) test, at the level of $p < 0.05$. All the statistical analyses were performed with R Project for Statistical Computing 3.6.0.

3. Results and Discussion

3.1. Ripening Study

The effects of ripening on the phenolic concentrations are shown in Figure 1. The total phenolic content was affected negatively by ripening stage, decreasing up to 49% at 3.2 of RI (from 566.74 ± 51.45 to 289.48 ± 17.43 mg·kg^{−1}) ($p \leq 0.05$). The same behaviour was observed for the secoiridoids' and phenolic alcohols; concentration. In contrast, the maximum concentration of flavones and lignans was reached at 3.2 of RI, whereas the highest phenolic acid content was obtained with olives from the second harvest with an RI of 1.2 (2.16 ± 0.06 mg·kg^{−1}).

The RI of the olive fruits affected the total amount of phenolic compounds in the EVOO, which was 43.64% lower ($p \leq 0.05$) when produced from the third harvest. This could be caused by endogenous enzymes in the olives, such as esterases, polyphenol oxidases, and β-glucosidases, which degrade phenols during the maturation process [13,14,16]. These results are in agreement with the literature [16,20–22], including a study carried out with 'Frantoio' and 'Manzanilla' EVOO, in which a gradual decrease in phenolic compounds was observed from the first to the fifth harvest [20]. In another study, however, this tendency did not begin below an RI of 2.5–3 [16]. It has also been suggested that the phenolic content is more influenced by the cultivar than the degree of fruit maturity [23]. It should be mentioned that the fruit of some olive cultivars ripens at an uneven rate, and therefore the

same harvest will contain olives of variable ripeness [24], rendering the RI a poor descriptor for analysis. The ‘Corbella’ cultivar exhibits a very uniform ripening pattern [25].

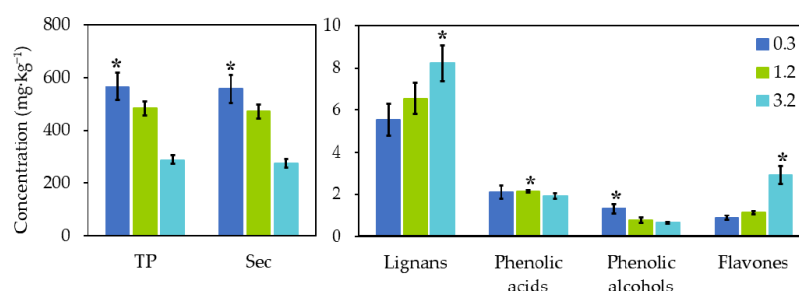


Figure 1. Effect of the ripening index on the total phenolic content and the phenolic composition of EVOO. TP: total phenols; SEC: secoiridoids. * indicates significant differences between phenolic concentration ($p < 0.05$).

The RI effect on the concentrations of individual phenolic compounds is shown in Table 1. The concentration of secoiridoids, the prevalent phenolic group found in EVOO and associated with many of its benefits [26], was greatly affected by the harvest time, as in other studies [13,27,28]. All the secoiridoids decreased as the RI increased, except for hydroxyoleuropein aglycone. The explanation may be that the enzymatic degradation of oleuropein and ligstroside results in a higher concentration of the aglycone.

Table 1. Phenolic compound contents ($\text{mg} \cdot \text{kg}^{-1}$) of EVOO produced from ‘Corbella’ olives with different crushing temperatures.

Group/Compound	Ripening Index		
	0.3 ± 0.07	1.2 ± 0.17	3.2 ± 0.27
Total Polyphenols	566.74 ± 51.45 ^a	483.72 ± 26.65 ^b	289.48 ± 17.43 ^c
Secoiridoids	556.81 ± 51.45 ^a	473.07 ± 26.17 ^b	275.72 ± 17.43 ^c
Elenolic acid	272.12 ± 9.71 ^a	228.82 ± 16.30 ^b	125.52 ± 9.89 ^c
Hydroxyelenolic acid	8.68 ± 0.38 ^a	8.79 ± 0.29 ^a	4.32 ± 0.21 ^b
Hydroxyoleuropein aglycone	1.11 ± 0.09 ^b	1.16 ± 0.07 ^b	2.03 ± 0.07 ^a
Ligstroside aglycone	15.07 ± 0.84 ^a	11.92 ± 0.47 ^b	9.00 ± 0.51 ^c
Methyloleuropein aglycone	0.92 ± 0.04 ^a	0.88 ± 0.02 ^a	0.87 ± 0.02 ^a
Oleacein	94.25 ± 6.29 ^a	78.72 ± 4.48 ^b	34.06 ± 2.06 ^c
Oleocanthal	9.14 ± 1.20 ^a	6.84 ± 0.31 ^b	2.36 ± 0.21 ^c
Oleuropein aglycone	155.51 ± 5.71 ^a	135.95 ± 2.98 ^a	97.56 ± 3.75 ^b
Phenolic alcohols	1.34 ± 0.21 ^a	0.78 ± 0.11 ^b	0.66 ± 0.04 ^c
Hydroxytyrosol	1.27 ± 0.08 ^a	0.68 ± 0.06 ^b	0.46 ± 0.05 ^c
Hydroxytyrosol acetate	0.08 ± 0.02 ^b	0.11 ± 0.02 ^b	0.21 ± 0.02 ^a
Flavones	0.90 ± 0.09 ^c	1.15 ± 0.08 ^b	2.92 ± 0.41 ^a
Apigenin	0.49 ± 0.01 ^c	0.82 ± 0.06 ^b	2.23 ± 0.2 ^a
Luteolin	0.41 ± 0.02 ^b	0.32 ± 0.04 ^b	0.69 ± 0.04 ^a
Phenolic acids	2.12 ± 0.32 ^{ab}	2.16 ± 0.06 ^a	1.93 ± 0.15 ^b
m-coumaric acid	1.53 ± 0.04 ^a	1.47 ± 0.03 ^a	1.42 ± 0.03 ^b
p-coumaric acid	0.59 ± 0.05 ^{ab}	0.69 ± 0.04 ^a	0.50 ± 0.04 ^b
Lignans	5.57 ± 0.76 ^b	6.56 ± 0.75 ^b	8.24 ± 0.85 ^a
Pinosresinol	5.57 ± 0.76 ^b	6.56 ± 0.75 ^b	8.24 ± 0.85 ^a

Different letters within the same row mean significant differences (p -value < 0.05) according to the Kruskal–Wallis test.

Among the phenolic alcohols, hydroxytyrosol decreased with maturation. In contrast, hydroxytyrosol acetate increased, probably because it can form part of more complex compounds such as oleacein, oleuropein, and verbascoside, and is released when they are

broken down [29]. Due to its powerful radical scavenging activity, hydroxytyrosol acetate plays an important role in the anti-inflammatory effect of EVOO and is able to downregulate cyclooxygenase-2 expression [30]. In the case of hydroxytyrosol, which is also a degradation product, its reduced concentration could be caused by its higher lability. This effect is of interest, as according to EFSA, the consumption of at least 5 mg of hydroxytyrosol and its derivatives is required to protect LDL particles from oxidative damage [5]. Therefore, to preserve the health benefits of EVOO, a higher RI is not recommended, as the resulting reduction of hydroxytyrosol far exceeds the increase in the acetate derivative.

In the case of flavones, an upward trend was observed, as in other studies [31,32]. Bengana et al., 2013 [33] reported an increase in the flavonoid concentration in EVOO produced from olives in the first period of maturation, although levels began to decline when the harvest was in December. This tendency was not observed in our work, as all the olives were collected before the end of October. The glycosidic forms of flavones found in the drupelet become more soluble in oil during ripening, when the bond with the sugar is broken down by β -glucosidase. Thus, with flavones in oil being retained to a higher degree than in water during the extraction, oils from late season olives have a higher flavone content. However, other studies report that while luteolin increased, apigenin levels remained unchanged in 'Hojiblanca', 'Picual', and 'Picudo' oil varieties [34] or even decreased in the 'Arbequina' variety [35]. Such variable maturation behaviour can be attributed to differences in composition and endogenous enzymes among varieties [23,36]. Additionally, accurate comparisons of results from different studies are not always possible, as some refer only to the harvest date but not the RI [23].

The level of phenolic acids showed a slight decrease from the first to the second RI, as reported elsewhere [32]. In contrast, other studies have found an increase, associated with the activity of hydrolytic enzymes in the drupelet [28,37].

Among the lignans, pinoresinol increased by approximately 40.75% over the three ripening stages, possibly because of the degradation of the coexisting acetoxypinoresinol [38], whose levels were below the detection limit. Pinoresinol is a very stable compound and is preserved during ripening. Lignans are reported to have an inhibitory activity against cancer cell growth and antiestrogenic effects [39,40].

3.2. Crushing Temperature Study

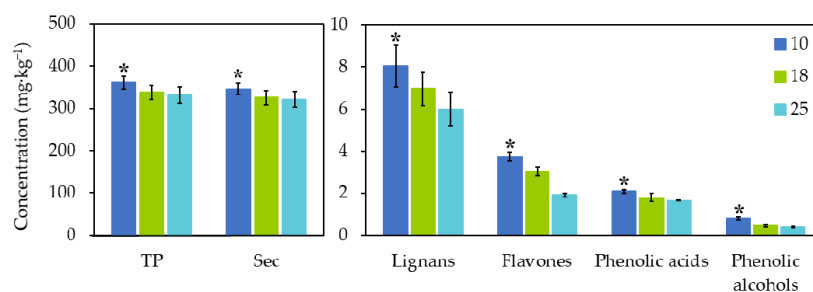
The crushing of the olive fruit is the first operation in the extraction of olive oil. The disruption of fruit tissues releases the oil and triggers the enzymatic reactions that shape the profile and concentration of phenolic and volatile compounds [41]. Table 2 presents the effect of the three different crushing temperatures on the phenolic profile of EVOO. As shown in Figure 2, the total phenols decreased with higher temperatures, with a significant difference observed between 10 and 18 °C (360.70 ± 15.00 to 337.55 ± 17.00 mg·kg⁻¹) ($p \leq 0.05$), but not between 18 and 25 °C. All the phenolic groups (secoiridoids, phenolic alcohols, flavones, phenolic acids, and lignans) followed the same trend ($p \leq 0.05$). Similarly, a study on olive oil of the 'Cima di Bitonto' cultivar, applying three crushing temperatures (12, 16, and 20 °C), reported a gradual decrease in the phenolic content [42]. The overall quality of EVOO, attributed to its content of minor compounds, can therefore be modulated by using the appropriate crushing conditions [43]. A study carried out by Caponio and Catalano [44] showed that higher temperatures in the crusher during olive processing led to a shorter preservation of the oils, mainly attributed to a higher degree of auto-oxidation.

The secoiridoids oleuropein aglycone and ligstroside aglycone underwent enzymatic degradation at higher crushing temperatures, resulting in the formation of oleacein and oleocanthal. During crushing and malaxation, β -glucosidases and esterases are released from the olive flesh and pit and can readily interact with phenols, the reaction kinetics increasing with the temperature [45]. A high concentration of oleocanthal and oleacein in EVOO might be desirable due to their health properties [8,26].

Table 2. Phenolic compound contents (mg·kg^{−1}) of EVOO produced from ‘Corbella’ olives using different crushing temperatures.

Group/Compound	Crushing Temperature (°C)		
	10	18	25
Total Polyphenols	360.70 ± 15.00 ^a	337.55 ± 17.00 ^b	331.23 ± 20.00 ^b
Secoiridoids	346.03 ± 14.00 ^a	325.30 ± 17.00 ^b	321.28 ± 19.00 ^b
Elenolic acid	191.00 ± 8.00 ^a	175.00 ± 10.00 ^{ab}	171.00 ± 4.00 ^b
Hydroxyelenolic acid	7.44 ± 0.50 ^a	6.06 ± 0.50 ^b	6.13 ± 0.30 ^b
Hydroxyoleuropein aglycone	2.14 ± 0.08 ^a	1.72 ± 0.10 ^b	1.74 ± 0.08 ^b
Ligstroside aglycone	10.00 ± 0.20 ^a	8.96 ± 0.50 ^b	8.47 ± 0.10 ^c
Methyloleuropein aglycone	0.87 ± 0.01 ^a	0.87 ± 0.01 ^a	0.87 ± 0.00 ^a
Oleacein	16.50 ± 0.60 ^c	29.70 ± 2.00 ^b	35.20 ± 1.00 ^a
Oleocanthal	0.95 ± 0.09 ^c	2.05 ± 0.1 ^b	2.42 ± 0.20 ^a
Oleuropein aglycone	117.10 ± 2.96 ^a	97.20 ± 5.00 ^b	95.10 ± 2.00 ^b
Phenolic alcohols	0.79 ± 0.10 ^a	0.45 ± 0.05 ^b	0.39 ± 0.04 ^b
Hydroxytyrosol	0.59 ± 0.04 ^a	0.28 ± 0.03 ^b	0.29 ± 0.04 ^b
Hydroxytyrosol acetate	0.20 ± 0.02 ^a	0.16 ± 0.01 ^b	0.10 ± 0.01 ^c
Flavones	3.74 ± 0.20 ^a	3.04 ± 0.20 ^b	1.91 ± 0.09 ^c
Apigenin	2.85 ± 0.20 ^a	2.28 ± 0.10 ^b	1.43 ± 0.07 ^c
Luteolin	0.89 ± 0.09 ^a	0.67 ± 0.04 ^b	0.49 ± 0.05 ^c
Phenolic acids	2.08 ± 0.09 ^a	1.79 ± 0.20 ^b	1.65 ± 0.01 ^b
m-coumaric acid	1.49 ± 0.04 ^a	1.43 ± 0.04 ^{ab}	1.38 ± 0.03 ^b
p-coumaric acid	0.60 ± 0.06 ^a	0.33 ± 0.02 ^b	0.27 ± 0.04 ^b
Lignans	8.06 ± 1.00 ^a	6.97 ± 0.80 ^{ab}	5.99 ± 0.80 ^b
Pinoresinol	8.06 ± 1.00 ^a	6.97 ± 0.80 ^{ab}	5.99 ± 0.80 ^b

Different letters within the same row mean signif cant differences (p-value < 0.05) according to the Kruskal–Wallis test.

**Figure 2.** Effect of crushing temperature on the total phenolic content and the phenolic composition of EVOO. TP: total phenols; Sec: secoiridoids. * indicates signif cant differences between phenolic concentration (p < 0.05).

The levels of the phenolic alcohols hydroxytyrosol and hydroxytyrosol acetate decreased by 50.77 and 49.02% at 18 and 25 °C, respectively. Hydroxytyrosol underwent a great change between 10 and 18 °C, whereas the acetate derivative declined progressively. The EVOO concentration of flavones at 25 °C was 48.93% lower than at 10 °C. Similarly, in a study of ‘Ayvalik’ oils, malaxation at 27 °C produced oils with a higher concentration of luteolin than at 37 and 47 °C [46]. Accordingly, higher processing temperatures reduce the content of flavones in olive oil.

The effect on the phenolic acids was comparable to that observed in total phenols, with the most substantial degradation recorded at 18 °C, and only a slight further reduction at 25 °C. These results reflect that at lower temperatures phenolic acids are stable or degrade very little. Finally, although less drastically, lignans also declined with higher temperatures, in contrast with another study that found their concentration was unaltered by changes in temperature during malaxation [47].

3.3. Crushing Size and Malaxation Study

Considerable research has been carried out to clarify which factors most affect the concentration of phenolic compounds in EVOO, with the aim of maximizing its health properties. In this context, many studies have investigated the impact of malaxation temperature and time, together with either the time of harvest [48], extraction techniques [49], or the degree of ripeness [27,50], on the concentration of total and individual phenols. Crushing size has been evaluated to a lesser extent, often together with the hammer mill rotor speed [41,51]. Therefore, by analyzing the influence of the temperature and time of malaxation, as well as the crushing size, our study opens a new three-factor approach to optimizing the phenol content of EVOO.

The effect of malaxation temperature (27, 32, and 37 °C), malaxation time (30 and 60 min) and crushing size (4 and 6 mm) on the total and individual phenol content is presented in Table 3. In general, the malaxation temperature affected the content of total and individual phenols, except for ligstroside aglycone and luteolin ($p > 0.05$), whereas the duration was the only factor that modified the concentration of all the compounds analyzed and consequently the total polyphenol concentration ($p < 0.05$). Crushing size did not affect the concentration of ligstroside and oleuropein aglycone, luteolin, and pinoresinol ($p > 0.05$).

Concerning the factorial interaction effects, temperature*time modified the concentration of hydroxybenzoic acid, oleocanthal, hydroxytyrosol, and apigenin, but to a low extent ($p < 0.05$). Mixing temperature * crushing size affected the final concentration of up to 70% of phenolic compounds and the total polyphenols. Finally, malaxation time*crushing size significantly affected the concentration of total polyphenols, hydroxybenzoic acid, oleocanthal, oleacein, oleuropein aglycone, and hydroxytyrosol ($p < 0.05$).

The temperature*time*size interaction affected the content of total phenols and oleocanthal, oleacein, oleuropein aglycone, hydroxytyrosol, and apigenin. The factorial design showed that the EVOOs with the highest phenolic contents were obtained after 30 min of malaxation at 37 °C, the amount being $513.43 \pm 70.89 \text{ mg}\cdot\text{kg}^{-1}$ for a crushing size of 4 mm and $503.21 \pm 66.72 \text{ mg}\cdot\text{kg}^{-1}$ for 6 mm; conversely, the lowest content was obtained at 32 °C (Figure 3). The final concentration of phenolic compounds in EVOO probably depends on the equilibrium between their transfer to the oil phase and oxidative degradation [52]. A similar positive influence of a higher malaxation temperature has been reported [17,49,52], although some studies found that temperatures beyond 30 °C resulted in a lower phenolic concentration due to an enhanced activity of endogenous peroxidase (POD) and polyphenol oxidase (PPO) enzymes [17,47,53–56]. In addition, a short malaxation time increased the total phenol content (Figure 3), as in other studies [49,56,57], whereas a long malaxation time is rarely reported to have a positive effect [58]. The benefits of a brief malaxation can be attributed to a reduced activity of the hydrolytic and oxidative enzymes (POD and PPO) and less diffusion of the phenols to the water phase [56,57]. In our study, it was also demonstrated that the crushing size (4 and 6 mm) barely affected the total phenol content when the malaxation conditions were 37 °C for 30 min, in accordance with results in the literature [41,43,50].

Table 3. Effect of malaxation temperature, malaxation time, crushing size, and their interaction on phenolic compound contents (mg·kg^{−1}) in EVOO produced from ‘Corbella’ olives.

Compound	MTE			MT			CS			MTE × MT			MTE × CS			MT × CS			MTE × MT × CS		
	p Value	F Value	p Value	p Value	F Value	p Value	p Value	F Value	p Value	p Value	F Value	p Value	p Value	F Value	p Value	p Value	F Value	p Value	p Value	F Value	
Total Polyphenols	<0.001	218.2	<0.001	<0.001	96.7	0.0013	11.39	0.1351	2.29	<0.001	21.93	<0.001	<0.001	32.83	<0.001	<0.001	32.67	<0.001	<0.001	32.67	
Ellenoic acid	<0.001	34.4	<0.001	<0.001	76.14	0.0179	5.92	0.1762	1.87	<0.001	37.95	<0.001	<0.001	1.57	<0.001	<0.001	1.75	<0.001	<0.001	1.75	
Hydroxyellenolic acid	<0.001	103.58	<0.001	<0.001	89.65	0.0019	10.59	0.0005	13.46	<0.001	4.21	0.0445	0.0013	11.36	0.0013	0.0013	0.72	0.0013	0.0013	0.72	
Ligstroside aglycone	0.1559	2.07	<0.001	<0.001	57.75	0.2206	1.53	0.4396	0.61	0.6632	0.19	0.3395	0.3395	0.93	0.3395	0.3395	2.59	0.1113	0.1113	2.59	
Oleacein	<0.001	1271.08	<0.001	<0.001	147.99	<0.001	35.66	0.4081	0.69	0.0001	18.05	0.0001	<0.001	66.25	<0.001	<0.001	60.79	<0.001	<0.001	60.79	
Oleocanthal	<0.001	432.54	<0.001	<0.001	45.1	<0.001	30.78	0.0008	12.38	0.0001	18.95	0.0001	<0.001	64.67	<0.001	<0.001	71.49	<0.001	<0.001	71.49	
Oleuropein aglycone	0.0002	15.88	<0.001	<0.001	50.93	0.6893	0.16	0.6681	0.19	0.8331	0.04	0.8331	0.04	7.65	0.0075	0.0075	8.39	<0.001	<0.001	8.39	
Hydroxytyrosol	<0.001	46.77	<0.001	<0.001	21.08	0.0032	9.44	0.023	5.44	<0.001	266.07	<0.001	<0.001	9.16	0.0036	0.0036	15.34	0.0002	0.0002	15.34	
Apigenin	0.0007	12.92	0.0001	0.0001	18.33	0.0191	5.8	0.0036	9.16	0.3479	0.89	0.3479	0.89	1.15	0.2888	0.2888	7.02	0.0103	0.0103	7.02	
Luteolin	0.1896	1.76	0.0171	0.0171	6.02	0.3164	1.02	0.6117	0.26	<0.001	25.01	<0.001	<0.001	4.69	0.1389	0.1389	2.34	0.1310	0.1310	2.34	
Pinosresinol	0.0012	11.49	0.0223	0.0223	5.5	0.1407	2.23	0.9473	<0.001	<0.001	32.07	<0.001	<0.001	0.82	0.3674	0.3674	0.99	0.3233	0.3233	0.99	

MTE: malaxation temperature; MT: malaxation time and CS: crushing size.

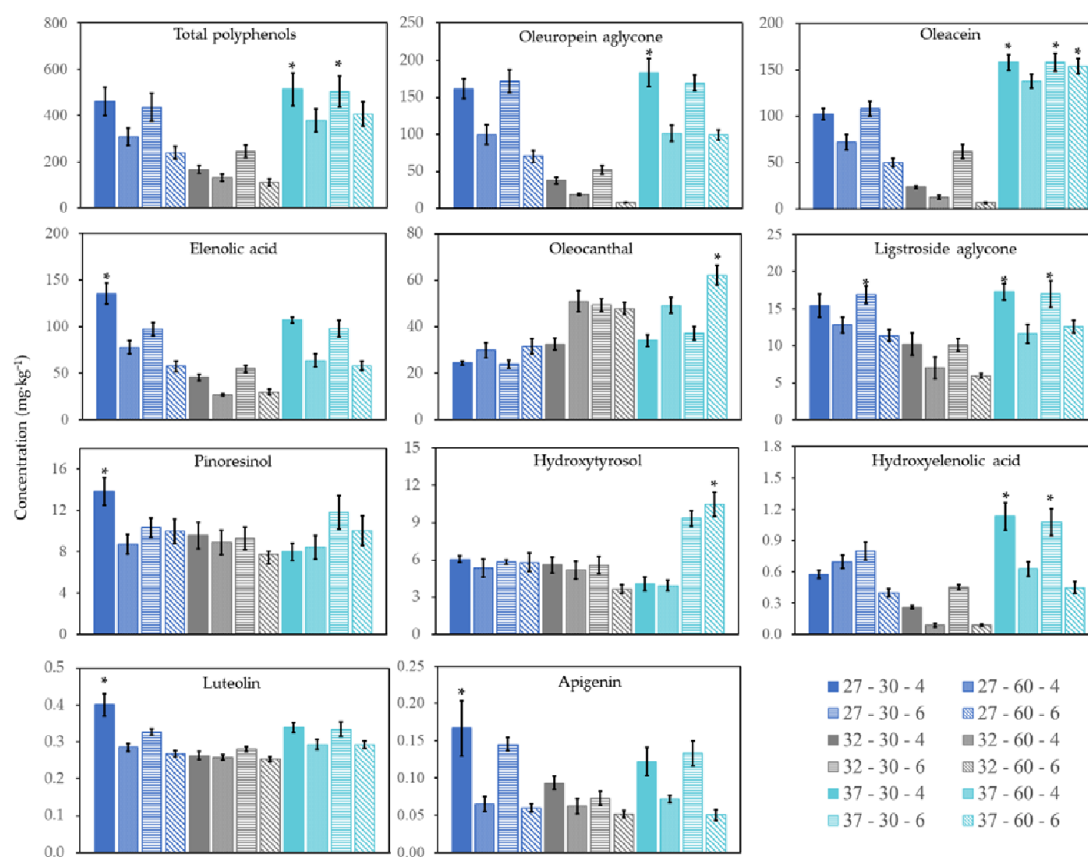


Figure 3. Effect of malaxation temperature (27, 32, and 37 °C), malaxation time (30 and 60 min), and crushing size (4 and 6 mm) on the total phenol content and the phenolic profile of EVOO. * indicates significant differences between phenolic concentration ($p < 0.05$).

Regarding the secoiridoids, the highest concentration of oleuropein aglycone was obtained after 30 min of malaxation at 37 °C applying a crushing size of 4 mm ($183.27 \pm 18.99 \text{ mg}\cdot\text{kg}^{-1}$). On the other hand, three treatments provided the best conditions to obtain an EVOO rich in ligstroside aglycone and oleacein. In the case of ligstroside aglycone, two treatments with the highest temperature (37 °C, 30 min, and 4 or 6 mm) and one with the lowest (27 °C, 30 min, and 6 mm) gave the best results. These findings are in line with the analysis of the factorial and interaction effects, where only the malaxation time significantly modified the concentration of this aglycone ($p < 0.05$). In the case of oleacein, its EVOO concentration was favored by the highest temperature (37 °C, 30 or 60 min, and 4 or 6 mm) (Figure 3). The optimal conditions for oleocanthal were 60 min at 37 °C with a crushing size of 6 mm. Finally, the highest concentration of elenolic acid ($135.52 \pm 11.33 \text{ mg}\cdot\text{kg}^{-1}$) was obtained using conditions of 27 °C, 30 min, and 4 mm and hydroxyelenolic acid with 37 °C, 30 min, and 4 or 6 mm.

Other studies have reported that higher malaxation temperatures may (i) increase the partition coefficient between oil and water phases in olive paste [59], (ii) reduce the activity of PPO enzymes [17], (iii) boost the release of phenols from the cell wall of polysaccharides

and other olive tissues [15,17], and (iv) increase enzymatic activity of β -glucosidases and esterases. β -glucosidases catalyze the hydrolysis of oleuropein and ligstroside, leading to their aglycone forms, and also contribute to the formation of oleacein and oleocanthal in combination with esterases [60]. The observed increase in concentration of most of the secoiridoids can be attributed to the reasons mentioned above.

Regarding duration, a longer malaxation noticeably reduced the content of secoiridoids, except for oleocanthal. An excessive malaxation time can result in undesired reactions catalyzed by PPO and phenoloxidases [17,57,61] by increasing olive paste exposure to air. Moreover, prolonged contact between the paste and water favors the diffusion of phenols into the aqueous phase [57,61]. Previous studies have reported a similar increase in oleocanthal, with other secoiridoids showing either a weak dependence on the malaxation duration [27,50] or a decreasing trend over time [48,57]. In summary, the oils with the highest secoiridoid content, except for elenolic acid, were produced by a malaxation temperature of 37 °C. These results are in agreement with Boselli et al. (2009), but again they disagree with the conclusions of other studies that describe a negative impact of higher temperatures on secoiridoids, probably due to an enhanced rate of degradation [52].

The hydroxytyrosol content was also affected by extraction conditions (Figure 3), the highest concentration ($10.45 \pm 0.98 \text{ mg} \cdot \text{kg}^{-1}$) being obtained by malaxation at 37 °C for 60 min and a crushing size of 6 mm. Contradictory results have been reported in the literature, in that a longer olive paste malaxation reduced the hydroxytyrosol concentration [57], as did a higher malaxation temperature [27].

Flavones (apigenin and luteolin) and lignans (pinoresinol) followed a similar pattern, concentrations being lowest at 32 °C and highest at 27 °C, in both cases with a malaxation time of 30 min and crushing size of 4 mm. Similar results have been recently reported for flavones [31,52,62], whereas other authors describe that the duration of malaxation has little effect on flavone and lignan content [57]. Other studies have found opposite trends, reporting that the pinoresinol content increased with temperature and malaxation time [31].

4. Conclusions

The total phenol content in the EVOO extracted from ‘Corbella’ olives decreased with fruit maturity, but flavones, hydroxyoleuropein aglycone, hydroxytyrosol acetate, and lignans increased. A negative correlation was found between the total phenols and higher crushing temperatures, although individual phenolic compounds such as oleocanthal and oleacein increased. In summary, to obtain a ‘Corbella’ EVOO rich in phenolic compounds, an early harvest and low crushing temperature should be employed. On the contrary, if a higher concentration of specific phenols such as oleocanthal or oleacein is desired, due to their health properties, a moderate crushing temperature should be used.

Regarding malaxation conditions, the results suggest that a higher temperature (37 °C) increased the total phenol content and the concentration of the most relevant phenols. A short malaxation time (30 min) enhanced the total phenols and oleuropein and ligstroside aglycone, whereas a longer process (60 min) increased oleocanthal concentration. Finally, a clear trend was not observed with respect to crushing size.

Based on these results, future research could be focused on studying the correlation between the enzymatic activity and ripening index or how the malaxation temperature affects olives collected at different times.

Author Contributions: Conceptualization, A.N., A.R.-A., and A.V.-Q.; methodology, A.N. and A.V.-Q.; software, J.L.-C. and M.P.; validation, E.E.-F., A.R.-A., and R.M.L.-R.; formal analysis, A.L.-Y., N.J.-R., J.L.-C., and M.P.; investigation, A.L.-Y., A.N., N.J.-R., J.L.-C., and A.V.-Q.; resources, E.E.-F., A.R.-A., and R.M.L.-R.; data curation, A.L.-Y., N.J.-R., J.L.-C., and M.P.; writing—original draft preparation, A.L.-Y., A.N., and N.J.-R.; writing—review and editing, J.L.-C., M.P., E.E.-F., A.R.-A., R.M.L.-R., and A.V.-Q.; visualization, A.L.-Y., A.N., and N.J.-R.; supervision, A.V.-Q.; project administration, E.E.-F., A.R.-A., and R.M.L.-R.; funding acquisition, E.E.-F., A.R.-A., and R.M.L.-R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by CICYT [AGL2016-75329-R], CIBEROBN from the Instituto de Salud Carlos III, ISCIII from the Ministerio de Ciencia, Innovación y Universidades, (AEI/ FEDER, UE) and Generalitat de Catalunya (GC) [2017SGR 196]. A.L-Y wishes to thank the Consejo Nacional de Ciencia y Tecnología (CONACYT) of Mexico for the doctoral scholarship. J. L-C thanks the Ministry of Science Innovation and Universities for the FPI contract (BES-2017-080017). A. V-Q thanks the Ministry of Science Innovation and Universities for the Ramon y Cajal contract (RYC-2016-19355). A. R-A and A.N. acknowledge financial support from the CERCA Program of the Generalitat of Catalonia.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article.

Acknowledgments: The authors wish to thank the CCiT-UB for use of the mass spectrometry equipment.

Conflicts of Interest: R.M.L.-R. reports receiving lecture fees from Cervenceros de España and receiving lecture fees and travel support from Adventia. The other authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

- Salas-Salvadó, J.; Becerra-Tomás, N.; García-Gavilán, J.F.; Bulló, M.; Barrubés, L. Mediterranean Diet and Cardiovascular Disease Prevention: What Do We Know? *Prog. Cardiovasc. Dis.* **2018**, *61*, 62–67. [CrossRef] [PubMed]
- García-Gavilán, J.F.; Bulló, M.; Canudas, S.; Martínez-González, M.A.; Estruch, R.; Giardina, S.; Fitó, M.; Corella, D.; Ros, E.; Salas-Salvadó, J. Extra virgin olive oil consumption reduces the risk of osteoporotic fractures in the PREDIMED trial. *Clin. Nutr.* **2018**, *37*, 329–335. [CrossRef]
- Estruch, R.; Ros, E.; Salas-Salvadó, J.; Covas, M.-I.; Corella, D.; Arós, F.; Gómez-Gracia, E.; Ruiz-Gutiérrez, V.; Fiol, M.; Lapetra, J.; et al. Primary Prevention of Cardiovascular Disease with a Mediterranean Diet. *N. Engl. J. Med.* **2013**, *368*, 1279–1290. [CrossRef] [PubMed]
- Yubero-Serrano, E.M.; Lopez-Moreno, J.; Gomez-Delgado, F.; Lopez-Miranda, J. Extra virgin olive oil: More than a healthy fat. *Eur. J. Clin. Nutr.* **2019**, *8*–17. [CrossRef] [PubMed]
- The European Commission. Commission Regulation (EU) No 1018/2013 of 23 October 2013 Amending Regulation (EU) No 432/2012 Establishing a List of Permitted Health Claims Made on Foods Other Than Those Referring to the Reduction of Disease Risk and to Children's Development and Health; 2013; Vol. L 113, pp. 6–12. Available online: http://eur-lex.europa.eu/pri/en/oj/dat/2003/l_285/l_28520031101en00330037.pdf (accessed on 20 September 2020).
- Pérez, M.; López-Yerena, A.; Lozano-castellón, J.; Olmo-cunillera, A.; Lamuela-raventós, R.M.; Martín-belloso, O.; Vallverdú-Queralt, A. Impact of novel technologies on virgin olive oil processing, consumer acceptance, and the valorization of olive mill wastes. *Antioxidants* **2021**, *10*, 417. [CrossRef] [PubMed]
- López-Yerena, A.; Ninot, A.; Lozano-Castellón, J.; Escribano-Ferrer, E.; Romero-Aroca, A.J.; Belaj, A.; Vallverdú-Queralt, A.; Lamuela-Raventós, R.M. Conservation of native wild ivory-white olives from the Medes islands natural reserve to maintain virgin olive oil diversity. *Antioxidants* **2020**, *9*, 1009. [CrossRef] [PubMed]
- Lozano-Castellón, J.; López-Yerena, A.; Rinaldi de Alvarenga, J.F.; Romero del Castillo-Alba, J.; Vallverdú-Queralt, A.; Escribano-Ferrer, E.; Lamuela-Raventós, R.M. Health-promoting properties of oleocanthal and oleacein: Two secoiridoids from extra-virgin olive oil. *Crit. Rev. Food Sci. Nutr.* **2019**, *60*, 2532–2548. [CrossRef] [PubMed]
- Vallverdú-Queralt, A.; Regueiro, J.; Rinaldi de Alvarenga, J.F.; Torrado, X.; Lamuela-Raventós, R.M. Home Cooking and Phenolics: Effect of Thermal Treatment and Addition of Extra Virgin Olive Oil on the Phenolic Profile of Tomato Sauces. *J. Agric. Food Chem.* **2014**. [CrossRef]
- Borges, T.H.; Serna, A.; López, L.C.; Lara, L.; Nieto, R.; Seiquer, I. Composition and antioxidant properties of Spanish extra virgin olive oil regarding cultivar, harvest year and crop stage. *Antioxidants* **2019**, *8*, 217. [CrossRef] [PubMed]
- López-Yerena, A.; Lozano-Castellón, J.; Olmo-Cunillera, A.; Tresserra-Rimbau, A.; Quifer-Rada, P.; Jiménez, B.; Pérez, M.; Vallverdú-Queralt, A. Effects of organic and conventional growing systems on the phenolic profile of extra-virgin olive oil. *Molecules* **2019**, *24*, 1986. [CrossRef]
- Taticchi, A.; Selvaggini, R.; Esposto, S.; Sordini, B.; Veneziani, G.; Servili, M. Physicochemical characterization of virgin olive oil obtained using an ultrasound-assisted extraction at an industrial scale: Influence of olive maturity index and malaxation time. *Food Chem.* **2019**, *289*, 7–15. [CrossRef] [PubMed]
- Ben Brahim, S.; Kelebek, H.; Ammar, S.; Abichou, M.; Bouaziz, M. LC–MS phenolic profiling combined with multivariate analysis as an approach for the characterization of extra virgin olive oils of four rare Tunisian cultivars during ripening. *Food Chem.* **2017**, *229*, 9–19. [CrossRef]

14. Clodoveo, M.L.; Hbaieb, R.H.; Kotti, F.; Mugnozza, G.S.; Gargouri, M. Mechanical strategies to increase nutritional and sensory quality of virgin olive oil by modulating the endogenous enzyme activities. *Compr. Rev. Food Sci. Food Saf.* **2014**, *13*, 135–154. [CrossRef] [PubMed]
15. Clodoveo, M.L. Malaxation: Influence on virgin olive oil quality. Past, present and future—An overview. *Trends Food Sci. Technol.* **2012**, *25*, 13–23. [CrossRef]
16. Peres, F.; Martins, L.L.; Ferreira-Dias, S. Influence of enzymes and technology on virgin olive oil composition. *Crit. Rev. Food Sci. Nutr.* **2017**, *57*, 3104–3126. [CrossRef] [PubMed]
17. Taticchi, A.; Esposto, S.; Veneziani, G.; Urbani, S.; Selvaggini, R.; Servili, M. The influence of the malaxation temperature on the activity of polyphenoloxidase and peroxidase and on the phenolic composition of virgin olive oil. *Food Chem.* **2013**, *136*, 975–983. [CrossRef] [PubMed]
18. Uceda, M.; Frías, L. Seasons of harvest. Changes on fruit oil content, oil composition and oil quality. In *Proceedings of the II Seminario Oleícola Internacional; IOOC: Cordoba, Spain, 1975*; pp. 25–46.
19. Lozano-Castellón, J.; López-Yerena, A.; Olmo-Cunillera, A.; Jáuregui, O.; Pérez, M.; Lamuela-Raventós, R.M.; Vallverdú-Queralt, A. Total analysis of the major secoiridoids in extra virgin olive oil: Validation of an uhplc-esi-ms/ms method. *Antioxidants* **2021**, *10*, 540. [CrossRef]
20. Alowairesh, B.; Singh, Z.; Fang, Z.; Kailis, S.G. Harvest time impacts the fatty acid compositions, phenolic compounds and sensory attributes of Frantoio and Manzanilla olive oil. *Sci. Hortic.* **2018**, *234*, 74–80. [CrossRef]
21. Matos, L.C.; Pereira, J.A.; Andrade, P.B.; Seabra, R.M.; Oliveira, M.B.P.P. Evaluation of a numerical method to predict the polyphenols content in monovarietal olive oils. *Food Chem.* **2007**, *102*, 976–983. [CrossRef]
22. Gómez-Rico, A.; Salvador, M.D.; La Greca, M.; Fregapane, G. Phenolic and volatile compounds of extra virgin olive oil (*Olea europaea* L. Cv. Cornicabra) with regard to fruit ripening and irrigation management. *J. Agric. Food Chem.* **2006**, *54*, 7130–7136. [CrossRef]
23. Bilušić, T.; Žanetić, M.; Ljubenković, I.; Generalić Mekinić, I.; Štambuk, S.; Bojović, V.; Soldo, B.; Magiatis, P. Molecular characterization of Dalmatian cultivars and the influence of the olive fruit harvest period on chemical profile, sensory characteristics and oil oxidative stability. *Eur. Food Res. Technol.* **2018**, *244*, 281–289. [CrossRef]
24. Romero, A.; Díaz, I.; Tous, J. Optimal harvesting period for “arbequina” olive cultivar in catalonia (spain). *Acta Hortic.* **2002**, *586*, 393–396. [CrossRef]
25. Tous, J.; Romero, A. Variedades del olivo. In *Fundación La Caixa; AEDOS: Barcelona, Spain, 1993*.
26. Castejón, M.L.; Montoya, T.; Alarcón, C.; Lastra, D. Potential Protective Role Exerted by Secoiridoids from *Olea europaea* L. in Cancer, Cardiovascular, Neurodegenerative, Aging—Related, and Immunoinflammatory Diseases. *Antioxidants* **2020**, *9*, 149. [CrossRef]
27. Lukić, I.; Krapac, M.; Horvat, I.; Godena, S.; Kosić, U.; Brkić Bubola, K. Three-factor approach for balancing the concentrations of phenols and volatiles in virgin olive oil from a late-ripening olive cultivar. *LWT Food Sci. Technol.* **2018**, *87*, 194–202. [CrossRef]
28. Amanpour, A.; Kelebek, H.; Selli, S. LC-DAD-ESI-MS/MS-based phenolic profiling and antioxidant activity in Turkish cv. Nizip Yaglik olive oils from different maturity olives. *J. Mass Spectrom.* **2019**, *54*, 227–238. [CrossRef] [PubMed]
29. Vilaplana-Pérez, C.; Auñón, D.; García-Flores, L.A.; Gil-Izquierdo, A. Hydroxytyrosol and Potential Uses in Cardiovascular Diseases, Cancer, and AIDS. *Front. Nutr.* **2014**, *1*, 1–11. [CrossRef]
30. Aparicio-Soto, M.; Sánchez-Fidalgo, S.; González-Benjumea, A.; Maya, I.; Fernández-Bolaños, J.G.; Alarcón-de-la-Lastra, C. Naturally occurring hydroxytyrosol derivatives: Hydroxytyrosyl acetate and 3,4-dihydroxyphenylglycol modulate inflammatory response in murine peritoneal macrophages. potential utility as new dietary supplements. *J. Agric. Food Chem.* **2015**, *63*, 836–846. [CrossRef]
31. De Torres, A.; Espínola, F.; Moya, M.; Alcalá, S.; Vidal, A.M.; Castro, E. Assessment of phenolic compounds in virgin olive oil by response surface methodology with particular focus on flavonoids and lignans. *LWT Food Sci. Technol.* **2018**, *90*, 22–30. [CrossRef]
32. Jiménez, B.; Sánchez-Ortiz, A.; Lorenzo, M.L.; Rivas, A. Influence of fruit ripening on agronomic parameters, quality indices, sensory attributes and phenolic compounds of Picudo olive oils. *Food Res. Int.* **2013**, *54*, 1860–1867. [CrossRef]
33. Bengana, M.; Bakhouch, A.; Lozano-Sánchez, J.; Amir, Y.; Youyou, A.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Influence of olive ripeness on chemical properties and phenolic composition of Chemlal extra-virgin olive oil. *Food Res. Int.* **2013**, *54*, 1868–1875. [CrossRef]
34. Brenes, M.; García, A.; García, P.; Rios, J.J.; Garrido, A. Phenolic compounds in Spanish olive oils. *J. Agric. Food Chem.* **1999**, *47*, 3535–3540. [CrossRef]
35. Artajo, L.S.; Romero, M.P.; Motilva, M.J. Transfer of phenolic compounds during olive oil extraction in relation to ripening stage of the fruit. *J. Sci. Food Agric.* **2006**, *86*, 518–527. [CrossRef]
36. Ye, J.H.; Wijesundera, C.; Shi, M. Effects of Agronomic and Oil Processing Conditions on Natural Antioxidative Phenolics in Olive (*Olea europaea* L.). *Austin J. Nutr. Food Sci.* **2014**, *2*.
37. Baccouri, O.; Guerfel, M.; Baccouri, B.; Cerretani, L.; Bendini, A.; Lercker, G.; Zarrouk, M.; Daoud Ben Miled, D. Chemical composition and oxidative stability of Tunisian monovarietal virgin olive oils with regard to fruit ripening. *Food Chem.* **2008**, *109*, 743–754. [CrossRef] [PubMed]

38. Olmo-Cunillera, A.; López-Yerena, A.; Lozano-Castellón, J.; Tresserra-Rimbau, A.; Vallverdú-Queralt, A.; Pérez, M. NMR spectroscopy: A powerful tool for the analysis of polyphenols in extra virgin olive oil. *J. Sci. Food Agric.* **2020**, *100*, 1842–1851. [CrossRef] [PubMed]
39. Fini, L.; Hotchkiss, E.; Fogliano, V.; Graziani, G.; Romano, M.; De Vol, E.B.; Qin, H.; Selgrad, M.; Boland, C.R.; Ricciardiello, L. Chemopreventive properties of pinoresinol-rich olive oil involve a selective activation of the ATM-p53 cascade in colon cancer cell lines. *Carcinogenesis* **2008**, *29*, 139–146. [CrossRef]
40. Owen, R.W.; Mier, W.; Giacosa, A.; Hull, W.E.; Spiegelhalder, B.; Bartsch, H. Identification of lignans as major components in the phenolic fraction of olive oil. *Clin. Chem.* **2000**, *46*, 976–988. [CrossRef] [PubMed]
41. Polari, J.J.; Wang, S.C. Hammer Mill Sieve Design Impacts Olive Oil Minor Component Composition. *Eur. J. Lipid Sci. Technol.* **2019**, *121*, 1900168. [CrossRef]
42. Caponio, F.; Pasqualone, A.; Gomes, T.; Catalano, P. Use of HPSEC analysis of polar compounds to assess the influence of crushing temperature on virgin olive oil's quality. *Eur. Food Res. Technol.* **2002**, *215*, 534–537. [CrossRef]
43. Inarejos-García, A.M.; Fregapane, G.; Salvador, M.D. Effect of crushing on olive paste and virgin olive oil minor components. *Eur. Food Res. Technol.* **2011**, *232*, 441–451. [CrossRef]
44. Caponio, F.; Catalano, P. Hammer crushers vs disk crushers: The influence of working temperature on the quality and preservation of virgin olive oil. *Eur. Food Res. Technol.* **2001**, *213*, 219–224. [CrossRef]
45. Abbattista, R.; Losito, I.; De Ceglie, C.; Basile, G.; Calvano, C.D.; Palmisano, F.; Cataldi, T.R.I. Structural characterization of the ligstroside aglycone isoforms in virgin olive oils by liquid chromatography–high-resolution Fourier-transform mass spectrometry and H/D exchange. *J. Mass Spectrom.* **2019**, *54*, 843–855. [CrossRef] [PubMed]
46. Jolayemi, O.S.; Tokatli, F.; Ozen, B. Effects of malaxation temperature and harvest time on the chemical characteristics of olive oils. *Food Chem.* **2016**, *211*, 776–783. [CrossRef] [PubMed]
47. Parenti, A.; Spugnoli, P.; Masella, P.; Calamai, L. The effect of malaxation temperature on the virgin olive oil phenolic profile under laboratory-scale conditions. *Eur. J. Lipid Sci. Technol.* **2008**, *110*, 735–741. [CrossRef]
48. Diamantakos, P.; Giannara, T.; Skarkou, M.; Melliou, E.; Magiatis, P. Influence of harvest time and malaxation conditions on the concentration of individual phenols in extra virgin olive oil related to its healthy properties. *Molecules* **2020**, *25*, 2449. [CrossRef] [PubMed]
49. Stefanoudaki, E.; Koutsaftakis, A.; Harwood, J.L. Influence of malaxation conditions on characteristic qualities of olive oil. *Food Chem.* **2011**, *127*, 1481–1486. [CrossRef]
50. Lukić, I.; Žanetić, M.; Jukić Špika, M.; Lukić, M.; Koprivnjak, O.; Brkić Bubola, K. Complex interactive effects of ripening degree, malaxation duration and temperature on Oblica cv. virgin olive oil phenols, volatiles and sensory quality. *Food Chem.* **2017**, *232*, 610–620. [CrossRef]
51. Polari, J.J.; Wang, S.C. Comparative Effect of Hammer Mill Screen Size and Cell Wall-Degrading Enzymes during Olive Oil Extraction. *ACS Omega* **2020**, *5*, 6074–6081. [CrossRef] [PubMed]
52. Boselli, E.; Di Lecce, G.; Strabbioli, R.; Pieralisi, G.; Frega, N.G. Are virgin olive oils obtained below 27 °C better than those produced at higher temperatures? *LWT Food Sci. Technol.* **2009**, *42*, 748–757. [CrossRef]
53. Servili, M.; Selvaggini, R.; Esposto, S.; Taticchi, A.; Montedoro, G.F.; Morozzi, G. Health and sensory properties of virgin olive oil hydrophilic phenols: Agronomic and technological aspects of production that affect their occurrence in the oil. *J. Chromatogr. A* **2004**, *1054*, 113–127. [CrossRef]
54. Ryan, D.; Antolovich, M.; Prenzler, P.; Robards, K.; Lavee, S. Biotransformations of phenolic compounds in *Olea europaea* L. *Sci. Hortic.* **2002**, *92*, 147–176. [CrossRef]
55. Ranalli, A.; Contento, S.; Schiavone, C.; Simone, N. Malaxing temperature affects volatile and phenol composition as well as other analytical features of virgin olive oil. *Eur. J. Lipid Sci. Technol.* **2001**, *103*, 228–238. [CrossRef]
56. Angerosa, F.; Mostallino, R.; Basti, C.; Vito, R. Influence of malaxation temperature and time on the quality of virgin olive oils. *Food Chem.* **2001**, *72*, 19–28. [CrossRef]
57. Jiménez, B.; Sánchez-Ortiz, A.; Rivas, A. Influence of the malaxation time and olive ripening stage on oil quality and phenolic compounds of virgin olive oils. *Int. J. Food Sci. Technol.* **2014**, *49*, 2521–2527. [CrossRef]
58. Polari, J.J.; García-Aguirre, D.; Olmo-García, L.; Carrasco-Pancorbo, A.; Wang, S.C. Interactions between hammer mill crushing variables and malaxation time during continuous olive oil extraction. *Eur. J. Lipid Sci. Technol.* **2018**, *120*, 1–19. [CrossRef]
59. Gomez-Rico, A.; Inarejos-Garcia, A.M.; Salvador, M.D.; Fregapane, G. Effect of malaxation conditions on phenol and volatile profiles in olive paste and the corresponding virgin olive oils (*Olea europaea* L. Cv. Cornicabra). *J. Agric. Food Chem.* **2009**, *57*, 3587–3595. [CrossRef] [PubMed]
60. Miho, H.; Moral, J.; López-González, M.A.; Díez, C.M.; Priego-Capote, F. The phenolic profile of virgin olive oil is influenced by malaxation conditions and determines the oxidative stability. *Food Chem.* **2020**, *314*, 126183. [CrossRef] [PubMed]
61. Ranalli, A.; Pollastri, L.; Contento, S.; Iannucci, E.; Lucera, L. Effect of olive paste kneading process time on the overall quality of virgin olive oil. *Eur. J. Lipid Sci. Technol.* **2003**, *105*, 57–67. [CrossRef]
62. Marx, Í.M.G.; Casal, S.; Rodrigues, N.; Pinho, T.; Veloso, A.C.A.; Pereira, J.A.; Peres, A.M. Impact of the malaxation temperature on the phenolic profile of cv. Cobrançosa olive oils and assessment of the related health claim. *Food Chem.* **2021**, *337*, 127726. [CrossRef]

4.2. Absorption and metabolism of phenolic compounds assessed by intestinal perfusion method

4.2.1. Article 6: Absorption and intestinal metabolic profile of oleocanthal in rats

Anallely López-Yerena, Anna Vallverdú-Queralt, Raf Mols, Patrick Augustijns, Rosa M. Lamuela-Raventós and Elvira Escribano-Ferrer

Pharmaceutics, 12(2), 134, 2020

Abstract

Background: OLC has emerged as a potential therapeutic agent against a variety of diseases due to its anti-inflammatory activity. However, most research has been focused on its health properties and the agronomic and processing factors that promote its presence in EVOO. However, its bioavailability information would help determine the mechanisms behind its biological activities; however, none has been reported to date.

Scope: The objectives of the present work are to study the intestinal absorption and metabolism of OLC using an *in situ* perfusion technique in rats involving simultaneous sampling from the luminal perfusate and mesenteric blood. As a reference, the highly permeable drug levofloxacin was included in the study. In addition, an intestinal metabolic profile of OLC was determined.

Methods and results: To identify the OLC metabolites in plasma and perfusion samples, we initially used LC–ESI–LTQ–Orbitrap–MS in FTMS mode, followed by a data dependent scan. The quantification of OLC and its metabolites was performed by ultra-high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (UHPLC–ESI–MS/MS). OLC was mostly metabolized by phase I metabolism, undergoing hydration, hydrogenation and hydroxylation. Phase II reactions (glucuronidation of hydrogenated OLC and hydrated metabolites) were observed in plasma samples. OLC was poorly absorbed in the intestine, as indicated by the low effective permeability coefficient ($2.23 \pm 3.16 \times 10^{-5}$ cm/s) and apparent permeability coefficient ($4.12 \pm 2.33 \times 10^{-6}$ cm/s) obtained relative to the values of the highly permeable reference compound levofloxacin. The extent of OLC absorption reflected by the area under the mesenteric blood-time curve normalized by the inlet concentration was also lower than that of levofloxacin (AUC 0.25 ± 0.04 vs. 0.64 ± 0.03 , respectively).

Conclusions: OLC is a PC with an incomplete oral bioavailability due to its relatively low absorption (16%) and high intestinal metabolism. Its poor absorption was indicated by a low effective permeability coefficient, apparent permeability coefficient and area under the mesenteric blood–time curve normalized by the inlet concentration in comparison with the reference standard compound levofloxacin.

Article

Absorption and Intestinal Metabolic Profile of Oleocanthal in Rats

Anallely López-Yerena ¹, Anna Vallverdú-Queralt ^{1,2}, Raf Mols ³, Patrick Augustijns ³, Rosa M. Lamuela-Raventós ^{1,2} and Elvira Escribano-Ferrer ^{2,4,*}

¹ Nutrition, Food Science and Gastronomy Department, XaRTA, Institute of Nutrition and Food Safety (INSA-UB), School of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain; naye.yerena@gmail.com (A.L.-Y.); avallverdu@ub.edu (A.V.-Q.); lamuela@ub.edu (R.M.L.-R.)

² CIBER Physiopathology of Obesity and Nutrition (CIBEROBN), Institute of Health Carlos III, 28029 Madrid, Spain

³ Drug Delivery and Disposition, KU Leuven, 3000 Leuven, Belgium; raf.mols@kuleuven.be (R.M.); patrick.augustijns@pharm.kuleuven.be (P.A.)

⁴ Department of Pharmacy and Pharmaceutical Technology and Physical Chemistry, Biopharmaceutics and Pharmacokinetics Unit, Institute of Nanoscience and Nanotechnology (IN2UB), Pharmacy and Food Sciences School, University of Barcelona, 08028 Barcelona, Spain

* Correspondence: escribano@ub.edu; Tel.: +34-93402-4578; Fax: +34 9340 35937

Received: 25 November 2019; Accepted: 30 January 2020; Published: 5 February 2020

Abstract: Oleocanthal (OLC), a phenolic compound of extra virgin olive oil (EVOO), has emerged as a potential therapeutic agent against a variety of diseases due to its anti-inflammatory activity. The aim of the present study is to explore its *in vivo* intestinal absorption and metabolism. An *in situ* perfusion technique in rats was used, involving simultaneous sampling from the luminal perfusate and mesenteric blood. Samples were analysed by UHPLC–MS–MS for the presence of oleocanthal (OLC) and its metabolites. OLC was mostly metabolized by phase I metabolism, undergoing hydration, hydrogenation and hydroxylation. Phase II reactions (glucuronidation of hydrogenated OLC and hydrated metabolites) were observed in plasma samples. OLC was poorly absorbed in the intestine, as indicated by the low effective permeability coefficient ($2.23 \pm 3.16 \times 10^{-5}$ cm/s) and apparent permeability coefficient ($4.12 \pm 2.33 \times 10^{-6}$ cm/s) obtained relative to the values of the highly permeable reference compound levofloxacin (LEV). The extent of OLC absorption reflected by the area under the mesenteric blood-time curve normalized by the inlet concentration (AUC) was also lower than that of LEV (0.25 ± 0.04 vs. 0.64 ± 0.03 , respectively). These results, together with the observed intestinal metabolism, suggest that OLC has a moderate-to-low oral absorption; but higher levels of OLC are expected to reach human plasma vs. rat plasma.

Keywords: bioavailability; metabolism; *in situ* perfusion; permeability; extra virgin olive oil; secoiridoids

1. Introduction

The secoiridoids are the most abundant and complex family of phenolic compounds in extra virgin olive oil (EVOO). The main secoiridoids compounds identified in EVOO are the monoaldehydic forms of oleuropein (3,4-DHPEA-EA) and ligstroside aglycones (*p*-HPEA-EA) and the dialdehydic forms of their decarboxymethylated derivatives, oleacein (3,4-DHPEA-EDA) and oleocanthal (*p*-HPEA-EDA) [1]. Since its identification in 1993 [2], oleocanthal (OLC) has been targeted by numerous *in vitro* and *in vivo* studies aiming to understand the health effects of EVOO

consumption [3]. The data obtained on OLC thus far have clearly demonstrated its anti-inflammatory activity [4] as well as its role in the prevention of various pathologies with an inflammatory component [5]. OLC also exerts a neuroprotective effect in conditions such as Alzheimer's disease [6,7] and has shown promising chemotherapeutic properties, reducing cell proliferation and promoting cell death through different mechanisms of action [8]. Antirheumatic activity has been demonstrated in *in vitro* studies, in which OLC ameliorated osteoarthritis and rheumatoid arthritis [3]. Additionally, OLC has been shown to be beneficial for cardiovascular health, improving endothelial function in patients with early atherosclerosis and reducing platelets in healthy men [3].

Given this broad and promising range of biological effects, most research on OLC has been focused on its health properties and the agronomic and processing factors that promote its presence in EVOO [9]. Information on the bioavailability of this phenolic compound would help determine the mechanisms behind its biological activities; however, none have been reported to date. The bioavailability of ingested phenolic compounds can be influenced by factors such as diet, genomic profile, enzymatic activity and colonic microflora [3], but it also depends on the extent of absorption and metabolism after ingestion. In this context, many *in vitro* and *in vivo* models for estimating human intestinal permeability and first-pass metabolism have been developed [10,11].

Before reaching the bloodstream, drugs and nutrients administered orally are usually absorbed from the small intestine. Although different factors (physicochemical, physiological and the matrix effect) can affect the rate and extent of absorption, the absorption of orally administered drugs is mainly determined by the solubility/dissolution of the molecule in the gastrointestinal environment as well as the permeability of the gastrointestinal wall [12]. *In situ* intestinal perfusion with venous sampling models facilitates a direct determination of drug absorption through the enterocytes on the basis of appearance kinetics in pre-hepatic blood [13]. This experimental model, which includes a mucus layer, blood irrigation and innervation, has been widely used due to its similarity with *in vivo* conditions [14]. It also allows the study of the role of transporters in absorption through the biorelevant expression of proteins (both transporters and intestinal enzymes) [12,15] and has full metabolic capacity for up to 120 min after intestinal isolation [16].

The objectives of the present work are to study the intestinal absorption and metabolism of OLC using an *in situ* perfusion technique in rats involving simultaneous sampling from the luminal perfusate and mesenteric blood. As a reference, the highly permeable drug levofloxacin (LEV) was included in the study. In addition, an intestinal metabolic profile of OLC was determined.

2. Materials and Methods

2.1. Reagents and Materials

OLC was purchased from PhytoLab GmbH (Vestenbergsgreuth, Germany). Phenol red, LEV, heparin sodium salt from porcine intestinal mucosa, Hanks' Balanced Salt Solution (HBBS) and HEPES 1 M solution were obtained from Sigma Aldrich (Madrid, Spain). Pentobarbital sodium 200 mg/mL (Dolethal) was purchased from Vétoquinol (Madrid, Spain) and isoflurane from Laboratorios Esteve (Barcelona, Spain). The reagents, methanol, acetonitrile and formic acid, were purchased from Sigma-Aldrich.

2.2. Animals

These studies were conducted following a protocol approved by the Animal Experimentation Ethics Committee of the University of Barcelona, Spain (trial no. CEEA 124/16) and Generalitat de Catalunya (no. 6435, 27 June 2019). For each compound, four Sprague–Dawley rats (body weight 306 ± 31 g) were used per intestinal perfusion with the mesenteric blood sampling experiment, and four (~350–400 g) were used as blood donors.

2.3. Single-Pass Intestinal Perfusion Studies

OLC was assayed at 0.1 mg/mL (328.94 μ M) in transport medium (TM pH 7, 9.7 g/L HBSS buffered with HEPES 10 mM). This concentration was chosen by taking into account both the OLC concentration in EVOO (around of 200 mg/kg) and the daily ingestion of EVOO recommended by the European Food Safety Authority (EFSA) (at least 5 mg of hydroxytyrosol and its derivatives per 20 g of olive oil) [17] and also considering the limit of quantification of the analytical technique. LEV was assayed at 3 mg/mL, according to its high dose strength, in 250 mL TM.

Single-pass intestinal perfusion was performed in anesthetized rats according to the method described by Brouwers et al. [15], with simultaneous sampling from the luminal perfusate and mesenteric blood.

2.3.1. Donor Blood

On the day of the perfusion, one or two rats (350–400 g) were anesthetised by isoflurane inhalation, and the whole blood was collected via cardiac puncture. The blood was diluted with heparin (50 u/mL TM) to 80% blood and kept in a 20 mL syringe for the in situ intestinal perfusion experiment.

2.3.2. Surgical Procedure

Anaesthesia was induced by intraperitoneal injection of pentobarbital sodium (60 mg/kg BW). The rats were then placed on a homeothermic blanket to maintain the body temperature at 37 °C. First, under general anaesthesia, the left jugular vein was cannulated with a heparinised (50 IU/mL) 0.5 × 0.9 mm/20 G catheter (Cavafix® MT, Braun Medical S.A., Barcelona, Spain) for infusion of donor blood. Second, a midline abdominal incision was made to isolate approximately 10 cm of the ileum using the ileocecal junction as a point of reference. Two glass cannulas (o.d. 4 mm, i.d. 3 mm, Duran®, Vidrafoc, Barcelona, Spain) were inserted at the proximal and distal ends of the isolated segment. The segment was rinsed by injection TM to remove the content until the solution came out clear. Next, the inlet cannula was connected to polyethylene tubing (i.d. 3 mm), which aspirated the tested solution (OLC or LEV) using a peristaltic pump (Minipuls3, M312 model, Gilson, Le Bel, France). Finally, the mesenteric vein draining blood from the intestinal segment was cannulated by inserting approximately 1 cm of a catheter (BD Insyte-W 24GA 0.7 × 19 mm, Becton Dickinson, Sangüesa S.A., Cornellà de Llobregat, Spain). The cannula was secured with a knot and connected to a silicone tube (i.d. 0.64 mm; o.d. 1.19 mm, Freudenberg Medical Europe, VWR International Eurolab S.L., Llinars del Vallés, Spain) that allowed blood to flow into the heparinised tubes. Care was taken to handle the small intestine gently to maintain normal blood supply. The exposed areas (both the jugular and abdomen) were wet with TM at 37 °C and covered with Parafilm® to keep them warm and moistened during the experiment.

2.3.3. Intestinal Perfusion

The experiments were carried out in a laboratory room with infrared light to avoid oxidation of OLC. The procedure was started by delivering the perfusion solution containing OLC (0.1 mg/mL) or LEV (3 mg/mL) (in separate experiments) and phenol red (0.1 mg/mL) at a flow rate of 1 mL/min to the intestinal segment previously cannulated. Simultaneously, the blank donor rat blood was supplied at a rate of 0.3 mL/min using a syringe pump (Injectomat MC Agilia, Fresenius Kabi España, Barcelona, Spain). Samples of both the intestinal lumen and mesenteric blood were collected at the same time. Simultaneously, the outflow perfusate was collected in 1.5 mL amber vials at 5 min intervals for 60 min, and the blood was collected in pre-weighted lithium-heparinised tubes (BD Vacutainer). At the end of the experiment, the animal was euthanised by injection of air into the jugular, and the length of the intestinal segment was measured. Next, the perfusion samples were centrifuged (7516 g for 10 min at 4 °C), and the supernatant was stored at −80 °C until analysis. The tubes with blood samples were weighted and centrifuged (7516.3 g for 10 min at 4 °C), and the plasma was collected and stored at −80 °C until solid-phase extraction (SPE).

The stability of the test products, sampled at different times, was tested in the perfusion solution at 37 °C for 60 min.

2.4. Oleocanthol Analysis

2.4.1. Solid-Phase Extraction (SPE) of OLC from Plasma

Extraction of OLC and its metabolites from plasma samples was carried out using an Oasis® HLB 30-mm (30 mg) 96-well plate (Waters, Wexford, Ireland), following the methodology proposed by Orrego-Lagarón and colleagues [18], with some modifications. Initially, the samples were thawed and centrifuged (7516 g, 10 min at 4 °C). The pre-treatment of samples consisted of diluting an exact volume of the supernatants to 1 mL with 0.1% of formic acid in water (*v/v*). The plate was activated with methanol and water containing 1% (*v/v*) formic acid. After loading the plasma mixture, the plates were cleaned by adding 1 mL 1% formic acid in water, followed by 1 mL methanol/water (5:95). The retained compounds were then eluted with 1 mL methanol acidified with 0.1% formic acid (*v/v*), and the resulting fraction was evaporated to dryness at room temperature under a stream of N₂. Finally, the samples were reconstituted with 100 µL of methanol containing 0.1% (*v/v*) formic acid. The extraction was performed in a darkened room with a red safety light to avoid oxidation of the analytes.

2.4.2. LC-ESI-LTQ-Orbitrap-MS

An LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) equipped with an ESI source in negative mode was used for accurate mass measurements. The operation parameters were the following: source voltage, 4 kV; capillary temperature, 275 °C (FT Automatic gain control (AGC) target 5×10^5 for MS mode and 5×10^4 for MSⁿ mode). The arbitrary units were used for sheath gas, auxiliary gas and sweep gas (20, 10 and 2, respectively). All samples were analysed in full scan mode at a resolving power of 30,000 at *m/z* 400, and data-dependent MS/MS events were acquired at a resolving power of 15,000. The most intense ions detected during full scan MS triggered data-dependent scanning. Ions that were not intense enough for a data-dependent scan were analysed in MSⁿ mode with the same orbitrap resolution (15,000 at *m/z* 400). An isolation width of 100 amu was used, and precursors were fragmented by collision-induced dissociation C-trap (CID) with normalized collision energy (35 V) and an activation time of 10 ms. The mass range in FTMS mode was from *m/z* 100 to 1000. The system was controlled by XCalibur software v2.0.7 (ThermoFisher Scientific).

An Accela chromatograph (Thermo Scientific, Hemel Hempstead, UK) equipped with a quaternary pump, a photodiode array detector (PDA) and a thermostated autosampler was used for the liquid chromatography analysis. The injection volume was 2 µL, the flow rate was set to 0.6 mL/min and the separations were carried out on an Acquity™ UPLC® BEH C18 Pre-Column (2.1 × 5 mm, i.d., 1.7 µm particle size) and an Acquity™ UPLC® BEH C18 Column (2.1 × 50 mm, i.d., 1.7 µm particle size) (Waters Corporation®, Wexford, Ireland) at 50 °C. The mobile phases consisted of methanol (A) and H₂O (B), both with 0.1% of formic acid. The elution was performed by means of an increasing linear gradient (*v/v*) of B (*t* (min), %B), as follows: (0, 100); (2, 100); (6, 46.4); (8, 0); (9, 0); (9.1, 100); (11, 100).

2.4.3. UHPLC-ESI-MS/MS

All luminal, plasma and stability samples were analysed by ultra-high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (UHPLC-ESI-MS/MS). The quantification of OLC and its metabolites was performed using an Acquity™ UPLC (Waters; Milford, Massachusetts, USA) coupled to an API 3000 triple-quadrupole mass spectrometer (ABSciex, Concord, Ontario, Canada) with a turbo ion spray source. The chromatographic separation of the parent compound and its metabolites were achieved using the same conditions (flow rate, column, injection volume and gradient conditions) as in LC-ESI-LTQ-Orbitrap-MS (Section 2.4.2).

The ionization of all compounds was performed using an electrospray interface operating in the negative mode [M–H] in the multiple monitoring mode (MRM). The arbitrary units were used for the nebulizer (10), curtain (12) and drying gas (450 °C) using N₂; the capillary voltage was –3500 V. The declustering, focusing and entrance potential and the collision energy were optimised to detect OLC and oleacein with the highest signal (see Table S1 for a detailed description of each compound). The system was controlled by Analyst version 1.4.2 software supplied by Applied Biosystems.

The calibration curves were prepared in TM and rat plasma (0–150 and 0–50 µg/mL, respectively) using OLC. The metabolites—in the absence of the reference standard—were evaluated by a screening method that includes a relative comparison of metabolite abundance (peak area ratio of metabolite/dosed parent compound OLC) [19,20].

2.5. Levofloxacin Analysis

A validated HPLC method was used to determine the concentration of LEV in luminal and plasma samples. The HPLC system consisted of two Waters 515 pumps, a 717 plus Waters auto sampler and a Waters dual absorbance detector 2487 with UV detection (Waters Chromatography S.A., Barcelona, Spain). The separation was carried out using a Hypersil® Elite C18 column (150 mm × 4.6 mm i.d., particle size 5 µm, ThermoFisher Scientific) with the flow rate at 0.8 mL/min. The mobile phase was isocratic, consisting of 1% triethylamine (pH 3.0 adjusted with phosphoric acid) and acetonitrile (86/14, v/v). The UV detection wavelength was set at 293 nm. The run time was 10 min for lumen samples and 12 min for plasma samples, and the injection volume was 10 µL. The calibration curves were prepared in TM and plasma and were linear over the concentration ranges 5–100 and 10–100 µg/mL, respectively.

For plasma samples, the procedure of Watabe et al. with some modifications was followed [21]. The standard solutions for the calibration curves were prepared from three stock standard solutions, using LEV (200 µg/mL and 1 mg/mL in TM) and ciprofloxacin as the internal standard (1 mg/mL in HCL 0.1 N). Different volumes of the LEV stock solutions (between 20 and 80 µL) and 16 µL of ciprofloxacin (1 mg/mL) were added to Eppendorf tubes, and drug-free rat plasma was incorporated to a total volume of 200 µL.

A liquid–liquid extraction was achieved using 200 µL calibration standards or samples mixed with 100 µL 6% (w/v) perchloric acid. Each mixture was vortexed for 30 s. Then, 100 µL of methanol was added to the above mixture, which was vortexed again for 30 s. The mixture was centrifuged at 9900 rpm for 12 min at room temperature, and 10 µL of the supernatant was injected into the HPLC. The final concentration of ciprofloxacin in the samples was 40 µg/mL. The retention times for LEV and ciprofloxacin were 7.8 ± 0.2 min and 8.7 ± 0.3 min, respectively.

2.6. Phenol Red Analysis

The concentration of phenol red in luminal samples from the in situ experiments with LEV was determined using a colorimetric assay. In each microplate well, 200 µL samples diluted 1/10 were added. The dilution was prepared with a 50 µL sample, NaOH 1 N (20 µL) and TM (430 µL). The absorbance at 558 nm was measured with a multidetector microplate reader (Biotek Instruments Inc., Winooski, VT, USA) equipped with Gen5 2.00 data analysis software. The calibration curves of phenol red were prepared in the same dissolution medium as the luminal samples at the concentrations of 24, 12, 6 and 3 µg/mL.

The concentration of phenol red in lumen samples from the in situ experiments with OLC was analysed by UHPLC–ESI–MS/MS in the same conditions as the OLC.

2.7. Data Analysis

The effective permeability coefficient (P_{eff} , cm/s) through the rat ileum was determined by Equation (1):

$$P_{\text{eff}} = \frac{-\Delta C_{\text{in}}}{2\pi RL} \times \ln \frac{C_{\text{in}}}{C_{\text{out,cor}}} \quad (1)$$

where $\emptyset_{in}$ is the perfusion solution flow (1 mL/min), C_{in} and $C_{out.cor}$ are the respective inlet and corrected outlet steady-state concentrations of the tested product, R is the radius of the intestinal segment (set to 0.2 cm) and L is the length of the intestinal segment determined after completion of the perfusion experiment.

The outlet concentrations were corrected for water transport by measuring the nonabsorbed and nonmetabolized phenol red marker according to Equation (2):

$$C_{out.cor} = C_{out} \times \frac{CPR_{in}}{CPR_{out}} \quad (2)$$

where C_{out} is the concentration of OLC or LEV in the perfusate at the specified time interval and CPR_{in} and CPR_{out} is the phenol red concentration in the inlet and outlet buffer solution at the specific time interval, respectively.

The apparent permeability coefficient (P_{app} , cm/s) was calculated using Equation (3):

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A \times C_0} \quad (3)$$

where Q is the cumulative amount of OLC or LEV appearing in the mesenteric blood as a function of time t in steady-state conditions, A is the surface area of the exposed intestinal segment and C_0 is the OLC or LEV initial concentration in the perfusate.

All in situ perfusion experiments were replicated in four rats, and the results were normalised to a 10-cm intestinal segment.

As a measure of the extent of absorption, the areas under the plasma concentration (normalised by the inlet concentration) time curves (AUC) for the intestinal perfusions were calculated using WinNonlin Professional software version 3.3 (Pharsight Corporation, Mountain View, USA) from time 0 to 60 min using the linear trapezoidal method.

Data are presented as mean and standard deviation (SD). Statistical analysis was performed using SAS (version 9.4). Statistical differences in the peak area ratio metabolite/parent OLC between different time periods were analysed using a one-way ANOVA. The P_{eff} , P_{app} and AUC of OLC and LEV were compared using a Mann–Whitney U -test. Differences were considered significant at $p < 0.05$.

3. Results and Discussion

3.1. Intestinal Metabolic Profile of Oleocanthal

3.1.1. Identification of Metabolites

To identify the OLC metabolites in plasma and perfusion samples, we initially used LC–ESI–LTQ–Orbitrap–MS in FTMS mode, followed by a data-dependent scan. Combining the FTMS scan and MSn experiments, four metabolites were identified in plasma and two metabolites (hydroxylated and hydrated) in perfusion samples. The proposed chemical structures are depicted in Figure 1. These metabolites and their retention times (RT), accurate masses, major fragments, error (mDa) and molecular formulas are shown in Table 1. Metabolite identification is important to reveal possible metabolic instability, causing extensive first-pass intestinal metabolism and poor oral bioavailability [22].

Table 1. Oleocanthal (OLC) and its metabolites identified in lumen and plasma samples using an LTQ–Orbitrap.

Compound	RT (min)	Accurate Mass	Major Fragments	Error (mDa)	Molecular Formula
OLC	5.8	303.1230	285.1125/179.0708	0.48	C ₁₇ H ₁₉ O ₅
OLC + OH	6.49	319.1180	153.0271/183.0665	0.16	C ₁₇ H ₁₉ O ₆
OLC + H ₂ O	6.36	321.1344	201.0759/183.0665	0.88	C ₁₇ H ₂₁ O ₆
OLC + H ₂ + glucuronide	5.83	481.1719	217.0859/185.0509	0.05	C ₂₃ H ₂₉ O ₁₁
OLC + H ₂ O + glucuronide	5.74	497.1666	321.1344/201.0759	0.17	C ₂₃ H ₂₉ O ₁₂

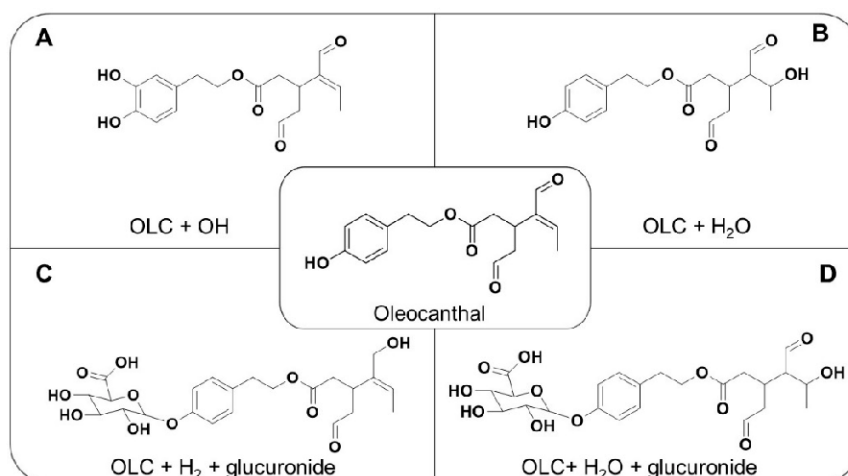


Figure 1. Chemical structure of oleocanthal and metabolites (A) = hydroxylated OLC; (B) = hydrated OLC; (C) = hydrogenated glucuronide; (D) = hydrated glucuronide).

3.1.2. Phase I Metabolism

The intestine is the most important extrahepatic site for drug biotransformation [10]. When crossing enterocytes, EVOO phenolic compounds are subjected to extensive first-pass metabolism through phase I/II biotransformation [23]. In our study, the two metabolites corresponding to phase I metabolism (hydration and hydroxylation identified in perfusion and plasma samples) are shown in Figures 2 and 3, respectively. Compared to OLC, the relative abundance of metabolites was higher in plasma than in the lumen.

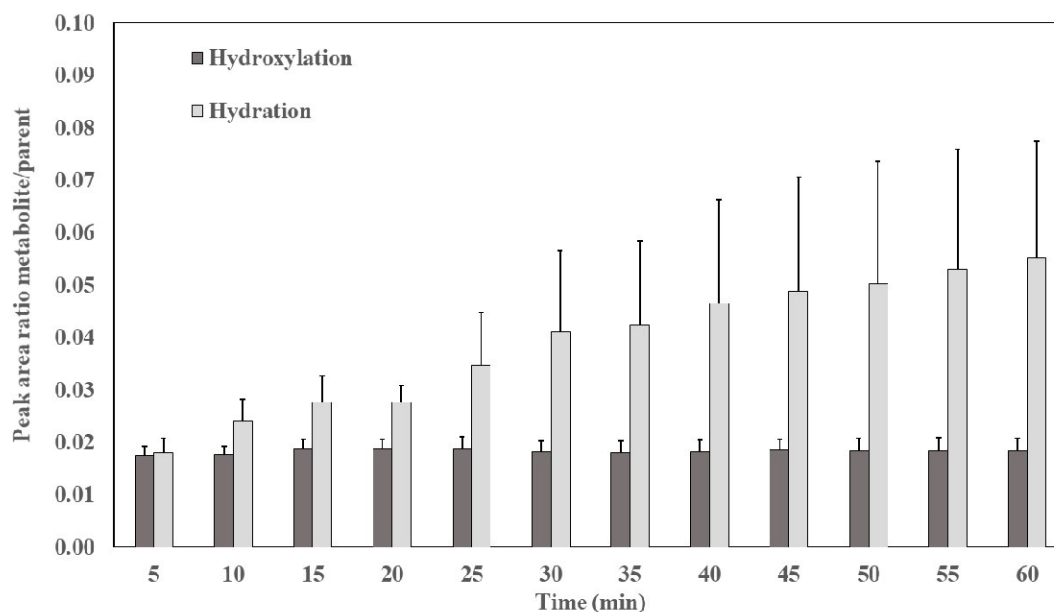


Figure 2. Peak area ratio metabolite/oleocanthal (OLC) as a function of time in lumen. Results are expressed as the mean $\pm$ standard deviation.

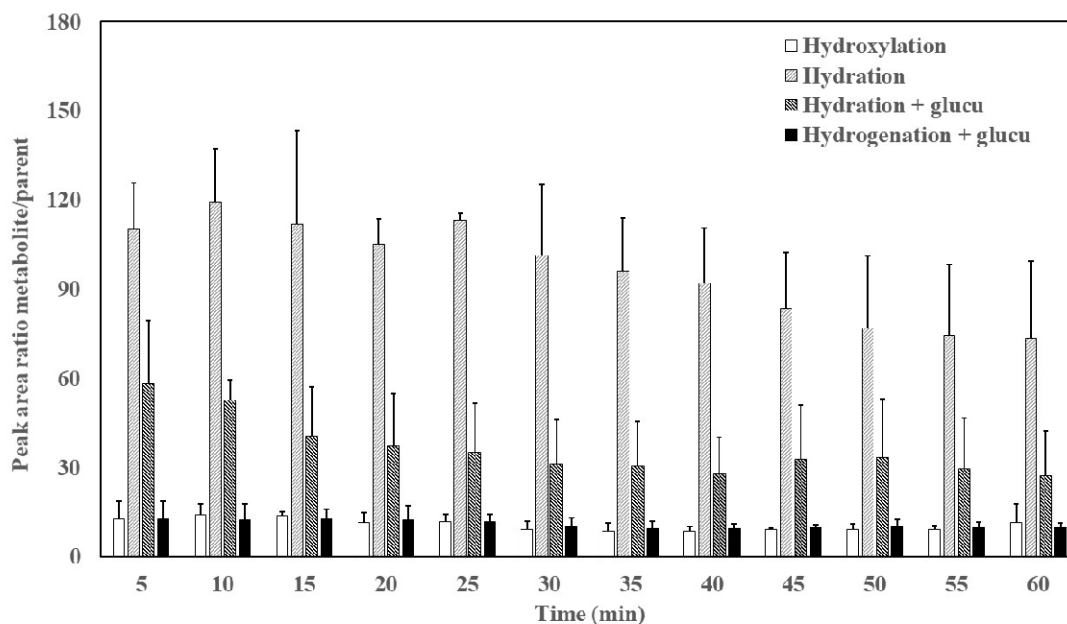


Figure 3. Peak area ratio metabolite/OLC as a function of time in plasma. Results are expressed as the mean $\pm$ standard deviation.

These results are consistent with the work of Garcia-Villalba et al. [24], who studied the urinary excretion of phenolic compounds after olive oil intake in humans. They identified, among others, hydroxylated and hydrated metabolites from phase I reactions in urine; however, the samples were discarded because the metabolites could have been related to hydroxytyrosol derivatives. Hydrogenated and hydrated (+glucuronidation) metabolites were additionally identified from phase II reactions with the same accurate mass. In the current study, we can confirm that they were direct metabolites of OLC. In another study [25], the hydrated metabolite was also identified in plasma samples after the ingestion of EVOO.

As shown in Figures 2 and 3, the most abundant phase I metabolite found in the samples was the hydrated form, with higher levels in plasma than in the lumen ($p < 0.05$). The metabolites in the lumen can originate from the microbiome, since many bacterial cytochromes P450 (CYP) are soluble [26], or from CYP of the enterocytes. This family of metabolic enzymes are present in the epithelium of the small intestine [27] and are responsible for the oxidative biotransformation of xenobiotics and other compounds [28]. The absorbed and metabolized OLC would be subsequently secreted to the intestinal lumen by efflux transporters.

The hydrogenated metabolites were found in a glucuronidated form in plasma but not in the lumen. Even when considering the differences between rat and human microsomal activity, hydrogenation seems to be an important phase I metabolic route for secoiridoids [25]. This reduction reaction is catalysed by NADPH-dependent aldo-keto reductases (AKR) located in the small intestine epithelium and can occur because OLC contains an open dialdehydic form of the attached elenolic acid molecule. During phase I metabolism, the reduction of aldehydes and ketones to primary and secondary alcohols, respectively, are formal functionalisation reactions and are involved in endogenous and xenobiotic compounds that have these carbonyl groups [29]. Moreover, the redox potential in the intestine favours the reduction reaction due to low oxygen tension, which provides a reducing environment, whereas oxidation is favoured in tissues such as the liver [30].

3.1.3. Phase II Metabolism

The appearance in plasma of metabolites arising from phase II reactions (glucuronidation of hydrogenated and hydrated metabolites) from time 0 (at the start of the in situ intestinal perfusion)

to 60 min are depicted in Figure 3. The main phase II metabolite detected in plasma samples was the hydrated metabolite with constant relative abundance over time ($p < 0.05$).

The glucuronidation of hydrogenated and hydrated metabolites likely occurs after oxidative-reductive metabolism because although drugs can undergo phase I and II reactions simultaneously, one of the pathways usually dominates [31]. The glucuronidation reaction mediates the transfer of a glucuronyl moiety from the ubiquitous co-substrate UDP-glucuronic acid to hydrophobic molecules with one or more electrophilic groups which serve as acceptors [32]. Similar results indicating that secoiridoids (3,4-DHPEA-EDA and 3,4-DHPEA-EA) undergo hydrogenation followed by glucuronidation have been previously reported in perfused segments of jejunum and ileum in rats [16]. Based on their high polarity and molecular weight, both phase II metabolites would be secreted to the mesenteric blood by transporters. A proposal of what could happen at the intestinal level is shown in Figure 4.

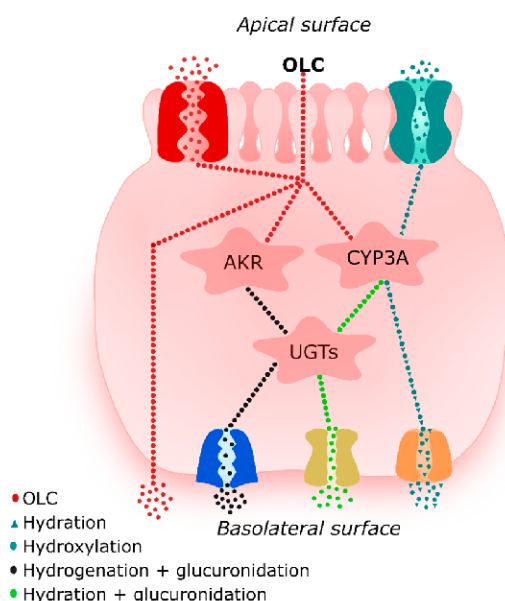


Figure 4. Tentative interactions of oleocanthal (OLC) with metabolic enzymes and transporters.

3.2. Perfusion Experiments and Intestinal Permeability of Oleocanthal

Prior to carrying out the intestinal permeability study, it is necessary to check the stability of the test compound and the reference standard in the perfusion solution at 37 °C during the test time (1 h). Both OLC and LEV remained stable in the perfusion solution ($p < 0.05$) during the experiments (Figure S1).

The intestinal permeability coefficient (P_{eff}) is widely used as part of a general screening process for orally administered drugs to study their intestinal absorption. It is important to determine the intestinal permeability of OLC, a component of EVOO (and therefore of the Mediterranean diet), of which many beneficial health properties have been reported [33]. In this work, to assess both the intestinal absorption and potential intestinal first-pass metabolism of OLC, simultaneous samples were collected from both the intestinal lumen and the mesenteric circulation. The concentration of OLC and the relative abundance of metabolites was determined (see Section 3.1). In addition, to ascertain if the results obtained for OLC imply high or low absorption/permeability, a group of rats perfused with LEV was also included in the study. According to the current Biopharmaceutics Classification System (BCS) [34], LEV is a highly permeable drug with an oral bioavailability close to 100% [35,36]. When assayed at 3 mg/mL in Caco-2 cells [35], it produced an apparent permeability coefficient similar to that of metoprolol, another reference drug of high permeability.

Surprisingly, despite the myriad of potential health benefits attributed to EVOO phenolic compounds, there is currently little information available on the intestinal permeability coefficients

of secoiridoids, especially OLC. Previous studies in rats have observed poor absorption of secoiridoids in the intestine perfused with oleuropein in iso-osmotic conditions, although it was significantly greater under hypotonic conditions (1.47×10^{-6} and 5.92×10^{-6} cm/s, respectively) [37]. Differentiated Caco-2 cell monolayers as a model system have been demonstrated to be highly permeable for hydroxytyrosol ($12.4 \pm 0.9 \times 10^{-6}$ cm/s) [38].

The permeability coefficients of OLC (0.1 mg/mL) and LEV (3 mg/mL) in the rat ileum, taking into account the disappearance of the test product in the lumen (P_{eff}) and appearance in the mesenteric circulation (P_{app}), are shown in Table 2.

Table 2. Permeability coefficients (P_{eff}) and apparent permeability coefficients (P_{app}) of oleocanthal and levofloxacin. Results are expressed as the mean $\pm$ SD of $n = 4$. Data are normalised to a 10-cm intestinal segment.

Test Compound	P_{eff} ($\times 10^{-5}$ cm/s)	P_{app} ($\times 10^{-6}$ cm/s)
OLC	2.23 ± 3.16	$4.12^* \pm 2.33$
LEV	7.64 ± 5.55	10.91 ± 6.27

* $p < 0.05$, Mann–Whitney U -test.

In Figure 5, the mean cumulative transport of OLC (A) and LEV (B), corrected for the length of the perfused segment, is illustrated as a function of time.

The difference between P_{eff} vs. P_{app} values is common in these types of studies; it has even been observed for theophylline where an absence of mass balance was observed, taking into account the mass lost from the perfusate and mass appearance in portal plasma [39]. Drug transfer from the lumen to the blood is not an instantaneous process: P_{eff} only refers to the resistance to transport from the lumen to the inside of the membrane, but not to the blood [40,41]. During the transport from the intestinal wall to the blood, different processes can occur, including accumulation in gut tissue, binding to transporters, gut wall metabolism, binding to red blood cells, and lymphatic transport [39]. Moreover, the rate of appearance in blood may be markedly dependent upon the blood flow rate. The difference in LEV values can be attributed to P-glycoprotein (P-gp) binding [42,43] and in the case of OLC, to microbiota and gut wall metabolism, according to the results reported in the previous section (Section 3.1). Moreover, as shown in Figure 5, the two compounds presented different accumulation profiles in mesenteric blood, with a lag time observed for OLC. This lag could be explained by the transit time in gut tissue due to phase I and phase II metabolic reactions in the enterocytes, which would delay appearance in the blood.

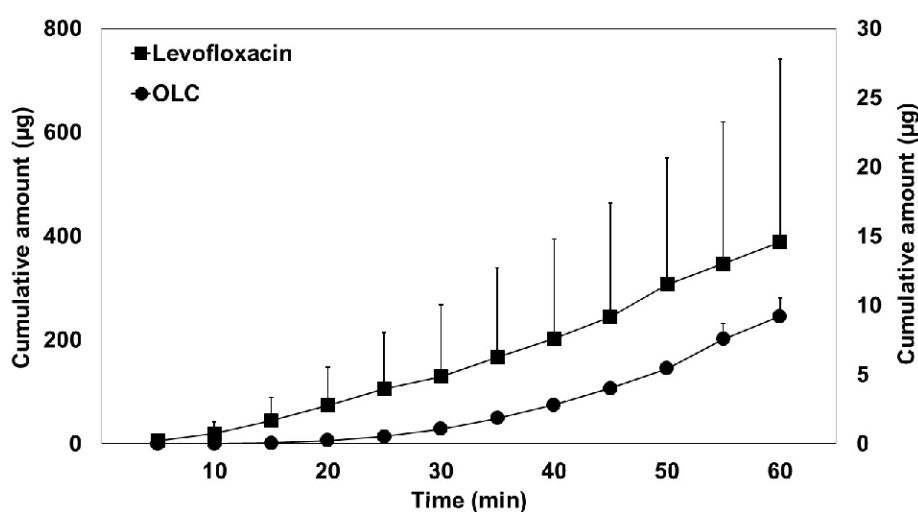


Figure 5. Transport of oleocanthal (OLC) (right Y-axis scale) and levofloxacin (left Y-axis scale) from the intestinal lumen to the mesenteric blood. Cumulative amount values are normalised to a 10-cm intestinal segment. Results are expressed as the mean $\pm$ standard deviation.

In our study, the decrease in concentration of LEV in the lumen samples was very low, resulting in lower absorption and permeability coefficient than expected. This low permeability could be attributed to LEV acting as a substrate for the efflux transporter P-gp, which would secrete the absorbed drug to the intestinal lumen. However, this finding observed in our in vivo study did not translate into a lower value of the apparent permeability coefficient in a study by Volpe [33] in vitro Caco-2 cells, where a P_{app} (apical-basolateral direction) similar to the highly permeable standard metoprolol was obtained.

Nevertheless, in this study, the values of P_{app} obtained for OLC are lower ($p < 0.05$) than for LEV. The lower extent of absorption is also reflected in the AUC calculated from the plasma concentrations in mesenteric blood (normalized by the input concentration). The lower OLC AUC values (AUC 0.25 ± 0.04 vs. 0.635 ± 0.03 for OLC and LEV, respectively) confirm that the intestinal membrane had low permeability for OLC, although its physicochemical characteristics ($\log P = 1.15$ and MW 304.34 g/mol) were not unfavourable for its absorption. The poor absorption of OLC in our experimental conditions (perfusion flow rate 1 mL/min), as well as its presence in the ileum (located next to the caecum, where bacterial density is high), could favour microbiome metabolism. Both circumstances, low absorption and high intestinal metabolism, can lead to incomplete bioavailability through a variety of mechanisms, including pre-systemic gastrointestinal and hepatic first-pass metabolism, saturation of carrier-mediated uptake processes, a low permeability constant, and P-gp-mediated transport out of the epithelial cells back into the lumen of the gastrointestinal tract [44]. According to its physicochemical properties (low solubility and relatively high lipophilicity), this compound meets the characteristics of BCS class 2. For these compounds, efflux transporters can affect their extent and rate of absorption [45], as observed in our study. The hypothesis that OLC could be a P-gp substrate should not be discarded, but more studies are needed to confirm it.

It should be noted that the human P_{eff} estimated for drugs transported by passive diffusion is, on average, 3.6 times higher in human in vivo than in rat in situ [11]; thus, higher levels of OLC are expected to reach human plasma.

4. Conclusions

In conclusion, OLC is a phenolic compound with an incomplete oral bioavailability due to its relatively low absorption (16%) and high intestinal metabolism. Its poor absorption was indicated by a low effective permeability coefficient, apparent permeability coefficient and area under the mesenteric blood–time curve normalized by the inlet concentration in comparison with the reference standard compound LEV. However, previous research has indicated that higher levels of OLC reach human plasma than in rats [11]. Regarding the metabolic profile, OLC was mostly metabolised by phase I hydration reactions. Hydrogenation and hydroxylation were also observed, and the hydrogenated and hydrated metabolites were then glucuronidated in phase II reactions.

This is the first in vivo study in rats to simultaneously assess the absorption and intestinal metabolism of OLC. Given the importance of phenolic compounds from EVOO, especially OLC on human health and its demonstrated moderated ability to be transported through the intestinal membrane, the development of dietary supplements containing OLC and excipients that favour their immediate release and absorption in the gastrointestinal tract should be considered, especially for populations with low intake of EVOO. In addition, further studies on OLC oral absorption and bioavailability are necessary, as well as studies on the biological relevance of the most abundant OLC metabolites.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: The declustering potential (DP), focusing potential (FP), collision energy (CE) and entrance potential (EP) settings for the oleocanthal and metabolites.

Author Contributions: Conceptualization, R.M.L.-R.; investigation, A.L.-Y. and E.E.-F.; methodology, A.L.-Y., R.M., P.A. and E.E.-F.; project administration, E.E.-F.; supervision, A.V.-Q. and R.M.L.-R.; visualization, R.M.; writing – original draft, A.L.-Y.; writing – review & editing, A.L.-Y., R.M. and P.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by CICYT [AGL2016- 75329-R]. A.L.-Y. wishes to thank the Consejo Nacional de Ciencia y Tecnología (CONACYT) of Mexico for the doctoral scholarship. A.V.-Q. thanks the Ministry of Science Innovation and Universities for the Ramon y Cajal contract (RYC-2016-19355).

Acknowledgments: The authors wish to thank the CCiT-UB for the mass spectrometry equipment.

Conflicts of Interest: R.M.L.-R. reports receiving lecture fees from Cerveceros de España and receiving lecture fees and travel support from Adventia. The other authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Celano, R.; Piccinelli, A.L.; Pugliese, A.; Carabetta, S.; Di Sanzo, R.; Rastrelli, L.; Russo, M. Insights into the Analysis of Phenolic Secoiridoids in Extra Virgin Olive Oil. *J. Agric. Food Chem.* **2018**, *66*, 6053–6063.
2. Montedoro, G.; Servili, M.; Baldioli, M.; Selvaggini, R.; Miniati, E.; Macchioni, A.; Agrarie, I.; Costanzo, V.S.; Agrarie, I.; Chimica, D. Simple and hydrolyzable compounds in virgin olive oil. 3. Spectroscopic Characterizations of the Secoiridoid Derivatives. *J. Agric. Food Chem.* **1993**, *41*, 2228–2234.
3. Lozano-Castellón, J.; López-Yerena, A.; Rinaldi de Alvarenga, J.F.; Castillo-Alba, J.R.; Vallverdú-Queralt, A.; Escribano-Ferrer, E.; Lamuela-Raventós, R.M. Health-promoting properties of oleocanthal and oleacein: Two secoiridoids from extra-virgin olive oil. *Crit. Rev. Food Sci. Nutr.* **2019**, 1–17, doi:10.1080/10408398.2019.1650715.
4. Beauchamp, G.K.; Keast, R.S.J.; Morel, D.; Lin, J.; Pika, J.; Han, Q.; Lee, C.-H.; Smith, A.B.; Breslin, P.A.S. Ibuprofen-like activity in extra-virgin olive oil. *Nature* **2005**, *437*, 45–46.
5. Francisco, V.; Ruiz-Fernández, C.; Lahera, V.; Lago, F.; Pino, J.; Skaltsounis, L.; González-Gay, M.A.; Mobasheri, A.; Gómez, R.; Scotese, M.; et al. Natural Molecules for Healthy Lifestyles: Oleocanthal from Extra Virgin Olive Oil. *J. Agric. Food Chem.* **2019**, *67*, 3845–3853.
6. Qosa, H.; Batarseh, Y.S.; Mohyeldin, M.M.; El Sayed, K.A.; Keller, J.N.; Kaddoumi, A. Oleocanthal Enhances Amyloid- β Clearance from the Brains of TgSwDI Mice and in Vitro across a Human Blood-Brain Barrier Model. *ACS Chem. Neurosci.* **2015**, *6*, 1849–1859.
7. Monti, M.C.; Margarucci, L.; Riccio, R.; Casapullo, A. Modulation of tau protein fibrillization by oleocanthal. *J. Nat. Prod.* **2012**, *75*, 1584–1588.
8. LeGendre, O.; Breslin, P.A.; Foster, D.A. (-)-Oleocanthal rapidly and selectively induces cancer cell death via lysosomal membrane permeabilization. *Mol. Cell. Oncol.* **2015**, *2*, e1006077.
9. López-Yerena, A.; Lozano-Castellón, J.; Olmo-Cunillera, A.; Tresserra-Rimbau, A.; Quifer-Rada, P.; Jiménez, B.; Pérez, M.; Vallverdú-Queralt, A. Effects of organic and conventional growing systems on the phenolic profile of extra-virgin olive oil. *Molecules* **2019**, *24*, 1986.
10. Yau, E.; Petersson, C.; Dolgos, H.; Peters, S.A. A comparative evaluation of models to predict human intestinal metabolism from nonclinical data. *Biopharm. Drug Dispos.* **2017**, *186*, 163–186.
11. Lennernäs, H. Human jejunal effective permeability and its correlation with preclinical drug absorption models. *J. Pharm. Pharmacol.* **1997**, *49*, 627–638.
12. Escribano, E.; García, X.; Salamanca, J.; Roig, C.; Queralt, J. Single-pass intestinal perfusion to establish the intestinal permeability of model drugs in mouse. *Int. J. Pharm.* **2012**, *436*, 472–477.
13. Griffin, B.; Driscoll, C.O. *Models of the Small Intestine*; Springer: Boston, MA, USA, 2007; pp. 34–76.
14. Caldeira, T.G.; Ruiz-Picazo, A.; Lozoya-Agullo, I.; Saúde-Guimarães, D.A.; González-Álvarez, M.; de Souza, J.; González-Álvarez, I.; Bermejo, M. Determination of intestinal permeability using in situ perfusion model in rats: Challenges and advantages to BCS classification applied to digoxin. *Int. J. Pharm.* **2018**, *551*, 148–157.
15. Brouwers, J.; Raf, M.; Annaert, P.; Augustijns, P. Validation of a Differential In Situ Perfusion Method with Mesenteric Blood Sampling in Rats for Intestinal Drug Interaction Profiling. *Biopharm. Drug Dispos.* **2010**, *31*, 278–285.
16. Pinto, J.; Paiva-Martins, F.; Corona, G.; Debnam, E.S.; Jose Oruna-Concha, M.; Vauzour, D.; Gordon, M.H.; Spencer, J.P.E. Absorption and metabolism of olive oil secoiridoids in the small intestine. *Br. J. Nutr.* **2011**, *105*, 1607–1618.
17. EFSA. Scientific Opinion on the substantiation of health claims related to polyphenols in olive and protection of LDL particles from oxidative damage (ID 1333, 1638, 1639, 1696, 2865), maintenance of normal blood HDL cholesterol concentrations (ID 1639), mainte. *EFSA J.* **2011**, *9*, 2033–2058.

18. Orrego-Lagarón, N.; Martínez-Huélamo, M.; Vallverdú-Queralt, A.; Lamuela-Raventos, R.M.; Escribano-Ferrer, E. High gastrointestinal permeability and local metabolism of naringenin: Influence of antibiotic treatment on absorption and metabolism. *Br. J. Nutr.* **2015**, *114*, 169–180.
19. Timmerman, P.; Anders Kall, M.; Gordon, B.; Laakso, S.; Freisleben, A.; Hucker, R. Best practices in a tiered approach to metabolite quantification: Views and recommendations of the European Bioanalysis Forum. *Bioanalysis* **2010**, *2*, 1185–1194.
20. Timmerman, P.; Blech, S.; White, S.; Green, M.; Delatour, C.; McDougall, S.; Mannens, G.; Smeraglia, J.; Williams, S.; Young, G. Best practices for metabolite quantification in drug development: Updated recommendation from the European Bioanalysis Forum. *Bioanalysis* **2016**, *8*, 1297–1305.
21. Watabe, S.; Yokoyama, Y.; Nakazawa, K.; Shinozaki, K.; Hiraoka, R.; Takeshita, K.; Suzuki, Y. Simultaneous measurement of pazufloxacin, ciprofloxacin, and levofloxacin in human serum by high-performance liquid chromatography with fluorescence detection. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2010**, *878*, 1555–1561.
22. Peterman, S.M.; Duczak, N.; Kalgutkar, A.S.; Lame, M.E.; Soglia, J.R. Application of a linear ion trap/orbitrap mass spectrometer in metabolite characterization studies: Examination of the human liver microsomal metabolism of the non-tricyclic anti-depressant nefazodone using data-dependent accurate mass measurements. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 363–375.
23. Serreli, G.; Deiana, M. Biological relevance of extra virgin olive oil polyphenols metabolites. *Antioxidants* **2018**, *7*, 11–13.
24. García-Villalba, R.; Carrasco-Pancorbo, A.; Nevedomskaya, E.; Fernández-Gutiérrez, A. Exploratory analysis of human urine by LC-ESI-TOF MS after high intake of olive oil: Understanding the metabolism of polyphenols. *Anal. Bioanal. Chem.* **2010**, *398*, 463–475.
25. Silva, S.; Garcia-aloy, M.; Figueira, M.E.; Combet, E.; Mullen, W.; Ros, M. High Resolution Mass Spectrometric Analysis of Secoiridoids and Metabolites as Biomarkers of Acute Olive Oil Intake—An Approach to Study Interindividual Variability in Humans. *Mol. Nutr. Food Res.* **2018**, *62*, 1700065.
26. Clarke, G.; Sandhu, K.V.; Griffin, B.T.; Dinan, T.G.; Cryan, J.F.; Hyland, N.P. Gut reactions: Breaking down xenobiotic–microbiome interactions. *Pharmacol. Rev.* **2019**, *71*, 198–224.
27. Dressman, J.B.; Thelen, K. Cytochrome P450-mediated metabolism in the human gut wall. *J. Pharm. Pharmacol.* **2009**, *61*, 541–558.
28. Spanogiannopoulos, P.; Bess, E.N.; Carmody, R.N.; Turnbaugh, P.J. The microbial pharmacists within us: A metagenomic view of xenobiotic metabolism. *Nat. Publ. Group* **2016**, *14*, 273–287.
29. Penning, T.M. The aldo-keto reductases (AKRs): Overview. *Chem.-Biol. Interact.* **2015**, *234*, 236–246.
30. Kang, M.J.; Kim, H.G.; Kim, J.S.; Oh, D.G.; Um, Y.J.; Seo, C.S.; Han, J.W.; Cho, H.J.; Kim, G.H.; Jeong, T.C.; et al. The effect of gut microbiota on drug metabolism. *Expert Opin. Drug Metab. Toxicol.* **2013**, *9*, 1295–1308.
31. Liu, Z.; Hu, M. Natural polyphenol disposition via coupled metabolic pathways. *Expert Opin. Drug Metab. Toxicol.* **2007**, *3*, 389–406.
32. Shipkova, M.; Wieland, E. Glucuronidation in therapeutic drug monitoring. *Clin. Chim. Acta* **2005**, *358*, 2–23.
33. Tresserra-Rimbau, A.; Lamuela-Raventos, R.M.; Moreno, J.J. Polyphenols, food and pharma. Current knowledge and directions for future research. *Biochem. Pharmacol.* **2018**, *156*, 186–195.
34. Amidon, G.L.; Lennernäs, H.; Shah, V.P.; Crison, J.R. A Theoretical Basis for a Biopharmaceutical Drug Classification: The Correlation of in Vitro Drug Product Dissolution and in Vivo Bioavailability. *Pharm. Res.* **1995**, *12*, 413–420.
35. Volpe, D.A. Permeability Classification of Representative Fluoroquinolones by a Cell Culture Method. *Aaps Pharmsci* **2004**, *6*, 1–6.
36. Fish, D.N.; Chow, A.T. The clinical pharmacokinetics of levofloxacin. *Clin. Pharmacokinet.* **1997**, *32*, 101–119.
37. Edgecombe, S.C.; Stretch, G.L.; Hayball, P.J. Oleuropein, an antioxidant polyphenol from olive oil, is poorly absorbed from isolated perfused rat intestine. *J. Nutr.* **2000**, *130*, 2996–3002.
38. Manna, C.; Galletti, P.; Maisto, G.; Cucciolla, V.; Angelo, S.D. Transport mechanism and metabolism of olive oil hydroxytyrosol in Caco-2 cells. *FEBS Lett.* **2000**, *470*, 341–344.
39. Dackson, K.; Stone, J.A.; Palin, K.J.; Charman, W.N. Evaluation of the mass balance assumption with respect to the two-resistance model of intestinal absorption by using in situ single-pass intestinal perfusion of theophylline in rats. *J. Pharm. Sci.* **1992**, *81*, 321–325.

40. Chiou, W.L. We may not measure the correct intestinal wall permeability coefficient of drugs: Alternative absorptive clearance concept. *J. Pharmacokinet. Biopharm.* **1995**, *23*, 323–331.
41. Chiou, W.L. The author replies to Lennernäs et al. *J. Pharmacokinet. Biopharm.* **1995**, *23*, 339–349.
42. Michot, J.M.; Seral, C.; Van Bambeke, F.; Mingeot-Leclercq, M.P.; Tulkens, P.M. Influence of efflux transporters on the accumulation and efflux of four quinolones (ciprofloxacin, levofloxacin, garenoxacin, and moxifloxacin) in J774 macrophages. *Antimicrob. Agents Chemother.* **2005**, *49*, 2429–2437.
43. Hua, W.; Hua, W. The Role of Transporters in the Pharmacokinetics of Antibiotics. *Adv. Pharmacoevidemiol. Drug Saf.* **2014**, *3*, 236–246.
44. Von Richter, O.; Greiner, B.; Fromm, M.F.; Fraser, R.; Omari, T.; Barclay, M.L.; Dent, J.; Somogyi, A.A.; Eichelbaum, M. Determination of in vivo absorption, metabolism, and transport of drugs by the human intestinal wall and liver with a novel perfusion technique. *Pharmacokinet. Drug Dispos.* **2001**, *70*, 217–227.
45. Wu, C.Y.; Benet, L.Z. Predicting drug disposition via application of BCS: Transport/absorption/elimination interplay and development of a biopharmaceutics drug disposition classification system. *Pharm. Res.* **2005**, *22*, 11–23.



© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).

4.2.2. Article 7: Oleacein intestinal permeation and metabolism in rats using an *in-situ* perfusion technique

Anallely López-Yerena, Maria Pérez, Anna Vallverdú-Queralt, Eleftherios Miliarakis, Rosa M. Lamuela-Raventós and Elvira Escribano-Ferrer

Pharmaceutics, 13(5), 719, 2021

Abstract

Background: OLEA is one of the most important EVOO PCs in terms of concentration and health-promoting properties, yet there are insufficient data on its absorption and metabolism. Several non-human models have been developed to assess the intestinal permeability of drugs, among them, SPIP, which is commonly used to investigate the trans-membrane transport of drugs *in situ*.

Scope: In this study, the SPIP model was used to monitor the intestinal permeability and metabolism of OLEA. For this purpose, the intestinal lumen and mesenteric blood plasma were used.

Methods and results: Samples of intestinal fluid and mesenteric blood were taken at different times and the ileum segment was excised at the end of the experiment for analysis by LC-ESI-LTQ-Orbitrap-MS. OLEA was mostly metabolized by phase I reactions, undergoing hydrolysis and oxidation, and metabolite levels were much higher in the plasma than in the lumen. The large number of metabolites identified, and their relatively high abundance indicates an important intestinal first-pass effect during absorption. According to the results, OLEA is well absorbed in the intestine, with an intestinal permeability similar to that of the highly permeable model compound naproxen. No significant differences were found in the percentage of absorbed OLEA and naproxen ($48.98 \pm 12.27\%$ and $43.96 \pm 7.58\%$, respectively).

Conclusions: This is the first *in situ* study to simultaneously assess the absorption and intestinal metabolism of OLEA in rats. The range and abundance of metabolites found in the perfusate and plasma suggest that the oral bioavailability of OLEA in rats is incomplete. The results indicate that the small intestine plays an important role in the bioavailability of OLEA, considering its high intestinal permeability and the metabolic reactions that contribute to its elimination. The metabolites arising from hydrolysis and hydroxylation were the main circulating metabolites of OLEA detected in plasma and the lumen. The higher metabolite levels in plasma suggests that the intestinal metabolism of OLEA occurs mainly during the transport of the compound across the intestinal membrane.



Article

Oleacein Intestinal Permeation and Metabolism in Rats Using an In Situ Perfusion Technique

Anallely López-Yerena ¹, Maria Pérez ^{1,2}, Anna Vallverdú-Queralt ^{1,3}, Eleftherios Miliarakis ⁴, Rosa M. Lamuela-Raventós ^{1,3} and Elvira Escribano-Ferrer ^{3,5,6,*}

- ¹ Department of Nutrition, Food Science and Gastronomy XaRTA, Faculty of Pharmacy and Food Sciences, Institute of Nutrition and Food Safety (INSA-UB), University of Barcelona, 08028 Barcelona, Spain; naye.yerena@gmail.com (A.L.-Y.); mariaperez@ub.edu (M.P.); avallverdu@ub.edu (A.V.-Q.); lamuela@ub.edu (R.M.L.-R.)
 - ² Laboratory of Organic Chemistry, Faculty of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain
 - ³ CIBER Physiopathology of Obesity and Nutrition (CIBEROBN), Institute of Health Carlos III, 28029 Madrid, Spain
 - ⁴ Department of Chemistry, Voutes Campus, University of Crete, 70013 Heraklion, Greece; leytmil@gmail.com
 - ⁵ Biopharmaceutics and Pharmacokinetics Unit, Department of Pharmacy and Pharmaceutical Technology and Physical Chemistry, Faculty of Pharmacy and Food Sciences, Institute of Nanoscience and Nanotechnology (IN2UB), University of Barcelona, 08028 Barcelona, Spain
 - ⁶ Pharmaceutical Nanotechnology Group I+D+I Associated Unit to CSIC, University of Barcelona, 08028 Barcelona, Spain
- * Correspondence: eescribano@ub.edu, Fax: +34-9340-35937



Citation: López-Yerena, A.; Pérez, M.; Vallverdú-Queralt, A.; Miliarakis, E.; Lamuela-Raventós, R.M.; Escribano-Ferrer, E. Oleacein Intestinal Permeation and Metabolism in Rats Using an In Situ Perfusion Technique. *Pharmaceutics* **2021**, *13*, 719. <https://doi.org/10.3390/pharmaceutics13050719>

Academic Editor: Im-Sook Song

Received: 27 April 2021

Accepted: 12 May 2021

Published: 14 May 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Abstract: Oleacein (OLEA) is one of the most important phenolic compounds in extra virgin olive oil in terms of concentration and health-promoting properties, yet there are insufficient data on its absorption and metabolism. Several non-human models have been developed to assess the intestinal permeability of drugs, among them, single-pass intestinal perfusion (SPIP), which is commonly used to investigate the trans-membrane transport of drugs in situ. In this study, the SPIP model and simultaneous luminal blood sampling were used to study the absorption and metabolism of OLEA in rats. Samples of intestinal fluid and mesenteric blood were taken at different times and the ileum segment was excised at the end of the experiment for analysis by LC–ESI–LTQ–Orbitrap–MS. OLEA was mostly metabolized by phase I reactions, undergoing hydrolysis and oxidation, and metabolite levels were much higher in the plasma than in the lumen. The large number of metabolites identified and their relatively high abundance indicates an important intestinal first-pass effect during absorption. According to the results, OLEA is well absorbed in the intestine, with an intestinal permeability similar to that of the highly permeable model compound naproxen. No significant differences were found in the percentage of absorbed OLEA and naproxen ($48.98 \pm 12.27\%$ and $43.96 \pm 7.58\%$, respectively).

Keywords: bioavailability; extra virgin olive oil; secoiridoids; metabolism; phenolic compounds; intestinal permeability

1. Introduction

The small intestine is the main site for drug absorption after oral administration [1,2], and the intestinal epithelial membrane is the principle physiological barrier that chemicals must cross to enter the bloodstream and become bioavailable [3]. Many in silico, in vitro, in situ, and in vivo models have been developed to investigate the transport mechanisms, intestinal permeability, and plasma pharmacokinetic profile of chemicals [4]. The in vivo models are the most clinically relevant because they include all the physiological factors that can affect absorption and bioavailability [1,4], but they are less useful for mechanistic studies. The in situ models, such as single-pass intestinal perfusion (SPIP), are commonly

used to investigate trans-membrane chemical transport. SPIP allows for intestinal effective permeability to be determined on the basis of the disappearance of the compound from the intestinal segment or its appearance in plasma after a venous sampling procedure [5–9], with the latter being especially suitable for poorly permeating substances [8]. Moreover, SPIP is the most similar alternative to an *in vivo* model in that it includes a mucus layer, blood irrigation, and innervation [4,6,7].

Intestinal permeability depends on the physicochemical properties and molecular structure and size of the drug or other xenobiotic molecules [2]. Lipophilicity, solubility, and the acid–base character can also affect the rate and extent of absorption, distribution, and transport through biological membranes [3]. In addition, the absorption rate is affected by the expression of transporter proteins and enzymes [1,3,10,11] that are highly region-dependent [2] and other biological factors [2,12].

The benefits of dietary phenolics have been extensively reported [13,14], especially as a protection from cardiovascular diseases. In extra virgin olive oil (EVOO), secoiridoids such as oleacein (OLEA) represent by far the most abundant group of phenolic compounds (>90%) [15] and are thought to be responsible for the healthy properties of the oil, although mediated by other components. In the review by Naruszewicz et al. (2015), OLEA is described as a substance with high pharmacological potential [16]. As well as various antioxidant and anti-inflammatory properties [17,18], OLEA exhibits anti-proliferative and anti-metastatic effects in the SH-SY5Y human neuroblastoma cell line [19].

Numerous studies have explored the bioavailability of phenolic compounds after the ingestion of EVOO or a phenolic extract, both in humans [20–25] and rats [26–29]. However, among the secoiridoids, most attention has been focused on oleuropein and hydroxytyrosol and its derivatives [30,31], and little information is available on OLEA. Thus, further animal and human studies using purified OLEA are needed to clarify whether the biological effects attributed to it are due to OLEA itself or its metabolites. In addition, as explained above, *in situ* studies are required to obtain detailed information on the intestinal permeability of OLEA and to verify the potential sites of its metabolization after oral administration.

To the best of our knowledge, no studies have been previously performed on pure OLEA using SPIP in rats. With this model, OLEA and its metabolites were simultaneously monitored in the intestinal lumen and mesenteric blood plasma, with the aim of shedding light on its intestinal permeability and metabolism. The resulting information will be useful for a better understanding of the biological effects attributed to OLEA. The highly permeable naproxen was included in the study as a reference drug.

2. Materials and Methods

2.1. Reagents and Materials

OLEA ($\geq 90\%$ purity) was purchased from Toronto Research Chemicals (North York, ON, Canada). Naproxen and phenol red were purchased from Sigma-Aldrich (Madrid, Spain). Heparin sodium salt from porcine intestinal mucosa, Hanks' balanced salt solution (HBBS), and HEPES 1M solution were also obtained from Sigma-Aldrich (Madrid, Spain). Pentobarbital sodium 200 mg/mL (Dolethal) was supplied by Vetoquinol (Madrid, Spain) and isoflurane by Laboratorios Esteve (Barcelona, Spain). The solvents acetonitrile and methanol, and the chemical formic acid were acquired from PanReac AppliChem (Panreac Quimica SLU, Barcelona, Spain). Finally, a Milli-Q purification system was used to obtain ultrapure water (Millipore, Bedford, MA, USA).

2.2. Work Solutions

The transport medium (TM; pH 7, 9.7 g/L HBSS buffered with HEPES 10 mM) was used to infuse the compounds via the intestine. The secoiridoid OLEA (Figure 1) was assayed at 0.15 mg/mL (468.3 μ M), a concentration chosen after taking into account both the OLEA concentration in EVOO (around 300 mg/kg) and the daily ingestion of EVOO recommended by the European Food Safety Authority (EFSA) (at least 5 mg of hydroxytyrosol and its derivatives per 20 g of olive oil) [32]. In accordance with its high

dose strength, naproxen was assayed at 2.2 mg/mL in 250 mL of TM, as recommended [33]. Red phenol was assayed at 0.1 mg/mL. The stability of these test compounds, sampled at different times, was monitored in the perfusion solution at 37 °C for 60 min.

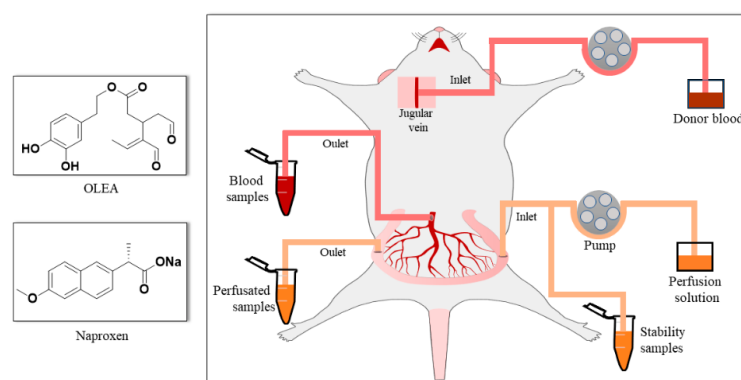


Figure 1. Chemical structure of the perfused compounds (OLEA and naproxen) and surgical procedure.

2.3. Animals

The Animal Experimentation Ethics Committee of the University of Barcelona, Spain (trial no. CEEA 124/16), and Generalitat de Catalunya (no. 6435, 27 June 2019) approved the study protocol. For each compound, four male Sprague-Dawley rats (Envigo RMS Spain SL, Sant Feliu de Codines, Barcelona, Spain) were used per intestinal perfusion experiment and four as blood donors (body weight 285 ± 6 g and 300–400 g, respectively). The animals arrived at the animal facility 10 days before the experiment and were kept with water and food ad libitum, with a 12 h light and dark cycle, at 21 °C and humidity-controlled ($55 \pm 10\%$).

2.4. Single-Pass Intestinal Perfusion Studies

The SPIP studies were performed in anesthetized rats according to the method described by López-Yerena and coworkers [6]; the donor blood and surgical procedure (Figure 1) were as described in that study. Regarding the procedure of donor blood, the rats were anesthetized by isoflurane inhalation (2.5%), and the whole blood was collected via cardiac puncture. The blood was diluted with heparin (50 u/mL TM) to 80% blood and kept in a 20 mL syringe for the in situ SPIP experiment. In the case of the rats that underwent surgery, the rats received intraperitoneal administration of pentobarbital sodium (Dolethal) at a dose of 60 mg/kg, and a maintenance dose of isoflurane (1.5%) was given 50 min after the induction dose. Body temperature was maintained at 37 ± 0.5 °C throughout the experiment by means of a homeothermic blanket. An ileal segment of approximately 7–10 cm was cannulated. The intestinal perfusion was started by delivering the perfusion solution containing OLEA or naproxen (in separate experiments) and phenol red at a flow rate of 0.2 mL/min to the cannulated intestinal segment, and the blood was supplied through the jugular vein at a rate of 0.3 mL/min. The phenol red included in the perfusion solution was used as non-absorbable marker for measuring water transport. To avoid the oxidation of OLEA, we carried out the SPIP experiments in a laboratory room with infrared light. Samples of both the intestinal lumen and mesenteric blood were collected simultaneously at 5 min intervals for 60 min. The outflow perfusate samples were collected in 2 mL amber vials and the blood was collected in pre-weighed lithium-heparinized tubes (BD Vacutainer). Next, the perfusate samples were centrifuged ($7516 \times g$ for 10 min at 4 °C), and the tubes with blood samples were weighed and centrifuged ($3000 \times g$ for 10 min at 4 °C). The supernatants were collected and immediately stored at -80 °C awaiting analysis by LC-ESI-LTQ-Orbitrap-MS.

2.5. Biological Sample Treatment

Plasma: The extraction of OLEA and its metabolites was carried out with protein precipitation. Initially, the samples were thawed and centrifuged ($11,000\times g$, 10 min at $4\text{ }^{\circ}\text{C}$). To precipitate proteins, we blended 100 μL of plasma with cold acetonitrile containing 2% of formic acid (1:5 *v/v*). Samples were vortex-mixed for 1 min and kept at $-20\text{ }^{\circ}\text{C}$ for 20 min. The samples were then centrifuged ($11,000\times g$, $4\text{ }^{\circ}\text{C}$, 10 min), and finally 100 μL of the supernatant was transferred to vials for analysis.

With regard to naproxen, the plasma samples were deproteinized following the methodology proposed by Elsinghorst and colleagues [34], with some modifications. Rat plasma samples (100 μL) were deproteinized by the addition of 200 μL of acetonitrile. After thorough vortex-mixing, the samples were centrifuged ($2733\times g$, $4\text{ }^{\circ}\text{C}$, 10 min), and the supernatant was mixed with 0.02 M ammonium acetate buffer (pH 4.0) (1:1 *v/v*). Again, they were vortex-mixed and after centrifugation, and 100 μL of the supernatant was transferred to vials for analysis by LC–MS/MS.

Stability study and lumen samples: The stability and perfusate OLEA samples were defrosted and centrifuged ($11,000\times g$, $4\text{ }^{\circ}\text{C}$, 10 min), and 100 μL of the upper phase was transferred to vials for analysis. The equivalent naproxen samples were thawed and centrifuged ($11,000\times g$, $4\text{ }^{\circ}\text{C}$, 10 min), and the upper phase was diluted with TM in a ratio of 5:95 (stability samples) and 1:9 (*v/v*) (perfusate samples) and transferred to vials.

Ileum tissue: The intestinal segment perfused with OLEA in the SPIP experiments was also analyzed to quantify the OLEA and its metabolites retained in the tissue. First, the intestinal segment was rinsed by several perfusions with TM and then cut into small sections and homogenized after the addition of water/acetonitrile (1:1 (*v/v*) with 0.1% ascorbic acid) with a small tissue disruptor (T10 basic ULTRA-TURRAX®, IKA laboratory technology, Staufen, Germany). The samples were sonicated in an ice bath (5 min), shaken for 1 min, and centrifuged ($11,000\times g$, $4\text{ }^{\circ}\text{C}$, 10 min). To precipitate proteins, we blended 100 μL of the upper layer with cold acetonitrile containing 2% of formic acid (1:3 *v/v*) [35]. Samples were vortex-mixed for 1 min, kept at $-20\text{ }^{\circ}\text{C}$ for 20 min, and centrifuged again ($11,000\times g$, $4\text{ }^{\circ}\text{C}$, 10 min) before analyzing the supernatant.

2.6. Analytical Technique

2.6.1. OLEA Analysis

The quantification of OLEA and red phenol in the samples and the profiling and structural characterization of OLEA metabolites was carried out using LC–ESI–LTQ–Orbitrap–MS. LC separation was performed using an Accela chromatograph (Thermo Scientific, Hemel Hempstead, UK) equipped with a quaternary pump, a photodiode array detector, and a thermostated autosampler. A 5- μL sample aliquot was injected onto an Acquity™ UPLC® BEH C₁₈ Column (2.1 $\times$ 100 mm, i.d., 1.7 μm particle size) coupled to an Acquity™ UPLC® BEH C₁₈ Pre-Column (2.1 $\times$ 5 mm, i.d., 1.7 μm particle size) (Waters Corporation®, Wexford, Ireland) with the column temperature set at $50\text{ }^{\circ}\text{C}$. Eluent A was 0.05% (*v/v*) formic acid in water, and eluent B was 0.05% formic acid in methanol. The total run time was 11 min. The elution gradient (0.6 mL/min) started at 0% B and was increased via a linear gradient to 53.6% B after 6 min. The gradient was then increased for 2 min to 100% B and held for 1 min before returning to 0% B for 1.9 min to re-equilibrate the column.

The mass spectrometer used for the analysis was an LTQ Orbitrap Velos (Thermo Scientific, Hemel Hempstead, UK) equipped with an electrospray (ESI) source. The ESI source was operated in negative mode [M–H] with the following conditions: source voltage, 4 kV; capillary temperature, $275\text{ }^{\circ}\text{C}$ (FT Automatic gain control (AGC) target 5–105 for MS mode and 5–104 for MSⁿ mode); sheath gas (ultra-pure nitrogen, >99.9%); flow rate 20; auxiliary gas flow rate 10; and sweep gas flow rate 2. In the case of the last 3 parameters, the arbitrary units were initially used, Fourier transform mass spectrometry (FTMS) mode was then used to analyze at a resolving power of 30,000 at m/z 600, and the data-dependent MS/MS events were acquired at a resolving power of 15,000 at m/z 600. The most intense ions detected in FTMS mode triggered data-dependent scanning. Ions that were not

intense enough for a data-dependent scan were analyzed in MSⁿ mode with the same orbitrap resolution (15,000 at m/z 600). Precursors were fragmented by collision-induced dissociation (CID) using a C-trap with normalized collision energy (35 V) and an activation time of 10 ms. The mass range in FTMS mode was from 100 to 600 (m/z). The system was controlled by Xcalibur 3.0 software (ThermoFisher Scientific, Hemel Hempstead, UK).

Accurate masses and the isotopic pattern (through the Formula Finder feature in Xcalibur 3.0 software (ThermoFisher Scientific, Hemel Hempstead, UK) were used to select the elemental composition of each OLEA derivative. In addition, metabolites were confirmed by comparison with those reported in the literature [21,22] and with a similar compound [6,36,37]. MSⁿ measurements were performed to obtain information about fragment ions generated in the linear ion trap within the same analysis.

The OLEA and phenol red calibration curves were prepared in TM (10–150 µg/mL). The OLEA calibration curves were also prepared in rat plasma (0.1–3 µg/mL) and ileum tissue (0.1–3 µg/mL). All calibration curves had an $R^2 > 0.97$. In the absence of a reference standard, OLEA derivatives were evaluated by considering the ratio between peak area metabolite and parent compound dosed (OLEA) [6].

2.6.2. Naproxen Analysis

All luminal, plasma, and stability samples were analyzed by ultra-high-performance liquid chromatography/ESI tandem mass spectrometry (UHPLC–ESI–MS/MS) following the procedure proposed by Elsinghorst et al. with some modifications [34]. The liquid chromatography system consisted of an AcquityTM UPLC (Waters; Milford, MA, USA). Chromatographic separations were performed on an XBridgeTM C₁₈ (4.6 × 50 mm, 5 µm particle size) column (Waters Corporation®, Wexford, Ireland). The mobile phase consisted of 0.02 M ammonium acetate buffer (pH 4.0) and acetonitrile (30/70, v/v) and was delivered at a flow rate of 1.0 mL/min (column temperature at 30 °C). A total of 10 µL of sample was injected.

The detection and quantification of naproxen and red phenol were performed using an AB SCIEX API 3000TM triple quadrupole mass spectrometer with a turbo ion spray source. Ionization was performed by ESI in the negative mode [M–H] in the multiple monitoring mode (MRM). Arbitrary units were used for the nebulizer (10), curtain (12), and drying gas (450 °C) using N₂; the capillary voltage was –3500 V. To detect naproxen and red phenol with the highest signal, we optimized the collision energy and the declustering, focusing, and entrance potential by direct infusion. The system was operated by Analyst version 1.4.2 software supplied by ABSciex (ABSciex, Framingham, MA, USA).

The calibration curves with naproxen and phenol red were prepared in TM (10–150 µg/mL) and in rat plasma (0.5–20 µg/mL). The samples were adequately diluted to be interpolated in the calibration curves. All calibration curves had an $R^2 > 0.98$.

2.7. Data Analysis

The equations used to determine the effective permeability coefficient, the correction of outlet concentrations, and the apparent permeability coefficient through the ileum are as follows:

$$P_{\text{eff}} = \frac{-O_{\text{in}}}{2 * \pi * R * L} * Ln \frac{C_{\text{out.cor}}}{C_{\text{in}}} \quad (1)$$

$$C_{\text{out.cor}} = C_{\text{out}} * \frac{CPR_{\text{in}}}{CPR_{\text{out}}} \quad (2)$$

$$P_{\text{app}} = \frac{dQ}{dt} * \frac{1}{A * C_0} \quad (3)$$

where O_{in} is the perfusion solution flow (0.2 mL/min), C_{in} and $C_{\text{out.cor}}$ are the respective inlet and corrected outlet steady-state concentrations of the tested product, R is the radius of the intestinal segment (set to 0.2 cm), and L is the length of intestinal segment determined after completion of the perfusion experiment.

The outlet concentrations were corrected for water transport by measuring the phenol red (PR) marker according to Equation (2), where C_{out} is the concentration of OLEA or naproxen in the perfusate at the specified time interval, and CPR_{in} and CPR_{out} are the phenol red concentrations in the inlet and outlet solutions at the specific time intervals, respectively.

The P_{app} was calculated using Equation (3), where Q is the cumulative number of tested compounds (OLEA or naproxen) appearing in the mesenteric blood as a function of time t in steady state conditions, A is the surface area of the exposed intestinal segment, and C_0 is the tested compounds initial concentration in the perfusate.

All in situ perfusion experiments were replicated in four rats. Data are presented as the arithmetic mean $\pm$ the standard deviation (SD). Statistical analysis was performed using Statgraphics Centurion XVI software (Statpoint Technologies Inc., Warrenton, VA, USA). The concentrations estimated at a range of times (stability samples) and the concentration of metabolites and OLEA at different times were compared using a parametric statistical assay (ANOVA test). Statistical differences in the concentration of metabolites between plasma and perfusion samples were analyzed using an ANOVA test, followed by the LSD post hoc test. The P_{eff} and P_{app} of OLEA and naproxen were compared using a Mann–Whitney U test. Differences were considered significant at $p < 0.05$.

3. Results and Discussion

3.1. Stability of OLEA

The stability of OLEA and the reference standard naproxen was evaluated prior to carrying out the intestinal permeability study, and both remained stable in the perfusion solution at 37 °C ($p > 0.05$) for the length of the experiments, i.e., over 60 min (Figure S1). The absence of degradation products such as M2 and elenolic acid was also verified, although traces of a hydrated form of OLEA (M5) were detected, probably due to interaction of the aldehyde groups with the aqueous TM during the analysis.

3.2. Qualitative and Quantitative Characterization of OLEA and Its Metabolites

The lumen, plasma, and ileum samples were analyzed by HPLC–ESI–LTQ–Orbitrap–MS to identify OLEA and its metabolites. The FTMS scan and MSⁿ experiments allowed for the identification of OLEA (M1), four phase I metabolites (M2, M3, M4, and M5), and six phase II metabolites (M6, M7, M8, M9, M10, and M11). These metabolites and their precursor ions (measured), tentative formula, mass error, retention times, and major fragments are presented in Table 1. An example of a chromatogram for each metabolite and the parent compound is shown in the supporting information (Figure S2), and the structure for the detected fragments is also proposed in Figure S3.

Table 1. Identification of OLEA and its metabolites in lumen, plasma, and ileum tissue samples by LTQ–Orbitrap–MS.

	Compound	Precursor Ion Measured m/z [M – H] [–]	Tentative Formula [M – H] [–]	Mass Error (ppm)	RT (min)	MS/MS
M1	OLEA	319.1184	C ₁₇ H ₁₉ O ₆	0.785	6.81	153/183
Phase I						
M2	OH-TY	153.0554	C ₈ H ₉ O ₃	0.779	3.67	123
M3	OLEA + H ₂	321.1337	C ₁₇ H ₂₁ O ₆	0.435	7.05	185/199/143
M4	OLEA + OH	335.1128	C ₁₇ H ₁₉ O ₇	0.271	6.82	131/199
M5	OLEA + H ₂ O	337.1282	C ₁₇ H ₂₁ O ₇	0.021	6.69	201/133
Phase II						
M6	OLEA + CH ₃	333.1348	C ₁₈ H ₂₁ O ₆	0.835	8.51	167
M7	OLEA + OH + CH ₃	349.1277	C ₁₈ H ₂₁ O ₇	–0.479	7.38	167/199
M8	OLEA + H ₂ O + CH ₃	351.1445	C ₁₈ H ₂₃ O ₇	1.771	7.20	215/167
M9	OLEA + H ₂ + Glucu	497.1665	C ₂₃ H ₂₉ O ₁₂	1.247	6.53	199/329
M10	OLEA + H ₂ O + Glucu	513.1621	C ₂₃ H ₂₉ O ₁₃	1.833	6.43	329/215
M11	OLEA + H ₂ O + CH ₃ + Glucu	527.1743	C ₂₄ H ₃₁ O ₁₃	0.963	6.50	343/201

RT: retention time; Glucu: glucuronic acid; OH-TY: hydroxytyrosol.

OLEA metabolites have been studied previously, but their structures have not been reported [21,22]. As mentioned above, on the basis of the fragmentation pattern of each detected metabolite and in comparison with related phenolic compounds [6,36], we proposed tentative structures for the OLEA derivatives (Figure 2).

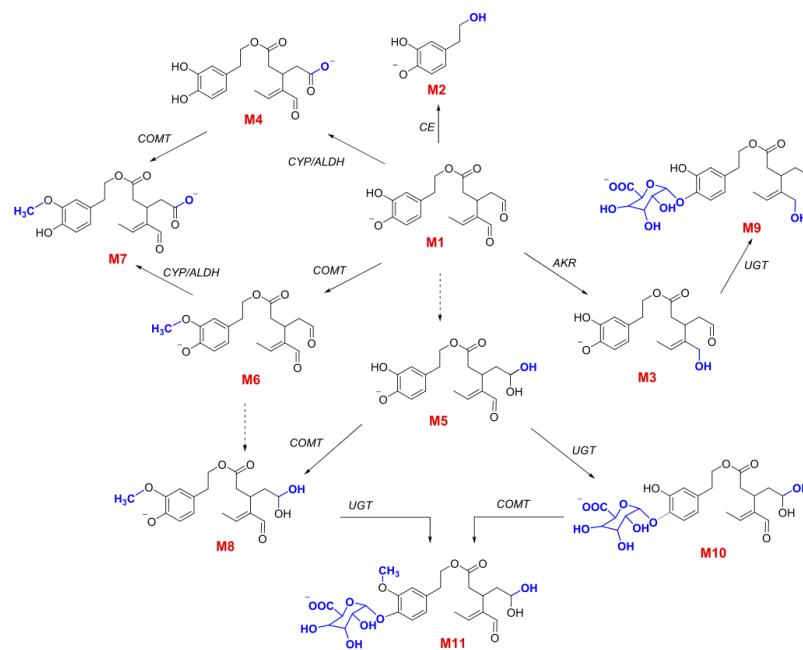


Figure 2. Proposed metabolic pathway of OLEA (M1) with phase I and phase II reactions. The chemical structures of M1 to M11 were identified in the plasma, lumen, and/or ileum samples after the SPIP study. CE: carboxylesterases; AKR: aldo-keto reductases; CYP3A: subfamily of cytochrome P450 enzymes; UGTs: glucuronosyltransferases; COMT: catechol-O-methyltransferase; ALDH: aldehyde dehydrogenase.

3.3. Phase I Metabolism

OLEA metabolites arising from phase I reactions both in lumen and plasma samples are shown in Figure 3. For the first time, the metabolic profile of OLEA in the ileum of rats after an SPIP assay has been evaluated. It is worth noting that the metabolite levels were much higher in plasma than the lumen, e.g., levels of M2 and M4 were 27- and 29-fold higher at 60 min, respectively. In the ileum tissue samples, the main metabolite was M2 ($p < 0.05$). The main phase I metabolites detected in plasma and lumen samples were M2 and M4, and its concentration was favored in time reaching the higher concentration at 55 and 60 min, respectively ($p < 0.05$). The large number of metabolites identified and their relatively high abundance (peak area metabolite/parent ratio up to 15 in plasma) indicates an important intestinal first-pass effect during the absorption of OLEA and that the metabolites formed are mainly transferred to the systemic circulation.

Hydrolysis is known to be a common process in phase I drug metabolism. Carboxylesterases (CEs) catalyze the hydrolysis of esters, thioesters, amides, and carbamates, with carboxylic acids and alcohols as the hydrolysis products [38,39]. Among the enzymes of this family, CES2 is present in the small intestine in both humans and rats [40]. The hydrolysis of OLEA can lead to the formation of hydroxytyrosol (M2) and elenolic acid [27]. In our study, M2 was found in lumen, plasma, and ileum tissue, but elenolic acid was not detected in any sample. Similar results were obtained in the study of Pinto et al. (2011), where the M2 metabolite was detected in Caco-2 cells and in the in vitro study of

isolated intestine (in the serosal fluid of the jejunum and ileum), but not elenolic acid [41]. Similarly, only the conjugated forms of M2 were detected in the stomach, intestinal and caecum content, and feces of rats fed for 21 days with a diet supplemented with an extract composed mainly of OLEA [27], although elenolic acid was found in plasma and urine. The absence of elenolic acid in our samples could be explained by its rapid absorption from the hydrolyzed OLEA fraction. In the study of Kano et al. (2016), M2 was also the main metabolite found in the plasma of portal blood after oral administration of OLEA to rats [29]. In addition, M2 was not detected in human urine after EVOO intake [21,22].

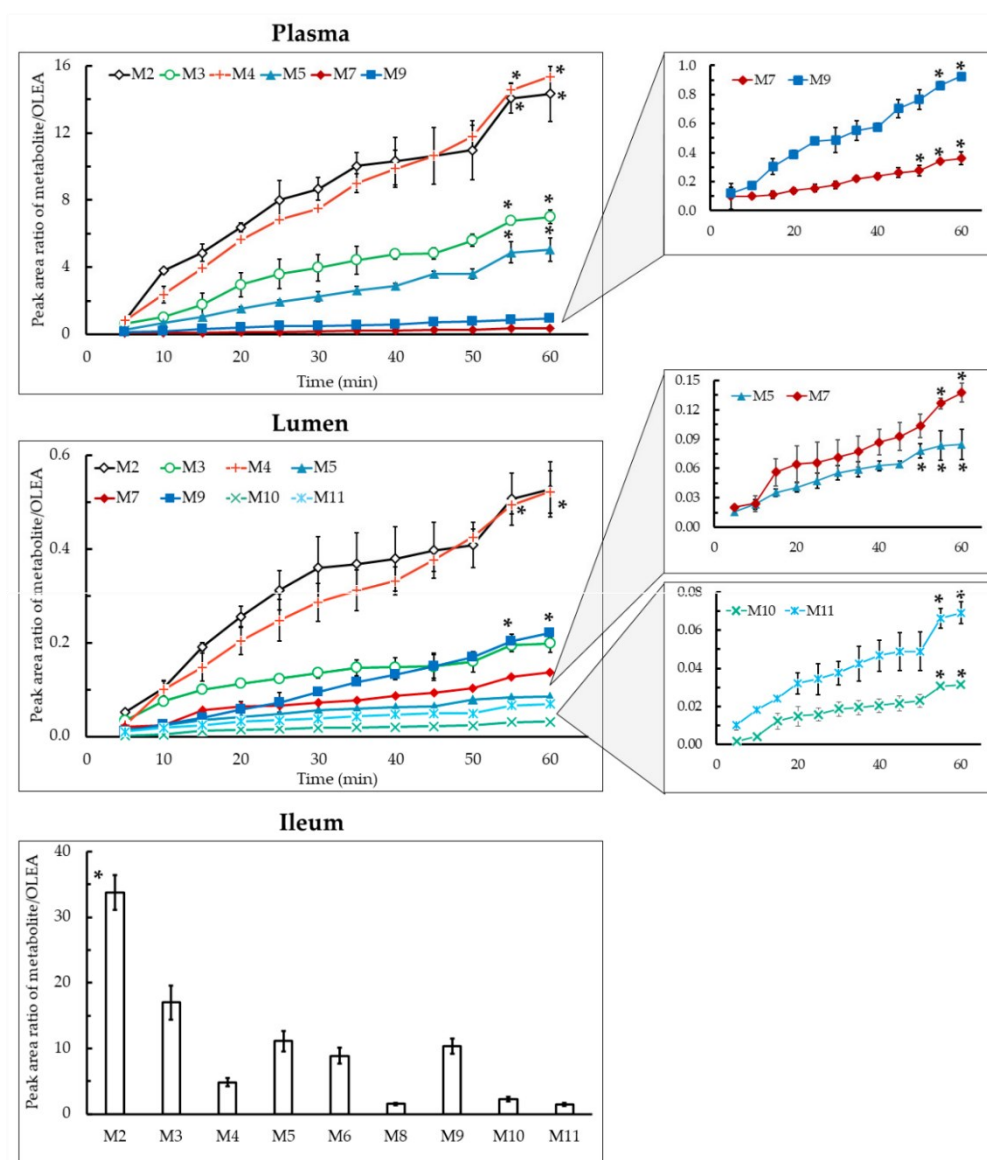


Figure 3. Peak area ratio of metabolite/OLEA as a function of time in plasma, lumen, and ileum tissue samples. Results are expressed as the mean $\pm$ standard deviation. * $p < 0.05$, one-way ANOVA.

Hydrogenated (M3), oxidated (M4), and hydrated (M5) forms of OLEA were detected in lumen, plasma, and ileum tissue (Figure 3). Major phase I enzymes include oxidases (especially monooxygenases), reductases, and hydrolases [10]. The hydrogenation of OLEA (M3) can arise from a reduction reaction catalyzed by NADPH-dependent aldo-keto reductases located in the small intestine epithelium. The reduction of aldehydes to primary alcohols can occur in OLEA because it contains the dialdehydic form of the linked enlenic acid [42]. In addition, the intestinal redox potential provides a reducing environment due to low oxygen tension, whereas oxidation is favored in tissues such as the liver [43]. Pinto et al. [41] proposed a structure for M3 but they were unable to confirm which of the carbonyl functional groups had undergone reduction, as the reaction at either site yielded similar fragmentation patterns. In our study, on the basis of the fragment detected (mass 143), we proposed a hydrogenation of the unsaturated aldehyde, although this cannot be confirmed without an NMR spectrum or the fragmentation analysis of a previously synthesized structure (Figure S3).

Cytochrome P450 enzymes (CYP), monooxygenases found in the epithelium of the small intestine [44], are responsible for the oxidative biotransformation of xenobiotics and other compounds [38,45]. In our work, the OLEA derivatives M4 and M5 could have been produced by the microbiome, as many bacterial CYP are soluble [46], or by the CYP expressed in the enterocytes, as shown in our luminal and tissue samples (Figure 3). However, non-CYP-mediated oxidative reactions can play an important role in the metabolism of xenobiotics [47]. Regarding carboxylic acids, as they are products of aldehyde oxidation, they could also be generated by aldehyde dehydrogenase enzyme catalysis. While only traces of M5 were detected in the TM (stability study), the high amount in both lumen and plasma samples indicates a metabolic reaction during its transport across the intestinal membrane. In 2010, Garcia-Villalba and co-workers found the same OLEA derivatives (M3, M4, and M5) in human urine after olive oil intake [21]. Although these derivatives were not found in plasma and urine samples of healthy volunteers in the study of Silva and co-workers [22], a hydrated OLEA metabolite was observed ($\text{OLE} + \text{CH}_3 + \text{H}_2\text{O} + \text{glucuronide}$). In fact, the metabolic profile of phenolic compounds from EVOO has been previously studied [23,26,41], but OLEA derivatives have not yet been reported.

3.4. Phase II Metabolism

Phase II biotransformation reactions, also known as conjugation reactions, generally serve as a detoxifying step in xenobiotic metabolism, increasing hydrophilicity and therefore excretion, as well as the metabolic inactivation of pharmacologically active compounds [48,49]. Phase II derivatives detected in plasma (M7 and M9), lumen (M7 and M9–M11), and ileum tissue samples (M6, M8, M9, and M11) are presented in Figure 3. The main product of phase II biotransformation reactions in all the samples was M9, with the highest relative abundance in plasma at 45–60 min, and in lumen samples at 55–60 min ($p < 0.05$).

It is well known that the enzyme that catalyzes *O*-methylation is catechol-*O*-methyl transferase, which mediates the transfer of a methyl moiety from the *S*-adenosyl-L-methionine cofactor to a hydroxyl group on the xenobiotic [50]. In rats and humans [51], catechol-*O*-methyltransferase is most active in the liver, kidney, intestine, and brain [52]. In agreement with the computational study carried out by Cuyàs et al. (2019), which concluded that meta-methylation at the O5 position of the catechol residue of OLEA occurs preferentially, we proposed the methylated derivative M6 (Figure 2) [50]. Among the three methylated metabolites, M6 and M8 were found in ileum tissue samples but not in the plasma and lumen (Figure 3), whereas M7 ($\text{OLEA} + \text{OH} + \text{CH}_3$) was detected in plasma and, after being secreted by an efflux membrane protein, in lumen samples. Previously reported results for these methylated forms are contradictory, being detected in human urine samples by Garcia-Villalba et al. [21] but not in other studies in rats [26,29,41]. Differences in experimental procedures may explain these discrepancies.

The glucuronidation reaction consists of transferring a glucuronyl moiety from the co-substrate UDP-glucuronic acid to one or more electrophilic groups of a hydrophobic molecules. The family of uridine diphosphate (UDP) glucuronosyltransferases are the enzymes that catalyze this reaction [53]. Glucuronidation often occurs as a secondary step after the production of primary metabolites in phase I reactions such as hydrolysis, hydroxylation, and dealkylation [54], as can be observed in M9 and M10, in which a glucuronic acid is attached to the hydrated and hydrogenated OLEA. Similarly, M11 arises from the addition of a methyl group and a glucuronic acid in a previously hydrated molecule of OLEA. M11 was detected in lumen and ileum tissue samples but not in plasma. The glucuronidation of hydrogenated OLEA has been previously reported in perfused segments of jejunum and ileum in rats [41]. Two different studies in humans obtained similar results: García-Villalba et al. [21] detected all glycoconjugates (M9, M10, and M11), while Silva et al. [22] only found M9 and M11. The bioavailability of phenolic compounds in EVOO depends not only on their concentration but also other dietary components and the individual genomic profile, which can affect enzymatic activity involved in the digestion and metabolism processes [22]. Polymorphism of conjugation enzymes or individual variations in digestive enzymes or bile salts could underlie the variations observed [1,2,17]. The aforementioned studies on humans reported three additional glycoconjugates (OLEA + glucuronide, OLEA + CH₃ + glucuronide, and OLEA + CH₃ + OH + glucuronide) not identified in our work, which may have been due to differences in the species and model used to evaluate the intestinal metabolism (in vivo vs. in situ models) [1]. The presence of these metabolites in humans can also be explained by the hepatic metabolism that OLEA or its derivatives may undergo after absorption.

In the present work, the observed phase I (M2–M5) and phase II (M7 and M9) OLEA derivatives in both lumen and plasma samples can be attributed to the presence of specific membrane transporters expressed in the apical (MDR1, BCRP, MRP2) or basal membrane (MRP1) of the enterocytes [55]. The OLEA derivatives recognized by these efflux transporters would thus be secreted to the intestinal lumen. On the basis of the results obtained, Figure 4 depicts the metabolic fate of OLEA in the small intestine, showing possible interactions with metabolic enzymes and carriers during transport across the enterocyte.

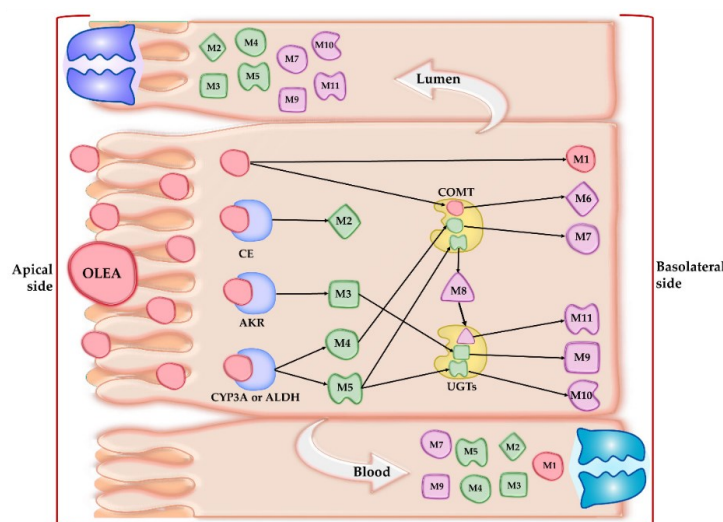


Figure 4. Tentative interactions of oleacein (OLEA) with metabolic enzymes and transporters. CE: carboxylesterases; AKR: aldo-keto reductases; CYP3A: subfamily of cytochrome P450 enzymes; UGTs: glucuronosyltransferases; COMT: catechol-O-methyltransferase; ALDH: aldehyde dehydrogenase.

3.5. Absorption Study

To investigate the intestinal permeability of OLEA, we carried out a comparative study with the anti-inflammatory drug naproxen. A highly permeable standard [55], naproxen has an oral bioavailability close to 100% [33] and was tested with the same in situ perfusion technique and conditions as OLEA.

The SPIP model is used in general screening for the intestinal membrane permeability of orally administered drugs and xenobiotics and to predict the effective permeability coefficient (P_{eff}). In this study, the ileum permeability of the tested compounds was based on luminal disappearance as well as appearance in mesenteric blood plasma, a suitable option for substances with low membrane permeability, as the differences in perfusate concentrations may be too small to determine accurately [8]. To obtain information about the intestinal metabolism of OLEA (see Sections 3.3 and 3.4), we analyzed perfusate and plasma samples for potential derivatives. The results of the absorption study are presented in Table 2, together with previously reported data for comparison. As can be observed, the P_{eff} values we obtained for naproxen agreed with those in the literature, indicating the validity of the methodology employed. However, as the variability between laboratories is relatively high, the data from individual studies should be interpreted separately [56].

Table 2. Intestinal effective permeability coefficient (P_{eff}), apparent permeability coefficient (P_{app}), and percentage of absorption after SPIP (mean $\pm$ SD, $n = 4$) for OLEA and the reference standard naproxen. Reported data are also included.

Compound	Segment	$P_{\text{eff}} \times 10^{-4}$ (cm/s) $\pm$ SD	$P_{\text{app}} \times 10^{-4}$ (cm/s) $\pm$ SD	Absorption (%)	Study
OLEA	Ileum	1.83 $\pm$ 0.18	0.607 * $\pm$ 0.202	48.98 $\pm$ 12.27	Current study
		1.47 $\pm$ 0.44	0.19 $\pm$ 0.018	43.96 $\pm$ 7.58	Current study
		1.17 $\pm$ 0.23			[57]
		1.78 $\pm$ 0.52			[56]
Naproxen	Jejunum	1.17 $\pm$ 0.23			[58]
		1.19 $\pm$ 0.12			[33]
		1.47 $\pm$ 0.25			[56]
		2.10 $\pm$ 0.41			[59]
	Colon	2.06 $\pm$ 1.04			[56]

* $p < 0.05$ differences OLEA vs. naproxen (Mann–Whitney U test).

Despite the broad range of promising biological effects of secoiridoids from EVOO [17], the P_{eff} value of OLEA has not been previously reported. The mean permeability ratio (P_{eff}) of OLEA/naproxen was 1.24, without significant differences between them ($p > 0.05$), which indicates that the intestinal permeability of OLEA is comparable with that of the highly permeable standard. No significant differences were found for the percentage of drug absorbed (48.98 $\pm$ 12.27% and 43.96 $\pm$ 7.58% for OLEA and naproxen, respectively). These results are coherent with the lipophilicity (expressed as log P (octanol/water)) and molecular weight of OLEA (1.53 [60]; 1.02 [16], and 320.3 g/mol, respectively), which favor intestinal membrane transport by passive mechanisms [61].

In the mesenteric blood plasma, in our experimental conditions, the P_{app} of naproxen was significantly lower than that of OLEA (Figure 5B). As naproxen has a complete oral bioavailability, this result could be explained by a higher retention in or interaction with the lipid membranes (apical and basolateral), attributable to its high lipophilicity (log $P = 3.18$ [62]) and molecular structure, rather than a presystemic intestinal metabolism. Other authors have described an affinity of naproxen and other nonsteroidal anti-inflammatory drugs (NSAIDs) for the phosphatidylcholine of biological membranes [63,64]. It would therefore have been interesting to extend the study time from 60 min to, for example, 90 min.

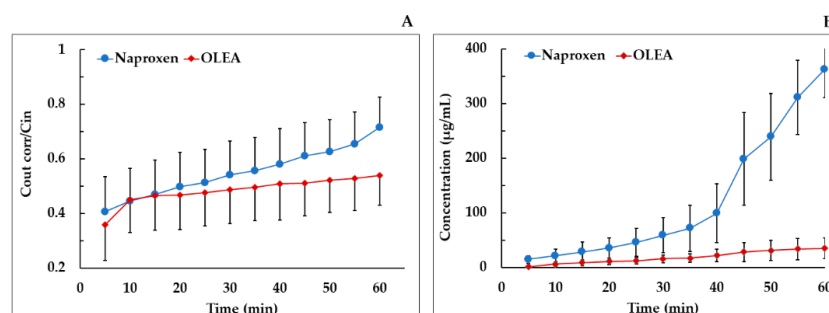


Figure 5. (A) Plot of the mean concentration ratio of the corrected outlet and inlet concentrations vs. time for naproxen and OLEA in SPIP in rats; (B) mean mesenteric plasma concentration of naproxen and OLEA vs. time. Error bars represent S.D.

To the best of our knowledge, this is the first study to report the intestinal and apparent permeability coefficients of OLEA in rats. Pinto et al. (2011), who investigated the metabolism of OLEA in an in vitro intestinal preparation from rats, also described its transport through the small intestine, observing OLEA in the receptor medium in an in vitro assay with Caco-2 cells [41]. Kano et al. (2016), who studied the absorption, metabolism, and excretion of OLEA after oral administration (300 mg/kg) in rats, did not observe OLEA in the portal plasma, possibly because its detection was hindered by binding to plasma components such as serum albumin, serum lipoprotein, and glycoprotein [29]; phenolic compounds and their metabolites are known to form complexes with plasma proteins [65]. Although further investigation is required, the amount of OLEA metabolites found in the perfusate and plasma in our study suggests that the bioavailability of this phenolic compound is incomplete. In conclusion, the important role of the small intestine in the bioavailability of OLEA has been demonstrated in terms of absorption and membrane transport as well as metabolic reactions that contribute to its elimination.

4. Conclusions

This is the first in situ study to simultaneously assess the absorption and intestinal metabolism of OLEA in rats. The SPIP model was used to determine the intestinal effective permeability of OLEA on the basis of its disappearance from the intestinal segment and its appearance in mesenteric blood. The range and abundance of metabolites found in the perfusate and plasma suggest that the oral bioavailability of OLEA in rats is incomplete. The results indicate that the small intestine plays an important role in the bioavailability of OLEA, considering its high intestinal permeability and the metabolic reactions that contribute to its elimination. The metabolites arising from hydrolysis (M2) and hydroxylation (M4) were the main circulating metabolites of OLEA detected in plasma and the lumen. The higher metabolite levels in plasma suggests that the intestinal metabolism of OLEA occurs mainly during the transport of the compound across the intestinal membrane.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/pharmaceutics13050719/s1>, Figure S1. Concentration of NAP (right Y-axis scale) and OLEA (left Y-axis scale) in the TM and 37 °C as a function of time in the stability study. Figure S2. Chromatogram and retention time of OLEA and its metabolites. Figure S3. Molecular structure of OLEA and derivatives and proposed fragments with their masses through LQT-Orbitrap-MS analysis.

Author Contributions: Conceptualization, A.L.-Y., E.E.-F., and R.M.L.-R.; methodology, A.L.-Y., E.E.-F., E.M., and A.V.-Q.; software, A.L.-Y., E.E.-F., and A.V.-Q.; validation, A.L.-Y., E.E.-F., and R.M.L.-R.; formal analysis, A.L.-Y. and E.E.-F.; investigation, A.L.-Y. and E.E.-F.; resources, R.M.L.-R.; data curation, A.L.-Y., E.E.-F., M.P., and A.V.-Q.; writing—original draft preparation, A.L.-Y. and E.E.-F.; writing—review and editing, A.L.-Y., E.M., and E.E.-F.; visualization, A.L.-Y., M.P., and E.E.-F.; supervision, E.E.-F. and R.M.L.-R.; project administration, E.E.-F. and R.M.L.-R.. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by CICYT [AGL2016-75329-R], CIBEROBN from the Instituto de Salud Carlos III, ISCIII from the Ministerio de Ciencia, Innovación y Universidades, (AEI/FEDER, UE), and Generalitat de Catalunya (GC) [2017SGR 196]. A.L.-Y. wishes to thank the Consejo Nacional de Ciencia y Tecnología (CONACYT) of Mexico for the doctoral scholarship. M.-P. thanks the Ministry of Science Innovation (MICIU/FEDER) for the project (RTI2018-093974-B-I00). A.V.-Q. thanks the Ministry of Science Innovation and Universities for the Ramon y Cajal contract (RYC-2016-19355). E.M. thanks the technical university of CRETA (TUC) and from Athens University of Economics and Business (AUEB) for the Erasmus scholarship.

Institutional Review Board Statement: The Animal Experimentation Ethics Committee of the University of Barcelona, Spain (trial no. CEEA 124/16), and Generalitat de Catalunya (no. 6435) approved the study protocol.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article or supplementary material. The data presented in this study are available in Figures 1–5. In addition, the information is also presented in Tables 1 and 2.

Acknowledgments: The authors wish to thank the CCiT-UB for the mass spectrometry equipment.

Conflicts of Interest: R.M.L.-R. reports receiving lecture fees from Cerveceros de España and receiving lecture fees and travel support from Adventia. The other authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Billat, P.-A.; Roger, E.; Faure, S.; Lagarce, F. Models for Drug Absorption from the Small Intestine: Where Are We and Where Are We Going? *Drug Discov. Today* **2017**, *22*, 761–775. [[CrossRef](#)] [[PubMed](#)]
2. Zhu, L.; Lu, L.; Wang, S.; Wu, J.; Shi, J.; Yan, T.; Xie, C.; Li, Q.; Hu, M.; Liu, Z. Oral Absorption Basics: Pathways and Physicochemical and Biological Factors Affecting Absorption. In *Developing Solid Oral Dosage Forms*; Academic Press: Cambridge, MA, USA, 2017; pp. 297–329. ISBN 978-0-12-802447-8.
3. Chmiel, T.; Mieszkowska, A.; Kempieńska-Kupczyk, D.; Kot-Wasik, A.; Namieśnik, J.; Mazerska, Z. The Impact of Lipophilicity on Environmental Processes, Drug Delivery and Bioavailability of Food Components. *Microchem. J.* **2019**, *146*, 393–406. [[CrossRef](#)]
4. Dahlgren, D.; Lennernäs, H. Intestinal Permeability and Drug Absorption: Predictive Experimental, Computational and In Vivo Approaches. *Pharmaceutics* **2019**, *11*, 411. [[CrossRef](#)] [[PubMed](#)]
5. Mudra, D.R.; Borchardt, R.T. Absorption Barriers in the Rat Intestinal Mucosa: 1. Application of an in Situ Perfusion Model to Simultaneously Assess Drug Permeation and Metabolism. *J. Pharm. Sci.* **2010**, *99*, 982–998. [[CrossRef](#)]
6. López-Yerena; Vallverdú-Queralt; Mols; Augustijns; Lamuela-Raventós; Escribano-Ferrer Absorption and Intestinal Metabolic Profile of Oleocanthal in Rats. *Pharmaceutics* **2020**, *12*, 134. [[CrossRef](#)]
7. Griffin, B.; O'Driscoll, C. Models of the small intestine. In *Drug Absorption Studies*; Springer: Boston, MA, USA, 2008; pp. 34–76.
8. Dahlgren, D.; Roos, C.; Peters, K.; Lundqvist, A.; Tannergren, C.; Sjögren, E.; Sjöblom, M.; Lennernäs, H. Evaluation of Drug Permeability Calculation Based on Luminal Disappearance and Plasma Appearance in the Rat Single-Pass Intestinal Perfusion Model. *Eur. J. Pharm. Biopharm.* **2019**, *142*, 31–37. [[CrossRef](#)]
9. Brouwers, J.; Mols, R.; Annaert, P.; Augustijns, P. Validation of a Differential in Situ Perfusion Method with Mesenteric Blood Sampling in Rats for Intestinal Drug Interaction Profiling. *Biopharm. Drug Dispos.* **2010**. [[CrossRef](#)] [[PubMed](#)]
10. Mao, Q.; Lai, Y.; Wang, J. Drug Transporters in Xenobiotic Disposition and Pharmacokinetic Prediction. *Drug Metab. Dispos.* **2018**, *46*, 561–566. [[CrossRef](#)]
11. Barthea, L.; Woodley, J.; Houin, G. Gastrointestinal Absorption of Drugs: Methods and Studies. *Fundam. Clin. Pharmacol.* **1999**, *13*, 154–168. [[CrossRef](#)]
12. Lennernäs, H. Intestinal Permeability and Its Relevance for Absorption and Elimination. *Xenobiotica* **2007**, *37*, 1015–1051. [[CrossRef](#)]
13. Tresserra-Rimbau, A.; Rimm, E.B.; Medina-Remón, A.; Martínez-González, M.A.; De la Torre, R.; Corella, D.; Salas-Salvado, J.; Gómez-Gracia, E.; Lapetra, J.; Arós, F. Inverse Association between Habitual Polyphenol Intake and Incidence of Cardiovascular Events in the PREDIMED Study. *Nutr. Metab. Cardiovasc. Dis.* **2014**, *24*, 639–647. [[CrossRef](#)] [[PubMed](#)]

14. Tresserra-Rimbau, A.; Rimm, E.B.; Medina-Remón, A.; Martínez-González, M.A.; López-Sabater, M.C.; Covas, M.I.; Corella, D.; Salas-Salvadó, J.; Gómez-Gracia, E.; Lapetra, J. Polyphenol Intake and Mortality Risk: A Re-Analysis of the PREDIMED Trial. *BMC Med.* **2014**, *12*, 1–11. [\[CrossRef\]](#) [\[PubMed\]](#)
15. López-Yerena, A.; Ninot, A.; Lozano-Castellón, J.; Escribano-Ferrer, E.; Romero-Aroca, A.J.; Belaj, A.; Vallverdú-Queralt, A.; Lamuela-Raventós, R.M. Conservation of Native Wild Ivory-White Olives from the MEDES Islands Natural Reserve to Maintain Virgin Olive Oil Diversity. *Antioxidants* **2020**, *13*, 1009. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Naruszewicz, M.; Czerwinska, M.; Kiss, A. Oleacein. Translation from Mediterranean Diet to Potential Antiatherosclerotic Drug. *Curr. Pharm. Des.* **2015**, *21*, 1205–1212. [\[CrossRef\]](#) [\[PubMed\]](#)
17. Lozano-Castellón, J.; López-Yerena, A.; Rinaldi de Alvarenga, J.F.; Romero del Castillo-Alba, J.; Vallverdú-Queralt, A.; Escribano-Ferrer, E.; Lamuela-Raventós, R.M. Health-Promoting Properties of Oleocanthal and Oleacein: Two Secoiridoids from Extra-Virgin Olive Oil. *Crit. Rev. Food Sci. Nutr.* **2020**, *60*, 2532–2548. [\[CrossRef\]](#)
18. Filipek, A.; Czerwińska, M.E.; Kiss, A.K.; Wrzosek, M.; Naruszewicz, M. Oleacein Enhances Anti-Inflammatory Activity of Human Macrophages by Increasing CD163 Receptor Expression. *Phytomedicine* **2015**, *22*, 1255–1261. [\[CrossRef\]](#)
19. Cirmi, S.; Celano, M.; Lombardo, G.E.; Maggisano, V.; Procopio, A.; Russo, D.; Navarra, M. Oleacein Inhibits STAT3, Activates the Apoptotic Machinery, and Exerts Anti-Metastatic Effects in the SH-SY5Y Human Neuroblastoma Cells. *Food Funct.* **2020**, *11*, 3271–3279. [\[CrossRef\]](#)
20. De Bock, M.; Thorstensen, E.B.; Derraik, J.G.B.; Henderson, H.V.; Hofman, P.L.; Cutfield, W.S. Human Absorption and Metabolism of Oleuropein and Hydroxytyrosol Ingested as Olive (*Olea europaea* L.) Leaf Extract. *Mol. Nutr. Food Res.* **2013**, *57*, 2079–2085. [\[CrossRef\]](#)
21. García-Villalba, R.; Carrasco-Pancorbo, A.; Nevedomskaya, E.; Mayboroda, O.A.; Deelder, A.M.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Exploratory Analysis of Human Urine by LC–ESI-TOF MS after High Intake of Olive Oil: Understanding the Metabolism of Polyphenols. *Anal. Bioanal. Chem.* **2010**, *398*, 463–475. [\[CrossRef\]](#)
22. Silva, S.; Garcia-Aloy, M.; Figueira, M.E.; Combet, E.; Mullen, W.; Bronze, M.R. High Resolution Mass Spectrometric Analysis of Secoiridoids and Metabolites as Biomarkers of Acute Olive Oil Intake-An Approach to Study Interindividual Variability in Humans. *Mol. Nutr. Food Res.* **2018**, *62*, 1700065. [\[CrossRef\]](#)
23. Rubió, L.; Farràs, M.; de La Torre, R.; Macià, A.; Romero, M.-P.; Valls, R.M.; Solà, R.; Farré, M.; Fitó, M.; Motilva, M.-J. Metabolite Profiling of Olive Oil and Thyme Phenols after a Sustained Intake of Two Phenol-Enriched Olive Oils by Humans: Identification of Compliance Markers. *Food Res. Int.* **2014**, *65*, 59–68. [\[CrossRef\]](#)
24. Khymenets, O.; Farré, M.; Pujadas, M.; Ortiz, E.; Joglar, J.; Covas, M.I.; de la Torre, R. Direct Analysis of Glucuronidated Metabolites of Main Olive Oil Phenols in Human Urine after Dietary Consumption of Virgin Olive Oil. *Food Chem.* **2011**, *126*, 306–314. [\[CrossRef\]](#)
25. Suárez, M.; Valls, R.M.; Romero, M.-P.; Macià, A.; Fernández, S.; Giral, M.; Solà, R.; Motilva, M.-J. Bioavailability of Phenols from a Phenol-Enriched Olive Oil. *Br. J. Nutr.* **2011**, *106*, 1691–1701. [\[CrossRef\]](#)
26. Serra, A.; Rubió, L.; Borràs, X.; Macià, A.; Romero, M.-P.; Motilva, M.-J. Distribution of Olive Oil Phenolic Compounds in Rat Tissues after Administration of a Phenolic Extract from Olive Cake. *Mol. Nutr. Food Res.* **2012**, *56*, 486–496. [\[CrossRef\]](#) [\[PubMed\]](#)
27. López de las Hazas, M.-C.; Piñol, C.; Macià, A.; Romero, M.-P.; Pedret, A.; Solà, R.; Rubió, L.; Motilva, M.-J. Differential Absorption and Metabolism of Hydroxytyrosol and Its Precursors Oleuropein and Secoiridoids. *J. Funct. Foods* **2016**, *22*, 52–63. [\[CrossRef\]](#)
28. Domínguez-Perles, R.; Auñón, D.; Ferreres, F.; Gil-Izquierdo, A. Gender Differences in Plasma and Urine Metabolites from Sprague–Dawley Rats after Oral Administration of Normal and High Doses of Hydroxytyrosol, Hydroxytyrosol Acetate, and DOPAC. *Eur. J. Nutr.* **2017**, *56*, 215–224. [\[CrossRef\]](#)
29. Kano, S.; Komada, H.; Yonekura, L.; Sato, A.; Nishiwaki, H.; Tamura, H. Absorption, Metabolism, and Excretion by Freely Moving Rats of 3,4-DHPEA-EDA and Related Polyphenols from Olive Fruits (*Olea europaea*). *J. Nutr. Metab.* **2016**, *2016*, 9104208. [\[CrossRef\]](#)
30. Radić, K.; Jurišić Dukovski, B.; Vitali Čepo, D. Influence of Pomace Matrix and Cyclodextrin Encapsulation on Olive Pomace Polyphenols' Bioaccessibility and Intestinal Permeability. *Nutrients* **2020**, *12*, 669. [\[CrossRef\]](#) [\[PubMed\]](#)
31. Edgecombe, S.C.; Stretch, G.L.; Hayball, P.J. Oleuropein, an Antioxidant Polyphenol from Olive Oil, Is Poorly Absorbed from Isolated Perfused Rat Intestine. *J. Nutr.* **2000**, *130*, 2996–3002. [\[CrossRef\]](#)
32. EFSA Scientific Opinion on the Substantiation of Health Claims Related to Polyphenols in Olive and Protection of LDL Particles from Oxidative Damage (ID 1333, 1638, 1639, 1696, 2865), Maintenance of Normal Blood HDL Cholesterol Concentrations (ID 1639), Mainte. *EFSA J.* **2011**, *9*, 2033–2058. [\[CrossRef\]](#)
33. Kim, J.-S.; Mitchell, S.; Kijek, P.; Tsume, Y.; Hilfinger, J.; Amidon, G.L. The Suitability of an in Situ Perfusion Model for Permeability Determinations: Utility for BCS Class I Biowaiver Requests. *Mol. Pharm.* **2006**, *3*, 686–694. [\[CrossRef\]](#)
34. Elsinghorst, P.W.; Kinzig, M.; Rodamer, M.; Holzgrabe, U.; Sörgel, F. An LC–MS/MS Procedure for the Quantification of Naproxen in Human Plasma: Development, Validation, Comparison with Other Methods, and Application to a Pharmacokinetic Study. *J. Chromatogr. B* **2011**, *879*, 1686–1696. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Polson, C.; Sarkar, P.; Incledon, B.; Raguvaran, V.; Grant, R. Optimization of Protein Precipitation Based upon Effectiveness of Protein Removal and Ionization Effect in Liquid Chromatography–Tandem Mass Spectrometry. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2003**, *785*, 263–275. [\[CrossRef\]](#)

36. López-Yerena, A.; Vallverdú-Queralt, A.; Mols, R.; Augustijns, P.; Lamuela-Raventós, R.M.; Escribano-Ferrer, E. Reply to “Comment on López-Yerena et al. ‘Absorption and Intestinal Metabolic Profile of Oleocanthal in Rats’” *Pharmaceutics* **2020**, *12*, 134. [CrossRef]
37. López-Yerena, A.; Vallverdú-Queralt, A.; Jáuregui, O.; García-Sala, X.; Lamuela-Raventós, R.M.; Escribano-Ferrer, E. Tissue Distribution of Oleocanthal and Its Metabolites after Oral Ingestion in Rats. *Antioxidants* **2021**, *10*, 688. [CrossRef] [PubMed]
38. Chen, G. Xenobiotic metabolism and disposition. In *An Introduction to Interdisciplinary Toxicology*; Academic Press: Cambridge, MA, USA, 2020; pp. 31–42.
39. Wang, D.; Zou, L.; Jin, Q.; Hou, J.; Ge, G.; Yang, L. Human Carboxylesterases: A Comprehensive Review. *Acta Pharm. Sin.* **2018**, *8*, 699–712. [CrossRef]
40. Taketani, M.; Shii, M.; Ohura, K.; Ninomiya, S.; Imai, T. Carboxylesterase in the Liver and Small Intestine of Experimental Animals and Human. *Life Sci.* **2007**, *81*, 924–932. [CrossRef]
41. Pinto, J.; Paiva-Martins, F.; Corona, G.; Debnam, E.S.; Jose Oruna-Concha, M.; Vauzour, D.; Gordon, M.H.; Spencer, J.P.E. Absorption and Metabolism of Olive Oil Secoiridoids in the Small Intestine. *Br. J. Nutr.* **2011**, *105*, 1607–1618. [CrossRef] [PubMed]
42. Penning, T.M. The Aldo-Keto Reductases (AKRs): Overview. *Chem. Biol. Interact.* **2015**, *234*, 236–246. [CrossRef]
43. Kang, M.J.; Kim, H.G.; Kim, J.S.; Oh, D.G.; Um, Y.J.; Seo, C.S.; Han, J.W.; Cho, H.J.; Kim, G.H.; Jeong, T.C.; et al. The Effect of Gut Microbiota on Drug Metabolism. *Expert Opin. Drug Metab. Toxicol.* **2013**, *9*, 1295–1308. [CrossRef]
44. Dressman, J.B.; Thelen, K. Cytochrome P450-Mediated Metabolism in the Human Gut Wall. *J. Pharm. Pharmacol.* **2009**, *61*, 541–558. [CrossRef] [PubMed]
45. Spanogiannopoulos, P.; Bess, E.N.; Carmody, R.N.; Turnbaugh, P.J. The Microbial Pharmacists within Us: A Metagenomic View of Xenobiotic Metabolism. *Nat. Publ. Group* **2016**, *14*, 273–287. [CrossRef] [PubMed]
46. Clarke, G.; Sandhu, K.V.; Griffin, B.T.; Dinan, T.G.; Cryan, J.F.; Hyland, N.P. Gut Reactions: Breaking Down Xenobiotic—Microbiome Interactions. *Pharmacol. Rev.* **2019**, 198–224. [CrossRef] [PubMed]
47. Strolin Benedetti, M.; Whomsley, R.; Baltes, E. Involvement of Enzymes Other than CYPs in the Oxidative Metabolism of Xenobiotics. *Expert Opin. Drug Metab. Toxicol.* **2006**, *2*, 895–921. [CrossRef]
48. Jancova, P.; Anzenbacher, P.; Anzenbacherova, E. Phase II Drug Metabolizing Enzymes. *Biomed. Pap.* **2010**, *154*, 103–116. [CrossRef]
49. Xu, C.; Li, C.Y.-T.; Kong, A.-N.T. Induction of Phase I, II and III Drug Metabolism/Transport by Xenobiotics. *Arch. Pharm. Res.* **2005**, *28*, 249–268. [CrossRef]
50. Cuyàs, E.; Verdura, S.; Lozano-Sánchez, J.; Viciano, I.; Llorach-Parés, L.; Nonell-Canals, A.; Bosch-Barrera, J.; Brunet, J.; Segura-Carretero, A.; Sanchez-Martinez, M.; et al. The Extra Virgin Olive Oil Phenolic Oleacein Is a Dual Substrate-Inhibitor of Catechol-O-Methyltransferase. *Food Chem. Toxicol.* **2019**, *128*, 35–45. [CrossRef]
51. Nissinen, E.; Tuominen, R.; Perhoniemi, V.; Kaakkola, S. Catechol-O-Methyltransferase Activity in Human and Rat Small Intestine. *Life Sci.* **1988**, *42*, 2609–2614. [CrossRef]
52. Taskinen, J.; Ethell, B.T.; Pihlavi, P.; Hood, A.M.; Burchell, B.; Coughtrie, M.W.H. Conjugation of Catechols by Recombinant Human Sulfotransferases, UDP-Glucuronosyltransferases, and Soluble Catechol O-Methyltransferase: Structure-Conjugation Relationships and Predictive Models. *Drug Metab. Dispos.* **2003**, *31*, 1187–1197. [CrossRef]
53. Shipkova, M.; Wieland, E. Glucuronidation in Therapeutic Drug Monitoring. *Clin. Chim. Acta* **2005**, *358*, 2–23. [CrossRef]
54. Yang, G.; Ge, S.; Singh, R.; Basu, S.; Shatzer, K.; Zen, M.; Liu, J.; Tu, Y.; Zhang, C.; Wei, J.; et al. Glucuronidation: Driving Factors and Their Impact on Glucuronide Disposition. *Drug Metab. Rev.* **2017**, *49*, 105–138. [CrossRef] [PubMed]
55. FDA. *Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System*; US Department of Health and Human Services Food and Drug Administration: Rockville, MD, USA, 2017.
56. Dubbelboer, I.R.; Dahlgren, D.; Sjögren, E.; Lennernäs, H. Rat Intestinal Drug Permeability: A Status Report and Summary of Repeated Determinations. *Eur. J. Pharm. Biopharm.* **2019**, *142*, 364–376. [CrossRef] [PubMed]
57. Fagerholm, U.; Lindahl, A.; Lennernäs, H. Regional Intestinal Permeability in Rats of Compounds with Different Physicochemical Properties and Transport Mechanisms. *J. Pharm. Pharmacol.* **1997**, *49*, 687–690. [CrossRef] [PubMed]
58. Zakeri-Milani, P.; Barzegar-Jalali, M.; Tajerzadeh, H.; Azarmi, Y.; Valizadeh, H. Simultaneous Determination of Naproxen, Ketoprofen and Phenol Red in Samples from Rat Intestinal Permeability Studies: HPLC Method Development and Validation. *J. Pharm. Biomed. Anal.* **2005**, *39*, 624–630. [CrossRef] [PubMed]
59. Lennernaäs, H. Human Intestinal Permeability. *J. Pharm. Sci.* **1998**, *87*, 403–410. [CrossRef] [PubMed]
60. ChemAxon. Available online: <https://foodb.ca/compounds/FBD016341> (accessed on 24 February 2021).
61. Avdeef, A. Physicochemical Profiling (Solubility, Permeability and Charge State). *Curr. Top. Med. Chem.* **2001**, *1*, 277–351. [CrossRef]
62. Hansch, C.; Leo, A.; Hoekman, D.; Livingstone, D. *Exploring QSAR: Hydrophobic, Electronic, and Steric Constants*; American Chemical Society: Washington, DC, USA, 1995; Volume 48.
63. Lichtenberger, L.M.; Zhou, Y.; Jayaraman, V.; Doyen, J.R.; O’Neil, R.G.; Dial, E.J.; Volk, D.E.; Gorenstein, D.G.; Boggara, M.B.; Krishnamoorti, R. Insight into NSAID-Induced Membrane Alterations, Pathogenesis and Therapeutics: Characterization of Interaction of NSAIDs with Phosphatidylcholine. *Biochim. Biophys. Acta BBA-Mol. Cell Biol. Lipids* **2012**, *1821*, 994–1002. [CrossRef]
64. Pereira-Leite, C.; Figueiredo, M.; Burdach, K.; Nunes, C.; Reis, S. Unraveling the Role of Drug-Lipid Interactions in NSAIDs-Induced Cardiotoxicity. *Membranes* **2021**, *11*, 24. [CrossRef]
65. López-Yerena, A.; Perez, M.; Vallverdú-Queralt, A.; Escribano-Ferrer, E. Insights into the Binding of Dietary Phenolic Compounds to Human Serum Albumin and Food-Drug Interactions. *Pharmaceutics* **2020**, *12*, 1123. [CrossRef]

4.3. Evidence of tissue distribution of olive oil phenols in rats

4.3.1. Article 8: Tissue distribution of oleocanthal and its metabolites after oral ingestion in rats

Anallely López-Yerena, Anna Vallverdú-Queralt, Olga Jáuregui, Xavier Garcia-Sala, Rosa M. Lamuela-Raventós, Elvira Escribano-Ferrer

Antioxidants, 10(5), 688, 2021

Abstract

Background: Claims for the potential health benefits of OLC, are based mainly on *in vitro* studies. It is important to mention that most of these studies do not take into account the matrix, the biological concentrations usually achieved when following a Mediterranean diet, metabolic transformations or the contemporary presence of more than one metabolite. In addition, little is known about the tissue availability of OLC, which is rapidly metabolized after ingestion.

Scope: The current work is therefore an attempt to improve our understanding of the health effects of OLC through a tissue distribution of OLC and its metabolites study in rats after the oral administration of OLC-enriched refined olive oil (ROO).

Methods and results: In this study, the distribution of OLC and its metabolites in rat plasma and tissues (stomach, intestine, liver, kidney, spleen, lungs, heart, brain, thyroid and skin) at 1, 2 and 4.5 h after the acute intake of a refined olive oil containing 0.3 mg/mL of OLC was examined by LC-ESI-LTQ-Orbitrap-MS. OLC was only detected in the stomach and intestine samples. Moreover, at 2 and 4.5 h, the concentration in the stomach decreased by 36% and 74%, respectively, and in the intestine by 16% and 33%, respectively. Ten OLC metabolites arising from phase I and phase II reactions were identified. The metabolites were widely distributed in rat tissues, and the most important metabolizing organs were the small intestine and liver.

Conclusions: The results demonstrate that under gastric conditions, OLC was hydrolyzed to tyrosol, whereas the non-hydrolyzed OLC underwent different phase I biotransformations in the stomach and small intestine. Once absorbed, the phase I metabolites were widely distributed throughout the organism or underwent further metabolic reactions. Although OLC was not detected in the tissues, its metabolites were found in the lungs, heart, brain, thyroids, spleen and skin in significant concentrations to potentially exert beneficial effects. The main circulating metabolites arising from phase II reactions were OLC + OH + CH₃ and OLC + H₂O + glucuronidation, suggesting that these metabolites are likely to significantly contribute to the beneficial health effects associated with the regular consumption of olive products. However, more studies are necessary to determine the concentrations and composition of OLC metabolites in human plasma and tissues, as well as pharmacodynamic studies that relate concentrations vs. effects.



Article

Tissue Distribution of Oleocanthal and Its Metabolites after Oral Ingestion in Rats

Anallely López-Yerena ¹, Anna Vallverdú-Queralt ^{1,2}, Olga Jáuregui ³, Xavier Garcia-Sala ⁴, Rosa M. Lamuela-Raventós ^{1,2} and Elvira Escribano-Ferrer ^{2,4,5,*}

- ¹ Department of Nutrition, Food Science and Gastronomy XaRTA, Faculty of Pharmacy and Food Sciences, Institute of Nutrition and Food Safety (INSA-UB), University of Barcelona, 08028 Barcelona, Spain; naye.yerena@gmail.com (A.L.-Y.); avallverdu@ub.edu (A.V.-Q.); lamuela@ub.edu (R.M.L.-R.)
- ² CIBER Physiopathology of Obesity and Nutrition (CIBEROBN), Institute of Health Carlos III, 28029 Madrid, Spain
- ³ Scientific and Technological Center of University of Barcelona (CCiTUB), 08028 Barcelona, Spain; ojauregui@ccit.ub.edu
- ⁴ Biopharmaceutics and Pharmacokinetics Unit, Department of Pharmacy and Pharmaceutical Technology and Physical Chemistry, Institute of Nanoscience and Nanotechnology (IN2UB), Faculty of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain; xgarciasala@ub.edu
- ⁵ Pharmaceutical Nanotechnology Group I+D+I Associated Unit to CSIC, University of Barcelona, 08028 Barcelona, Spain
- * Correspondence: eescribano@ub.edu; Fax: +34-9340-35937



Citation: López-Yerena, A.; Vallverdú-Queralt, A.; Jáuregui, O.; Garcia-Sala, X.; Lamuela-Raventós, R.M.; Escribano-Ferrer, E. Tissue Distribution of Oleocanthal and Its Metabolites after Oral Ingestion in Rats. *Antioxidants* **2021**, *10*, 688. <https://doi.org/10.3390/antiox10050688>

Academic Editors: Joanna Orazc and Dorota Żyżelewicz

Received: 8 April 2021
Accepted: 25 April 2021
Published: 27 April 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Abstract: Claims for the potential health benefits of oleocanthal (OLC), a dietary phenolic compound found in olive oil, are based mainly on in vitro studies. Little is known about the tissue availability of OLC, which is rapidly metabolized after ingestion. In this study, the distribution of OLC and its metabolites in rat plasma and tissues (stomach, intestine, liver, kidney, spleen, lungs, heart, brain, thyroid and skin) at 1, 2 and 4.5 h after the acute intake of a refined olive oil containing 0.3 mg/mL of OLC was examined by LC-ESI-LTQ-Orbitrap-MS. OLC was only detected in the stomach and intestine samples. Moreover, at 2 and 4.5 h, the concentration in the stomach decreased by 36% and 74%, respectively, and in the intestine by 16% and 33%, respectively. Ten OLC metabolites arising from phase I and phase II reactions were identified. The metabolites were widely distributed in rat tissues, and the most important metabolizing organs were the small intestine and liver. The two main circulating metabolites were the conjugates OLC + OH + CH₃ and OLC + H₂O + glucuronic acid, which may significantly contribute to the beneficial health effects associated with the regular consumption of extra virgin olive oil. However, more studies are necessary to determine the concentrations and molecular structures of OLC metabolites in human plasma and tissues when consumed with the presence of other phenolic compounds present in EVOO.

Keywords: extra virgin olive oil; polyphenols; metabolism; bioaccumulation; LC-ESI-LTQ-Orbitrap

1. Introduction

According to the World Health Organization (WHO), the main causes of death worldwide in 2016 were ischemic heart disease, stroke, chronic obstructive pulmonary disease, lower respiratory infections, Alzheimer's disease and other dementias and diabetes mellitus [1]. In 2018, the Mediterranean diet, a model of eating based on the traditional foods and drinks of the countries surrounding the Mediterranean Sea [2], was recommended by the WHO for its numerous health benefits, including effectiveness in reducing non-communicable diseases [3]. The main source of fat in the Mediterranean diet is extra virgin olive oil (EVOO), whose minority compounds include phenolic substances such as phenolic acids, phenolic alcohols, flavonoids, secoiridoids and lignans [4,5]. Among the secoiridoids, which contain elenolic acid or its derivatives in their molecular structures [6], oleocanthal (OLC) has proven biological activity, including antioxidant and antimicrobial

properties [7,8], anti-inflammatory effects (more potent than ibuprofen) [9], anticancer properties (melanoma, breast, liver and colon cancer and leukemia) [10–12] and neuroprotective effects [13,14].

Recognized by the human body as xenobiotics, phenolic compounds have a relatively low bioavailability compared with micro and macronutrients [15]. Bioavailability depends on multiple factors, including gastrointestinal digestion, absorption and metabolism, as well as tissue distribution [16], which plays a fundamental role in the pharmacokinetic behavior of drugs and other xenobiotics and conditions the pharmacological and toxicological responses of the organism [17]. Studies in animal models help to determine the tissue distribution and accumulation of compounds and their metabolites and identify potential sites of action [18]. To date, the extensive research on the bioavailability of phenolic compounds from EVOO has focused mainly on absorption or metabolism in humans [19–24] and rats [25–28]. Regarding OLC, a low intestinal absorption (16%) and intestinal metabolism *in vivo*, indicating limited systemic exposure, has been described by only one study [29], whereas none of the investigations aiming to understand the bioavailability of EVOO phenolic compounds have explored OLC tissue distribution. Recently, the OLC reactivity with amino acids with high preference to glycine was also reported [30].

It should also be noted that most of the *in vitro* studies on the health benefits of OLC do not take into account the matrix, the biological concentrations usually achieved when following a Mediterranean diet, metabolic transformations or the contemporary presence of more than one metabolite [31]. The current work is therefore an attempt to improve our understanding of the health effects of OLC through a tissue distribution study in rats after the oral administration of OLC-enriched refined olive oil (ROO). The distribution of OLC metabolites in multiple tissues and at different times post-administration was also evaluated.

2. Materials and Methods

2.1. Reagents and Materials

OLC (purity > 90) was purchased from PhytoLab GmbH (Vestenbergsgreuth, Germany), and its purity was verified by ^1H NMR, as in a previous study [32]. The reagents of methanol, acetonitrile (ACN) and formic acid were purchased from Sigma-Aldrich. Ultrapure water was obtained using a Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2. Animals and Diets

The studies were conducted following a protocol approved by the Animal Experimentation Ethics Committee of the University of Barcelona, Spain (trial no. CEEA 124/16) and Generalitat de Catalunya (no. 6435). Male Sprague Dawley rats (body weight 249 ± 8 g) were purchased from Envigo RMS Spain S.L. (Sant Feliu de Codines, Barcelona, Spain). The animals were acclimatized in animal housing in an air-conditioned room at 22 ± 2 °C under a 12 h/12 h cycle of light and darkness and given free access to a commercial diet (Teklad 2014, Envigo RMS Spain S.L.) and water for at least 10 days before the study. The rats were fasted for over 12 h before the experiments but were allowed free access to water.

2.3. Tissue Distribution Experiments

Before the distribution experiment, the ROO (vehicle) was analyzed by LC-ESI-LTQ-Orbitrap-MS to make sure it was free of phenolic compounds. The liquid–liquid extraction of phenolic compounds of ROO was performed following the previously described procedures [33]. The chromatographic and mass spectrometer conditions were the same as those used for the identification of metabolites (Section 2.3). After verifying that the ROO was free of phenols, OLC was added to obtain a final concentration of 0.3 mg/mL. The OLC concentration was chosen while taking into account both the OLC concentration in commercial EVOO (~300 mg/kg) and the daily ingestion of EVOO recommended by the

European Food Safety Authority of at least 5 mg of hydroxytyrosol and its derivatives per 20 g of olive oil [34].

The rats were divided into four groups—a control group (no administration) and three dosed groups—to study the distribution of OLC at three different time points: 1, 2 and 4.5 h post-administration. On treatment day, the rats received the ROO containing OLC by oral gavage (2.5 mL/300 g body weight). The volume for gavage administration was established following a good practice guide for substance administration [35] and was lower than the maximum volume of lipid vehicles per kg of rat body weight that prevented elevated plasma corticosterone levels [36].

The rats were anesthetized with isoflurane (IsoFloR, Veterinaria Esteve, Barcelona, Spain) and euthanized by exsanguination via cardiac puncture at 1, 2 or 4.5 h after ROO ingestion. The control group was maintained under fasting conditions without olive oil ingestion and then similarly euthanized. The blood was collected in EDTA tubes, and plasma samples were obtained by centrifugation ($3000 \times g$ for 10 min at 4°C) and stored at -80°C until analysis. Different tissues and organs (heart, liver, spleen, lungs, kidney, brain, thyroid, stomach, small intestine and skin) were removed from the rats and placed in pre-weighed tubes. The small intestine samples consisted of a pool of duodenum, jejunum and ileum segments, but the main region used was the jejunum. The procedure was to locate the plica duodenocolica [37] and, from there up to about 5 cm of the intestine before reaching the cecum, to remove it from the animal. The collected tissues were washed with physiological serum at 37°C and placed between absorbent paper to remove the rest of the serum. In the case of the intestine, the luminal contents were first removed by applying pressure with the index finger and thumb, and then the lumen was washed by perfusing with the physiological serum at 37°C . It was then placed between absorbent paper to remove the remains of the washings. In order to avoid compound oxidation, the collected tissues were kept on ice at all times. Finally, the samples were weighed and stored at -80°C until analysis. For each group, four animals were used.

2.4. Liquid–Liquid Extraction for Bioanalysis

The tissues were homogenized with a small tissue disruptor (T 10 basic ULTRA-TURRAX®, IKA laboratory technology, Staufen Germany) in a 1:1 ratio (*w/v*) with water:ACN (1:1 (*v/v*) with 0.1% ascorbic acid). The samples were sonicated for 5 min in an ice bath and shaken for 1 min (Vortex-Genie 2). Each homogenate was centrifuged for 10 min ($11,000 \times g$ at 4°C). A volume of 100 μL of the upper layer was blended with cold ACN containing 2% formic acid in a 1:3 ratio (*v/v*) in order to precipitate the proteins [38]. The samples were homogenized for 1 min and kept at -20°C for 20 min before centrifugation ($11,000 \times g$ at 4°C for 10 min). Finally, 100 μL of supernatant was transferred to vials for analysis. Three replicates were evaluated for each sample.

The plasma samples were thawed and centrifuged ($11,000 \times g$ at 4°C at 10 min). Plasma (100 μL) was mixed with cold ACN containing 2% formic acid in a ratio of 1:5 (*v/v*) to precipitate proteins, homogenized for 1 min and kept at -20°C for 20 min. The samples were then centrifuged ($11,000 \times g$ at 4°C for 10 min), and finally, 100 μL of each organic phase was transferred to vials for analysis. Three replicates were evaluated for each sample.

2.5. LC-ESI-LTQ-Orbitrap-MS Equipment and Conditions

The LC system consisted of an Accela chromatograph (Thermo Scientific, Hemel Hempstead, UK) with a quaternary pump, a photodiode array detector (PDA) and a thermostated autosampler. The separations were carried out on an Acquity™ UPLC® BEH C₁₈ pre-column (2.1 $\times$ 5 mm, i.d., 1.7 μm particle size) and an Acquity™ UPLC® BEH C₁₈ column (2.1 $\times$ 100 mm, i.d., 1.7 μm particle size) (Waters Corporation®, Ireland). The mobile phases consisted of H₂O (A) and methanol (B), both with 0.05% of formic acid. The elution was performed by means of an increasing linear gradient (*v/v*) of B (t (min), %B), as follows: (0, 0); (2, 0); (6, 53.6); (8, 100); (9, 100); (9.1, 0); and (11, 0). The injection volume was 5 μL , the flow rate was set to 0.6 mL/min, and the column temperature was 50°C .

An LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) equipped with an ESI source in negative mode was used to obtain high-resolution mass spectra for the identification of OLC and its derivatives. The operation parameters were the following: source voltage = 4 kV and capillary temperature = 275 °C (FT automatic gain control (AGC) target 5·10⁵ for MS mode and 5·10⁴ for MSn mode). The sheath gas, auxiliary gas and sweep gas were set at 20, 10 and 2 arbitrary units, respectively. All samples were analyzed in Fourier transform mass spectrometry (FTMS) mode at a resolving power of 30,000 at 600 m/z, and data-dependent MS/MS events were acquired at a resolving power of 15,000 at 600 m/z. The most intense ions detected during FTMS-MS triggered data-dependent scanning. Ions that were not intense enough for a data-dependent scan were analyzed in MSn mode with the same orbitrap resolution (15,000 at 600 m/z). Precursors were fragmented by collision-induced C-trap dissociation with normalized collision energy (35 V) and an activation time of 10 ms. The mass range in FTMS mode was from 100 to 600 m/z. The system was controlled by XCalibur software v2.0.7 (ThermoFisher Scientific).

The OLC calibration curves were prepared in each isolated tissue matrix (heart, liver, spleen, lung, kidney, brain, thyroid, stomach, small intestine and skin) and in plasma (0.1–3 µg/mL). The concentrations of OLC metabolites were calculated using the peak area of the standard OLC and expressed as OLC equivalents. All calibration curves presented $R^2 > 0.98$.

The elemental composition of each metabolite was selected according to the accurate masses and the isotopic pattern (through the Formula Finder feature in XCalibur software v2.0.7 and searched for in the Dictionary of Natural Products (Chapman & Hall/CRC) and the MOTO database (<http://appliedbioinformatics.wur.nl/moto>, accessed on 23 April 2021). MSn measurements were performed to obtain information on the fragment ions generated in the linear ion trap within the same analysis. Finally, metabolites were confirmed by comparing MS/MS spectra, with fragments reported in the literature as the main tool for the putative identification of OLC [20,24,29].

2.6. Statistical Analysis

Data are presented as the mean and standard deviation (SD). Statistical analysis was performed using SAS (version 9.4). The assumption of normalization was checked with standardized bias and standardized kurtosis. Statistical differences in the concentration of metabolites and OLC at different post-administration times (1, 2 and 4.5 h) for each compound and tissue were evaluated using one-way ANOVA, followed by the LSD post hoc test. Differences were considered significant at $p < 0.05$.

3. Results and Discussion

OLC and its metabolites were not detected in the plasma and tissues of the control group. In the groups receiving ROO, OLC was only detected in the stomach and small intestine samples, with the maximum concentration (C_{max}) being at 1 h of consumption ($p < 0.05$) (Figure 1). In the stomach samples, the concentration decreased by 36% and 74% at 2 h and 4.5 h, respectively, which could be due to gastric emptying and the fact that OLC decomposition increases with gastric residence time, although in the fasted (pH 1.5) and fed (pH 2–3) conditions and normal physiological time frames (up to 4 h), some would remain intact and enter the small intestine unhydrolyzed [39]. The concentration of the OLC that reached the intestine decreased by 16% and 33% at 2 h and 4.5 h post-ingestion, respectively (Figure 1), which can be attributed mainly to the intestinal metabolism and less to intestinal absorption [29].

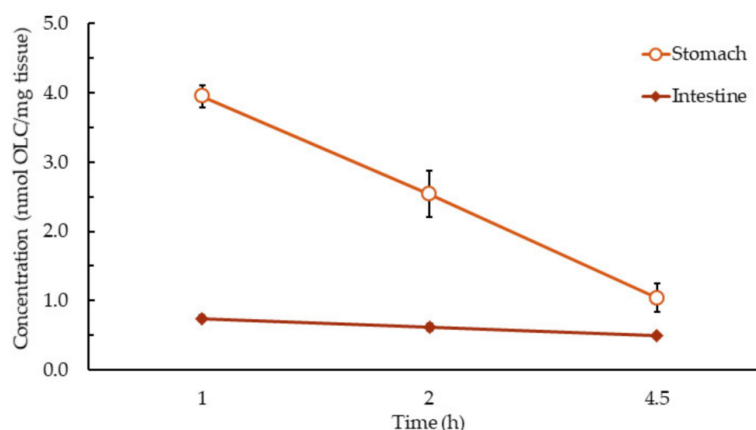


Figure 1. OLC concentrations in the small intestine and stomach (nmol/g tissue), calculated as nmol OLC equivalents. Results are expressed as the mean $\pm$ standard deviation.

3.1. Metabolite Identification

Ten metabolites arising from phase I and phase II reactions were identified in the rat plasma and tissues. Accurate and comprehensive identification by LC-ESI-LTQ-Orbitrap-MS showed that the OLC metabolites were widely distributed in different tissues. The corresponding molecular formulas, MS/MS fragments, mass measurement errors and retention times are shown in the Supporting Information (S1). In addition, an example of a chromatogram for each metabolite and the parent compound is shown in Figure S1.

3.1.1. Phase I Metabolism

The first step in the digestion of dietary lipids in the stomach involves their dispersal into finely divided emulsion particles, generating a lipid–water interface [40]. Previous studies have demonstrated that phenolic compounds are transferred to the aqueous or oily phase according to their polarity [41]. In the case of OLC, its amphiphilic characteristics result in a partition between the oily and aqueous phase, tending toward a higher concentration in the latter (68.7%) due to its polar functional groups [42]. At the same time, OLC can be partially modified in the acidic environment of the stomach, where OLC was hydrolyzed into tyrosol (TY) and only detected in the stomach and intestinal samples, with the highest concentrations observed at 1 h of intragastric administration (Figure 2A). Reports on secoiridoid hydrolysis in the literature are contradictory. Thus, whereas *in vitro* and *in vivo* studies described secoiridoid compounds undergoing a rapid hydrolysis under gastric conditions, leading to an increase in hydroxytyrosol and TY concentrations [24,41], an *in vitro* digestion study observed good OLC stability in the gastric phase [42]. According to our results, the hydrolysis of OLC can occur due to either acidic conditions or the presence of nonlipolytic carboxylic ester hydrolases, which hydrolyze carboxylic esters cleaved into an acid and an alcohol [43]. In our study, the observation of TY in samples of the stomach and intestine but not the other organs can be attributed to its metabolization by enzymes of the gastrointestinal mucosal epithelium before entering the systemic circulation. However, TY metabolites were not detected, possibly because they were at a very low concentration.

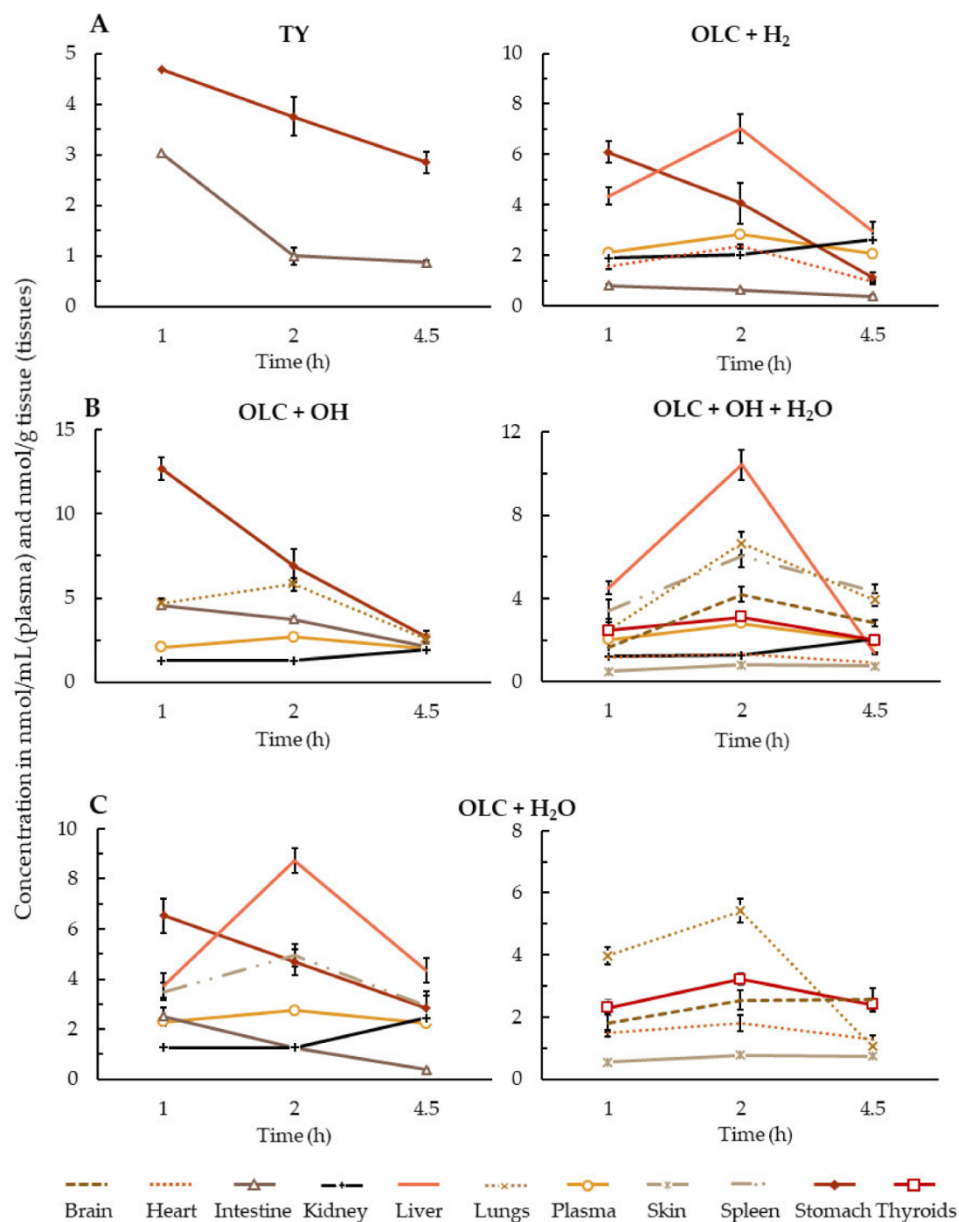


Figure 2. Phase I metabolites of OLC in plasma (nmol/mL) and tissues (nmol/g tissue), calculated as nmol OLC equivalents. (A): TY and OLC + H₂ metabolites; (B): OLC + OH and OLC + OH + H₂O metabolites; (C): OLC + H₂O metabolite. Results are expressed as the mean $\pm$ standard deviation.

Non-hydrolyzed OLC in the acidic conditions of the stomach can undergo different phase I biotransformations. The metabolite OLC + H₂ was identified in the plasma, stomach, intestine, liver, kidney and heart samples (Figure 2A), with a C_{max} (the highest concentration observed among the three evaluation sample times) in the plasma, liver and

heart at 2 h of ROO intake ($p < 0.05$), in the stomach and small intestine at 1 h and in the kidney at 4.5 h. In another study, small traces of OLC + H₂ were detected in human urine at 2 h after a high intake of olive oil [20]. Recently, the same metabolite was identified in the plasma and lumen samples in an intestinal perfusion study in rats [29], whereas it was not found in a screening of secoiridoid metabolites in humans after an acute intake of EVOO [24]. Recently, it was suggested that the reduction OLC is catalyzed by NADPH-dependent aldo-keto reductases (AKR) located in the small intestine epithelium, and it can occur because OLC contains an open dialdehydic form of the attached elenolic acid molecule [29].

A hydroxylated metabolite, OLC + OH, was found in the plasma, stomach, intestine, kidney and lungs (Figure 2B). The C_{max} in the stomach and small intestine samples was at 1 h, at 2 h in the plasma and at 4.5 h in the lungs and in the kidney ($p < 0.05$). Only one previous study [29] has identified this metabolite in rat plasma and lumen samples, while in another it was reported in human urine, although there were doubts about whether it was in fact a hydroxytyrosol derivative such as oleacein [20]. In our study, OLC + OH was not detected in the main metabolizing organ, the liver, where it would have rapidly undergone other metabolic reactions, such as the addition of another OH group, methylation or glucuronidation. Its presence in the plasma, lungs and the brain could be due to enzymatic deconjugation, which occurs with glucuronidated metabolites of quercetin [44] or hydroxytyrosol [45].

The only metabolite present in all the samples was the hydrated form of OLC (Figure 2C). The C_{max} in the stomach and small intestine was at 1 h, in the kidney at 4.5 h ($p < 0.05$), in the brain and skin at 2 h and 4.5 h (the differences were insignificant) and in the plasma and the rest of the tissues at 2 h post-ingestion. Other studies have reported this metabolite in plasma [24,29].

A new OLC metabolite, OLC + OH + H₂O, which was not previously reported in either human or animal experiments [20,23–25,29], was identified in all tissues except the stomach and small intestine (Figure 2B). The C_{max} in most of the samples was at 2 h post-consumption of ROO, at 4.5 h in the kidney and at both 2 h and 4.5 h in the skin. This metabolite could be formed from the hydroxylated and hydrated metabolites identified in the stomach and small intestine following further metabolic reactions in the liver (+ OH or + H₂O). In our study, the presence of OLC + OH + H₂O could be attributed to the hepatic expression of CYP enzymes responsible for the majority of phase I-dependent drug metabolism and for the metabolism of a huge variety of dietary constituents and endogenous chemicals [46].

In the stomach, small intestine and liver [47,48], CYP functions act as a barrier against drugs and chemical compounds, catalyzing and introducing an oxygen atom into substrate molecules, which often results in dealkylated and hydroxylated metabolites [48].

3.1.2. Phase II Metabolism

OLC that escapes first-pass metabolism and the phase I metabolites can undergo other biotransformations. Our results suggest that the hydroxylated (OLC + OH) and hydrated (OLC + H₂O) forms of OLC were subject to the addition of a methyl group (OLC + OH + CH₃ and OLC + H₂O + CH₃). Catechol-O-methyltransferases (COMT) are conjugating enzymes that catalyze the transfer of a methyl group from S-adenosyl-L-methionine to phenolic compounds with an O-diphenolic moiety. This reaction usually takes place at the 3' position of the compound, although a minor proportion of 4'-O-methylated product is also possible [49]. OLC + OH + CH₃ was the only conjugated metabolite detected in all analyzed tissues (Figure 3A). The COMT enzymes are most active in the liver and kidneys but are also active in the pylorus of the stomach and in the small intestine, especially the duodenum and ileum [50], a behavior that would explain the presence of this conjugated metabolite in our samples. In contrast, OLC + H₂O + CH₃ was found in almost all samples, except in the stomach and intestine, probably due to the high activity of COMT enzymes in the liver. The C_{max} of both conjugated OLC derivatives (OLC + OH + CH₃ and OLC +

H₂O + CH₃) in the plasma and most of the tissues (liver, spleen, hearts, lungs and thyroids) were reached at 2 h after ROO ingestion ($p < 0.05$) and at 4.5 h in the kidney ($p < 0.05$). In the skin, the C_{max} was reached at 2 h and with a similar concentration at 4.5 h ($p > 0.05$). The only previous study to determine the intestinal metabolic profile of OLC did not detect either of these metabolites [29], whereas other in vivo studies observed the same trend found here [20,23–25].

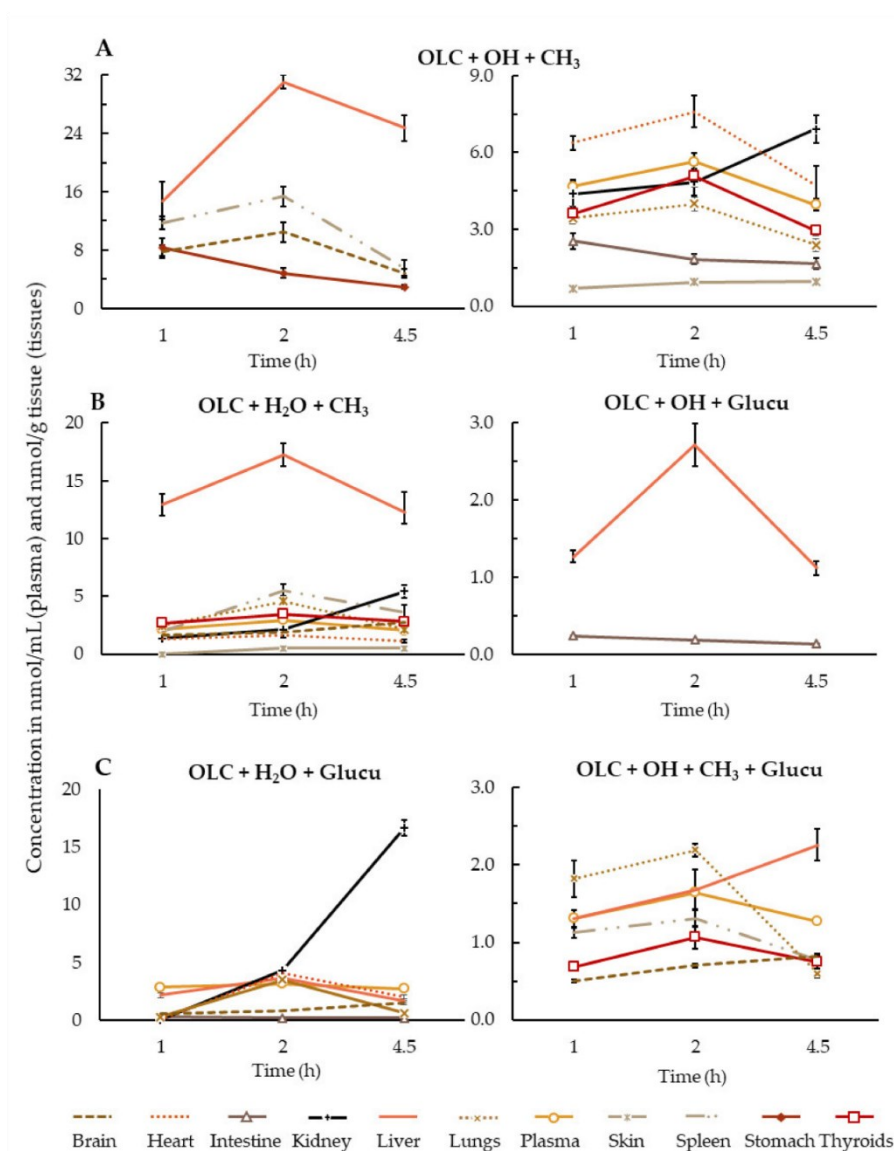


Figure 3. Phase II metabolites in plasma (nmol/mL) and tissues (nmol/g tissue), calculated as nmol OLC equivalents. (A): OLC + OH + CH₃ metabolite; (B): OLC + H₂O + CH₃ and OLC + OH + glucuronidation metabolites; (C): OLC + H₂O + glucuronidation and OLC + OH + CH₃ + glucuronidation metabolites. Results are expressed as the mean $\pm$ standard deviation.

A frequent phase II reaction is catalyzed by uridine-diphosphate glucuronosyl transferase (UGT), an enzyme located in the endoplasmic reticulum that mediates the transfer of glucuronic acid from uridine diphosphate to lipophilic compounds [51]. It is known that UGT interacts with structures that have one glucuronidation site and two hydrophobic sites separated by 3 or 6.2 Å of aliphatic hydroxyls, carboxylic acid and amines [52,53]. Although the liver is the main site of glucuronidation [54], there is clear evidence for the expression of UGT in the stomach [54], and UGT1A8 and UGT1A10 are exclusively expressed in the small intestine [53]. Figure 3 also shows the metabolites containing a glucuronic acid. OLC + OH + glucu was only detected in the small intestine, where UGT enzymes are expressed, and the liver (Figure 3B). The $C_{\max}$ in the small intestine was at 1 h after ROO intake, and in the liver, it was at 2 h ($p < 0.05$). In contrast, OLC + H₂O + glucu was more widespread (Figure 3C), with a $C_{\max}$ in the small intestine at 1 h of ROO intake, at 2 h in the plasma, liver, heart and lungs and at 4.5 h in the kidney and brain ($p < 0.05$). OLC + H₂O + CH₃ or OLC + H₂O + glucu underwent further metabolism, resulting in OLC + OH + CH₃ + glucu, which was found in the plasma, liver, spleen, lungs, thyroid and brain (Figure 3C). The three phase II metabolites detected in our samples had not been previously identified in rat tissues [25] or human urine [20,22,24] or plasma [23]. In a recent study on OLC absorption, only the OLC + H₂O + glucu metabolite was identified in plasma [29]. Nevertheless, the glucuronidation of OLC, which was not observed here, had been detected in human urine after the intake of EVOO [20,24].

3.2. Brief Summary of OLC Metabolite Distribution by Tissue

Our results show that the OLC metabolites were widely distributed in all the tested tissues, even in the brain and skin (Figures 2 and 3). The liver, small intestine and plasma were the biological samples with the greatest variety of metabolites.

In the stomach samples, in addition to OLC, four metabolites from the phase I reactions and one from the phase II reactions were detected (Figures 2 and 3). The presence of a TY metabolite in the stomach indicates that OLC underwent a rapid hydrolysis under gastric conditions. The $C_{\max}$ of all the metabolites was observed at 1 h of ROO intake.

Besides OLC, a wide range of its metabolites was found in the small intestine, where concentrations were lower than in the stomach. It is well known that the small intestine is the main site where xenobiotic absorption occurs [55,56], and the intestinal epithelial membrane is the principle physiological barrier that chemicals must cross to enter the bloodstream [57]. Phenolic compounds can be metabolized in the small intestine by numerous pathways involving both phase I and phase II reactions. Accordingly, four phase I and three phase II derivatives were identified (Figures 2 and 3). For all the metabolites, the $C_{\max}$ was observed at 1 h of ROO intake. These results confirm that OLC is subjected to a high intestinal metabolism, as previously reported [29].

According to the results obtained, the liver (in addition to the small intestine) represents the main metabolizing organ for OLC. For most of the metabolites detected in the liver, the $C_{\max}$ was at 2 h. Up to five phase II metabolites were found at high concentrations, in accordance with the functionality of this organ in xenobiotic and phenolic compound metabolism. The results are consistent with those obtained in a tissue distribution study in rats after olive cake ingestion, in which the highest number and concentration of metabolites were detected in the liver [25].

OLC was not found in the plasma, which could be explained by its relatively low absorption (16%) and high intestinal metabolism [29]. In the latter study, OLC was detected in mesenteric blood plasma, but the compound was administered directly to the small intestine, thus avoiding passing through the stomach and the liver, where OLC hydrolysis and metabolism can potentially take place, respectively. Although OLC was not detected in the plasma, four phase I and four phase II metabolites were identified in the plasma with a $C_{\max}$ at 2 h. This study demonstrates for the first time that OLC + OH + CH₃ is the main circulating OLC metabolite in rats after OLC consumption, followed by OLC + H₂O + glucu. The systemic exposure of these metabolites can reflect tissue exposure [58]. It is worth

noting that most in vitro and in vivo studies or clinical trials determining the effects of OLC have focused on the control and progression of different types of cancer, cardiovascular diseases, neurodegeneration, anti-aging processes and immunoinflammatory diseases, but the biological concentrations of OLC [6,59] and its major circulating metabolites were not considered.

The potential protective effect of OLC against Alzheimer's disease has been investigated in both in vitro and in vivo studies [13,14,60]. However, for the first time, it was demonstrated that only the metabolites (Figures 2 and 3) were able to cross the blood–brain barrier in rats—at the dose of OLC administered—and undergo brain uptake. The results showed brain accumulation of the OLC + OH + CH₃ metabolite, suggesting that this metabolite could be able to exert beneficial effects, specifically neuroprotective activity, by the reduction of the oxidative stress at the neuronal level. However, in the last decade, several studies have been focused on the evaluation of the potential health benefits of OLC, and the data available about the biological relevance of its metabolites are still limited. Future studies about the effective brain accumulation of the main biological OLC metabolites are needed to determine their potential to protect neuronal cells.

Based on the number and high concentration of OLC metabolites detected in the lungs, this organ is an important site of extrahepatic OLC metabolism. It is well known that the lungs contain drug-metabolizing enzymes, with low levels of monooxidases, transferases and esterases found in the endothelial cells, which may contribute to the elimination of drugs and xenobiotics [60,61]. A wide range of OLC derivatives (six) were also found in the heart, more than in the spleen and thyroids (5). In all these samples, OLC + OH + CH₃ was the main metabolite detected. Various drug-metabolizing enzymes are also expressed by macrophages and lymphocytes present in the white pulp region of the spleen, explaining the presence of metabolites in this tissue. More information about the metabolite functionality in the spleen is required [25], although a role of the spleen in lipid metabolism has been reported [62].

The wide range of metabolites detected in the kidney indicates that renal excretion is a major pathway of elimination for OLC metabolites in rats. In addition, accumulating evidence indicates that specific CYP, UGT and possibly other drug-metabolizing enzymes contribute to renal drug and chemical metabolic clearance, thereby modulating intrarenal exposure to these compounds, as well as regulating the activity of physiological mediators [63].

Even though the skin was the tissue where the fewest metabolites arrived, this study also contributes to providing a new perspective for understanding how the mechanism through EVOO phenolic compounds acts in the body.

The findings found in this study suppose a breakthrough in the research to understand the positive correlation of olive oil phenolic compound consumption and the prevention and improvement of some diseases. Nevertheless, further experiments are required to investigate the effect of the main circulating metabolites on the functions of the target tissues, such as those in the brain, heart, skin and spleen.

4. Conclusions and Remarks

There is still a lack of information on the distribution and accumulation of olive oil phenolic compounds in the body. In this study, the distribution and accumulation of OLC metabolites in rat tissues was studied for the first time. The results demonstrate that under gastric conditions, OLC was hydrolyzed to TY, whereas the non-hydrolyzed OLC underwent different phase I biotransformations in the stomach and small intestine. Once absorbed, the phase I metabolites were widely distributed throughout the organism or underwent further metabolic reactions. Although OLC was not detected in the tissues, its metabolites were found in the lungs, heart, brain, thyroids, spleen and skin in sufficient concentrations to exert beneficial effects. The main circulating metabolites arising from phase II reactions were OLC + OH + CH₃ and OLC + H₂O + glucu, suggesting that these metabolites are likely to significantly contribute to the beneficial health effects associated

with the regular consumption of olive products. However, more studies are necessary to determine the concentrations and composition of OLC metabolites in human plasma and tissues, as well as pharmacodynamic studies that relate concentrations vs. effects.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/antiox10050688/s1>, Table S1. Identification of OLC and its metabolites in plasma and tissues through LTQ-Orbitrap-MS. Figure S1. Extracted ion chromatograms of the OLC and its identified metabolites in different tissues.

Author Contributions: Conceptualization, A.L.-Y., E.E.-F. and R.M.L.-R.; methodology, A.L.-Y., E.E.-F., O.J. and A.V.-Q.; software, A.L.-Y., E.E.-F. and A.V.-Q.; validation, A.L.-Y., E.E.-F. and R.M.L.-R.; formal analysis, A.L.-Y. and E.E.-F.; investigation, A.L.-Y. and E.E.-F.; resources, R.M.L.-R.; data curation, A.L.-Y., E.E.-F., O.J. and A.V.-Q.; writing—original draft preparation, A.L.-Y. and E.E.-F.; writing—review and editing, A.L.-Y., X.G.-S. and E.E.-F.; visualization, A.L.-Y., X.G.-S. and E.E.-F.; supervision, E.E.-F. and R.M.L.-R.; project administration, E.E.-F. and R.M.L.-R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by CICYT [AGL2016-75329-R], CIBEROBN from the Instituto de Salud Carlos III, ISCIII from the Ministerio de Ciencia, Innovación y Universidades, (AEI/FEDER, UE) and Generalitat de Catalunya (GC) [2017SGR 196]. A.L.-Y. wishes to thank the Consejo Nacional de Ciencia y Tecnología (CONACYT) of Mexico for the doctoral scholarship. A.V.-Q. thanks the Ministry of Science Innovation and Universities for the Ramon y Cajal contract (RYC-2016-19355).

Institutional Review Board Statement: The studies were conducted following a protocol approved by the Animal Experimentation Ethics Committee of the University of Barcelona, Spain (trial no. CEEA 124/16) and Generalitat de Catalunya (no. 6435).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article or supplementary material. The data presented in this study are available in Figures 1–3.

Acknowledgments: The authors wish to thank the CCiT-UB for the mass spectrometry equipment.

Conflicts of Interest: R.M.L.-R. reports receiving lecture fees from Cerveceros de España and receiving lecture fees and travel support from Adventia. The other authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. World Health Organisation. The Top 10 Causes of Death. Available online: <https://www.who.int/en/news-room/fact-sheets/detail/the-top-10-causes-of-death> (accessed on 9 December 2020).
2. Dinu, M.; Pagliai, G.; Casini, A.; Sofi, F. Mediterranean Diet and Multiple Health Outcomes: An Umbrella Review of Meta-Analyses of Observational Studies and Randomised Trials. *European J. Clin. Nutr.* **2018**, *72*, 30–43. [CrossRef]
3. Renzella, J.; Townsend, N.; Jewell, J.; Breda, J.; Roberts, N.; Rayner, M.; Wickramasinghe, K. What National and Subnational Interventions and Policies Based on Mediterranean and Nordic Diets Are Recommended or Implemented in the WHO European Region, and Is There Evidence of Effectiveness in Reducing Noncommunicable Diseases. World Health Organization, Regional Office for Europe: Geneva, Switzerland, 2018; ISBN 92-890-5301-1.
4. Romani, A.; Ieri, F.; Urciuoli, S.; Noce, A.; Marrone, G.; Nediani, C.; Bernini, R. Health Effects of Phenolic Compounds Found in Extra-Virgin Olive Oil, By-Products, and Leaf of *Olea Europaea* L. *Nutrients* **2019**, *11*, 1776. [CrossRef] [PubMed]
5. Piroddi, M.; Albin, A.; Fabiani, R.; Giovannelli, L.; Luceri, C.; Natella, F.; Rosignoli, P.; Rossi, T.; Taticchi, A.; Servili, M.; et al. Nutrigenomics of Extra-Virgin Olive Oil: A Review. *BioFactors* **2017**, *43*, 17–41. [CrossRef] [PubMed]
6. Lozano-Castellón, J.; López-Yerena, A.; Rinaldi de Alvarenga, J.F.; Romero del Castillo-Alba, J.; Vallverdú-Queralt, A.; Escribano-Ferrer, E.; Lamuela-Raventós, R.M. Health-Promoting Properties of Oleocanthal and Oleacein: Two Secoiridoids from Extra-Virgin Olive Oil. *Crit. Rev. Food Sci. Nutr.* **2020**, *60*, 2532–2548. [CrossRef]
7. Nikou, T.; Liaki, V.; Stathopoulos, P.; Sklirou, A.D.; Tsakiri, E.N.; Jakschitz, T.; Bonn, G.; Trougakos, I.P.; Halabalaki, M.; Skaltsounis, L.A. Comparison Survey of EVOO Polyphenols and Exploration of Healthy Aging-Promoting Properties of Oleocanthal and Oleacein. *Food Chem. Toxicol.* **2019**, *125*, 403–412. [CrossRef]
8. Cicerale, S.; Lucas, L.J.; Keast, R.S.J. Antimicrobial, Antioxidant and Anti-Inflammatory Phenolic Activities in Extra Virgin Olive Oil. *Curr. Opin. Biotechnol.* **2012**, *23*, 129–135. [CrossRef] [PubMed]
9. Beauchamp, G.K.; Keast, R.S.J.; Morel, D.; Lin, J.; Pika, J.; Han, Q.; Lee, C.-H.; Smith, A.B.; Breslin, P.A.S. Ibuprofen-like Activity in Extra-Virgin Olive Oil. *Nature* **2005**, *437*, 45–46. [CrossRef]

10. LeGendre, O.; Breslin, P.A.S.; Foster, D.A. (-)-Oleocanthal Rapidly and Selectively Induces Cancer Cell Death via Lysosomal Membrane Permeabilization. *Mol. Cell. Oncol.* **2015**, *2*. [\[CrossRef\]](#)
11. Khanfar, M.A.; Bardaweel, S.K.; Akl, M.R.; El Sayed, K.A. Olive Oil-Derived Oleocanthal as Potent Inhibitor of Mammalian Target of Rapamycin: Biological Evaluation and Molecular Modeling Studies. *Phytother. Res.* **2015**, *29*, 1776–1782. [\[CrossRef\]](#)
12. Siddique, A.B.; Ebrahim, H.Y.; Akl, M.R.; Ayoub, N.M.; Goda, A.A.; Mohyeldin, M.M.; Nagumalli, S.K.; Hananeh, W.M.; Liu, Y.Y.; Meyer, S.A.; et al. (-)-Oleocanthal Combined with Lapatinib Treatment Synergized against HER-2 Positive Breast Cancer in Vitro and in Vivo. *Nutrients* **2019**, *11*, 412. [\[CrossRef\]](#)
13. Qosa, H.; Batarseh, Y.S.; Mohyeldin, M.M.; El Sayed, K.A.; Keller, J.N.; Kaddoumi, A. Oleocanthal Enhances Amyloid- β Clearance from the Brains of TgSwDI Mice and in Vitro across a Human Blood-Brain Barrier Model. *ACS Chem. Neurosci.* **2015**, *6*, 1849–1859. [\[CrossRef\]](#)
14. Monti, M.C.; Margarucci, L.; Riccio, R.; Casapullo, A. Modulation of Tau Protein Fibrillization by Oleocanthal. *J. Nat. Prod.* **2012**, *75*, 1584–1588. [\[CrossRef\]](#) [\[PubMed\]](#)
15. Orazc, J.; Nebesny, E.; Zyzelewicz, D.; Budryn, G.; Luzak, B. Bioavailability and Metabolism of Selected Cocoa Bioactive Compounds: A Comprehensive Review. *Crit. Rev. Food Sci. Nutr.* **2019**, 1–39. [\[CrossRef\]](#)
16. Angelino, D.; Cossu, M.; Marti, A.; Zanoletti, M.; Chiavaroli, L.; Brighenti, F.; Del Rio, D.; Martini, D. Bioaccessibility and Bioavailability of Phenolic Compounds in Bread: A Review. *Food Funct.* **2017**, *8*, 2368–2393. [\[CrossRef\]](#) [\[PubMed\]](#)
17. Lanao, J.; Fraile, M. Drug Tissue Distribution: Study Methods and Therapeutic Implications. *Curr. Pharm. Des.* **2005**, *11*, 3829–3845. [\[CrossRef\]](#) [\[PubMed\]](#)
18. Singh, G. Preclinical Drug Development. In *Pharmaceutical Medicine and Translational Clinical Research*; Academic Press: Waltham, MA, USA, 2017; ISBN 9780128021033.
19. de Bock, M.; Thorstensen, E.B.; Derraik, J.G.B.; Henderson, H.V.; Hofman, P.L.; Cutfield, W.S. Human Absorption and Metabolism of Oleuropein and Hydroxytyrosol Ingested as Olive (*Olea Europaea* L.) Leaf Extract. *Mol. Nutr. Food Res.* **2013**, *57*, 2079–2085. [\[CrossRef\]](#) [\[PubMed\]](#)
20. García-Villalba, R.; Carrasco-Pancorbo, A.; Nevedomskaya, E.; Mayboroda, O.A.; Deelder, A.M.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Exploratory Analysis of Human Urine by LC–ESI-TOF MS after High Intake of Olive Oil: Understanding the Metabolism of Polyphenols. *Anal. Bioanal. Chem.* **2010**, *398*, 463–475. [\[CrossRef\]](#) [\[PubMed\]](#)
21. Rubió, L.; Farràs, M.; de La Torre, R.; Macià, A.; Romero, M.-P.; Valls, R.M.; Solà, R.; Farré, M.; Fitó, M.; Motilva, M.-J. Metabolite Profiling of Olive Oil and Thyme Phenols after a Sustained Intake of Two Phenol-Enriched Olive Oils by Humans: Identification of Compliance Markers. *Food Res. Int.* **2014**, *65*, 59–68. [\[CrossRef\]](#)
22. Khymenets, O.; Farré, M.; Pujadas, M.; Ortiz, E.; Joglar, J.; Covas, M.I.; de la Torre, R. Direct Analysis of Glucuronidated Metabolites of Main Olive Oil Phenols in Human Urine after Dietary Consumption of Virgin Olive Oil. *Food Chem.* **2011**, *126*, 306–314. [\[CrossRef\]](#)
23. Suárez, M.; Valls, R.M.; Romero, M.-P.; Macià, A.; Fernández, S.; Giral, M.; Solà, R.; Motilva, M.-J. Bioavailability of Phenols from a Phenol-Enriched Olive Oil. *Br. J. Nutr.* **2011**, *106*, 1691–1701. [\[CrossRef\]](#)
24. Silva, S.; Garcia-aloy, M.; Figueira, M.E.; Combet, E.; Mullen, W.; Ros, M. High Resolution Mass Spectrometric Analysis of Secoiridoids and Metabolites as Biomarkers of Acute Olive Oil Intake — An Approach to Study Interindividual Variability in Humans. *Mol. Nutr. Food Res.* **2018**, *1700065*. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Serra, A.; Rubió, L.; Borràs, X.; Macià, A.; Romero, M.-P.; Motilva, M.-J. Distribution of Olive Oil Phenolic Compounds in Rat Tissues after Administration of a Phenolic Extract from Olive Cake. *Mol. Nutr. Food Res.* **2012**, *56*, 486–496. [\[CrossRef\]](#)
26. Pinto, J.; Paiva-Martins, F.; Corona, G.; Debnam, E.S.; Jose Oruna-Concha, M.; Vauzour, D.; Gordon, M.H.; Spencer, J.P.E. Absorption and Metabolism of Olive Oil Secoiridoids in the Small Intestine. *Br. J. Nutr.* **2011**, *105*, 1607–1618. [\[CrossRef\]](#)
27. López de las Hazas, M.-C.; Piñol, C.; Macià, A.; Romero, M.-P.; Pedret, A.; Solà, R.; Rubió, L.; Motilva, M.-J. Differential Absorption and Metabolism of Hydroxytyrosol and Its Precursors Oleuropein and Secoiridoids. *J. Funct. Foods* **2016**, *22*, 52–63. [\[CrossRef\]](#)
28. Domínguez-Perles, R.; Auñón, D.; Ferreres, F.; Gil-Izquierdo, A. Gender Differences in Plasma and Urine Metabolites from Sprague–Dawley Rats after Oral Administration of Normal and High Doses of Hydroxytyrosol, Hydroxytyrosol Acetate, and DOPAC. *Eur. J. Nutr.* **2017**, *56*, 215–224. [\[CrossRef\]](#)
29. López-Yerena, A.; Vallverdú-Queralt, A.; Mols, R.; Augustijns, P.; Lamuela-Raventós, R.M.; Escribano-Ferrer, E. Absorption and Intestinal Metabolic Profile of Oleocanthal in Rats. *Pharmaceutics* **2020**, *12*, 134. [\[CrossRef\]](#)
30. Darakjian, L.I.; Rigakou, A.; Brannen, A.; Qusa, M.H.; Tasiakou, N.; Diamantakos, P.; Reed, M.N.; Panizzi, P.; Boersma, M.D.; Melliou, E.; et al. Spontaneous In Vitro and In Vivo Interaction of (-)-Oleocanthal with Glycine in Biological Fluids: Novel Pharmacokinetic Markers. *ACS Pharmacol. Transl. Sci.* **2021**, *4*, 179–192. [\[CrossRef\]](#)
31. Serreli, G.; Deiana, M. In Vivo Formed Metabolites of Polyphenols and Their Biological Efficacy. *Food Funct.* **2019**, *10*, 6999–7021. [\[CrossRef\]](#)
32. López-Yerena, A.; Vallverdú-Queralt, A.; Mols, R.; Augustijns, P.; Lamuela-Raventós, R.M.; Escribano-Ferrer, E. Reply to “Comment on López-Yerena et al. ‘Absorption and Intestinal Metabolic Profile of Oleocanthal in Rats’ *Pharmaceutics* 2020, 12, 134.”. *Pharmaceutics* **2020**, *12*, 1221. [\[CrossRef\]](#)
33. Lozano-Castellón, J.; López-Yerena, A.; Olmo-Cunillera, A.; Jáuregui, O.; Pérez, M.; Lamuela-Raventós, R.M.; Vallverdú-Queralt, A. Total Analysis of the Major Secoiridoids in Extra Virgin Olive Oil: Validation of an UHPLC-ESI-MS/MS Method. *Antioxidants* **2021**, *10*, 540. [\[CrossRef\]](#)

34. Panel, E.; Nda, A. Scientific Opinion on the Substantiation of Health Claims Related to Polyphenols in Olive and Protection of LDL Particles from Oxidative Damage (ID 1333, 1638, 1639, 1696, 2865), Maintenance of Normal Blood HDL Cholesterol Concentrations (ID 1639), Mainte. *EFSA J.* **2011**, *9*, 1–25. [\[CrossRef\]](#)
35. Diehl, K.-H.; Hull, R.; Morton, D.; Pfister, R.; Rabemampianina, Y.; Smith, D.; Vidal, J.-M.; Vorstenbosch, C.V.D. A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes. *J. Appl. Toxicol.* **2001**, *21*, 15–23. [\[CrossRef\]](#)
36. Brown, A.P.; Dinger, N.; Levine, B.S. Stress Produced by Gavage Administration in the Rat. *Contemp. Top. Lab. Anim. Sci.* **2000**, *39*, 17–21.
37. Ishida, M.; Sakata, N.; Ise, I.; Ono, T.; Shimura, M.; Ishii, K.; Murakami, M.; Takadate, T.; Aoki, T.; Kudo, K. The Comparative Anatomy of the Folds, Fossae, and Adhesions around the Duodenojejunal Flexure in Mammals. *Folia Morphol.* **2018**, *77*, 286–292. [\[CrossRef\]](#)
38. Polson, C.; Sarkar, P.; Incledon, B.; Raguvanan, V.; Grant, R. Optimization of Protein Precipitation Based upon Effectiveness of Protein Removal and Ionization Effect in Liquid Chromatography–Tandem Mass Spectrometry. *J. Chromatogr. B* **2003**, *785*, 263–275. [\[CrossRef\]](#)
39. Corona, G.; Tzounis, X.; Dessi, M.A.; Deiana, M.; Edward, S.; Visioli, F.; Spencer, J.P.E.; Corona, G.; Tzounis, X.; Dessi, M.A.; et al. The Fate of Olive Oil Polyphenols in the Gastrointestinal Tract: Implications of Gastric and Colonic Microflora-Dependent Biotransformation. *Free Radic. Res.* **2006**, *40*, 647–658. [\[CrossRef\]](#)
40. Singh, H.; Ye, A.; Horne, D. Structuring Food Emulsions in the Gastrointestinal Tract to Modify Lipid Digestion. *Prog. Lipid Res.* **2009**, *48*, 92–100. [\[CrossRef\]](#)
41. Quintero-Flórez, A.; Pereira-Caro, G.; Sánchez-Quezada, C.; Moreno-Rojas, J.M.; Gaforio, J.J.; Jimenez, A.; Beltrán, G. Effect of Olive Cultivar on Bioaccessibility and Antioxidant Activity of Phenolic Fraction of Virgin Olive Oil. *Eur. J. Nutr.* **2017**, *1*–22. [\[CrossRef\]](#)
42. Soler, A.; Romero, M.P.; Macià, A.; Saha, S.; Furniss, C.S.M.; Kroon, P.A.; Motilva, M.J. Digestion Stability and Evaluation of the Metabolism and Transport of Olive Oil Phenols in the Human Small-Intestinal Epithelial Caco-2/TC7 Cell Line. *Food Chem.* **2010**, *119*, 703–714. [\[CrossRef\]](#)
43. Yassine Ben, A.; Verger, R.; Abousalham, A. Lipases or Esterases: Does It Really Matter? Toward a New Bio-Physico-Chemical Classification. In *Lipases and Phospholipases*; Humana Press: Totowa, NJ, USA, 2012; pp. 31–51.
44. Menendez, C.; Dueñas, M.; Galindo, P.; González-Manzano, S.; Jimenez, R.; Moreno, L.; Zarzuelo, M.J.; Rodríguez-Gómez, I.; Duarte, J.; Santos-Buelga, C.; et al. Vascular Deconjugation of Quercetin Glucuronide: The Flavonoid Paradox Revealed? *Mol. Nutr. Food Res.* **2011**, *55*, 1780–1790. [\[CrossRef\]](#)
45. Rubió, L.; Serra, A.; Macià, A.; Piñol, C.; Romero, M.-P.; Motilva, M.-J. In Vivo Distribution and Deconjugation of Hydroxytyrosol Phase II Metabolites in Red Blood Cells: A Potential New Target for Hydroxytyrosol. *J. Funct. Foods* **2014**, *10*, 139–143. [\[CrossRef\]](#)
46. Dressman, J.B.; Thelen, K. Cytochrome P450-Mediated Metabolism in the Human Gut Wall. *J. Pharm. Pharmacol.* **2009**, *61*, 541–558. [\[CrossRef\]](#) [\[PubMed\]](#)
47. Canaparo, R.; Finnström, N.; Serpe, L.; Nordmark, A.; Muntoni, E.; Eandi, M.; Rane, A.; Zara, G.P. Expression of CYP3A Isoforms and P-Glycoprotein in Human Stomach, Jejunum and Ileum. *Clin. Exp. Pharmacol. Physiol.* **2007**, *34*, 1138–1144. [\[CrossRef\]](#)
48. Srinivas, M. Cytochrome P450 Enzymes, Drug Transporters and Their Role in Pharmacokinetic Drug-Drug Interactions of Xenobiotics: A Comprehensive Review. *Open J. Chem.* **2017**, *001*–011. [\[CrossRef\]](#)
49. Heleno, S.A.; Martins, A.; Queiroz, M.J.R.P.; Ferreira, I.C.F.R. Bioactivity of Phenolic Acids: Metabolites versus Parent Compounds: A Review. *Food Chem.* **2015**, *173*, 501–513. [\[CrossRef\]](#) [\[PubMed\]](#)
50. Karhunen, T.; Tilgmann, C.; Ulmanen, I.; Julkunen, I.; Panula, P. Distribution of Catechol-O-Methyltransferase Enzyme in Rat Tissues. *J. Histochem. Cytochem.* **1994**, *42*, 1079–1090.
51. Meech, R.; Mackenzie, P.I. Structure and Function of Uridine Diphosphate Glucuronosyltransferases. *Clin. Exp. Pharmacol. Physiol.* **1997**, *24*, 907–915. [\[CrossRef\]](#)
52. Moroy, G.; Martiny, V.Y.; Vayer, P.; Villoutreix, B.O.; Miteva, M.A. Toward in Silico Structure-Based ADMET Prediction in Drug Discovery. *Drug Discov. Today* **2012**, *17*, 44–55. [\[CrossRef\]](#)
53. Pereira De Sousa, I.; Bernkop-Schnürch, A. Pre-Systemic Metabolism of Orally Administered Drugs and Strategies to Overcome It. *J. Control. Release* **2014**, *192*, 301–309. [\[CrossRef\]](#)
54. Rowland, A.; Miners, J.O.; Mackenzie, P.I. The UDP-Glucuronosyltransferases: Their Role in Drug Metabolism and Detoxification. *Int. J. Biochem. Cell Biol.* **2013**, *45*, 1121–1132. [\[CrossRef\]](#) [\[PubMed\]](#)
55. Billat, P.-A.; Roger, E.; Faure, S.; Lagarde, F. Models for Drug Absorption from the Small Intestine: Where Are We and Where Are We Going? *Drug Discov. Today* **2017**, *22*, 761–775. [\[CrossRef\]](#)
56. Zhu, L.; Lu, L.; Wang, S.; Wu, J.; Shi, J.; Yan, T.; Xie, C.; Li, Q.; Hu, M.; Liu, Z. Oral Absorption Basics: Pathways and Physicochemical and Biological Factors Affecting Absorption. In *Developing Solid Oral Dosage Forms*; Academic Press: Waltham, MA, USA, 2017; pp. 297–329. ISBN 978-0-12-802447-8.
57. Chmiel, T.; Mieszkowska, A.; Kempńska-Kupczyk, D.; Kot-Wasik, A.; Namieśnik, J.; Mazerska, Z. The Impact of Lipophilicity on Environmental Processes, Drug Delivery and Bioavailability of Food Components. *Microchem. J.* **2019**, *146*, 393–406. [\[CrossRef\]](#)
58. López-Yerena, A.; Perez, M.; Vallverdú-Queralt, A.; Escribano-Ferrer, E. Insights into the Binding of Dietary Phenolic Compounds to Human Serum Albumin and Food-Drug Interactions. *Pharmaceutics* **2020**, *12*, 1123. [\[CrossRef\]](#) [\[PubMed\]](#)

59. Castejón, M.L.; Montoya, T.; Alarcón-de-la-Lastra, C.; Sánchez-Hidalgo, M. Potential Protective Role Exerted by Secoiridoids from *Olea Europaea* L. in Cancer, Cardiovascular, Neurodegenerative, Aging-Related, and Immunoinflammatory Diseases. *Antioxidants* **2020**, *9*, 149. [[CrossRef](#)] [[PubMed](#)]
60. Chan, J.M. Drug Metabolism and Pharmacogenetics. In *Pharmacology and Physiology for Anesthesia*. Elsevier: Amsterdam, The Netherlands, 2019; pp. 70–90. ISBN 978-0-323-48110-6.
61. Yilmaz, Y.; Williams, G.; Walles, M.; Manevski, N.; Krähenbühl, S.; Camenisch, G. Comparison of Rat and Human Pulmonary Metabolism Using Precision-Cut Lung Slices (PCLS). *Drug Metab. Lett.* **2019**, *13*, 53–63. [[CrossRef](#)]
62. Fatouros, M.; Bourantas, K.; Bairaktari, E.; Elisaf, M.; Tsolas, O.; Cassiounis, D. Role of the Spleen in Lipid Metabolism. *Br. J. Surg.* **1995**, *82*, 1675–1677. [[CrossRef](#)]
63. Miners, J.; Yang, X.; Knights, K.; Zhang, L. The Role of the Kidney in Drug Elimination: Transport, Metabolism, and the Impact of Kidney Disease on Drug Clearance. *Clin. Pharmacol. Ther.* **2017**, *102*, 436–449. [[CrossRef](#)] [[PubMed](#)]

4.3.1. Article 9: LC-ESI-LTQ-Orbitrap-MS for profiling the distribution of oleacein and its metabolites in rat tissues

Anallely López-Yerena, Anna Vallverdú-Queralt, Rosa M. Lamuela-Raventós, Elvira Escribano-Ferrer

Send to Antioxidants journal

Background: OLEA, also termed 3,4-DHPEA-EDA, plays a significant role in the organoleptic qualities of EVOO, and its antioxidant properties improve the oil shelf life. In addition, OLEA is also reported to have biological and pharmacological activities with wide-ranging health benefits.

Scope: The purpose of this work was to study the distribution of OLEA and its metabolites in rat plasma and different tissues, namely brain, heart, kidney, liver, lung, small intestine, spleen, stomach, skin, and thyroid, after the acute intake of a refined olive oil containing 0.3 mg/mL of OLEA. For this purpose, a distribution kinetics study was carried out. The plasma and tissues were collected at 1, 2 and 4.5 h after the intervention, and analyzed by LC-ESI-LTQ-Orbitrap-MS.

Methods and results: Unmetabolized OLEA was detected in the stomach, small intestine, liver, plasma and, most notably, the heart. This finding may be useful for the development of new applications of OLEA for cardiovascular disease prevention. Also noteworthy are the high levels of hydroxytyrosol (OH-TY) and OLEA + CH₃ found in the small intestine, liver, and plasma, and the detection of nine OLEA metabolites, five of them arising from conjugation reactions. Liver, heart, spleen, and lungs were the target tissues where the metabolites were most distributed. However, important to note that OH-TY was not able to reach any target tissue.

Conclusions: OLEA was found in the small intestine, liver, plasma, and most interestingly, the heart. This finding may be useful for the development of new applications of OLEA to prevent cardiovascular diseases. The results may also shed light on why OLEA is active in vivo, even if its blood concentration is low. Also noteworthy are the high levels of OH-TY and OLEA + CH₃ found in the small intestine, liver, and plasma, and the detection of nine OLEA metabolites, five of them arising from conjugation reactions. The liver, heart, spleen, and lungs were the target tissues where the metabolites were most detected. These results give a new perspective on the metabolism and tissue distribution of OLEA and contribute to understanding the mechanisms underlying its effect in human health. In summary, after the oral administration of OLEA, its metabolites were more widely distributed in tissues and plasma than the parent compound.



Article

LC-ESI-LTQ-Orbitrap-MS for profiling the distribution of oleacein and its metabolites in rat tissues

Anallely López-Yerena ¹, Anna Vallverdú-Queralt ^{1,2}, Rosa M. Lamuela-Raventós ^{1,2}, Elvira Escribano-Ferrer ^{2,3*}

¹ Department of Nutrition, Food Science and Gastronomy XIA Faculty of Pharmacy and Food Sciences, Institute of Nutrition and Food Safety (INSA-UB), University of Barcelona, 08028 Barcelona, Spain; naye.yerena@gmail.com (A.L.-Y.); avallverdu@ub.edu (A.V.-Q.); lamuela@ub.edu (R.M.L.-R.)

² CIBER Physiopathology of Obesity and Nutrition (CIBEROBN), Institute de Salud Carlos III, 28029 Madrid, Spain.

³ Biopharmaceutics and Pharmacokinetics Unit, Department of Pharmacy and Pharmaceutical Technology and Physical Chemistry, Institute of Nanoscience and Nanotechnology (IN2UB), Faculty of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain.

⁴ Pharmaceutical Nanotechnology Group I+D+I Associated Unit to CSIC, University of Barcelona, 08028 Barcelona, Spain.

* Correspondence: eescribano@ub.edu; Fax: +34-9340-35937

Abstract: The purpose of this work was to study the distribution of oleacein (OLEA) and its metabolites in rat plasma and different tissues, namely brain, heart, kidney, liver, lung, small intestine, spleen, stomach, skin, and thyroid, after the acute intake of a refined olive oil containing 0.3 mg/mL of OLEA. For this purpose, a distribution kinetics study was carried out. The plasma and tissues were collected at 1, 2 and 4.5 h after the intervention, and analyzed by LC-ESI-LTQ-Orbitrap-MS. Unmetabolized OLEA, was detected in the stomach, small intestine, liver, plasma and, most notably, the heart. This finding may be useful for the development of new applications of OLEA for cardiovascular disease prevention. Also noteworthy are the high levels of hydroxytyrosol (OH-TY) and OLEA + CH₃ found in the small intestine, liver, and plasma, and the detection of nine OLEA metabolites, five of them arising from conjugation reactions. Liver, heart, spleen, and lungs were the target tissues where the metabolites were most distributed. However, important to note that OH-TY was not able to reach any target tissue. These results shed further light on the metabolism and tissue distribution of OLEA and contribute to understanding the mechanisms underlying its effect in human health.

Keywords: Biotransformation; extra virgin olive oil; hydroxytyrosol; phenolic compound; secoiridoid,

Citation: Lastname, F.; Lastname, F.; Lastname, F. Title. *Antioxidants* **2021**, *10*, x. <https://doi.org/10.3390/xxxxx>

Academic Editor: Firstname Lastname

Received: date

Accepted: date

Published: date

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors. Submitted for possible open access publication under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Oleacein (OLEA), also termed 3,4-DHPEA-EDA, plays a significant role in the organoleptic qualities of extra virgin olive oil (EVOO), and its antioxidant properties improve the oil shelf life [1,2]. One of the most abundant compounds in EVOO, the concentrations of this secoiridoid can reach up to 1834 ± 110 mg/kg [3,4]. OLEA is also reported to have biological and pharmacological activities with wide-ranging health benefits. In 2011, a study showed that OLEA may offer far more protection against reactive oxygen/nitrogen species-induced injury of human biomolecules than its parent compound oleuropein [5]. It offers protection against cardiovascular diseases, decreasing the risk of atherosclerosis [6–8], ischemic stroke [9] and hypertension [10]. Anti-cancer effects have also been reported, including antiproliferative and apoptosis-inducing activity in leukemia cells [11] and reduction of viability and migration of non-melanoma skin cancer cells [12]. An in vitro study found that OLEA has significant anti-tumor activity by promoting cell cycle

arrest and apoptosis [13]. Moreover, OLEA has been associated with healthy aging in both in vitro and in vivo studies [14]. Anti-inflammatory properties give OLEA a protective role against several metabolic abnormalities, including dyslipidemia, insulin resistance [15,16], and glucose intolerance.

Despite the numerous studies on the biological activity of OLEA (referencia), its tissue distribution is not well documented. To date, only one study has focused on elucidating the absorption of OLEA, through simultaneous sampling from the luminal perfusate and mesenteric blood, and its metabolism during enterocytic transfer in the ileum [17]. In addition, the stability of OLEA under low pH conditions and permeability in Caco-2 cells and in isolated rat intestine has been studied [18] as well as its fecal microbial metabolism in vivo [19]. The distribution and accumulation of EVOO polyphenols [20] and oleocanthal [21] has also been determined in rat tissues. The health-enhancing properties of OLEA cannot be fully understood without further analysis of its absorption, tissue distribution, metabolism, and excretion.

In this context, we carried out a qualitative and quantitative evaluation of OLEA and its metabolites in a range of biological samples taken from rats at different times (0, 1, 2 and 4.5 h) after the administration of refined olive oil (ROO) containing OLEA. For the first time, OLEA and its metabolites were studied in organs of the digestive system (stomach, small intestine, and liver), excretory tissue (kidney), and several target tissues (brain, lungs, heart, spleen, thyroids, and skin).

2. Materials and Methods

2.1. Chemicals and reagents

The reference standard OLEA ($\geq 90\%$) was purchased from Toronto Research Chemicals (North York, ON, Canada) and hydroxytyrosol (OH-TY) was obtained from Extrasynthese (Genay, France). The reagents methanol, acetonitrile (ACN) and formic acid were acquired from AppliChem, Panreac Quimica SLU (Barcelona, Spain). Ultrapure water was obtained using a Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2. Animals

In this study, male Sprague-Dawley rats (body weight 227 ± 11 g) were obtained from Envigo RMS Spain SL (Sant Feliu de Codines, Barcelona, Spain). The animals were acclimated for at least 1 week before the initiation of dosing with free access to food and water. They were kept at 22 ± 2 °C in an air-conditioned room on a 12-h light–12-h dark cycle. Before the experiments, the animals were fasted overnight with water available ad libitum. The experiments were performed in compliance with the Animal Experimentation Ethics Committee of the University of Barcelona, Spain and were approved by the Generalitat de Catalunya (trial no. CEEA 124/16 and no. 6435, respectively).

2.3. Refined olive oil

Before the experiment, the ROO (used as a vehicle to dissolve OLEA for intragastric administration) was analyzed by LC-ESI-LTQ-Orbitrap-MS to make sure it was free of phenolic compounds. The liquid–liquid extraction of phenolic compounds in the ROO was performed following previously described procedures [22]. The chromatographic and mass spectrometer conditions were the same as used for the identification of metabolites (Section 2.6). After the absence of phenolic compounds was confirmed, the OLEA was added to obtain a final concentration of 0.3 mg/mL. This concentration was chosen based on its concentration in commercial EVOO (around 300 mg/kg) and the daily ingestion recommended by the European Food Safety Authority of at least 5 mg of hydroxytyrosol and its derivatives per 20 g of olive oil [23].

2.4. Dosage information

The rats were divided into three different groups according to the time of euthanasia: 1 h (n = 4), 2 h (n = 5) and 4.5 h (n=4). At 0 h, ROO loaded with OLEA was supplied to the rats (2.5 mL/300 g body weight) by intragastric administration. Additionally, a control group of rats was maintained under fasting conditions without olive oil ingestion and then similarly euthanized. The gavage volume was established following a good practice guide for substance administration [24] and was lower than the maximum volume of lipid vehicles per kg of rat body weight that prevents elevated plasma corticosterone levels [25].

The rats were anesthetized with isoflurane (IsoFlo, veterinaria Esteve, Barcelona, Spain) and euthanized by exsanguinations at the aforementioned time points after the ROO ingestion. Blood samples were collected in EDTA tubes, centrifuged to obtain the plasma (3000 × g, 10 min at 4 °C), and stored at −80 °C until analysis. The gastrointestinal tract (including stomach and small intestine), liver, kidney, heart, lungs, spleen, brain, thyroids, and skin were removed from rats at the designated time points. All tissues were washed with saline solution (NaCl 0.9%) and dried with filter paper. In the case of the intestine, the portion removed was 15 cm from 2 cm posterior to the pyloric sphincter, and the intestine was also washed internally by perfusing the isotonic saline solution several times. Rat tissues were accurately weighed and stored at −80 °C until analysis.

2.5. Sample pre-treatment for OLEA metabolite analysis

For extraction, all samples were slowly thawed in ice. To avoid compound oxidation, the samples were handled in a room with filtered light and kept on ice throughout. The clean-up of the biological matrix (protein precipitation) was performed using a previously developed method [21].

2.5.1. Plasma

Plasma samples were centrifuged for 10 min (11000 × g, 4 °C). Briefly, 100 µL of each sample was deproteinized with ice-cold ACN acidified with formic acid (2%) in a 1:5 (v/v) ratio. The samples were then homogenized for 1 min and kept at −20 °C for 20 min. After that, samples were centrifuged (11000 × g, 4 °C, 10 min) and finally, 100 µL of each organic phase was transferred to vials for analysis.

2.5.2. Tissues

A small tissue disruptor (T 10 basic ULTRA-TURRAX®, IKA laboratory technology, Staufen Germany) was used to homogenize the tissues in a 1:1 ratio (w/v) with water:ACN (1:1 (v/v) with 0.1% ascorbic acid). The samples were then sonicated for 5 min in an ice bath, shaken for 1 min (Vortex-Genie 2) and centrifuged for 10 min (11000 × g, 4 °C). To precipitate the proteins, a volume of 100 µL of the upper layer was blended with cold ACN acidified with formic acid (2%) in a 1:3 (v/v) ratio. After that, the samples were homogenized for 1 min and kept at −20 °C for 20 min. Finally, the samples were centrifuged for 10 min (11000 × g, 4 °C) and 100 µL of supernatant was transferred to vials for analysis.

2.6. Instruments and analytical conditions

The metabolic profile was determined by analyzing the samples with LC-ESI-LTQ-Orbitrap-MS. The equipment was an Accela chromatograph (Thermo Scientific, Hemel Hempstead, UK) with a quaternary pump, a photodiode array detector (PDA) and a thermostated autosampler coupled with a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) equipped with an ESI source in negative mode.

Chromatographic separation was performed using an Acquity™ UPLC® BEH C₁₈ Pre-Column (2.1 × 5 mm, i.d., 1.7 µm particle size) and an Acquity™ UPLC® BEH C₁₈ Column (2.1 × 100 mm, i.d., 1.7 µm particle size) (Waters Corporation®, Ireland) maintained at 50 °C. The mobile phases consisted of methanol (A) and H₂O (B), both with 0.1% of formic acid. The elution was performed by means of an increasing linear gradient (v/v) of B (t (min), %B), as follows: (0, 100); (2, 100); (6, 46.4); (8, 0); (9, 0); (9.1, 100); (11, 100). The injection volume and the flow rate were set to 5 µL and 0.6 mL/min, respectively.

The instrumental conditions for the LTQ Orbitrap Velos mass spectrometer were the same as reported in our previous study [17]. The identification of OLEA and OH-TY was achieved using their respective commercially available standards. The identification of the other metabolites was based on chromatographic elution time, chemical composition, and MS/MS fragmentation pattern, also as performed previously [17].

For quantification purposes, the calibration curves were prepared with OLEA in plasma and each blank isolated tissue (brain, heart, kidney, liver, lung, small intestine, spleen, stomach, skin, and thyroid) in concentrations of 0.1 to 3 µg/mL. All calibration curves presented $R^2 > 0.97$. The concentrations of all OLEA metabolites were calculated using the peak area of the parent molecule and were expressed as nmol OLEA equivalents.

2.7. Data analysis

All statistical analyses were performed with Statgraphics Centurion XVI software (Statpoint Technologies Inc., Warrenton, VA, USA). Data are presented as mean and standard deviation (SD). The assumption of normalization was checked with standardized Bias and standardized Kurtosis. Statistical differences in the concentration of metabolites and OLEA at different post-administration times (1, 2 and 4.5 h) for each compound and tissue were evaluated using a one-way ANOVA, followed by the LSD post-hoc test. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Pre-systemic metabolism

The concentrations of OLEA and its derivatives in the stomach, small intestine and liver expressed as nmol/g tissue are presented in Table 1. The highest concentration (C_{max}) of OLEA in the stomach (8.73 ± 0.29 nmol/g tissue) was found at 1 h after acute ingestion of ROO, and it had significantly decreased by 16% at 2 h and by 34% at 4.5 h ($p < 0.05$). One explanation for this reduction is gastric emptying, as OLEA was observed in the small intestine at 1 h post-ingestion. Another possible cause is the acidic environment of the stomach and/or the presence of nonlipolytic carboxylester hydrolases, which can contribute to a hydrolysis of OLEA, resulting in OH-TY and elenolic acid (EA) [26]. Contradictory results about the gastric stability of OLEA have been reported. In a recent in vivo study with rats by Pinto et al. [18], OLEA was found to undergo a complete degradation in the gastric region, and only a partial hydrolysis after incubation at pH 2.0 (releasing OH-TY), with about 67% of OLEA remaining after 4 h. A partial hydrolysis was also observed in our study, as the amount of OLEA reaching the small intestine was lower than in the stomach at the same time point (Table 1). The C_{max} of OLEA in the small intestine was reached after 1 h of ROO intake, decreasing successively thereafter (by 25% and 77% at 2 h and 4.5 h, respectively) ($p < 0.05$). This reduction is mainly attributable to its good permeability through the intestinal epithelium, as reported recently [17], and transport to the liver (where notably OLEA was detected only at 1 h post-ingestion). The low concentration of OLEA in the liver vs. the small intestine is explained by the partial intestinal metabolism of OLEA during the absorption [17].

The metabolites OH-TY and EA released in the stomach were transported to the small intestine, where they were absorbed, similar amounts being found in the liver. Both compounds are weak basic organic aromatic molecules with very low molecular weights (<250 g/mol) and moderate lipophilicity ($\log P \sim 0.9$), which favors their absorption through passive mechanisms [26,27]; their detection in plasma confirmed their absorption (Table 2). Current data suggest OH-TY is the only biologically active derivative of OLEA [28,29], so the detection of free OH-TY in the liver is noteworthy, pointing to its potential application in the prevention of certain hepatic and renal diseases. Our results are in line with other studies that demonstrate that OH-TY can reach target tissues [27].

As shown in Table 1, the metabolite arising from the hydrogenation of OLEA (OLEA + H₂) was detected in the stomach and small intestine, with the C_{max} in both tissues at 2 h ($p < 0.05$). In previous studies, this metabolite was not identified in the stomach, small intestine or liver [19,20], although it has been detected in human urine [30] and rat plasma [17]. The hydrogenation of an aldehyde could be attributed to aldo-keto reductases (AKRs) that primarily reduce aldehydes and ketones to primary and secondary alcohols, respectively [31]. In our study, the absence of the hydrogenated metabolite in the liver could be attributed to other biotransformations once it reaches this tissue, such as glucuronidation. It is well known that conjugation reactions lead to the formation of a covalent bond joining a functional group in the parent compound or phase I metabolite [32].

The metabolite OLEA + H₂O was detected in the stomach, small intestine, and liver samples. The C_{max} in the two first tissues was reached at 1 h, and in the liver at 2 h ($p < 0.05$), reflecting the time required for absorption and transport. These results support those described by García-Villalba et al. (2010) [30] and López Yerena et al. (2021) [17], who identified this metabolite in human urine and rat plasma, respectively. However, in other studies of secoiridoid metabolism, this metabolite has not been quantified [18–20,33].

Regarding OLEA derivatives arising from conjugation reactions, a methylated metabolite (OLEA + CH₃) was detected in the stomach, small intestine, and the liver, with higher concentrations in the latter two organs. In the liver, it was the metabolite with the highest concentration. The catechol-O-methyltransferase (COMT) enzymes, most active in the liver and kidneys, but also in the pylorus of the stomach and in the small intestine [34], are responsible for the methylation of available OLEA. Glycoconjugated metabolites, on the other hand, require the activity of uridine-disphosphate glucuronosyl transferases (UGT). Although these enzymes are present in the stomach [35] and UGT1A8 and UGT1A10 are exclusively expressed in the small intestine [36], the liver is the main site of glucuronidation [35], which could explain why we found metabolites with a glucuronide acid only in the liver and not in the stomach and small intestine. Regarding metabolites not derived from hydrolysis reactions, their concentrations were very low (or even absent) in the stomach and increased in the intestine and liver, in accordance with the higher qualitative and quantitative enzyme endowment of these organs. These metabolites were also detected in the intestinal lumen and ileal tissue by Lopez-Yerena [17]. The fact that we did not find the metabolites OLEA + H₂O + CH₃, OLEA + H₂ + glucu and OLEA + H₂O + CH₃ + glucu in the small intestine (Table 1), yet they were detected in the aforementioned study (at very low concentrations), can be mainly attributed to methodological differences (in situ vs. oral administration).

Table 1. Concentration of OLEA and phase I and phase II metabolites in the stomach, small intestine, and liver, expressed as nmol OLEA equivalents/g tissue. Results are shown as the mean $\pm$ standard deviation.

Metabolite	Time (h)	Stomach	Small Intestine	Liver
OLEA	1	8.73 $\pm$ 0.29 ^a	2.24 $\pm$ 0.14 ^a	0.28 $\pm$ 0.02
	2	7.39 $\pm$ 0.25 ^b	1.69 $\pm$ 0.25 ^b	n.d.
	4.5	5.77 $\pm$ 0.54 ^c	0.51 $\pm$ 0.10 ^c	n.d.
OH-TY	1	13.37 $\pm$ 1.27 ^a	4.71 $\pm$ 0.32 ^a	3.34 $\pm$ 0.08 ^b
	2	7.81 $\pm$ 0.69 ^b	3.79 $\pm$ 0.29 ^b	3.83 $\pm$ 0.21 ^a
	4.5	5.23 $\pm$ 0.37	2.70 $\pm$ 0.21 ^c	2.51 $\pm$ 0.07 ^c
EA	1	7.53 $\pm$ 0.56 ^a	1.81 $\pm$ 0.05 ^a	1.07 $\pm$ 0.06 ^b
	2	4.07 $\pm$ 0.36 ^b	0.67 $\pm$ 0.08 ^b	1.34 $\pm$ 0.08 ^a
	4.5	1.81 $\pm$ 0.13 ^c	0.16 $\pm$ 0.02 ^c	0.76 $\pm$ 0.02 ^c

OLEA + H ₂	1	1.34 ± 0.01 ^a	5.31 ± 0.48 ^a	n.d.
	2	0.82 ± 0.01 ^b	3.01 ± 0.35 ^b	n.d.
	4.5	0.81 ± 0.01 ^b	2.16 ± 0.10 ^c	n.d.
OLEA + H ₂ O	1	1.90 ± 0.18 ^a	1.29 ± 0.14 ^a	0.33 ± 0.02 ^b
	2	1.66 ± 0.12 ^a	0.96 ± 0.06 ^b	0.77 ± 0.01 ^a
	4.5	0.80 ± 0.03 ^b	0.53 ± 0.01 ^c	0.26 ± 0.04 ^c
OLEA + CH ₃	1	0.46 ± 0.05 ^a	3.69 ± 0.19 ^a	3.88 ± 0.45 ^b
	2	0.41 ± 0.01 ^a	2.99 ± 0.46 ^b	5.79 ± 0.36 ^a
	4.5	0.26 ± 0.00 ^b	2.26 ± 0.22 ^c	1.90 ± 0.24 ^c
OLEA + H ₂ O + CH ₃	1	n.d.	n.d.	1.37 ± 0.12 ^b
	2	n.d.	n.d.	1.78 ± 0.07 ^a
	4.5	n.d.	n.d.	1.10 ± 0.07 ^c
OLEA + H ₂ + glucu	1	n.d.	n.d.	0.77 ± 0.05 ^b
	2	n.d.	n.d.	1.18 ± 0.06 ^a
	4.5	n.d.	n.d.	0.46 ± 0.05 ^c
OLEA + H ₂ O + CH ₃ + glucu	1	n.d.	n.d.	0.38 ± 0.03 ^b
	2	n.d.	n.d.	0.69 ± 0.04 ^a
	4.5	n.d.	n.d.	0.26 ± 0.01 ^c

Different letters in each metabolite and tissue indicate significant differences in concentration between 1, 2 and 4.5 h ($p < 0.05$). n.d. not detected

3.2. Plasma

Table 2 shows the concentration of OLEA and its phase I and II metabolites detected in rat plasma after the intragastric administration of OLEA in ROO. As can be observed, OLEA reached the systemic circulation unchanged, being found in the plasma at all three sampling times. The highest concentration was at 1 h post-ROO intake (1.37 ± 0.20 nmol/mL), and some remained at 4.5 h (0.67 ± 0.10 nmol/mL). Our results are in accordance with those of Silva et al., [33] who identified unmetabolized OLEA in human plasma after EVOO intake. Recently, OLEA was also detected in mesenteric rat plasma after in situ perfusion of the small intestine [17]; this method avoided the pass through the stomach where hydrolysis can potentially take place, but the OLEA would still have to pass through the liver to reach the systemic circulation. In contrast with the present results, OLEA was not identified in human plasma after olive oil intake [30], or in rat plasma after the administration of a phenolic extract from olive cake [20] or a diet supplemented with OLEA [19]. The presence of OLEA in plasma at all sample times, unlike in the liver, where it was only detected at 1 h, could be due to an in situ deconjugation in red blood cells, as suggested for sulphates and glucuronidates of OH-TY [37].

The metabolites circulating in plasma at the highest concentrations were methylated OLEA (OLEA + CH₃) and OH-TY. The C_{max} of methylated OLEA was highest at 2 h post-ingestion of ROO (3.50 ± 0.18 ng/mL), representing 25% of the total metabolites identified at this time point ($p < 0.05$). The second most abundant, OH-TY, represented 20, 22% and 17% of the total metabolites at 1, 2 and 4.5 h, respectively. Our results are similar to those of Serra et al. (2012) [20], who linked the high percentage of total phenyl alcohol metabolites (e.g., OH-TY and tyrosol) in plasma with the high content of OLEA in the administered olive cake extract, which acted as a source of OH-TY. The detection of free OH-TY in plasma may shed light on the health benefits of the phenolic content of EVOO. Interest in OH-TY has grown since 2012, when the European Food Safety Agency (EFSA) Panel on Dietetic Products, Nutrition, and Allergies (NDA) established a cause-and-effect

relationship between the consumption of olive oil polyphenols and protection of LDL particles from oxidative damage [28]. Based on this scientific opinion, “Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress” was included in the list of authorized health claims in the European Union [29], being the only relating polyphenols with health.

Other metabolites arising from phase I (EA, OLEA + H₂ and OLEA + H₂O) and phase II reactions (OLEA + H₂O + CH₃, OLEA + H₂ + glucu, OLEA + H₂O + glucu, OLEA + H₂O + CH₃ + glucu) were also detected in plasma samples. For all metabolites, the C_{max} was reached at 2 h after ROO consumption. In another study, OLEA + H₂O + CH₃ + glucu was also identified in human plasma [33].

Table 2. Mean concentration of OLEA and its phase I and phase II metabolites $\pm$ standard deviation in plasma. Results are expressed as nmol OLEA equivalents/g tissue.

Metabolites (nmol/mL)	Time (h)		
	1	2	4.5
OLEA	1.37 $\pm$ 0.20 ^a	0.65 $\pm$ 0.07 ^b	0.67 $\pm$ 0.10 ^b
OH-TY	2.11 $\pm$ 0.10 ^b	3.02 $\pm$ 0.16 ^a	1.62 $\pm$ 0.14 ^c
EA	0.43 $\pm$ 0.03 ^b	0.76 $\pm$ 0.04 ^a	0.16 $\pm$ 0.02 ^c
OLEA + H ₂	0.87 $\pm$ 0.02 ^b	1.08 $\pm$ 0.02 ^a	0.87 $\pm$ 0.02 ^b
OLEA + H ₂ O	0.91 $\pm$ 0.03 ^b	1.14 $\pm$ 0.02 ^a	0.84 $\pm$ 0.02 ^c
OLEA + CH ₃	2.52 $\pm$ 0.24 ^b	3.50 $\pm$ 0.18 ^a	2.80 $\pm$ 0.22 ^b
OLEA + H ₂ O + CH ₃	1.04 $\pm$ 0.01 ^b	1.35 $\pm$ 0.05 ^a	0.91 $\pm$ 0.02 ^c
OLEA + H ₂ + glucu	0.88 $\pm$ 0.04 ^b	1.10 $\pm$ 0.04 ^a	0.71 $\pm$ 0.02 ^c
OLEA + H ₂ O + glucu	1.26 $\pm$ 0.03 ^b	1.44 $\pm$ 0.04 ^a	1.03 $\pm$ 0.03 ^c
OLEA + H ₂ O + CH ₃ + glucu	0.57 $\pm$ 0.01 ^b	0.65 $\pm$ 0.01 ^a	0.50 $\pm$ 0.01 ^b

Different letters in each metabolite and tissue indicate significant differences in concentration between 1, 2 and 4.5 h ($p < 0.05$).

3.3. Accumulation and distribution of OLEA derivatives in rat tissues

To date, only two studies have focused on the metabolism and distribution of secoiridoids, including OLEA, in target tissues of rats [19,20]. The vehicles they used to administer the phenolic compounds (pellets containing a secoiridoid extract and a phenolic extract from olive cake dispersed in water, respectively) could be seen as a drawback when attempting to elucidate the health effects of EVOO. In our study, ROO was chosen as the vehicle to better simulate the conditions of EVOO consumption. In addition, the metabolite profile was determined by UPLC coupled with high-resolution mass spectrometry (HRMS), using Orbitrap technology to overcome the limitations of the low-resolution MS techniques (triple quadrupole) of the two previous studies [19,20,38]. The main results are shown in Table 3.

3.3.1. Heart

The heart was the only organ where unmetabolized OLEA was detected without significant differences in concentration between the time points ($p > 0.05$) (0.44 ± 0.07 , 0.45 ± 0.04 and 0.44 ± 0.06 nmol/g tissue, at 1, 2 and 4.5 h, respectively). The effect of OLEA on the progression of different types of cardiovascular diseases has been studied in vitro. These include biological effects such as the ability to regulate low-density lipoprotein (LDL) oxidation and myeloperoxidase (MPO) activity, to reduce the expression of adhesion molecules through angiotensin II production, and to confer protection to erythrocytes from oxidative hemolysis [5–9]. Although the concentrations detected in the heart (≤ 0.5 nmol/g tissue) were very low compared with the results of in vitro studies (50 and 100 mM [6] and 10–20 mM [7]), this is the first demonstration that OLEA is able to reach the

heart, where it may exert a beneficial effect. However, more studies are necessary to determine if the concentration detected in our study would be sufficient for OLEA to play a preventive and curative role against cardiovascular injury. In addition to OLEA, seven metabolites were also detected (Table 3).

3.3.2. Spleen, lungs and thyroids

Seven metabolites in the spleen and lungs and six in thyroids were detected (Table 3), all with a C_{max} at 2 h after ROO ingestion. The activity of metabolizing enzymes in the endothelial cells of lungs [39,40] and their expression in macrophages and lymphocytes in the white pulp region of the spleen [20] could explain the presence of these metabolites in these tissues.

3.3.3. Brain

The main metabolites found in the brain were from phase II reactions. The only phase I metabolite quantified was the hydrated form of OLEA vs five conjugated metabolites detected in greater number and concentration. After 1 h of ROO ingestion, the main metabolites identified were OLEA + CH_3 , OLEA + H_2 + glucu, and OLEA + H_2O + glucu. Neurodegenerative diseases normally begin in the elderly, caused by a significant accumulation of reactive oxygen species in the brain due to unknown causes. In neurodegeneration, mitochondrial dysfunction, neuroinflammation, and in some pathologies, protein aggregation and neuronal death culminate in brain dysfunction [41]. Recently, Gutiérrez-Miranda et al. [42] suggested that OLEA may provide protective effects against autoimmune encephalomyelitis by reducing leukocyte infiltration into the central nervous system, preventing the formation of inflammatory mediators, and inhibiting the elevation of the oxidative stress status. According to the results of our study, in which free OLEA was not detected in the brain, more research is needed to determine the bioactivity of the main metabolites at the physiological concentrations achieved after regular consumption of EVOO, or the potential intracellular deconjugation of the metabolites in the target tissue.

3.3.4. Kidneys

As shown in Table 3, only conjugated metabolites were detected in the kidneys, which is the only organ where the highest concentration of metabolites was found at 4.5 h post-ROO consumption ($p < 0.05$). As kidneys are an elimination organ, hydrophilic metabolites are likely to accumulate there for excretion. At 4.5h, the main metabolite identified was OLEA + H_2O + glucu, with a C_{max} of 0.91 ± 0.05 nmol/g tissue. The wide range of metabolites detected indicates that renal excretion is a major pathway of OLEA metabolite elimination in rats. The presence of metabolites in the kidneys could also be attributed to the activity of metabolic enzymes (CYP and UGT). By regulating renal drug and chemical metabolic clearance, they modulate intra-renal exposure to compounds as well as the activity of physiological mediators [43]. In 2010, Garcia-Villalba et al. [30] identified the same metabolites in human urine 2 h after the intake of olive oil.

3.3.5. Skin

The skin is the least studied tissue in distribution studies after a phenolic compound ingestion. In a recent study of oleocanthal distribution, four metabolites were detected in the skin [21], whereas we identified only OLEA + CH_3 . The concentration of this metabolite increased over time: 0.09 ± 0.01 , 0.15 ± 0.02 and 0.25 ± 0.02 nmol/g tissue at 1, 2 and 4.5 h, respectively, achieving a C_{max} at 4.5 h post-ingestion (results not included in the table). The skin is a relatively poorly perfused tissue compared to well-perfused tissues like the lungs, heart and thyroid gland, so it absorbs compounds less rapidly [44]. Despite the detection of only one metabolite in the skin, this finding may help to shed new light on the mechanisms underlying EVOO phenolic compound activity in the body, specifically in the area of skin disorder prevention.

Table 3. Mean $\pm$ standard deviation of tissue concentration of metabolites, expressed as nmol OLEA equivalents/g tissue, following the acute intake of OLEA (0.3 mg/mL) in ROO.

Compounds	Time (h)	Heart	Spleen	Thyroids	Lungs	Brain	Kidney
OLEA	1	0.44 $\pm$ 0.07 ^a	n.d.	n.d.	n.d.	n.d.	n.d.
	2	0.45 $\pm$ 0.04 ^a	n.d.	n.d.	n.d.	n.d.	n.d.
	4.5	0.44 $\pm$ 0.06 ^a	n.d.	n.d.	n.d.	n.d.	n.d.
OLEA + H ₂	1	0.17 $\pm$ 0.02 ^b	0.30 $\pm$ 0.05 ^c	0.10 $\pm$ 0.01 ^c	0.49 $\pm$ 0.04 ^c	n.d.	n.d.
	2	0.30 $\pm$ 0.04 ^a	0.54 $\pm$ 0.03 ^a	0.24 $\pm$ 0.01 ^a	0.70 $\pm$ 0.05 ^a	n.d.	n.d.
	4.5	0.18 $\pm$ 0.01 ^b	0.34 $\pm$ 0.02 ^b	0.12 $\pm$ 0.01 ^b	0.52 $\pm$ 0.01 ^b	n.d.	n.d.
OLEA + H ₂ O	1	0.07 $\pm$ 0.01 ^b	0.42 $\pm$ 0.03 ^b	0.12 $\pm$ 0.02 ^b	0.49 $\pm$ 0.02 ^b	0.13 $\pm$ 0.03 ^b	n.d.
	2	0.17 $\pm$ 0.02 ^a	0.61 $\pm$ 0.07 ^a	0.24 $\pm$ 0.02 ^a	1.02 $\pm$ 0.12 ^a	0.22 $\pm$ 0.02 ^a	n.d.
	4.5	0.07 $\pm$ 0.01 ^b	0.42 $\pm$ 0.02 ^b	0.13 $\pm$ 0.03 ^b	0.42 $\pm$ 0.05 ^b	0.10 $\pm$ 0.01 ^b	n.d.
OLEA + CH ₃	1	0.20 $\pm$ 0.02 ^b	0.31 $\pm$ 0.03 ^b	0.21 $\pm$ 0.02 ^b	0.35 $\pm$ 0.03 ^b	0.32 $\pm$ 0.03 ^b	0.17 $\pm$ 0.01 ^b
	2	0.36 $\pm$ 0.03 ^a	0.39 $\pm$ 0.04 ^a	0.31 $\pm$ 0.02 ^a	0.45 $\pm$ 0.04 ^a	0.52 $\pm$ 0.04 ^a	0.20 $\pm$ 0.02 ^b
	4.5	0.25 $\pm$ 0.03 ^b	0.27 $\pm$ 0.03 ^b	0.24 $\pm$ 0.01 ^b	0.31 $\pm$ 0.02 ^b	0.30 $\pm$ 0.03 ^b	0.34 $\pm$ 0.04 ^a
OLEA + H ₂ O + CH ₃	1	0.11 $\pm$ 0.01 ^b	0.18 $\pm$ 0.03 ^c	0.14 $\pm$ 0.01 ^b	0.62 $\pm$ 0.06 ^b	0.17 $\pm$ 0.02 ^b	0.13 $\pm$ 0.02 ^c
	2	0.18 $\pm$ 0.03 ^a	0.68 $\pm$ 0.03 ^a	0.22 $\pm$ 0.03 ^a	0.84 $\pm$ 0.04 ^a	0.35 $\pm$ 0.02 ^a	0.23 $\pm$ 0.03 ^b
	4.5	0.08 $\pm$ 0.01 ^c	0.44 $\pm$ 0.04 ^b	0.09 $\pm$ 0.09 ^c	0.48 $\pm$ 0.04 ^c	0.15 $\pm$ 0.02 ^b	0.43 $\pm$ 0.06 ^a
OLEA + H ₂ + glucu	1	0.13 $\pm$ 0.02 ^b	0.08 $\pm$ 0.01 ^b	0.17 $\pm$ 0.02 ^b	0.24 $\pm$ 0.01 ^b	0.28 $\pm$ 0.03 ^b	0.10 $\pm$ 0.02 ^c
	2	0.20 $\pm$ 0.01 ^a	0.18 $\pm$ 0.01 ^a	0.28 $\pm$ 0.01 ^a	0.37 $\pm$ 0.01 ^a	0.40 $\pm$ 0.03 ^a	0.35 $\pm$ 0.13 ^b
	4.5	0.11 $\pm$ 0.01 ^b	0.08 $\pm$ 0.01 ^b	0.07 $\pm$ 0.01 ^c	0.11 $\pm$ 0.02 ^c	0.09 $\pm$ 0.02 ^c	0.64 $\pm$ 0.03 ^a
OLEA + H ₂ O + glucu	1	0.42 $\pm$ 0.07 ^a	0.30 $\pm$ 0.03 ^b	0.21 $\pm$ 0.02 ^b	0.32 $\pm$ 0.02 ^b	0.28 $\pm$ 0.03 ^b	0.20 $\pm$ 0.03 ^b
	2	0.43 $\pm$ 0.06 ^a	0.43 $\pm$ 0.03 ^a	0.35 $\pm$ 0.05 ^a	0.45 $\pm$ 0.03 ^a	0.41 $\pm$ 0.03 ^a	0.25 $\pm$ 0.04 ^b
	4.5	0.17 $\pm$ 0.02 ^b	0.13 $\pm$ 0.02 ^c	0.01 $\pm$ 0.02 ^c	0.15 $\pm$ 0.02 ^c	0.10 $\pm$ 0.02 ^c	0.91 $\pm$ 0.05 ^a
OLEA + H ₂ O + CH ₃ + glucu	1	0.05 $\pm$ 0.00 ^c	0.11 $\pm$ 0.02 ^b	n.d.	0.06 $\pm$ 0.01 ^b	0.04 $\pm$ 0.00 ^b	0.05 $\pm$ 0.00 ^c
	2	0.20 $\pm$ 0.01 ^a	0.42 $\pm$ 0.04 ^a	n.d.	0.10 $\pm$ 0.02 ^a	0.08 $\pm$ 0.01 ^a	0.14 $\pm$ 0.02 ^b
	4.5	0.13 $\pm$ 0.01 ^b	0.05 $\pm$ 0.00 ^c	n.d.	0.04 $\pm$ 0.01 ^b	0.05 $\pm$ 0.01 ^b	0.40 $\pm$ 0.02 ^a

Different letters in each metabolite and tissue indicate significant differences in concentration between 1, 2 and 4.5 h ($p < 0.05$). n.d. not detected

4. Conclusions

In this work, the distribution of OLEA and derived metabolites in rat plasma and ten organs/tissues was investigated using an LC-ESI-LTQ-Orbitrap-MS system. After a single oral administration of refined olive oil (by gastric gavage), OLEA was found to be hydrolyzed in the stomach. Unmetabolized OLEA, was detected in the small intestine, liver, plasma, and most interestingly, the heart. This finding may be useful for the development of new applications of OLEA to prevent cardiovascular diseases. The results may also shed light on why OLEA is active in vivo, even if its blood concentration is low.

Also noteworthy are the high levels of OH-TY and OLEA + CH₃ found in the small intestine, liver, and plasma, and the detection of nine OLEA metabolites, five of them arising from phase II reactions. The liver, heart, spleen, and lungs were the target tissues where the metabolites were most detected. In summary, after the oral administration of OLEA, its metabolites were more widely distributed in tissues and plasma than the parent compound.

Author Contributions: Conceptualization, A.L.-Y., E.E.-F. and R.M.L.-R.; methodology, A.L.-Y., E.E.-F., and A.V.-Q.; software, A.L.-Y., E.E.-F. and A.V.-Q.; validation, A.L.-Y., E.E.-F. and R.M.L.-R.; formal analysis, A.L.-Y. and E.E.-F.; investigation, A.L.-Y. and E.E.-F.; resources, R.M.L.-R.; data curation, A.L.-Y., E.E.-F., and A.V.-Q.; writing—original draft preparation, A.L.-Y. and E.E.-F.; writing—review and editing, A.L.-Y., and E.E.-F.; visualization, A.L.-Y., and E.E.-F.; supervision, E.E.-

F. and R.M.L.-R.; project administration, E.E.-F. and R.M.L.-R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by CICYT (AGL2016-75329-R), CIBEROBN from the Instituto de Salud Carlos III, ISCIII from the Ministerio de Ciencia, Innovación y Universidades (AEI/FEDER, UE) and Generalitat de Catalunya (GC) (2017SGR 196). A.L.-Y. thanks the Consejo Nacional de Ciencia y Tecnología (CONACYT) of Mexico for the doctoral scholarship. A.V.-Q. thanks the Ministry of Science Innovation and Universities for the Ramon y Cajal contract (RYC-2016-19355).

Institutional Review Board Statement: The animal experiments were performed in compliance with the Animal Experimentation Ethics Committee of the University of Barcelona, Spain and were approved by the Generalitat de Catalunya (trial no. CEEA 124/16 and no. 6435, respectively).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article. The data presented in this study are available in Table 1, Table 2 and Table 3.

Acknowledgments: The authors wish to thank the CCiT-UB for the mass spectrometry equipment.

Conflicts of Interest: R.M.L.-R. reports receiving lecture fees from Cerveceros de España and receiving lecture fees and travel support from Adventia. The other authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

- Andrewes, P.; Busch, J.L.H.C.; De Joode, T.; Groenewegen, A.; Alexandre, H. Sensory Properties of Virgin Olive Oil Polyphenols: Identification of Deacetoxyligstroside Aglycon as a Key Contributor to Pungency. *J. Agric. Food Chem.* **2003**, *51*, 1415–1420, doi:10.1021/jf026042j.
- López-Yerena, A.; Ninot, A.; Lozano-Castellón, J.; Escribano-Ferrer, E.; Romero-Aroca, A.J.; Belaj, A.; Vallverdú-Queralt, A.; Lamuela-Raventós, R.M. Conservation of Native Wild Ivory-White Olives from the MEDES Islands Natural Reserve to Maintain Virgin Olive Oil Diversity. *Antioxidants* **2020**, *9*, 1009, doi:10.3390/antiox9101009.
- Miho, H.; Díez, C.M.; Mena-Bravo, A.; Sánchez de Medina, V.; Moral, J.; Melliou, E.; Magiatis, P.; Rallo, L.; Barranco, D.; Priego-Capote, F. Cultivar Influence on Variability in Olive Oil Phenolic Profiles Determined through an Extensive Germplasm Survey. *Food Chem.* **2018**, *266*, 192–199, doi:10.1016/j.foodchem.2018.06.002.
- Miho, H.; Moral, J.; López-González, M.A.; Díez, C.M.; Priego-Capote, F. The Phenolic Profile of Virgin Olive Oil Is Influenced by Malaxation Conditions and Determines the Oxidative Stability. *Food Chem.* **2020**, *314*, 126183, doi:10.1016/j.foodchem.2020.126183.
- Czerwińska, M.; Kiss, A.K.; Naruszewicz, M. A Comparison of Antioxidant Activities of Oleuropein and Its Dialdehydic Derivative from Olive Oil, Oleacein. *Food Chem.* **2012**, *131*, 940–947, doi:10.1016/j.foodchem.2011.09.082.
- Czerwińska, M.E.; Kiss, A.K.; Naruszewicz, M. Inhibition of Human Neutrophils NEP Activity, CD11b/CD18 Expression and Elastase Release by 3,4-Dihydroxyphenylethanol-Elenolic Acid Dialdehyde, Oleacein. *Food Chem.* **2014**, *153*, 1–8, doi:10.1016/j.foodchem.2013.12.019.
- Filipek, A.; Czerwińska, M.E.; Kiss, A.K.; Wrzosek, M.; Naruszewicz, M. Oleacein Enhances Anti-Inflammatory Activity of Human Macrophages by Increasing CD163 Receptor Expression. *Phytomedicine* **2015**, *22*, 1255–1261, doi:10.1016/j.phymed.2015.10.005.
- Naruszewicz, M.; Czerwińska, M.; Kiss, A. Oleacein. Translation from Mediterranean Diet to Potential Antiatherosclerotic Drug. *Curr. Pharm. Des.* **2015**, *21*, 1205–1212, doi:10.2174/1381612820666141007141137.
- Filipek, A.; Czerwińska, M.E.; Kiss, A.K.; Polański, J.A.; Naruszewicz, M. Oleacein May Inhibit Destabilization of Carotid Plaques from Hypertensive Patients. Impact on High Mobility Group Protein-1. *Phytomedicine* **2017**, *32*, 68–73, doi:10.1016/j.phymed.2017.06.004.

10. Hansen, K.; Adersen, A.; Christensen, S.B.; Jensen, S.R.; Nyman, U.; Smitt, U.W. Isolation of an Angiotensin Converting Enzyme (ACE) Inhibitor from *Olea Europaea* and *Olea Lancea*. *Phytomedicine* **1996**, *2*, 319–325, doi:10.1016/s0944-7113(96)80076-6. 418–420
11. Fabiani, R.; Rosignoli, P.; De Bartolomeo, A.; Fuccelli, R.; Servili, M.; Montedoro, G.F.; Morozzi, G. Oxidative DNA Damage Is Prevented by Extracts of Olive Oil, Hydroxytyrosol, and Other Olive Phenolic Compounds in Human Blood Mononuclear Cells and HL60 Cells. *J. Nutr.* **2008**, *138*, 1411–1416, doi:10.1093/jn/138.8.1411. 421–423
12. Polini, B.; Digiaco, M.; Carpi, S.; Bertini, S.; Gado, F.; Saccomanni, G.; Macchia, M.; Nieri, P.; Manera, C.; Fogli, S. Oleocanthal and Oleacein Contribute to the in Vitro Therapeutic Potential of Extra Virgin Oil-Derived Extracts in Non-Melanoma Skin Cancer. *Toxicol. In Vitro* **2018**, *52*, 243–250, doi:10.1016/j.tiv.2018.06.021. 424–426
13. Juli, G.; Oliverio, M.; Bellizzi, D.; Cantafio, M.E.G.; Grillone, K.; Passarino, G.; Colica, C.; Nardi, M.; Rossi, M.; Procopio, A.; et al. Anti-Tumor Activity and Epigenetic Impact of the Polyphenol Oleacein in Multiple Myeloma. *Cancers* **2019**, *11*, 1–14, doi:10.3390/cancers11070990. 427–429
14. Nikou, T.; Liaki, V.; Stathopoulos, P.; Sklirou, A.D.; Tsakiri, E.N.; Jakschitz, T.; Bonn, G.; Trougakos, I.P.; Halabalaki, M.; Skaltsounis, L.A. Comparison Survey of EVOO Polyphenols and Exploration of Healthy Aging-Promoting Properties of Oleocanthal and Oleacein. *Food Chem. Toxicol.* **2019**, *125*, 403–412, doi:10.1016/j.fct.2019.01.016. 430–432
15. Carpi, S.; Scoditti, E.; Massaro, M.; Polini, B.; Manera, C.; Digiaco, M.; Salsano, J.E.; Poli, G.; Tuccinardi, T.; Doccini, S.; et al. The Extra-Virgin Olive Oil Polyphenols Oleocanthal and Oleacein Counteract Inflammation-Related Gene and Mirna Expression in Adipocytes by Attenuating Nf-Kb Activation. *Nutrients* **2019**, *11*, doi:10.3390/nu11122855. 433–435
16. Lepore, S.M.; Maggiasano, V.; Bulotta, S.; Mignogna, C.; Arcidiacono, B.; Procopio, A.; Brunetti, A.; Russo, D.; Celano, M. Oleacein Prevents High Fat Diet-Induced a Diposity and Ameliorates Some Biochemical Parameters of Insulin Sensitivity in Mice. *Nutrients* **2019**, *11*, 8–10, doi:10.3390/nu11081829. 436–438
17. López-Yerena, A.; Pérez, M.; Vallverdú-Queralt, A.; Miliarakis, E.; Lamuela-Raventós, R.M.; Escribano-Ferrer, E. Oleacein Intestinal Permeation and Metabolism in Rats Using an In Situ Perfusion Technique. *Pharmaceutics* **2021**, *13*, 719. 439–440
18. Pinto, J.; Paiva-Martins, F.; Corona, G.; Debnam, E.S.; Jose Oruna-Concha, M.; Vauzour, D.; Gordon, M.H.; Spencer, J.P.E. Absorption and Metabolism of Olive Oil Secoiridoids in the Small Intestine. *Br. J. Nutr.* **2011**, *105*, 1607–1618, doi:10.1017/S000711451000526X. 441–443
19. López de las Hazas, M.C.; Piñol, C.; Macià, A.; Romero, M.P.; Pedret, A.; Solà, R.; Rubió, L.; Motilva, M.J. Differential Absorption and Metabolism of Hydroxytyrosol and Its Precursors Oleuropein and Secoiridoids. *J. Funct. Foods* **2016**, *22*, 52–63, doi:10.1016/j.jff.2016.01.030. 444–446
20. Serra, A.; Rubió, L.; Borràs, X.; Macià, A.; Romero, M.P.; Motilva, M.J. Distribution of Olive Oil Phenolic Compounds in Rat Tissues after Administration of a Phenolic Extract from Olive Cake. *Mol. Nutr. Food Res.* **2012**, *56*, 486–496, doi:10.1002/mnfr.201100436. 447–449
21. López-Yerena, A.; Vallverdú-Queralt, A.; Jáuregui, O.; Garcia-Sala, X.; Lamuela-Raventós, R.M.; Escribano-Ferrer, E. Tissue Distribution of Oleocanthal and Its Metabolites after Oral Ingestion in Rats. *Antioxidants* **2021**, *10*, 688, doi:10.3390/antiox10050688. 450–452
22. Lozano-Castellón, J.; López-Yerena, A.; Olmo-Cunillera, A.; Jáuregui, O.; Pérez, M.; Lamuela-Raventós, R.M.; Vallverdú-Queralt, A. Total Analysis of the Major Secoiridoids in Extra Virgin Olive Oil: Validation of an UHPLC-ESI-MS/MS Method. *Antioxidants* **2021**, *10*, 540, doi:10.3390/antiox10040540. 453–455
23. Panel, E.; Nda, A. Scientific Opinion on the Substantiation of Health Claims Related to Polyphenols in Olive and Protection of LDL Particles from Oxidative Damage (ID 1333, 1638, 1639, 1696, 2865), Maintenance of Normal Blood HDL Cholesterol Concentrations (ID 1639), Mainte. *EFSA J.* **2011**, *9*, 1–25, doi:10.2903/j.efsa.2011.2033. 456–458

24. Diehl, K.-H.; Hull, R.; Morton, D.; Pfister, R.; Rabemampianina, Y.; Smith, D.; Vidal, J.-M.; Vorstenbosch, C.V.D. A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes. *J. Appl. Toxicol.* **2001**, *21*, 15–23, doi:10.1002/jat.727. 459–461
25. Brown, A.P.; Dinger, N.; Levine, B.S. Stress Produced by Gavage Administration in the Rat. *Contemp. Top. Lab. Anim. Sci.* **2000**, *39*, 17–21. 462–463
26. Serreli, G.; Deiana, M. Biological Relevance of Extra Virgin Olive Oil Polyphenols Metabolites. *Antioxidants* **2018**, *7*, 170, doi:10.3390/antiox7120170. 464–465
27. Manna, C.; Galletti, P.; Maisto, G.; Cucciolla, V.; D'Angelo, S.; Zappia, V. Transport Mechanism and Metabolism of Olive Oil Hydroxytyrosol in Caco-2 Cells. *FEBS Lett.* **2000**, *470*, 341–344, doi:10.1016/S0014-5793(00)01350-8. 466–467
28. EFSA Panel on Dietetic Products, N. and A. (NDA) Scientific Opinion on the Substantiation of Health Claims Related to Polyphenols in Olive and Protection of LDL Particles from Oxidative Damage (ID 1333, 1638, 1639, 1696, 2865), Maintenance of Normal Blood HDL Cholesterol Concentrations (ID 1639), Maintenance of Normal Blood Pressure (ID 3781), “Anti-inflammatory Properties” (ID 1882), “Contributes to the Upper Respiratory Tract Health” (ID 3468), “Can Help to Maintain a Normal Function of Gastrointestinal Tract” (3779), and “Contributes to Body Defences against External Agents” (ID 3467) Pursuant to Article 13 (1) of Regulation (EC) No 1924/2006. *EFSA J.* **2011**, *9*, 2033. 468–473
29. Commission Regulation (EU) 432/2012 of 16 May 2012 Establishing a List of Permitted Health Claims Made on Foods, Other than Those Referring to the Reduction of Disease Risk and to Children's Development and Health. *O.J.* **2012**, *L136/1*.; 474–475
30. García-Villalba, R.; Carrasco-Pancorbo, A.; Nevedomskaya, E.; Mayboroda, O.A.; Deelder, A.M.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Exploratory Analysis of Human Urine by LC-ESI-TOF MS after High Intake of Olive Oil: Understanding the Metabolism of Polyphenols. *Anal. Bioanal. Chem.* **2010**, *398*, 463–475, doi:10.1007/s00216-010-3899-x. 476–478
31. Jin, Y.; Penning, T.M. Aldo-Keto Reductases and Bioactivation/Detoxication. *Annu. Rev. Pharmacol. Toxicol.* **2007**, *47*, 263–292, doi:10.1146/annurev.pharmtox.47.120505.105337. 479–480
32. Iyanagi, T. Molecular Mechanism of Phase I and Phase II Drug - Metabolizing Enzymes : Implications for Detoxification. *Int. Rev. Cytol.* **2007**, *260*, 35–112, doi:10.1016/S0074-7696(06)60002-8. 481–482
33. Silva, S.; Garcia-Aloy, M.; Figueira, M.E.; Combet, E.; Mullen, W.; Bronze, M.R. High Resolution Mass Spectrometric Analysis of Secoiridoids and Metabolites as Biomarkers of Acute Olive Oil Intake-An Approach to Study Interindividual Variability in Humans. *Mol. Nutr. Food Res.* **2018**, *62*, 1700065, doi:10.1002/mnfr.201700065. 483–485
34. Karhunen, T.; Tilgmann, C.; Ulmanen, I.S.M.; Julkunen, I. Distribution of Catechol-O-Methyltransferase Enzyme in Rat Tissues'. **1994**, *42*, 1079–1090. 486–487
35. Rowland, A.; Miners, J.O.; Mackenzie, P.I. The UDP-Glucuronosyltransferases: Their Role in Drug Metabolism and Detoxification. *Int. J. Biochem. Cell Biol.* **2013**, *45*, 1121–1132, doi:10.1016/j.biocel.2013.02.019. 488–489
36. Pereira De Sousa, I.; Bernkop-Schnürch, A. Pre-Systemic Metabolism of Orally Administered Drugs and Strategies to Overcome It. *J. Controlled Release* **2014**, *192*, 301–309, doi:10.1016/j.jconrel.2014.08.004. 490–491
37. Rubió, L.; Serra, A.; Macià, A.; Piñol, C.; Romero, M.-P.; Motilva, M.-J. In Vivo Distribution and Deconjugation of Hydroxytyrosol Phase II Metabolites in Red Blood Cells: A Potential New Target for Hydroxytyrosol. *J. Funct. Foods* **2014**, *10*, 139–143, doi:10.1016/j.jff.2014.06.001. 492–494
38. López-Yerena, A.; Domínguez-López, I.; Vallverdú-Queralt, A.; Pérez, M.; Jáuregui, O.; Escribano-Ferrer, E.; Lamuela-Raventós, R.M. Metabolomics Technologies for the Identification and Quantification of Dietary Phenolic Compound Metabolites: An Overview. *Antioxidants* **2021**, *10*, 846. 495–497
39. Chan, J.M. Drug Metabolism and Pharmacogenetics. In *Pharmacology and Physiology for Anesthesia*; Elsevier, 2019; pp. 70–90 ISBN 978-0-323-48110-6. 498–499

40. Yilmaz, Y.; Williams, G.; Walles, M.; Manevski, N.; Krähenbühl, S.; Camenisch, G. Comparison of Rat and Human Pulmonary Metabolism Using Precision-Cut Lung Slices (PCLS). *Drug Metab. Lett.* **2019**, *13*, 53–63, doi:10.2174/1872312812666181022114622. 500
41. de las Hazas, M.-C.L.; Godinho-Pereira, J.; Macià, A.; Almeida, A.F.; Ventura, M.R.; Motilva, M.-J.; Santos, C.N. Brain Uptake of Hydroxytyrosol and Its Main Circulating Metabolites: Protective Potential in Neuronal Cells. *J. Funct. Foods* **2018**, *46*, 110–117. 502
42. Gutiérrez-Miranda, B.; Gallardo, I.; Melliou, E.; Cabero, I.; Álvarez, Y.; Magiatis, P.; Hernández, M.; Nieto, M.L. Oleacein Attenuates the Pathogenesis of Experimental Autoimmune Encephalomyelitis through Both Antioxidant and Anti-Inflammatory Effects. **2020**, *22*. 504
43. Miners, J.; Yang, X.; Knights, K.; Zhang, L. The Role of the Kidney in Drug Elimination: Transport, Metabolism, and the Impact of Kidney Disease on Drug Clearance. *Clin. Pharmacol. Ther.* **2017**, *102*, 436–449, doi:10.1002/cpt.757. 506
44. Rowland, M.; Tozer, T.N. *Clinical Pharmacokinetics and Pharmacodynamics: Concepts and Applications*; 4th ed.; Wolters Kluwer Health/Lippincott William & Wilkins: Philadelphia, Pa., 2019; ISBN 978-0-7817-5009-7. 508

GLOBAL DISCUSSION

5. GLOBAL DISCUSSION

The beneficial effects of a Mediterranean diet on human health in reducing the risk of cardiovascular disease have been attributed mainly to its high content in EVOO. Although oleic acid is considered important for these effects, other biological properties depend on or are enhanced by other minor components ^[8] such as PCs.

Between PCs presents in EVOO, the SECs are a family that have caught the attention of the scientific community for their protective role in diseases with an important pathogenic contribution of oxidative and peroxidative damage ^[17,44]. In this group, OLC and OLEA are by far the most studied compounds focusing on their plausible mechanisms of the action involved.

The concentration of PCs in EVOO is affected by a very complex multivariate interaction between agronomic, biochemical, and technological factors. With respect to the first factor, the genetic origin of the fruits, the geographical area of cultivation, the climate, and agronomic practices are determining factors in the composition of the olive fruit ^[93,94]. Regarding to technological factors, the regulation of the main variables (temperature, oxygen, enzymatic activities and the disruption level of olive cell tissues), during the mechanical extraction of EVOO, has a impact on parameters associated with quality ^[95,96]. The crushing is a determinant factor in the release and activation of endogenous enzymes (β -glycosidases and oxidoreductases) and the malaxation in the modulation of the enzymatic activity ^[20,96–98]. Given the health-promoting properties both OLC and OLEA, the scientific community and olive oil industry have focused their efforts in optimizing EVOO extraction maintaining the balance between oil yield and oil quality.

The biological activities of SECs not only depend on the initial concentration of these compounds in the oil, but the health benefits of ingesting SEC are greatly dependent on their bioaccessibility and bioavailability in the digestive tract and circulatory system. The bioavailability is a multifactorial consequence that includes gastrointestinal digestion, absorption, tissue distribution and elimination, either by metabolism and/or by excretion. The absorption, distribution and elimination of dietary phytochemicals depend on their intestinal permeability and the influence of pre-systemic enzymes and/or transporters ^[99].

5.1. Phenolic compounds: towards obtaining olive oil with higher nutritional value

To date, several studies have shown that the phenolic composition of EVOO depends on a very complex multivariate interaction between agronomic, biochemical, and technological factors ^[93]. The Article 3 (Antioxidants, 9 (10), 1009, 2020), summarizes the work carried out, in which one of the objectives was the characterization of the phenolic profile in the fruits with unique characteristics obtained from ivory-white wild olive trees of MI. The exploration of the natural habitat of the MI was to evaluate the native oleaster olive fruit as a resource to maintain olive oil

biodiversity in the Mediterranean basin. The mentioned study is part of an ongoing project related with the valorization of local cultivated and wild olive genetic resources in Catalonia (northeastern Spain). It is important to note that, like other commercial varieties, SECs were the main PCs family detected in the EVOO of this cultivar. The OLEA and OLC concentration represented the 56 al 31% of total polyphenols, respectively. High concentration of these compounds was expected since they are responsible for the bitter and pungent test. In addition, very high SECs contents are interesting from the health point of view, due to its anti-inflammatory activity that can be relevant in many pathologies. Physicochemical characterization of these fruits and olive oil revealed unique sensory physicochemical and stability characteristics, highlighting the need to maintain the biodiversity of olive oil and food authenticity of this traditional Mediterranean food.

The growing system appears another very interesting agronomical factor that affects the PCs. Organic agriculture is associated with a natural increase in the amount of defense substances, as the plant is exposed to greater stress in the absence of synthetic pesticides. In this sense, the aim of the Article 4 (Molecules, 24 (10), 1986, 2019) was to compare the content of PCs in 'Hojiblanca' EVOO produced by organic and conventional production systems under the same environmental conditions. The results demonstrated that total polyphenols content was significantly higher in oil obtained from organic agriculture ($p < 0.05$). The SECs, synthesized through the secologanin pathway without the need for nitrogen or phosphorus, represented 91–92% of the PCs and were higher in oils from olives cultivated under organic conditions. In this group, OLC is included, which was satisfactorily analyzed by LC-MS/MS, as demonstrated by results obtained by qNMR. In contrast, the concentrations of lignans and phenolic acids were higher under the conventional system, as their synthesis is through the phenylpropanoid pathway via shikimic acid, which requires nitrogen and phosphorus. The cultivation method appears to have no effect on the concentration of the case of phenolic alcohols and flavones. When the effect of the ripening stage of the olive fruit was assessed, both the total polyphenols and SECs concentrations were found to decrease with maturation in both production systems, whereas the flavone content increased. Olive maturation was also associated with a decline in certain compounds: lignans in organic EVOO and phenolic acids and phenolic alcohols in conventional samples. Additional information taking in account the effect of seasonality is required. It is also necessary to corroborate the potentially greater beneficial effects of organic food on human health compared to those produced conventionally through randomized, controlled dietary intervention trials. However, organic food may be recommended, not only for its health benefits, but also because its production has less of an environmental impact.

As mentioned above, the technological factors also affect the phenolic composition in EVOO. In the Article 5 (Antioxidants, 10 (6), 877, 2021), it was evaluated the effect of ripening indices and

crushing temperatures on phenolic profile. Malaxation parameters (time and temperature) and crushing size content was also evaluated with the aim of obtaining an EVOO with higher PCs content. The study was carried out with olives from the 'Corbella' cultivar, native to Bages in the center-north of Catalonia, an ancient variety that was abandoned and at risk of extinction. The total phenol content in the EVOO decreased with fruit maturity. These results are in line with those obtained in the previous publication.

The concentration of some compounds (flavones, hydroxyoleuropein aglycone, hydroxytyrosol acetate, and lignans) was favoured by increasing the fruit maturity. A negative correlation was found between the total phenols and higher crushing temperatures, although individual PCs such as OLC and OLEA increased. In summary, to obtain a 'Corbella' EVOO rich in PCs, an early harvest and low crushing temperature should be employed and to obtain higher concentration of OLC and OLEA a moderate crushing temperature should be used. Regarding malaxation conditions, the results suggest that a higher temperature (37 °C) increased the total phenol content and the concentration of the most relevant phenols. A short malaxation time (30 min) enhanced the total phenols and oleuropein and ligstroside aglycone, whereas a longer process (60 min) increased OLC concentration. Finally, a clear trend was not observed with respect to crushing size. Based on these results, future research could be focused on studying the correlation between the enzymatic activity and ripening index or how the malaxation temperature affects olives collected at different times.

5.2. Absorption and metabolism of phenolic compounds assessed by intestinal perfusion method

EVOO is an interesting source of bioactive compounds that might contribute synergistically or complementarily to the health benefits associated to its consumption. Despite the importance of understanding the bioavailability of PCs in relation to the concentration and chemical structure of circulating bioactive metabolites and their contribution to the health effects of EVOO consumption, there is very little information on their absorption and metabolism, aspects that condition the bioavailability. For this reason, one of our objectives was to study the intestinal absorption and metabolism of OLC (Article 6, *Pharmaceutics*, 12 (2), 134, 2020) and OLEA (Article 7, *Pharmaceutics*, 13(5), 719, 2021) using an IPVS in rats. As a reference, the highly permeable drug levofloxacin (for OLC) and naproxen (for OLEA) was included in the study. In addition, an intestinal metabolic profile of SECs was also determined.

Regarding to OLC, this is the first *in vivo* study in rats to simultaneously assess its absorption and intestinal metabolism, and the results demonstrated that OLC has an incomplete oral bioavailability due to its relatively low absorption (16%) and high intestinal metabolism. OLC poor absorption was indicated by the low effective permeability coefficient, apparent permeability

coefficient and area under the mesenteric blood–time curve normalized by the inlet concentration in comparison with the reference standard compound (levofloxacin). OLC was mostly metabolized by phase I metabolism, undergoing hydration, hydrogenation and hydroxylation. Phase II reactions (glucuronidation of hydrogenated OLC and hydrated metabolites) were observed in plasma samples. Given the biological relevance of OLC on human health and its demonstrated moderated ability to be transported through the intestinal membrane, the development of dietary supplements containing OLC and excipients that favour their immediate release and absorption in the gastrointestinal tract should be considered, especially for populations with low intake of EVOO.

Respect to OLEA, the range and abundance of metabolites found in the perfusate and plasma suggest that its oral bioavailability is incomplete. The results indicate that the small intestine plays an important role in the bioavailability of OLEA, considering its high intestinal permeability ($48.98 \pm 12.27\%$) and the metabolic reactions that contribute to its elimination. The metabolites arising from hydrolysis (hydroxytyrosol) and hydroxylation were the main circulating metabolites of OLEA detected in plasma and the lumen. The higher metabolite levels in plasma suggests that the intestinal metabolism of OLEA occurs mainly during the transport of the compound across the intestinal membrane.

5.3. Evidence of tissue distribution of olive oil phenols in rats

The distribution and accumulation of PCs of olive oil are still not well understood. However, the relationship between the ingestion of EVOO and its beneficial effect could be explained in how the parent compounds and its metabolites are distributed in target tissues, beyond just evaluating plasma and urine ^[65,100]. To provide a new perspective for understanding the mechanism through EVOO PCs acts in the body, the present work aims to study the distribution of OLC (Article 8, Antioxidants, 10(5), 688, 2021) and OLEA (Article 9, Antioxidants under review) and its derived metabolites in rat plasma and organs/tissues. The distribution was investigated with LC-ESI-LTQ-Orbitrap-MS system after the oral administration of OLC or OLEA-enriched refined olive oil.

In the case of OLC, our results demonstrated that under gastric conditions, it was hydrolyzed to tyrosol, whereas the non-hydrolyzed OLC underwent different phase I biotransformations in the stomach and small intestine. It is important to note that the OLC was not able to reach the systemic circulation or target tissues where it could exert a protective effect. This result is consistent with the previous absorption study, which showed low intestinal absorption and high pre-systemic metabolism. Add to this the passage of OLC through the aggressive stomach pH conditions associated with oral administration, it is consistent with OLC not reaching the systemic circulation in an unchanged form. However, its metabolites were found in the lungs, heart, brain, thyroids,

spleen and skin in significant concentrations to potentially exert beneficial effects. The main circulating metabolites arising from phase II reactions were $OLC + OH + CH_3$ and $OLC + H_2O + glucu$, suggesting that these metabolites are likely to significantly contribute to the beneficial health effects associated with the regular consumption of olive products.

The information obtained for OLEA demonstrated that at gastric level, this SEC was hydrolyzed in stomach, obtaining hydroxytyrosol. Unlike OLC unmetabolized OLEA was detected in stomach, small intestine, liver, plasma and heart, and is consistent with the observed higher intestinal absorption. The identification of unmetabolized OLEA in heart was noteworthy. To date, this form appears to be the only biologically active form, and thus, it provides relevant information for optimizing the potential applications of OLEA to prevent certain cardiovascular diseases. These results could help us to understand why OLEA is active *in vivo*, even if its blood concentration is low. Also noteworthy are the elevated levels of OH-TY and $OLEA + CH_3$ found in the small intestine, liver and plasma. Furthermore, from the nine metabolites detected, five of them were due to conjugation reactions. Liver, heart, spleen and lungs were the target tissues where the metabolites were more distributed. In summary, the OLEA derived metabolites were more widely distributed in tissues and plasma after oral administration those OLEA.

The results obtained contribute to understand the health effects of OLC and OLEA in the organism through its wide distribution to organs and tissues, either of the metabolites (in the case of OLC), or of the parent compound and metabolites (in the case of OLEA). However, more studies are necessary to determine the concentrations and composition of OLC or OLEA metabolites in human plasma and tissues, as well as pharmacodynamic studies that relate concentrations vs. effects.

CONCLUSIONS

6. CONCLUSIONS

The main conclusions drawn from the stated objectives and the results obtained in the different studies that make up this doctoral thesis are set out below.

6.1. Phenolic compounds: towards obtaining olive oil with higher nutritional value

1. Physicochemical characterization of ivory-white olive fruits and olive oil revealed unique sensory physicochemical and stability characteristics, highlighting the need to maintain the biodiversity of olive oil and food authenticity of this traditional Mediterranean food.
2. The phenolic concentration and specially the content of SECs in EVOO from 'Hojiblanca' cultivar is favoured when the fruits are grown in organic systems vs. conventional production systems.
3. Regardless of cultivation system, the concentration of total PCs and SECs were found to decrease with maturation in EVOO from 'Hojiblanca' cultivar.
4. The total phenol content and SECs in the EVOO extracted from 'Corbella' olives decreased with fruit maturity.
5. In general, a negative correlation was found between the total phenols content and higher crushing temperatures. However, some PCs, in particular OLC and OLEA, increased. In summary, to obtain a 'Corbella' EVOO rich in PCs, low crushing temperature should be employed. On the contrary, if a higher concentration of specific phenols such as OLC or OLEA is desired, due to their health properties, a moderate crushing temperature should be used.
6. Higher malaxation temperature (37°C) increased the total phenol content and the concentration of the most relevant phenols. A short malaxation time (30 min) enhanced the total phenols and oleuropein and ligstroside aglycone, whereas a longer process (60 min) increased OLC concentration. On the other hand, no clear trend was observed with regard to crushing size.

6.2. Absorption and metabolism of phenolic compounds assessed by intestinal perfusion method

1. A moderate-to-low oral absorption of OLC was observed due to a low effective permeability coefficient (P_{eff}), low apparent permeability coefficient (P_{app}) and low area under the mesenteric blood-time curve (AUC) normalized by the inlet concentration in comparison with the reference standard compound highly permeable levofloxacin. As OLC show favourable physicochemical properties for absorption through the intestinal membrane, it is suggested OLC as a substrate of an efflux transporter. More studies are needed to confirm this hypothesis.

2. A high number of OLC-derived metabolites were detected and quantified in plasma and in the intestinal lumen, indicating a high intestinal first-pass effect. Both low permeability and high pre-systemic metabolism contribute to its incomplete bioavailability.
3. OLEA showed high intestinal permeability, as its intestinal permeability coefficient (Peff) and apparent permeability coefficient (Papp) were not statistically different from the standard compound, naproxen.
4. Despite its high intestinal permeability, OLEA show incomplete oral bioavailability in rats due to the range and abundance of metabolites found in the perfusate and plasma samples.
5. OLC was mostly metabolized by phase I hydration reactions and in the case of OLEA, metabolites arising from hydrolysis (tyrosol) and hydroxylation (OLC + OH) were the main circulating metabolites detected in plasma and the lumen.

6.3. Evidence of tissue distribution of olive oil phenols in rats

1. Under gastric conditions, OLC was hydrolyzed to tyrosol and OLEA to hydroxytyrosol.
2. OLC did not reach the systemic circulation and was only detected in stomach and small intestine. This information confirms its moderate-to-low bioavailability attributed to its low absorption and high metabolism.
3. Although OLC was not detected in the tissues, its metabolites were found in the lungs, heart, brain, thyroids, spleen and skin in significant concentrations to potentially exert beneficial effects. The main circulating metabolites arising from phase II reactions were $\text{OLC} + \text{OH} + \text{CH}_3$ and $\text{OLC} + \text{H}_2\text{O} + \text{glucu}$, suggesting that these metabolites are likely to significantly contribute to the beneficial health effects associated with the regular consumption of olive products.
4. OLEA was detected in the small intestine, liver, plasma, and most interestingly, the heart. This finding may be useful for the development of new applications of OLEA to prevent cardiovascular diseases. The results may also shed light on why OLEA is active *in vivo*, even if its blood concentration is low.
5. The liver, heart, spleen, and lungs were the target tissues for OLEA metabolites because were the organs with the highest levels.
6. Despite OLEA was detected in different organs after the oral administration, its metabolites were more widely distributed in tissues and plasma than the parent compound.

REFERENCES

7. REFERENCES

- [1] I. Julca, M. Marcet-Houben, F. Cruz, J. Gómez-Garrido, B. S. Gaut, C. M. Díez, I. G. Gut, T. S. Alioto, P. Vargas, T. Gabaldón, *BioRxiv* **2020**, DOI 10.1007/s40278-013-5396-4.
- [2] P. S. Green, *KEW Bulletin* **2002**, 57, 91–140.
- [3] V. Uylaşer, G. Yildiz, **2014**, 8398, DOI 10.1080/10408398.2011.626874.
- [4] International Olive Council, “Designations and definitions of olive oils,” available at <https://www.internationaloliveoil.org/olive-world/olive-oil/>, **n.d.**
- [5] R. Estruch, E. Ros, J. S. Salvadó, M. Covas, D. Corella, F. Arós, E. G. Gracia, V. R. Gutiérrez, M. Fiol, J. Lapetra, R. M. L. Raventos, L. S. Majem, X. Pintó, J. Basora, M. A. Muñoz, J. V. Sorlí, J. A. Martínez, M. Fitó, *New England Journal of Medicine* **2018**, 34, 1–14.
- [6] J. F. García-Gavilán, M. Bulló, S. Canudas, M. A. Martínez-González, R. Estruch, S. Giardina, M. Fitó, D. Corella, E. Ros, J. Salas-Salvadó, *Clinical Nutrition* **2018**, 37, 329.335.
- [7] C. Jimenez-Lopez, M. Carpena, C. Lourenço-Lopes, M. Gallardo-Gomez, J. M. Lorenzo, F. J. Barba, M. A. Prieto, J. Simal-Gandara, *Foods* **2020**, 9, 1014.
- [8] E. M. Yubero-Serrano, J. Lopez-Moreno, F. Gomez-Delgado, J. Lopez-Miranda, *European Journal of Clinical Nutrition* **2019**, 8–17.
- [9] M. Servili, B. Sordini, S. Esposto, S. Urbani, G. Veneziani, I. Di Maio, R. Selvaggini, A. Taticchi, *Antioxidants* **2013**, 3, 1–23.
- [10] A. García, E. Rodríguez-Juan, G. Rodríguez-Gutiérrez, J. J. Rios, J. Fernández-Bolaños, *Food Chemistry* **2016**, 197, 554–561.
- [11] A. López-Yerena, J. Lozano-Castellón, A. Olmo-Cunillera, A. Tresserra-Rimbau, P. Quifer-Rada, B. Jiménez, M. Pérez, A. Vallverdú-Queralt, *Molecules* **2019**, 24, DOI 10.3390/molecules24101986_rfseq1.
- [12] A. López-Yerena, A. Ninot, J. Lozano-Castellón, E. Escribano-Ferrer, A. J. Romero-Aroca, A. Belaj, A. Vallverdú-Queralt, R. M. Lamuela-Raventós, *Antioxidants* **2020**, 9, 1–13.
- [13] R. Celano, A. L. Piccinelli, A. Pugliese, S. Carabetta, R. Di Sanzo, L. Rastrelli, M. Russo, *Journal of Agricultural and Food Chemistry* **2018**, 66, 6053–6063.
- [14] J. Lozano-Castellón, A. López-Yerena, A. Olmo-Cunillera, O. Jáuregui, M. Pérez, R. M. Lamuela-Raventós, A. Vallverdú-Queralt, *Antioxidants* **2021**, 10, 1–13.
- [15] M. Baldioli, M. Servili, G. Perretti, G. F. Montedoro, *Journal of the American Oil Chemists' Society* **1996**, 73, 1589–1593.
- [16] J. Lozano-Castellón, A. López-Yerena, J. F. Rinaldi de Alvarenga, J. Romero del Castillo-Alba, A. Vallverdú-Queralt, E. Escribano-Ferrer, R. M. Lamuela-Raventós, *Critical Reviews in Food Science and Nutrition* **2019**, 60, 2532–2548.
- [17] M. L. Castejón, T. Montoya, C. Alarcón-de-la-lastra, M. Sánchez-hidalgo, *Antioxidants* **2020**, 9, DOI 10.3390/antiox9020149.
- [18] B. Jiménez, A. Sánchez-Ortiz, A. Rivas, *International Journal of Food Science and Technology* **2014**, 49, 2521–2527.

-
- [19] M. Pérez, A. López-yerena, J. Lozano-castellón, A. Olmo-cunillera, R. M. Lamuela-raventós, O. Martin-belloso, A. Vallverdú-queralt, *Antioxidants* **2021**, *10*, 1–19.
 - [20] M. L. Clodoveo, *Trends in Food Science and Technology* **2012**, *25*, 13–23.
 - [21] P. Diamantakos, T. Giannara, M. Skarkou, E. Melliou, P. Magiatis, *Molecules* **2020**, *25*, DOI 10.3390/MOLECULES25102449.
 - [22] H. Miho, J. Moral, D. Barranco, C. A. Ledesma-Escobar, F. Priego-Capote, C. M. Díez, *Food Chemistry* **2021**, *342*, 128357.
 - [23] H. Miho, C. M. Díez, A. Mena-Bravo, V. Sánchez de Medina, J. Moral, E. Melliou, P. Magiatis, L. Rallo, D. Barranco, F. Priego-Capote, *Food Chemistry* **2018**, *266*, 192–199.
 - [24] K. B. Bubola, M. Lukić, I. Mofardin, A. Butumović, O. Koprivnjak, *LWT* **2017**, *84*, 370–377.
 - [25] M. L. Castejón, T. Montoya, C. Alarcón, D. Lastra, **2020**, DOI 10.3390/antiox9020149.
 - [26] T. Nikou, V. Liaki, P. Stathopoulos, A. D. Sklirou, E. N. Tsakiri, T. Jakschitz, G. Bonn, I. P. Trougakos, M. Halabalaki, L. A. Skaltsounis, *Food and Chemical Toxicology* **2019**, *125*, 403–412.
 - [27] S. Cicerale, L. Lucas, R. Keast, *Current Opinion in Biotechnology* **2012**, *23*, 129–135.
 - [28] G. K. Beauchamp, R. S. J. Keast, D. Morel, J. Lin, J. Pika, Q. Han, C.-H. Lee, A. B. Smith, P. A. S. Breslin, *Nature* **2005**, *437*, 45–46.
 - [29] O. LeGendre, P. A. Breslin, D. A. Foster, *Molecular & Cellular Oncology* **2015**, *2*, e1006077.
 - [30] M. A. Khanfar, S. K. Bardaweel, M. R. Akl, K. A. El Sayed, *Phytotherapy Research* **2015**, *29*, 1776–1782.
 - [31] A. B. Siddique, H. Y. Ebrahim, M. R. Akl, N. M. Ayoub, A. A. Goda, M. M. Mohyeldin, S. K. Nagumalli, W. M. Hananeh, Y.-Y. Liu, S. A. Meyer, *Nutrients* **2019**, *11*, 412.
 - [32] H. Qosa, Y. S. Batarseh, M. M. Mohyeldin, K. A. El Sayed, J. N. Keller, A. Kaddoumi, *ACS Chemical Neuroscience* **2015**, *6*, 1849–1859.
 - [33] M. C. Monti, L. Margarucci, R. Riccio, A. Casapullo, *Journal of Natural Products* **2012**, *75*, 1584–1588.
 - [34] M. Czerwińska, A. K. Kiss, M. Naruszewicz, *Food Chemistry* **2012**, *131*, 940–947.
 - [35] M. Servili, S. Esposto, R. Fabiani, S. Urbani, A. Taticchi, F. Mariucci, R. Selvaggini, G. F. Montedoro, *Inflammopharmacology* **2009**, *17*, 76–84.
 - [36] S. Carpi, E. Scoditti, M. Massaro, B. Polini, C. Manera, M. Digiacomo, J. E. Salsano, G. Poli, T. Tuccinardi, S. Doccini, F. M. Santorelli, M. A. Carluccio, M. Macchia, M. Wabitsch, R. De Caterina, P. Nieri, *Nutrients* **2019**, *11*, DOI 10.3390/nu11122855.
 - [37] M. Naruszewicz, M. Czerwinska, A. Kiss, *Current Pharmaceutical Design* **2014**, *21*, 1205–1212.
 - [38] A. Filipek, M. E. Czerwińska, A. K. Kiss, M. Wrzosek, M. Naruszewicz, *Phytomedicine* **2015**, *22*, 1255–1261.
 - [39] M. Naruszewicz, M. E. Czerwiska, A. K. Kiss, *Current Pharmaceutical Design* **2015**, *21*, 1–8.
 - [40] A. Filipek, M. E. Czerwińska, A. K. Kiss, J. A. Polański, M. Naruszewicz,

-
- Phytomedicine* **2017**, *32*, 68–73.
- [41] K. Hansen, A. Adersen, S. B. Christensen, S. R. Jensen, U. Nyman, U. W. Smitt, *Phytomedicine* **1996**, *2*, 319–325.
 - [42] L. Marconato, A. Facchinetti, C. Zanardello, E. Rossi, R. Vidotto, K. Capello, E. Melchioti, P. Laganga, R. Zamarchi, M. Vascellari, *Cancers* **2019**, *11*, 163.
 - [43] S. M. Lepore, V. Maggisano, S. Bulotta, C. Mignogna, B. Arcidiacono, A. Procopio, A. Brunetti, D. Russo, M. Celano, *Nutrients* **2019**, *11*, 8–10.
 - [44] J. Lozano-Castellón, A. López-Yerena, J. F. Rinaldi de Alvarenga, J. R. Castillo-Alba, A. Vallverdú-Queralt, E. Escribano-Ferrer, R. M. Lamuela-Raventós, *Critical Reviews in Food Science and Nutrition* **2019**, *0*, 1–17.
 - [45] E. P. Gutiérrez-Grijalva, D. L. Ambriz-Pérez, N. Leyva-López, R. I. Castillo-López, J. B. Heredia, *Revista Espanola de Nutricion Humana y Dietetica* **2016**, *20*, 140–147.
 - [46] M. J. Rein, M. Renouf, C. Cruz-hernandez, L. Actis-goretta, S. K. Thakkar, S. Pinto, *British Journal of Clinical Pharmacology* **2012**, *75*, 588–602.
 - [47] H. Singh, A. Ye, D. Horne, *Progress in Lipid Research* **2009**, *48*, 92–100.
 - [48] A. Quintero-Flórez, G. Pereira-Caro, C. Sánchez-Quezada, J. M. Moreno-Rojas, J. J. Gaforio, A. Jimenez, G. Beltrán, *European Journal of Nutrition* **2017**, *1475*, 1–22.
 - [49] A. Soler, M. P. Romero, A. Macià, S. Saha, C. S. M. Furniss, P. A. Kroon, M. J. Motilva, *Food Chemistry* **2010**, *119*, 703–714.
 - [50] P. A. Billat, E. Roger, S. Faure, F. Lagarce, *Drug Discovery Today* **2017**, *22*, 761–775.
 - [51] L. Zhu, L. Lu, S. Wang, J. Wu, J. Shi, T. Yan, C. Xie, Q. Li, M. Hu, Z. Liu, *Oral Absorption Basics: Pathways and Physicochemical and Biological Factors Affecting Absorption*, **2017**.
 - [52] E. Escribano, X. García, J. Salamanca, C. Roig, J. Queralt, *International Journal of Pharmaceutics* **2012**, *436*, 472–477.
 - [53] T. Chmiel, A. Mieszkowska, D. Kempńska-Kupczyk, A. Kot-Wasik, J. Namieśnik, Z. Mazerska, *Microchemical Journal* **2019**, *146*, 393–406.
 - [54] G. R. Velderrain-Rodríguez, H. Palafox-Carlos, A. Wall-Medrano, J. F. Ayala-Zavala, C. Y. O. Chen, M. Robles-Sánchez, H. Astiazaran-García, E. Alvarez-Parrilla, G. A. González-Aguilar, *Food & function* **2014**, *5*, 189–197.
 - [55] G. Lemmens, A. Van Camp, S. Kourula, T. Vanuytsel, P. Augustijns, *Pharmaceutics* **2021**, *13*, 161.
 - [56] V. Vasiliou, K. Vasiliou, D. W. Nebert, *Human genomics* **2009**, *3*, 1–10.
 - [57] D. Dahlgren, H. Lennernäs, *Pharmaceutics* **2019**, *11*, DOI 10.3390/pharmaceutics11080411.
 - [58] Z. Luo, Y. Liu, B. Zhao, M. Tang, H. Dong, L. Zhang, B. Lv, L. Wei, *Journal of pharmacological and toxicological methods* **2013**, *68*, 208–216.
 - [59] B. Griffin, C. O. Driscoll, **2007**, 34–76.
 - [60] D. Dahlgren, C. Roos, K. Peters, A. Lundqvist, C. Tannergren, E. Sjögren, M. Sjöblom, H. Lennernäs, *European Journal of Pharmaceutics and Biopharmaceutics* **2019**, *142*, 31–37.
 - [61] D. Dahlgren, C. Roos, A. Lundqvist, C. Tannergren, P. Langguth, M. Sjöblom, E.

- Sjögren, H. Lennernäs, *Molecular pharmaceutics* **2017**, *14*, 4243–4251.
- [62] J. Pinto, F. Paiva-Martins, G. Corona, E. S. Debnam, M. Jose Oruna-Concha, D. Vauzour, M. H. Gordon, J. P. E. Spencer, *British Journal of Nutrition* **2011**, *105*, 1607–1618.
- [63] K. Radić, B. J. Dukovski, D. V. Čepo, *Nutrients* **2020**, *12*, DOI 10.3390/nu12030669.
- [64] S. C. Edgecombe, G. L. Stretch, P. J. Hayball, *Journal of Nutrition* **2000**, *130*, 2996–3002.
- [65] A. Serra, L. Rubió, X. Borràs, A. Macià, M. P. Romero, M. J. Motilva, *Molecular Nutrition and Food Research* **2012**, *56*, 486–496.
- [66] G. Singh, *Preclinical Drug Development*, Elsevier Inc. **2017**.
- [67] S. D'Angelo, C. Manna, V. Migliardi, O. Mazzoni, P. Morrica, G. Capasso, G. Pontoni, P. Galletti, V. Zappia, *Drug Metabolism and Disposition* **2001**, *29*, 1492–1498.
- [68] A. K. Shakya, B. O. Al-Najjar, P. K. Deb, R. R. Naik, R. K. Tekade, *First-Pass Metabolism Considerations in Pharmaceutical Product Development*, Elsevier Inc. **2018**.
- [69] J. M. Chan, *Drug Metabolism and Pharmacogenetics*, Elsevier Inc. **2019**.
- [70] Q. Mao, Y. Lai, J. Wang, *Drug Metabolism and Disposition* **2018**, *46*, 561–566.
- [71] C. Xu, C. Y.-T. Li, A.-N. T. Kong, *Archives of pharmacal research* **2005**, *28*, 249–68.
- [72] M. S. Benedetti, R. Whomsley, E. Baltes, *Expert Opinion on Drug Metabolism and Toxicology* **2006**, *2*, 895–921.
- [73] K. Thelen, J. B. Dressman, **2009**, 541–558.
- [74] L. Rubió, A. Macià, R. M. Valls, A. Pedret, M.-P. Romero, R. Solà, M.-J. Motilva, *Food Chemistry* **2012**, *134*, 1132–1136.
- [75] R. García-Villalba, A. Carrasco-Pancorbo, E. Nevedomskaya, O. A. Mayboroda, A. M. Deelder, A. Segura-Carretero, A. Fernández-Gutiérrez, *Analytical and Bioanalytical Chemistry* **2010**, *398*, 463–475.
- [76] S. Silva, M. Garcia-Aloy, M. E. Figueira, E. Combet, W. Mullen, M. R. Bronze, *Molecular Nutrition & Food Research* **2018**, *62*, 1700065.
- [77] L. Rubió, R.-M. Valls, A. Macià, A. Pedret, M. Giralt, M.-P. Romero, R. De La Torre, M.-I. Covas, R. Solà, M.-J. Motilva, *Food Chemistry* **2012**, *135*, 2922–2929.
- [78] M. Suárez, R. M. Valls, M.-P. Romero, A. Macià, S. Fernández, M. Giralt, R. Solà, M.-J. Motilva, *British Journal of Nutrition* **2011**, *106*, 1691–1701.
- [79] L. Rubió, A. Macià, R. M. Valls, A. Pedret, M.-P. Romero, R. Solà, M.-J. Motilva, *Food Chemistry* **2012**, *134*, 1132–1136.
- [80] M. Suárez, M. P. Romero, A. Macià, R. M. Valls, S. Fernández, R. Solà, M. J. Motilva, *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences* **2009**, *877*, 4097–4106.
- [81] M. C. López de las Hazas, C. Piñol, A. Macià, M. P. Romero, A. Pedret, R. Solà, L. Rubió, M. J. Motilva, *Journal of Functional Foods* **2016**, *22*, 52–63.
- [82] M. C. López de las Hazas, L. Rubió, A. Kotronoulas, R. de la Torre, R. Solà, M. J. Motilva, *Molecular Nutrition and Food Research* **2015**, *59*, 1395–1399.

-
- [83] F. N. Bazoti, E. Gikas, A. Tsarbopoulos, *Biomedical Chromatography* **2010**, *24*, 506–515.
- [84] R. Domínguez-Perles, D. Auñón, F. Ferreres, A. Gil-Izquierdo, *European Journal of Nutrition* **2017**, *56*, 215–224.
- [85] A. Have, H. Health, M. Marhuenda-muñoz, E. P. Laveriano-santos, A. Tresserra-rimbau, **n.d.**, 1–15.
- [86] S. Silva, M. Garcia-Aloy, M. E. Figueira, E. Combet, W. Mullen, M. R. Bronze, *Molecular Nutrition and Food Research* **2018**, *62*, 1–11.
- [87] O. Khymenets, M. Farré, M. Pujadas, E. Ortiz, J. Joglar, M. I. Covas, R. De La Torre, *Food Chemistry* **2011**, *126*, 306–314.
- [88] O. Khymenets, M. C. Crespo, O. Dangles, N. Rakotomanomana, C. Andres-Lacueva, F. Visioli, *Journal of Functional Foods* **2016**, *23*, 278–282.
- [89] L. Rubió, M. Farràs, R. de La Torre, A. Macià, M.-P. Romero, R. M. Valls, R. Solà, M. Farré, M. Fitó, M.-J. Motilva, *Food Research International* **2014**, *65*, 59–68.
- [90] W. Sun, Z. Chen, J. Hong, J. Shi, *Biology* **2021**, *10*, 20.
- [91] A. López-Yerena, I. Domínguez-López, A. Vallverdú-Queralt, M. Pérez, O. Jáuregui, E. Escribano-Ferrer, R. M. Lamuela-Raventós, *Antioxidants* **2021**, *10*, 846.
- [92] U. Catalan, L. Barrubés, R. M. Valls, R. Sola, L. Rubio, *Genomics, proteomics & bioinformatics* **2017**, *15*, 236–245.
- [93] A. Taticchi, R. Selvaggini, S. Esposto, B. Sordini, G. Veneziani, M. Servili, *Food Chemistry* **2019**, *289*, 7–15.
- [94] A. López-Yerena, J. Lozano-Castellón, A. Olmo-Cunillera, A. Tresserra-Rimbau, P. Quifer-Rada, B. Jiménez, M. Pérez, A. Vallverdú-Queralt, *Molecules* **2019**, *24*, DOI 10.3390/molecules24101986_rfseq1.
- [95] S. Ben Brahim, H. Kelebek, S. Ammar, M. Abichou, M. Bouaziz, *Food Chemistry* **2017**, *229*, 9–19.
- [96] M. L. Clodoveo, R. H. Hbaieb, F. Kotti, G. S. Mugnozza, M. Gargouri, *Comprehensive Reviews in Food Science and Food Safety* **2014**, *13*, 135–154.
- [97] F. Peres, L. L. Martins, S. Ferreira-Dias, *Critical Reviews in Food Science and Nutrition* **2017**, *57*, 3104–3126.
- [98] A. Taticchi, S. Esposto, G. Veneziani, S. Urbani, R. Selvaggini, M. Servili, *Food Chemistry* **2013**, *136*, 975–983.
- [99] U. S. F. and D. Administration, *Rockville: FDA, Center for Drug Evaluation and Research.[Google Scholar]* **2003**.
- [100] M.-C. López de las Hazas, J. Godinho-Pereira, A. Macià, A. F. Almeida, M. R. Ventura, M.-J. Motilva, C. N. Santos, *Journal of Functional Foods* **2018**, *46*, 110–117.

ANEXES

8. ANEXES

8.1. Other publications

Article 10, Scientific Paper: Lozano-Castellón, J., **López-Yerena, A.**, Olmo-Cunillera, A., Jáuregui, O., Pérez, M., Lamuela-Raventós, R. M., & Vallverdú-Queralt, A. (2021). Total Analysis of the Major Secoiridoids in Extra Virgin Olive Oil: Validation of an UHPLC-ESI-MS/MS Method. *Antioxidants*, 10(4), 540. **Equal contributor**

Article 11, Review: Pérez, M., **López-Yerena, A.**, Lozano-Castellón, J., Olmo-Cunillera, A., Lamuela-Raventós, R. M., Martín-Belloso, O., & Vallverdú-Queralt, A. (2021). Impact of Novel Technologies on Virgin Olive Oil Processing, Consumer Acceptance, and the Valorization of Olive Mill Wastes. *Antioxidants*, 10(3), 417.

Article 12, Letter: **López-Yerena, A.**, Vallverdú-Queralt, A., Mols, R., Augustijns, P., Lamuela-Raventós, R. M., & Escribano-Ferrer, E. (2020). Reply to “Comment on López-Yerena et al. ‘Absorption and Intestinal Metabolic Profile of Oleocanthal in Rats’ *Pharmaceutics* 2020, 12, 134”. *Pharmaceutics*, 12(12), 1221.

Article 13, Scientific Paper: Perez, M., **Lopez-Yerena, A.**, & Vallverdú-Queralt, A. (2020). Traceability, authenticity and sustainability of cocoa and chocolate products: a challenge for the chocolate industry. *Critical Reviews in Food Science and Nutrition*, 1-15. **Equal contributor**

Article 14, Review: Olmo-Cunillera, A., **López-Yerena, A.**, Lozano-Castellón, J., Tresserra-Rimbau, A., Vallverdú-Queralt, A., & Pérez, M. (2020). NMR spectroscopy: a powerful tool for the analysis of polyphenols in extra virgin olive oil. *Journal of the Science of Food and Agriculture*, 100(5), 1842-1851.

Article 15, Review: Lozano-Castellón, J., **López-Yerena, A.**, Rinaldi de Alvarenga, J. F., Romero del Castillo-Alba, J., Vallverdú-Queralt, A., Escribano-Ferrer, E., & Lamuela-Raventós, R. M. (2020). Health-promoting properties of oleocanthal and oleacein: Two secoiridoids from extra-virgin olive oil. *Critical reviews in food science and nutrition*, 60(15), 2532-2548. **Equal contributor.**

Article 16, Scientific Paper: **López-Yerena, A.**, Guerra-Ramírez, D., Jácome-Rincón, J., Espinosa-Solares, T., Reyes-Trejo, B., Famiani, F., & Cruz-Castillo, J. G. (2018). Initial evaluation of fruit of accessions of *Persea schiedeana* Nees for nutritional value, quality and oil extraction. *Food chemistry*, 245, 879-884.

Article 17, Book chapter: Inés Domínguez-López, Maria Pérez, **Anallely López-Yerena**, Julián Lozano-Castellón, Alexandra Olmo-Cunillera, Anna Vallverdú-Queralt a and Rosa M. Lamuela-

Raventós. Fats and Associated Compounds: Human health and the consumption of fat-associated compounds: tyrosol, hydroxytyrosol, oleuropein, oleacein and oleocanthal. *Under review

Article 18, Review: Maria Perez, Inés Domínguez-López, **Anallely López-Yerena**, Anna Vallverdú Queralta, Current strategies to guarantee the authenticity of coffee. Under review in Critical Reviews in Food Science and Nutrition

8.2. Conference communications

Poster: Anallely López-Yerena, Rosa M. Lamuela-Raventós, Elvira Escribano-Ferrer. Absorption profile of oleocanthal in rats, 12th World Meeting on Pharmaceutics Biopharmaceutics and Pharmaceutical Technology. 11-14 May, 2021.

Poster: Anallely López-Yerena, Rosa M. Lamuela-Raventós, Xavier García-Sala, Elvira Escribano-Ferrer. Influence of the luminal perfusion rate in the determination of the effective intestinal permeability coefficient of levofloxacin, 12th World Meeting on Pharmaceutics Biopharmaceutics and Pharmaceutical Technology. 11-14 May, 2021.

Poster: López-Yerena Anallely, Anna Vallverdú-Queralta, Rosa M. Lamuela-Raventós, Elvira Escribano-Ferrer. Biodistribution of oleacein in rats after oral administration of virgin olive oil. A preliminary study. V WORKSHOP INSA UB 2019, Al·lèrgies i intoleràncies alimentàries: de la sospita a la taula. November, 2019.

Poster: Anallely López-Yerena, Julián Lozano-Castellón, Anna Vallverdú-Queralta, María Pérez Bosch, Paola Quifer Rada, Elvira Escribano, Brígida Jiménez and Rosa M. Lamuela-Raventós. Quantitative method for determination of oleocanthal and oleacein in virgin olive oils by UPLC-MS. XVII Congreso de la Sociedad Española de Nutrición, June, 2018.

Poster: López-Yerena Anallely, Lozano-Castellón, Julián, Vallverdú-Queralta, Anna, Escribano-Ferrer, Elvira, Romero-Aroca, Agustí J., Ninot-Cort, Antònia, Lamuela-Raventós, Rosa M. The influence of the extraction process on oleocanthal content of extra virgin olive oil. IV WORKSHOP INSA UB 2018, Ciència i propietats del cava i el vi, November, 2018.

8.3. Participation in projects

Title: Efecte immunomodulador i transmissió materna de derivats secoiridoides de l'oli d'oliva verge extra, Principal Investigators: Maria José Rodríguez y Anna Vallverdú Queralta, Funding agency: Instituto de Investigación en Nutrición y Seguridad Alimentaria de la Universidad de Barcelona (INSA-UB)

Title: Effect of diet polyphenols on the insulin sensitivity, inflammation markers and diabetes mellitus type 2, Project reference: AGL2016-75329-R, Principal Investigator: Rosa María Lamuela Raventós, Funding agency: Ministerio de Economía y Competitividad; National

Programme for Research Aimed at the Challenges of Society 2016. Duration: 30/12/2016-29/12/2020.