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Metabolism and Bioavailability of Olive Oil Polyphenols

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1. Introduction

The significance of virgin olive oil (VOO), hinged to its many virtues in both gastronomy and health, is nowadays undeniable. Their protective effects are attributed to its high content of monounsaturated fatty acids and to the presence of some minor components, which add up to 2% of the weight. Among its several minor constituents, polar phenolic compounds, usually characterized as polyphenols, have become the subject of intensive research because of their biological activities, their influence on the organoleptic properties of VOO and their contribution to its oxidative stability (Bendini et al., 2007).

The phenolic fraction of VOO consists of a heterogeneous mixture of compounds belonging to several families with varying chemical structures. A brief description of the main classes of phenolic compounds contained in VOO is given below:

- *Phenolic acids.* There are two main series of these acids, depending on the carbon skeleton: benzoic acids (C6-C1: 3-hydroxybenzoic, *p*-hydroxybenzoic, protocatechuic, gentisic, vanillic, syringic and gallic acids) and cinnamic acids (C6-C3: *o*-coumaric, *p*-coumaric, caffeic, ferulic and sinapic acids).
- *Phenolic alcohols.* The two most important in VOO are hydroxytyrosol (Hyty) and tyrosol (Ty), although two Hyty derivatives, its acetate and its glucoside, can be also found. Hyty and Ty only differ in a hydroxyl group in the *meta* position.
- *Secoiridoids.* They are present exclusively in plants of the Olearaceae family. The olives mainly contain the polar oleuropein (Ol) and ligstroside (Lig) glycosides. Ol is the ester of elenolic acid (EA) with Hyty, and Lig is the ester of EA with Ty. Ol and Lig aglycones (Ol Agl and Lig Agl, respectively) are formed by removal of the glucose moiety from glycosides by endogenous β-glucosidases during ripening, oil extraction and storage.
- *Lignans.* (+)-1-Pinoresinol, (+)-1-hydroxypinoresinol and (+)-1-acetoxypinoresinol are the most reported compounds in olive oil.
- *Flavonoids.* The main flavonoids present in VOO are apigenin and luteolin, which are originated from their corresponding glucosides present in the drupe.

The qualitative and quantitative composition of VOO hydrophilic phenols is strongly affected by the agronomic and technological conditions of production (Servili et al., 2004). Among agronomic parameters, the cultivar, the fruit ripening degree, the agronomic

techniques used and the pedoclimatic conditions are the aspects more extensively studied (Tovar et al., 2001; Uceda et al., 1999). Moreover, by modulating technology, it is possible to some extent to optimize the transfer of some polar minor constituents into the oil or reduce their level (Boskou, 2009). The influence of variety, extraction system, ripening degree and storage in the polyphenolic content of a VOO has been extensively discussed in the literature (Boskou, 2009; Uceda et al., 1999).

Wide ranges of total polar phenols concentration have been reported in olive oils (50-1000 mg/kg), although the most usual value is found between 100-350 mg/kg (Boskou et al., 2006). In general, the most abundant phenolic compounds in VOO are aglycones deriving from secoiridoids. Trying to establish levels of individual phenols, Servili & Montedoro (2002) calculated average values of 7 phenolic compounds from a considerable number of samples of industrial olive oils. They concluded that Hyty and Ty were found only in trace amounts (less than 10 mg/kg oil) and the most abundant phenols were decarboxylated Ol Agl (63-840 mg/kg), Ol Agl (85–310 mg/kg), and decarboxylated Lig Agl (15-33 mg/kg). Brenes et al. (2002) published values ranging from 3-67 mg/kg for 1-acetoxypinoresinol, and from 19-41 mg/kg for pinoresinol in 5 Spanish olive oils, data that can be completed with the researches carried out by Romero et al. (2002) and Tovar et al. (2001). Levels of luteolin have been found to be around 10 mg/kg in some Spanish olive oils (Brenes et al., 1999) or ranging between 0.2-7 mg/kg for Greek oils (Murkovic et al., 2004). Carrasco-Pancorbo et al. (2006) developed a method to quantify 14 individual phenols belonging to different families in 7 Spanish extra-virgin olive oils (EVOOs). They also quantified them, finding the following contents (mg/kg): simple phenols: 6.8-11.5; complex phenols: 70.5-799.5; lignans: 0.81-20.6; and flavonoids: 1.4-8.6.

Intake of olive oil in the Mediterranean countries is estimated to be 30–50 g/day, based on the per capita olive oil consumption of 10–20 kg/year in Greece, Italy and Spain (Boskou, 2000; Food and Agricultural Organization, 2000). A daily consumption of 50 g olive oil with a concentration of 180 mg/kg of phenols would result in an estimated intake of about 9 mg of olive oil phenols per day (de la Torre, 2008; Vissers et al., 2004), of which at least 1 mg is derived from free Hyty and Ty, and 8 mg are related to their elenolic esters and also to Ol Agl and Lig Agl (de la Torre, 2008). Some other estimations have been made. For the Greek population (Dilis & Trichopolou, 2009), the daily per-capita intake is about 17 mg. Vissers et al. (2004) estimated that about 1 mg of the phenol intake per day (6 mmol) is derived from Hyty and Ty, about 8 mg (23 mmol) from the aglycones, and so the total phenol intake would be about 29 mmol.

2. Bioavailability of olive oil polyphenols

Accumulating evidence suggests that VOO may have health benefits; it can be considered as an example of a functional food containing a variety of components that may contribute to its overall therapeutic characteristics (Stark & Madar, 2002; Visioli & Bernardini, 2011). To explore and determine the mechanisms of action of olive oil polyphenols and their role in disease prevention, understanding the factors that constrain their release from the olive oil, their extent of absorption, and their fate in the organism is crucial. These issues can be described under the term *bioavailability,* borrowed from the field of pharmacology, redefined as "that fraction of an oral dose, either parent compound or active metabolite, from a particular preparation that reaches the systemic circulation" (Stahl et al., 2002). To simplify this definition, D'Archivio et al. (2010) explained that it simply means how much of the

ingested amount of polyphenols is able to exert its beneficial effects in the target tissues. It is important to realize that the most abundant phenolic compounds in our diet are not necessarily those that have the best bioavailability profile, either because they have a lower intrinsic activity or because they are poorly absorbed from the intestine, highly metabolized, or rapidly eliminated. In addition, the metabolites that are found in blood and target organs, resulting from digestive or hepatic activity, may differ from the native compounds in terms of biological activity (Manach et al., 2004).

Although the information concerning the bioavailability of most olive oil polyphenols is limited, intensive research has been carried out in the past decade. This fact is reflected in the number of reviews published since 2002 (Corona et al., 2009; Covas et al., 2009; de la Torre, 2008; Fitó et al., 2007; Tuck & Hayball, 2002; Visioli et al., 2002; Vissers et al., 2004). To address the bioavailability of olive oil phenolic compounds, we have reviewed *in vitro* and *in vivo (*both animal and human) studies on the absorption, transport, metabolism and excretion of olive oil phenolic compounds.

2.1 Absorption and disposition

Direct evidence on bioavailability of olive oil phenolic compounds has been obtained by measuring the concentration of the polyphenols and their metabolites in biological fluids, mostly plasma and urine, after ingestion of pure compounds or of olive oil, either pure or enriched with the phenolics under study. The majority of research regarding the bioavailability of olive oil polyphenols has been focused on three major phenolics: Hyty, Ty and Ol, as can be seen in **Tables 1** and **2**.

After ingestion, olive oil polyphenols can be partially modified in the acidic environment of the stomach. The effect of such environment on aglycone secoiridoids has been examined *in vitro* by incubating the compounds at 37 ºC in simulated gastric pH conditions and during normal physiological time frames (Corona et al., 2006; Pinto et al., 2011). Although hydrolysis takes place releasing free phenolic alcohols, a significant amount remains intact and thus, enters the small intestine unmodified. Ol Agl and its dialdehydic form, however, are likely not absorbed as such in the small intestine; the major metabolites detected using the perfused rat intestine model were the glucuronide conjugates of the reduced form of both compounds (Pinto et al., 2011).

Manna et al. (2000) carried out studies on the transport kinetics of radiolabeled Hyty using differentiated Caco-2 cells. The only metabolite found in the culture medium was the methylated derivative (i.e. homovanillic alcohol - HVAlc). They also demonstrated that Hyty was transported across the membrane of the human enterocytes by a bidirectional passive diffusion mechanism. Caco-2/TC7 cell monolayers have been used to study the metabolism of other olive oil polyphenols, such as Ty, *p*-coumaric acid, pinoresinol, luteolin (Soler et al., 2010) and Hyty acetate (Mateos et al., 2011). Results showed that the methylated conjugates are the main metabolites and that the acetylation of Hyty significantly increases its transport across the small intestinal epithelial cell barrier, enhancing the delivery of Hyty to the enterocytes.

To study the potential hepatic metabolism of olive oil phenols, human hepatoma HepG2 cells were incubated for 2 and 18 h with Ty, Hyty and Hyty acetate (Mateos et al., 2005). Extensive uptake and metabolism of Hyty and Hyty acetate were observed, with scarce metabolism of Ty. Hyty acetate was converted into free Hyty and then metabolized;

Tested Phenol	Model system[a]	Methods	Metabolites Detected	Study Outcome	Ref.
[^{14}C] Hyty	Caco-2 cell monolayers	Transport kinetics: incubation with increasing concentrations (50-500 µM) at 37 and 4 ^{o}C for 2 min. Transepithelial transport: incubation with 100 µM Hyty, glucose and mannitol	HVAlc	Hyty transport occurs via a passive diffusion mechanism, bidirectionally and in a dose-dependent manner. Hyty is quantitatively absorbed in the intestine	Manna et al., 2000
Ol glycoside	Isolated rat intestine	In situ intestinal perfusion technique: infusion of aqueous solution (1 mM, 50 µl/min) at 37 ^{o}C during 40 min in both iso-osmotic and hypotonic luminal conditions		Ol in aqueous solution can be absorbed, albeit poorly, from isolated perfused rat intestine. The P_{app} of Ol in hypotonic conditions is significantly higher	Edge-combe et al., 2000
Hyty, Ty, Hyty-Ac	Hepatoma HepG2 cells	Cell uptake and metabolism of phenols: incubation with 100 µM at 37 ^{o}C for 2 and 18 h	Hyty mono-gluc and methyl-gluc, HVA, Ty gluc, Hyty-Ac mono-gluc	Extensive uptake and hepatic metabolism of Hyty and Hyty-Ac with scarce metabolism of Ty; main derivatives formed: glucuronidated and methylated conjugates	Mateos et al., 2005
Hyty, Ty, Ol	Caco-2 cell monolayers and rat segments of jejunum and ileum		Hyty and Ty gluc, HVAlc, Hyty glutathionylated	Hyty and Ty were transferred across the cell monolayers and rat segments of intestine and were subjected to classic phase I/II biotransformation. No absorption of Ol	Corona et al., 2006
Hyty, Ty, *p*-coumaric acid, pinoresinol, luteolin	Caco-2/TC7 cell monolayers	Phenols metabolism: incubation with 40, 50 and 100 µM at 37 ^{o}C for 1, 6 and 24 h. Transport experiments in the AP, cellular and BL compartments: AP loading of phenol at 100 µM	*Hyty:* methyl, sulfate, methyl-sulfate. *Ty:* methyl, sulfate. *p-Coumaric acid:* disulfate, methyl. *Pinoresinol:* gluc, sulfate. *Luteolin:* gluc, methyl, methyl-gluc,	Limited intestinal metabolism. Major metabolites: methylated conjugates. Time-dependent efflux of various free and conjugated forms, showing preferential AP to BL transport after 24 h of incubation	Soler et al., 2010
Hyty, Hyty-Ac	Caco-2/TC7 cell monolayers	Metabolism experiments and transport experiments in the AP and BL compartments: incubation with 50 µM at 37 ^{o}C for 1, 2 and 4 h	*Hyty:* HVAlc. *Hyty-Ac:* Hyty, HVAlc, mono-gluc.	Hyty-Ac is better absorbed than free Hyty and serves to enhance delivery of Hyty to the enterocytes for subsequent metabolism and BL efflux)	Mateos et al., 2011
Ol Agl, dialdehydic form of Ol Agl	Human Caco-2 cell monolayers and isolated lumen of rat intestine (jejunum and ileum)	Transport experiments using Caco-2 cells: incubation with 50, 100 and 200 µM at 37 ^{o}C for 2 h; AP loading. Transport experiments using rat intestine: perfusion of methanol solution (100 µM) at 37 ^{o}C during 80 min	Hyty, HVAlc, Hyty and HVAlc gluc, gluc conjugates of the reduced forms of tested compounds	Caco-2 cells expressed limited metabolic activity. Major metabolites using the perfused rat intestine model: gluc of the reduce forms. Secoiridoids in the parental form were little absorbed in the small intestine	Pinto et al., 2011

[a] Caco-2 cells: model system of the human intestinal epithelium; HepG2 cells: model system of the human liver; TC7 cells: spontaneously differentiating clone derived from the original Caco-2 cell population.
Abbreviations: AP: apical; BL: basolateral; gluc: glucuronide; Hyty: hydroxytyrosol; Hyty-Ac: hydroxytyrosol acetate; HVA: homovanillic acid; HVAlc homovanillic alcohol; Ol: oleuropein; Ol Agl: oleuropein aglycone; P_{app}: apparent permeability coefficient; Ty: tyrosol.

Table 1. *In vitro* studies carried out with olive oil polyphenols.

glucurono- and methyl-, but no sulfo-conjugates, were found. Olive oil phenols are metabolized by the liver as well, as suggested by these results.

The colonic metabolism of olive oil polyphenols is scarcely reported. Corona et al. (2006) demonstrated that secoiridoids, which appear not to be absorbed in the small intestine, suffer bacterial catabolism in the large intestine with Ol undergoing rapid degradation by the colonic microflora producing Hyty as the major end product.

It is essential to establish whether olive oil phenolics are absorbed in the intestine *in vivo* and how they are distributed in the organism. **Table 1** shows the *in vivo* bioavailability studies of olive oil polyphenols carried out so far. For practical reasons, rats are used as the model of choice for *in vivo* studies. Bai et al. (1998) studied the absorption and pharmacokinetics of Hyty in rats, finding that the absorption of Hyty after the ingestion of a single dose is very fast. The metabolic fate of Hyty and Ty in rats has been also evaluated by administration of the radiolabeled polyphenols. Hyty appeared in plasma at maximum levels 5 min after oral administration, although the proportion of free aglycones in some tissues differed to that observed in plasma (D'Angelo et al., 2001). In all of the investigated tissues, Hyty was enzymatically converted in oxidized and/or methylated derivatives, whereas the major urinary products were sulfo-conjugates. Tuck et al. (2001) compared the elimination of Hyty and Ty in rat urine within 24 h after administration, both orally (in oil- and water-based solutions) and intravenously (in saline). When orally administrated, polyphenols will be subjected to first-pass metabolism, so that the contribution of intestinal metabolism will be quite relevant. If the administration is intravenous, only hepatic contribution to its disposition will be seen. Results showed that Hyty and Ty can be absorbed into the systemic circulatory system after oral dosing and that their bioavailability when administered as an olive oil solution is almost complete. Later, urine samples were re-examined and Hyty and five of its metabolites were detected (Tuck et al., 2002). Three were conclusively identified as monosulfate and 3-*O*-glucuronide conjugates of Hyty, and homovanillic acid (HVA), and one was tentatively identified as *O*-glucuronide conjugate of HVA. Although there is no disagreement between studies, a major limitation is that they were done with rats and some researches suggest that comparisons between the model species might not be adequate. Visioli et al. (2003) observed a 25 fold higher basal excretion of Hyty and of its main metabolites in rats than humans.

In a well-designed approach, Vissers et al. (2002) measured the absorption and urinary excretion of olive oil polyphenols in healthy ileostomy subjects and subjects with a colon after the ingestion of increasing doses of extracted phenols. Only a small amount of the ingested compounds was recovered in the urine, supporting the hypothesis that humans absorb a major fraction of the olive oil phenols consumed. Furthermore, the comparison between the absorbed polyphenols in normal and ileostomy subject showed similar results, which implies that the small intestine is the major site for the absorption of those compounds. Free Hyty and Ty and their glucurono-conjugates were the only metabolites detected in the urine samples. Another study carried out in human subjects assessed quantitatively the uptake of phenolics from olive oils containing different amounts of Ty and Hyty (Visioli et al., 2000). It was observed that these compounds were absorbed in a dose-dependent manner, that they were excreted in urine as glucuronide conjugates and that, as the concentration of phenols administered increased, the proportion of conjugation with glucuronic acid also increased. Upon re-examination of samples two more metabolites of Hyty were identified: HVA and HVAlc (Caruso et al., 2001).

Administrated Polyphenol	Administration and Dose	Biological Sample	Concentration in Plasma	Excretion in Urine[a]	Metabolites Detected	Other Measurements	Analysis Methods	Ref.
Synthetic Hyty in 0.5% tragacanth solution	Oral, 1 ml single dose: Hyty 10 mg /ml	Rat plasma	Hyty 0.89-3.26 µg/ml (after 10 min)				GC-MS	Bai et al., 1998
Olive oil enriched with increasing concentrations of phenols	Oral, 50 ml single dose. Phenolic content (mg/l): total phenols 487.5-1950; Hyty 20-84; Ty 36-140	Human urine		Total Hyty 30-60%; Total Ty 20-22%	Hyty and Ty gluc		GC-MS	Visioli et al., 2000
Synthetic Hyty in aqueous solution	Oral, single dose: Hyty: 20 mg/kg	Rat plasma	Hyty 1.91 µg/ml (after 10 min)				HPLC-UV (280 nm)	Ruiz-Gutiérrez et al., 2000
EVOO	Oral (a) Sustained doses for 1 month of 50 g EVOO/day; (b) 100 g single dose	Human plasma				Plasma antioxidant capacity. Hyty, Ty and vitamin E content in LDL		Bonanome et al., 2000
Olive mill waste water extracts with increasing concentrations of Hyty	Oral, single doses *(a)* 1 mg/kg of extract: 41.4 µg/kg of Hyty *(b)* 5 mg/kg of extract: 207 µg/kg of Hyty *(c)* 10 mg/kg of extract: 414 µg/kg of Hyty	Rat plasma and urine			Hyty gluc	Plasma antioxidant capacity		Visioli et al., 2001
Radiolabeled synthetic Hyty and Ty in different solutions	*(a)* Oral, single dose; 225 mg oil-based solution (23.5 mg Hyty or 14.7 mg Ty in 1300 mg EVOO) or water-based solution (25.5 mg Hyty or 14.4 mg Ty in 1300 mg water) *(b)* IV, 950 mg saline solution (6.5 mg Hyty or 9.8 mg Ty added to 5 ml of 9 g/l NaCl)	Rat urine and feces		*(a)* Oral in oil (%): Hyty 94.1, Ty 72.9 ; oral in water, Hyty 70.9, Ty 53.2; *(b)* IV (%): Hyty 94.9, Ty 74.4	Gluc and sulfate conjugates	Feces: Hyty: < 3% and Ty: 25-30% of administered amount (after 24 h)	HPLC-radiometric detection	Tuck et al., 2001
Radiolabeled synthetic Hyty	IV, 0.3 mg single dose: 1.5 mg Hyty/kg body weight	Rat blood, urine, feces, tissues and GI content	Hyty: 8% of administered radioactivity (after 5 min), 6% associated with plasma and 1.9% with cell fraction	Hyty: 90% of administrated radioactivity (after 5 h)	Sulfo-conjugated, HVAlc, HVA, DOPAC, DOPAL	Hyty (% of administered radioactivity): brain 0.89, heart 0.39, kidney 0.8, liver 3.19, lung 0.53, skeletal muscle 61, GI content 9 (after 5 min). Feces: 3.2% (after 5 h). Measurements of detected metabolites in urine and tissues as well	Radioactivity measures, HPLC-UV for metabolite identification	D'Angelo et al., 2001
Olive oil enriched with increasing concentrations of phenols	Oral, 50 ml single dose (mg): total Ol Agl 12.6-39.5; free Hyty 1.9-7.1; total Hyty 7-23.2	Human urine		% of total metabolites: Hyty 16.8-23.7% ; HVA 53.9-61.8 %; HVAlc 22.0-22.4 %	HVAlc, HVA		GC/LC-MS	Caruso et al., 2001

Administrated Polyphenol	Administration and Dose	Biological Sample	Concentration in Plasma	Excretion in Urine[a]	Metabolites Detected	Other Measurements	Analysis Methods	Ref.
EVOO	Oral, 50 ml single dose: 1650 µg of Ty	Human urine		Ty 17-43%	Ty conjugates		GC-MS	Miró-Casas et al., 2001a
VOO	Ora; 50 ml single dose: 1055 µg of Hyty, and 655 µg of Ty	Human urine		Hyty 32-98.8%; Ty 12.1-52%; total free Hyty and Ty ~15%	Hyty and Ty conjugates		GC-MS	Miró-Casas et al., 2001b
Aqueous and oil solutions of radiolabelled synthetic Hyty	*(a)* Oral, oil solution *(b)* IV, aqueous solution (For a detailed description see Tuck et al., 2001)	Rat urine		*(a)* Free Hyty 4.10 %; Hyty sulfate 48.42%; Hyty gluc 9.53%; HVA 10.26%; other metabolites: 20.27%; *(b)* Free Hyty 2.35 %; Hyty sulfate 34.24%; Hyty gluc 3.58%; HVA 18.69%; other metabolites 30.87%	Hyty monosulfate, Hyty 3-O-gluc, HVA	Determination of the radical scavenging ability of authentic HVA and HVAlc and of each metabolite using DPPH radical scavenging test.	HPLC-radiometric detection; HPLC-MS/MS, ^{1}H NMR for metabolite identification	Tuck et al., 2002
Supplements containing nonpolar and polar phenols extracted from EVOO, and Ol glycoside (commercially available capsules)	Oral, single doses: 100 mg of phenols *(a)* Ileostomy subjects. Phenolic content (µmol): nonpolar 371; polar 498; Ol glycoside 190; *(b)* Subjects with a colon. Phenolic content (µmol): nonpolar 382; polar 526	Human urine and ileostomy effluent		*(a)* Nonpolar 12%; polar 6%; Ol glycoside 16% *(b) N*onpolar 6%; polar 5%	Hyty and Ty in free form or gluc conjugated	Total excretion in ileostomy effluent over 24 h (µmol): nonpolar < 127; polar < 153; Ol glycoside < 51	HPLC-MS/MS, GC-MS, HPLC-DAD	Vissers et al., 2002
VOO from Arbequina cultivar	Oral *(a)* 50 ml single dose (µg): Ty 1720; Hyty 1370 *(b)* 25 ml /day sustained doses for 1 week (µg): Ty 860; Hyty 685	Human urine		*(a)* Ty 16.9%; Hyty 78.5% *(b)* Ty 19.4%; Hyty 121.5% (at the end of the sustained period)			GC-MS	Miró-Casas et al., 2003a
VOO	Oral, 25 ml single dose (mg/l): free Hyty 6.2; Hyty after acidic treatment 49.3	Human urine and plasma	Hyty conjugate 25.83 µg/l (after 32 min); HVAlc 3.94 µg/l (after 53 min)	Different results according to hydrolytic treatment (µg): acidic conditions: Hyty 714.7, HVAlc 188.0; enzymatic hydrolysis: Hyty 479.6, HVAlc 122.9 (after 12 h)	HVAlc, Hyty gluc		GC-MS	Miró-Casas et al., 2003b
EVOO and synthetic Hyty in ROO and low-fat yogurt	Oral, single dose *(a)* Rats: 50.3 µg total Hyty 0.5 ml in EVOO (201.2 µg/kg) *(b)* Humans: 3.2 mg total Hyty in 30 ml EVOO (45.7 µg/kg); 7 mg Hyty in 30 ml ROO; 20 mg synthetic Hyty in 125 g yogurt.	Rat and human urine		% of total Hyty administered: *(a)* Hyty + HVAlc 7.6 *(b)* EVOO: Hyty + HVAlc 44.2. ROO: Hyty + HVAlc 23.0; Yogurt: Hyty + HVAlc 6.7	HVAlc		GC/LC-MS	Visioli et al., 2003

Administrated Polyphenol	Administration and Dose	Biological Sample	Concentration in Plasma	Excretion in Urine[a]	Metabolites Detected	Other Measurements	Analysis Methods	Ref.
Ol in soya oil and distilled water	Oral, 350g single dose (Ol 100 mg/kg)	Rat plasma and urine	Ol 200 ng/ml (after 2 h)	Ol gluc 91%; Hyty gluc 97%	Ol and Hyty gluc		LC-MS/MS	Del Boccio et al., 2003
Ol in saline solution	IV, 100 µl single dose: 25 mg/kg of Ol in NaCl (0.9%, w/v)	Rat plasma	Approximate values: 3.5 µg/ml Ol; 20 ng/ml Hyty (after 10 min)				HPLC-fluorescence detection	Tan et al., 2003
EVOO and pure Ol (isolated from olive tree leaves)	Oral, sustained doses for 80 days (g/kg) *(a)* EVOO 50 *(b)* Ol 0.15	Rat urine		ng/ml: *(a)* free Ty 321; free Hyty 253.2; total Ty 1855.6; total Hyty 404.3 *(b)* free Ty 183.6; free Hyty 154.4; total Ty 814.5; total Hyty 1036.7	Gluc conjugates		GC-MS/MS (for urine samples) and HPLC-DAD (for EVOO extracts)	Bazoti et al.,2005
EVOO with increasing concentrations of polyphenols	Oral, sustained doses for 4 days. 25 ml EVOO: phenol content (mg/kg) *(a)* high 486 *(b)* moderate 133 *(c)* low 10 (%: Hyty 6.3, Ty 5.3, Ol Agl 40.0; Lig Agl 26.2; luteolin 11.7; apigenin 2.6)	Human urine		Approximate values (mmol): *(a)* Ty 1.2; Hyty 0.5; HVAlc 0.2 *(b)*Ty 2.8; Hyty 2.2; HVAlc 0.6; *(c)*Ty 3.5; Hyty 4.8; HVAlc 1.0			HPLC	Weinbrenner et al., 2004
VOO, COO or ROO from Picual cultivar	Oral, 25 ml sustained doses for 3 weeks. Total phenols in olive oil (mg/kg): VOO 366; COO 164; ROO 2.7	Human plasma and urine				Detection of etheno-DNA adducts in plasma. Measurement in 24 h urine of Ty and Hyty as biomarkers of the type of olive oil ingested	LC-MS/MS	Hillestrom et al., 2006
VOO from Picual cultivar	Oral, 50 ml VOO single dose	Human blood			Hyty mono-gluc, Hyty monosulfate, Ty gluc, Ty and HVA sulfate	Metabolites in LDL (ng/mg apo-B): Hyty mono-gluc 2.11; Hyty monosulfate 24.27; Ty gluc 1.16; Ty sulfate 14.87; HVA sulfate 27.16 (after 1 h)	HPLC-DAD-MS/MS	de la Tore-Carbot et al., 2006
EVOO from Picual cultivar	Oral, 50 ml single dose. Phenolic content (µg/ml): total 648; Hyty 70.6; Ty 27.01	Human blood			Hyty mono-gluc isomers, Hyty monosulfate, Ty and HVA sulfate, Ty gluc	Metabolites in LDL (ng/mg apo-B): total phenolics 105.43; Hyty mono-gluc I 2.45; Hyty mono-gluc II 2.55; Hyty monosulfate 34.22; Ty gluc 0.96; Ty sulfate: 17.23; HVA sulfate 48.02 (after 1 h)	HPLC-DAD-MS/MS	de la Tore-Carbot et al., 2007
VOO	Oral, 30 ml single dose: total phenols 400 mg/kg	Human plasma	11 free phenolicss and 9 metabolites (after 1 and 2 h)		Hyty and apigenin gluc; Hyty, Ty, HVA, vanillin, vanillic acid, dihydroferulic acid and coumaric acid sulfates		UPLC-ESI-MS/MS	Suárez et al., 2009

Administrated Polyphenol	Administration and Dose	Biological Sample	Concentration in Plasma	Excretion in Urine[a]	Metabolites Detected	Other Measurem
EVOO enriched with Ol and pure Ol (isolated from olive tree leaves)	Oral, sustained doses for 80 days *(a)* EVOO: 1.1 g/kg *(b)* Ol supplement: 0.33 mg/kg	Rat plasma	After enzymatic treatment (ng/ml): *(a)* HVAlc 61.9 *(b)* HVAlc 53.5; (Hyty not detected)		HVAlc	
Spanish VOO (Picual cultivar) and ROO	Oral, 25 ml/day sutained doses for 3 weeks *(a)* VOO, phenol concentration (mg/l): total 629; Hyty 63.5; Ty 24.4; Ol derivatives 327.2; Lig derivatives 208; other 6 phenolics quantified *(b)* ROO	Human blood			Hyty, Ty and HVA sulfates	LDL composition consumption of
EVOO (50% v/v Arbequina and Picual cultivars)	Oral, 50 ml single dose: Phenolic content (mg/kg): Hyty 8.31; Ty 5.33; pinoresinol 3.25; luteolin 2.65; apigenin 0.64; EA 34.91; Lig Agl 40.58	Human urine		28 phase I and 32 and II metabolites	Phase I reactions: hydrogenation, hydroxylation, hydration, methylation Phase II reactions: gucuronidation, sulfoconjugation	Excretion kinetics metabolites ident in human urine a
Spanish VOO	Oral, 50 ml single dose. Phenolic content in µmol: Hyty 21.96; Ty 15.20; HVAlc 0.27	Human plasma and urine		% of ingested Hyty: Total Hyty 9.2; free Hyty 1.8; Hyty-4′-*O*-gluc 3.1; Hyty-3′-*O*-gluc 4.3; total Ty 12.9; free Ty 3.3; Ty-4′-*O*-gluc 9.6; Total HVAlc 4.5; free HVAlc: 1.4; HVAlc -4′-*O*-gluc: 3.1	HVAlc; Hyty, Ty and HVAlc gluc	Control of compl dietary recomme analyzing the pla concentrations of HVAlc

[a] Percentage of administered amount after 24 h, unless otherwise indicated

Abbreviations: apo-B: apolipoprotein-B; COO: common olive oil; DAD: diode array detector; DOPAC: 3,4-dihydr dihydroxyphenylacetaldehyde; DPPH: 2,2-diphenyl-1-picrylhydrazyl; EA: elenolic acid; EVOO: extra virgin oliv chromatography; GI: gastrointestinal; HPLC: high performance liquid chromatography; Hyty: hydroxytyrosol; homovanillic alcohol; IV: intravenous; LC: liquid chromatography; LDL: low-density lipoproteins; Lig Agl: ligst spectrometry; Ol: oleuropein; P_{app}: apparent permeability coefficient; ROO: refined olive oil; Ty: tyrosol; UV: ult

Table 2. Bioavailability of olive oil polyphenols in animals and humans.

A major limitation of the commented human studies is that they used phenolics extracts or olive oil samples artificially enriched with phenolics extracts, and therefore extrapolation of these results to typical olive oil consumption may not be realistic. Further studies have been performed administering VOO at doses close to that used in the Mediterranean countries (30-50 g/day) (Bonanome et al., 2000; de la Torre-Carbot et al., 2006, 2007; García-Villalba et al., 2010; Khymenets et al., 2011; Miró-Casas et al., 2001a, 2001b, 2003a, 2003b; Suárez et al, 2009). Results confirmed that Hyty and Ty are mainly excreted in their glucurono-conjugated form; in fact, the role of glucuronidation in metabolism of main olive oil phenols can be evaluated in about 65-75% of totally recovered in urine after dietary VOO consumption (Khymenets et al., 2011; Miró-Casas et al., 2003b), which suggests an extensive first-pass intestinal/hepatic metabolism of the compounds ingested. Suárez et al. (2009) considered for the first time the absorption and disposition of flavonoids and lignans after the ingestion of VOO. Besides the presence of those VOO polyphenols in their conjugated forms, an important variability in the concentrations was observed between the plasma samples obtained from different volunteers. This variability may be attributed to differences in the expression of metabolizing enzymes due to genetic variability within the population. The most comprehensive study regarding the identification of metabolites in human urine of practically all the olive oil polyphenols described was reported by García-Villalba et al. (2010). These authors were able to achieve the tentative identification of 60 metabolites; the most abundant were those containing a catechol group, such as Hyty and the secoiridoids Ol Agl and deacetoxy-Ol Agl. Phenolic compounds were subjected to various phase I and phase II reactions, mainly methylation and glucuronidation. The report suggests that most of the olive oil polyphenols are absorbed to a greater or lesser extent, although absorption and metabolism seems to differ greatly among the different compounds.

2.2 Conjugation and nature of metabolites

Low doses of polyphenols are delivered through human diet and, generally, do not escape first-pass metabolism. As a result, most olive oil polyphenols undergo structural modifications, i.e. conjugation process; in fact, conjugates are the predominant forms in plasma. Once absorbed, olive oil polyphenols are subjected to three main types of conjugation: methylation, glucuronidation and, to a lesser extent, sulfation, through the respective action of catechol-O-methyl transferases (COMT), uridine-5′-diphosphate glucuronosyltransferases (UDPGT) and sulfotransferases (SULT) (Manach et al., 2004).

Recently, García-Villalba et al. (2010) carried out a broad study of the metabolites of most olive oil phenolic compounds excreted in human urine, showing that most polyphenols were absorbed, metabolized and excreted to a lesser or greater extent. It was initially suggested in literature that Ol Agl and Lig Agl were hydrolyzed in the gastrointestinal tract (GI) tract and then, the resulting polar phenols, Hyty and Ty, were absorbed and metabolized (Vissers et al., 2002). Nevertheless, the results obtained in later experiments with Caco-2 cells (Pinto et al., 2011) and humans (García-Villalba et al., 2010), showed that, at least, part of the secoiridoids can be absorbed and metabolized; reduction (hydrogenation) is the most probable metabolic pathway of these compounds. Hydroxylation and hydration are also possible pathways for the secoiridoids. In fact, they can precede or follow the action of COMT on compounds such as Hyty, deacetoxy-Ol Agl, and Ol Agl. Some compounds can even suffer a double hydroxylation before or after the methylation (García-Villalba et al., 2010).

A notable metabolic pathway for Hyty is the methylation, giving rise to the formation of HVAlc (Caruso et al., 2001; Bazoti et al., 2010; Manna et al., 2000; Visioli et al, 2003). Oxidation and methylation-oxidation, rendering 3,4-dihydroxyphenilacetic acid (DOPAC) and HVA, respectively, have been also proposed (D'Angelo et al., 2001). It is noteworthy that many of the reported metabolites of Hyty are also the major molecular species deriving from dopamine metabolism (HVA, DOPAC, 3,4-dihydroxyphenyl acetaldehyde - DOPAL); in fact, Hyty can be also called DOPET, a well-known dopamine metabolite.

Besides, olive oil phenolic compounds and most of their corresponding phase I metabolites can be subsequently subjected to phase II reactions, preferentially glucuronoconjugation (García-Villalba et al., 2010). The presence of glucuronoconjugates of phenolic compounds belonging to most of chemical classes families described in olive oil has been widely detected in both urine and plasma, whereas the presence of sulfated metabolites has scarcely been reported in literature.

The metabolism of olive oil lignans has not been reported in detail so far and one of the few references appeared only recently (Soler et al., 2010). In this study, pinoresinol glucuronide and sulfate conjugates were identified after incubation of free pinoresinol using differentiated Caco-2/TC7 cell monolayers.

As far as flavonoids are concerned, products of methylation and glucuronidation have been observed (Soler et al., 2010; Suárez et al., 2009). Methyl-monoglucuronides of apigenin and luteolin have been identified as well (García-Villalba et al., 2010).

2.3 Binding of olive oil polyphenols to lipoproteins

Several reports converge on the *in vitro* ability of olive oil phenolic compounds to bind low density lipoproteins (LDL) and to protect them against oxidation (Covas et al., 2000; Visioli et al., 1995). Moreover, both animal and human *in vivo* studies (Coni et al., 2000; Marrugat et al., 2004) have provided evidence on the effects of olive oil ingestion on LDL composition and the incorporation of olive oil phenolics and their metabolites in LDL. In one of the first studies, Bonanome et al. (2000) determined the presence of Hyty and Ty in human lipoprotein fractions after olive oil ingestion. Both compounds were recovered in all of the fractions, except in the very low density lipoproteins one; concentrations peaked between 1 and 2 h. Covas et al. (2006) demonstrated that the postprandial oxidative stress can be modulated by the olive oil phenolic content and that the degree of LDL oxidation decreases in a dose-dependent manner with the phenol concentration of the olive oil ingested. They arrived to these conclusions administering a single dose of olive oil, but similar results were obtained in studies using sustained doses; olive oil consumption for 1 week led to an increase in the total phenolic content of LDL (Gimeno et al., 2002). In a later study, volunteers were requested to ingest virgin, common or refined olive oil daily for 3 weeks (Gimeno et al., 2007). The concentration of total phenolic compounds in LDL was directly correlated with the phenolic concentration of the oils and with the resistance of LDL to their *in vitro* oxidation.

De la Torre-Carbot et al. (2006, 2007) developed a rapid method for the determination in LDL of Ty, Hyty and several of their metabolites. The presence of these compounds in LDL strengthens claims that these compounds can act as *in vivo* antioxidants. The effect of the intake of virgin and refined olive oils after long-term ingestion of real-life doses on the

content of the metabolites in LDL was examined as well (de la Torre-Carbot et al., 2010). The phenols in VOO modulated the LDL content of 3 phenolic metabolites, Hyty, Ty, and HVA sulfates; the concentration of these compounds increased significantly after the ingestion of VOO, in contrast to the refined one. In parallel, the ingestion of VOO significantly reduced LDL and plasma oxidative markers, which suggests that the metabolic activities of phenols can be related to the capacity of these compounds to remain bound to LDL.

2.4 Plasma concentration and tissue uptake

In 1998, Bai et al. reported for the first time the absorption of Hyty into the bloodstream. Hyty was administered orally to rats and appeared in plasma 2 min after, reaching the highest level at 5-10 min. Its concentration was low compared to the administered amount. The experiment, however, did not take into account the presence of metabolites.

After this first approach, different methods for the simultaneous detection of Ol and Hyty in rat plasma have been optimized. Ruiz-Gutiérrez et al. (2000) determined Hyty in overnight-fasted rat plasma after its oral administration. Ol and Hyty plasma concentrations were measured after oral administration of a single dose of Ol to rats using soya oil and distilled water as administration vehicle (Del Boccio et al., 2003). Analysis of plasma showed the presence of unmodified Ol, reaching a peak value within 2 h, with a small amount of Hyty. In another study, Ol and Hyty plasma levels were monitored in rats after intravenous dosing of Ol (Tan et al., 2003). The dosing profile showed that at 10 min both Ol and Hyty were rapidly distributed.

Studies in which phenolic ingestion is closer to typical dietary patterns may be more appropriate for estimating bioavailability than the administration of pure compounds. Recently (Bazoti et al., 2010), the simultaneous quantification of Ol and its major metabolites in rat plasma was carried out after a control diet of 80 days supplemented with Ol or with EVOO. Basal levels of HVAlc were detected in the blood stream after the enzymatic treatment of the samples with β-glucuronidase. Before the enzymatic treatment, HVAlc was detected below the limits of quantification in plasma samples of rats supplement with Ol. Hyty was not detected, which indicates that it was metabolized to HVAlc. Ol was detected below the LOQ before and after the enzymatic treatment. These results are in accordance with the study made by Del Boccio et al. (2003), who demonstrated that Ol was rapidly metabolized and eliminated.

Miró-Casas et al. (2003b) quantified Hyty and HVAlc in human plasma and urine after real-life doses of VOO. Both compounds appeared rapidly in plasma mainly as glucuronides, with peak concentrations at 30 min for Hyty and 50 min. for HVAlc after the ingestion, supporting the premise that the small intestine is the major site of absorption for these compounds (Vissers et al., 2002).

Most of the studies described so far have centered their attention on Hyty, Ty and Ol derivatives. In a recent work, the absorption and disposition of other olive oil polyphenols (flavonoids and lignans) have been considered (Suárez et al., 2009). Samples were obtained from healthy humans 1 and 2 h after the ingestion of VOO. The major compounds identified and quantified in plasma corresponded to metabolites of Ty, although Ty sulfate was only detected in one subject, and especially Hyty, as glucuronide and sulfate conjugates. HVA sulfate could be the direct product of the Hyty methylation, and vanillin sulfate and vanillic

acid sulfate could be formed as products of alcohol dehydrogenase and aldehyde dehydrogenase activities. Suárez & co-workers also found hydroxybenzoic acid in all the plasma samples. The glucuronide metabolite of apigenin was tentatively quantified in all the samples analyzed, but showing a considerable inter-individual variation. Lignans (pinoresinol and acetoxypinoresinol) could not be detected in the plasma samples even in glucuronide or sulfate conjugated forms.

Once the polyphenols reach the bloodstream, they are able to penetrate tissues, particularly those in which they are metabolized. The nature of the tissular metabolites may be different from that of blood metabolites; data are still very scarce, even in animals, and their ability to accumulate within specific target tissues needs to be further investigated. An article written by D'Angelo et al. (2001) studied the fate of radiolabelled ^{14}C Hyty intravenously injected in rats in different biological fluids (plasma, urine and feces) and tissues (brain, heart, kidney, liver, lung, skeletal muscle and GI content). The pharmacokinetic analysis indicated a fast and extensive uptake of the molecule by the organs and tissues investigated, with a preferential renal uptake. Over 90% of the administered radioactivity was excreted in urine after 5 h and about 5% was detectable in feces and GI content. Less than 8% of the administered radioactivity was still present in the blood stream 5 min after injection. Regarding tissues, the time course analysis indicated that the highest level of radioactivity was detected 5 min after injection, followed by a rapid decrease. It is worth noting that Hyty is able to cross the blood-brain barrier, even though its brain uptake is lower compared with other organs. In all the investigated tissues, Hyty was enzimatically converted in four oxidized and/or methylated derivatives (HVAlc, HVA, DOPAC, DOPAL) and sulfoconjugated derivatives. Enzymatic methylation is presumably operative in the brain, HVAlc representing 41.9% of the detected, labeled species. This reflects the key role of COMT in the central nervous system. The occurrence in the analyzed organs of both labeled DOPAL and DOPAC implies a sequential oxidation of Hyty ethanol side chain catalyzed by alcohol, and aldehyde dehydrogenase, respectively. Labeled HVA, the product of both methylation and oxidation, was also identified. Sulfoconjugated metabolites were mainly found in plasma (43.3%) and urine (44.1%).

As data on plasma concentration of olive oil phenols are still scarce, an alternative is to look at olive oil phenols excreted in urine; these may provide information on the form in which phenols are present in plasma.

2.5 Elimination

The amount and form in which the olive oil phenols are excreted in urine may give an insight into their metabolism in the human body. The first experimental evidence of the absorption of Ty and Hyty from olive oil in humans was obtained by Visioli et al. (2000) from a single oral dose of 50 ml of phenolic-enriched olive oil. The proportions of Hyty and Ty recovered in glucuronidase-hydrolyzed urine, with respect to ingested dose, were in the ranges of 30–60% and 20–22%, respectively. This paper postulated that Hyty and Ty were dose-dependently absorbed in humans and excreted in urine as glucuronide conjugates.

Miró-Casas et al. (2001a) measured the urinary recovery of administered Ty during the 24 h after EVOO ingestion. Maximal Ty values were obtained in the 0-4 h urine samples and

decrease to reach basal values after 8-12 h. Ty was excreted in urine mainly in its glucuro-conjugated form, with only 6–11% excreted in the free form. In a later study (Miró-Casas et al., 2001b), the simultaneous determination of Hyty and Ty in human urine after the intake of VOO was reported. Like the previous study, Hyty and Ty levels in urine rose after VOO consumption, reaching a peak at 0–4 h and returning to basal values at 12–24 h. After hydrolytic treatment, the amount of total compounds recovered in 24 h urine was also determined for Hyty and Ty. Recoveries ranged between 32-98% for Hyty and 12.1-52% for Ty. Both compounds were mainly excreted in conjugated form since only 5.9% Hyty and 13.8% Ty of the total amount excreted were in free form. The hydrolysis procedure applied in this study was limited because it did not provide specific information about the type of conjugation involved. This paper also postulated that Ol is not the main source of Hyty after ingestion of olive oil. The absorption of Hyty and Ty was later confirmed in an experiment using single and sustained doses of VOO (Miró-Casas et al., 2003a). Urinary recoveries of Ty were similar for both cases; however, mean recovery values for Hyty after ingestion of 25 ml/day VOO for one week, were 1.5-fold of those obtained after a 50 ml single dose.

Vissers et al. (2002) studied the absorption of Hyty, Ty and, for the first time, Ol and Lig Agl, in ileostomy subjects and in volunteers with a colon. The results showed that 55-66% of the ingested olive oil phenols were absorbed in ileostomy subjects, which implies that most phenols are absorbed in the small intestine. Excreted phenolics, mainly in the form of Hyty and Ty, were determined to be 5–16% of the total ingested. Similar levels of Hyty and Ty were found in the urine of subjects with and without a colon, confirming that olive oil phenols are absorbed mainly in the small intestine. The obtained values, lower than those reported by others, could be underestimations because metabolites of olive oil phenols were not considered. In this work it is also suggested that an important step in the metabolism of the Ol glycoside and Ol and Lig-aglycones is their transformation into Hyty or Ty. This was supported by finding that 15% of an Ol glycoside supplement administered to healthy human subjects was excreted in urine as Hyty and Ty.

Tuck et al. (2001) investigated the *in vivo* fate of tritium labeled Hyty and Ty after intravenous (in saline, tail vein) and oral dosing (in oil- and water-based solutions) to rats. For both Hyty and Ty, the elimination of radioactivity in urine within 24 h for the intravenously and orally administered oil-based dosing was significantly greater (95 and 75%, respectively) than the oral aqueous dosing method (74 and 53%, respectively). The majority of the excreted dose was eliminated from the body within 2 h, when intravenously dosed, and within 4 h for both oral dosings. Later, urine samples collected after 24 h were re-examined (Tuck et al., 2002). After oral oil dosing Hyty represented 4.10% of compound eliminated, monosulfate conjugate 48.42%, glucuronide conjugate 9.53%, HVA 10.26% and other possible metabolites 20.27%. Other study with rats supplemented with Ol (Ol rats) or with EVOO (EVOO rats) was developed for the simultaneous determination of Hyty, Ty and EA in rat urine (Bazoti et al., 2005). The urinary levels of free Ty and Hyty were higher in EVOO rats than in Ol rats. When the urine sample were treated with β-glucuronidase, the total amount of metabolites measured for the EVOO rat was higher for Ty but lower for Hyty than in Ol rats. EA was not detected, probably because of its further metabolism to simpler molecules. Nevertheless, as already mentioned, caution should be taken interpreting the results achieved from rats (Visioli et al., 2003).

The urinary excretion of HVAlc and HVA in humans was reported for the first time by Caruso et al. (2001) after the intake of different VOOs. HVAlc contributes to 22% of the total excretion of Hyty and its metabolites, and HVA 56%. The excretion of both metabolites correlated with the administered dose of Hyty. Even at low doses, HVAlc and HVA were excreted. In a later study, Miró-Casas et al. (2003b) observed how urinary amounts of Hyty and HVAlc increased in response to VOO ingestion, reaching the maximum peak at 0-2 h. Urinary recovery 12 h after olive oil ingestion was rather different depending on the hydrolytic treatment applied. Under acidic conditions recoveries were higher for both Hyty and HVAlc than with enzymatic hydrolysis. It was apparent that 65% of Hyty was in its glucuronoconjugated form and 35% in other conjugated forms.

To understand the impact of glucuronidation on the metabolic pathway of olive oil phenols, the simultaneous determination of Ty, Hyty, HVAlc and their corresponding *O*-glucuronides in human urine was carried out by Khymenets et al. (2011). It was the first time that the glucuronides of these compounds have been directly identified and quantified in urine samples of volunteers supplemented with EVOO, because previous methods measured either free or total phenols after hydrolysis. The maximum excretion of Hyty and Ty occurred during the first 6 h after administration, which is in agreement with earlier reported data. The free Ty and Hyty, as well as HVAlc, were detected at significant concentrations in all urine samples collected 6 h after EVOO acute intake. Concentrations of *O*-glucuronide metabolites (4-*O*-gluc-Ty, 4-*O*-gluc-Hyty, 3-*O*-gluc-Hyty, and 4-*O*-gluc-HVAlc) were substantially higher in 6 h postprandial samples when compared to their parent compounds. About 13% of the consumed olive oil polyphenols were recovered in 24 h urine, 75% of which were in the form of glucuronides and 25% as free compounds.

In another recent work, specific information about the type of conjugates in human urine was provided (García-Villalba et al., 2010). The authors were able to indentify more than 60 metabolites. This was the first report in which metabolites of simple phenols, flavonoids, lignans and secoiridoids have been found in human urine samples. Phenolic compounds were subjected to different phase I (hydrogenation, hydroxylation, methylation) and phase II (mainly glucuronidation) reactions. Ten metabolites were identified as possible biomarkers of olive oil intake and their levels in urine after the olive oil ingestion were monitored, finding the highest level of most of them 2 h after administration.

In summary, data on urinary excretion indicate that at least 5% of ingested olive oil phenols is recovered in urine mainly as glucuronidated Ty and Hyty. The remaining phenols are metabolized into other compounds, such as *O*-methylated Hyty. Monosulfate conjugates might be other metabolites, as shown in two rat studies; however, if olive oil phenols are also metabolized into these conjugates in humans remains to be elucidated.

3. Need and difficulties of carrying out bioavailability studies of polyphenols

In this section, we will discuss some common mistakes that can be made when bioavailability studies are carried out, the difficulties that the analyst can find and the limitations of some of the studies made so far. **Figure 1** gives a general idea of the most important topics commented in the section.

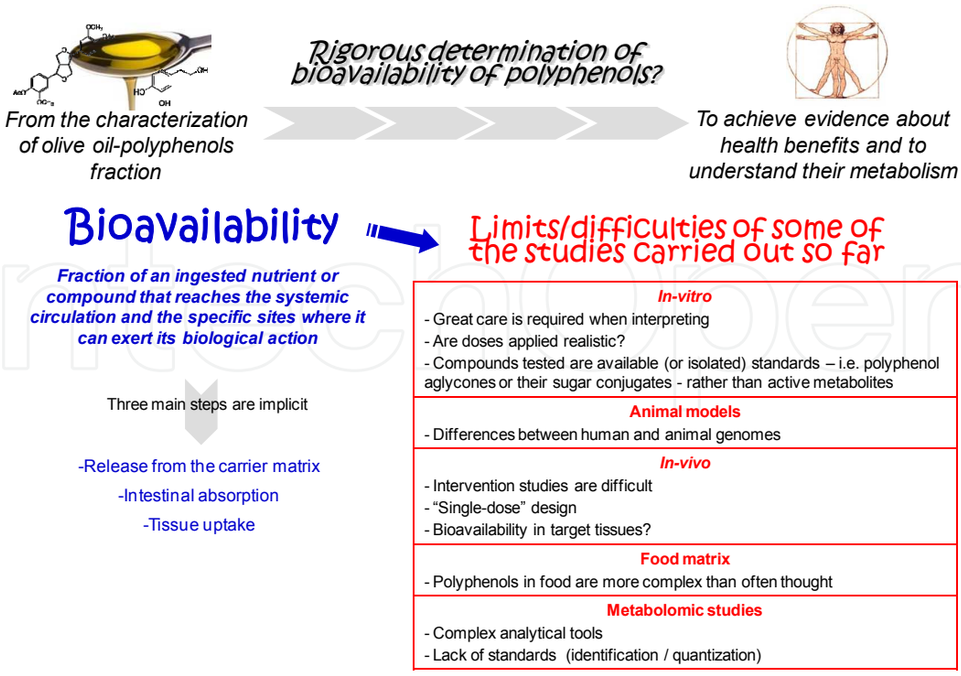

Fig. 1. Definition of bioavailability and the limits affecting bioavailability studies of polyphenols

Since intervention studies are very difficult to carry out, in many cases the researchers have to turn to *in vitro* or animal studies. *In vitro* studies are a pillar of pharmacological research and build the bases for future *in vivo* assays; however, the interpretation and extrapolation of the achieved data have to be made very carefully (Kroon et al., 2004). When the biological activity of polyphenols is assessed by using culture cells as tissue models, in most of the cases, cells are treated with aglycones or polyphenols-rich extract derived from plants or, in this case, from olive oil, and data are reported at concentrations which elicited a response. It is absolutely necessary to bear in mind that plasma and tissues are not exposed *in vivo* to polyphenols in these forms: the molecular forms reaching the peripheral circulation and tissues are different from those present in the olive oil (Day et al., 2001). Moreover, the polyphenols concentration tested should be of the same order as the maximum plasma concentration attained after a polyphenol-rich meal (0.1-10 µmol/l).

Matters of practicality determine the use of rats rather than humans as the model of choice for *in vivo* studies, although interspecies variability renders comparisons between the model species (animals, humans) complex and sometimes questionable (Visioli et al., 2003), since the rats and rodents in general are not the best model for the study of dietary problem of human metabolism.

When *in vivo* studies are carried out, we can say that most of researches have investigated the kinetics and extent of polyphenol absorption by measuring plasma concentrations and/or urinary excretion among adults after the ingestion of a single dose of polyphenol, provided as pure compound, plant extract, or whole food/beverage. Using this "single-dose" design, the increase in the blood concentration is transitional and reflects mainly the ability of the organism to take up the polyphenol from the food matrix. Consequently, most

of the data from humans presented in the literature on the bioavailability refer only to the release of the polyphenols from the food matrix and their consequent absorption (D'Archivio et al., 2010; Vissers et al., 2004).

To address the bioavailability of olive oil phenols, we should exclude studies without a control diet and studies in which the amount of ingested phenols is not reported or could not be estimated (Miró-Casas et al., 2001a; Visioli et al., 2000a; Vissers et al., 2004). In other words, it is essential to characterize in depth the polyphenolic extract of the olive oil before starting bioavailability studies to assure their usefulness; since this fraction is quite complex and heterogeneous, it represents another requirement which difficults the whole process.

Advances in the understanding of olive oil polyphenols metabolism have been made possible by improvements in the analytical methodologies used, particularly high-resolution chromatographic systems with mass spectrometry as detector (Bai et al., 1998; Del Boccio et al., 2003; García-Villalba et al., 2010; Khymenets et al., 2011; Miró-Casas et al., 2003b). Performing metabolomic studies is challenging and requires measurements of a very high quality using powerful platforms. Even if the analyst uses proper tools, the fully structural assignment of the metabolites under study is sometimes very difficult due to the lack of the metabolite standards; fact which makes difficult the correct quantification too (D'Archivio et al., 2010; García-Villalba et al., 2010). The amount of information about the sample under study achieved in metabolomic studies is considerable, that is why for meaningful interpretation the appropriate statistical tools must be employed to manipulate the large raw data sets in order to provide understandable and workable information (Manach et al., 2009).

A very interesting review written by D'Archivio et al. (2010) gives a critical overview about the difficulties and the controversies surrounding the studies aimed at determining the bioavailability of polyphenols. Summarizing, there are some essential steps to be followed to establish conclusive evidences for the effectiveness of polyphenols in disease prevention and in human health improvement: 1) determination of the distribution of these compounds in our diet, estimating their content in each food; 2) identification of which of the existing polyphenols are likely to provide the greatest effects in the context of preventive nutrition, and 3) assesment of the bioavailability of polyphenols and their metabolites, to evaluate their biological activity in target issues.

Even though the bioavailability studies are properly designed, we have to be aware of how many different endogenous and exogenous variables are involved and the difficulties that have to be faced. The main factors recognized as affecting olive oil polyphenols bioavailability can be grouped in the following categories: factors related to the polyphenol characteristics, food/food processing related factors, external factors and factors related to the host, as it can be observed in **Figure 1.** An in-depth discussion of every factor influencing the bioavailability of olive oil polyphenols has been made by Manach et al. (2004) and Cicerale et al. (2009).

4. Conclusions

To explore and understand the mechanism of action of olive oil polyphenols and their role in disease prevention and human health improvement, extensive studies of absorption, metabolism, excretion, toxicity, and efficacy are needed. Although *in vitro* studies can be

very useful and provide valuable information, they have to be completed with extensive *in vivo* research. The first requirement for a beneficiary dietary compound is that it enters into the blood circulation; therefore to demonstrate *in vivo* effects of olive oil phenolics it is necessary to assess first their *bioavailability*.

Analysis of plasma and urine provide valuable information on the identity and pharmacokinetics of circulating metabolites after ingestion. Since the metabolites sequestered in body tissues are not usually taken into account, results from urine samples could be an underestimation. There have been several studies which have determined the metabolites of the various olive oil polyphenols (mainly Hyty, Ty, and Ol) in human plasma and urine after oral intake, although the information is still scarce. The conjugation mechanisms that occur in the small intestine and later in the liver are highly efficient. The resulting metabolites are mainly glucuronate and sulfate conjugates with or without methylation across the catechol group (many are multiply conjugated).

Bioavailability studies are gaining increasing interest as food industries are continually involved in developing new products, defined as ''functional'' by virtue of the presence of specific antioxidants or phytochemicals. The difference between functional foods and medicines calls for moderation when the "medicinal" properties of individual food items, be it olive oil, are indicated. The correct message should be to select foods whose components have proven, albeit limited in magnitude, biological activities and build a balanced diet round them, to reduce several chronic diseases.

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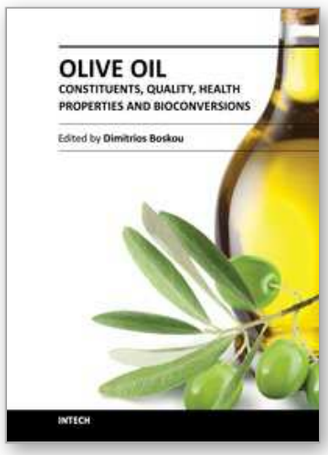

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The health-promoting effects attributed to olive oil, and the development of the olive oil industry have intensified the quest for new information, stimulating wide areas of research. This book is a source of recently accumulated information. It covers a broad range of topics from chemistry, technology, and quality assessment, to bioavailability and function of important molecules, recovery of bioactive compounds, preparation of olive oil-based functional products, and identification of novel pharmacological targets for the prevention and treatment of certain diseases.

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