

## Bioavailability of phenols from a phenol-enriched olive oil

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### Abstract

Phenolic compounds are one of the main reasons behind the healthy properties of virgin olive oil (VOO). However, their daily intake from VOO is low compared with that obtained from other phenolic sources. Therefore, the intake of VOO enriched with its own phenolic compounds could be of interest to increase the daily dose of these beneficial compounds. To evaluate the effectiveness of enrichment on their bioavailability, the concentration of phenolic compounds and their metabolites in human plasma (0, 60, 120, 240 and 300 min) from thirteen healthy volunteers (seven men and six women, aged 25 and 69 years) was determined after the ingestion of a single dose (30 ml) of either enriched virgin olive oil (EVOO) (961.17 mg/kg oil) or control VOO (288.89 mg/kg oil) in a cross-over study. Compared with VOO, EVOO increased plasma concentration of the phenol metabolites, particularly hydroxytyrosol sulphate and vanillin sulphate ( $P < 0.05$ ). After the consumption of VOO, the maximum concentration of these peaks was reached at 60 min, while EVOO shifted this maximum to 120 min. Despite these differences, the wide variability of results indicates that the absorption and metabolism of olive oil phenols are highly dependent on the individual.

**Key words:** Bioavailability; Human plasma; Phenol-enriched olive oil; Plasma phenol metabolites

Over the last decade, epidemiological studies have proven that a Mediterranean diet, rich in vegetables, fruit and legumes, is correlated with a low incidence of CHD and cancer<sup>(1)</sup>. Within this nutritional pattern, virgin olive oil (VOO) plays an important role, being the main source of fat in the diet in this area. The beneficial effects of VOO were initially attributed to its fatty acid composition, rich in MUFA. Nevertheless, further studies have highlighted the importance of its phenolic composition, especially after the publication of the results of the EUROLIVE project, which demonstrated that the increase in the HDL-cholesterol level and the decrease in the lipid oxidation damage after VOO consumption were highly correlated with its phenolic content<sup>(2,3)</sup>. Thus, phenolic compounds from VOO (mainly phenolic alcohols, secoiridoid derivatives, phenolic acids, lignans and flavonoids) have been reported to have anti-oxidant<sup>(4)</sup>, anti-inflammatory<sup>(5)</sup>, anti-atherogenic<sup>(6)</sup> and anti-carcinogenic properties<sup>(7)</sup>.

The metabolic fate of phenolic compounds after ingestion has been the subject of study by the scientific community to find out the mechanisms through which they exert their

activity into the organism. It is known that polyphenols suffer strong metabolism phase I and phase II, in which they are hydrolysed (phase I) and later conjugated (phase II) into their glucuronidated, methylated and sulphated forms in order to be absorbed<sup>(8)</sup>. In the case of VOO, studies have focused on some specific compounds such as hydroxytyrosol and tyrosol as they have been reported as the most biologically active. Thus, Visioli *et al.*<sup>(9)</sup> were the first researchers who identified these compounds in urine, both in the conjugated and in their free forms. In addition, this study showed that hydroxytyrosol and tyrosol are dose-dependently absorbed in humans. Later, the same research group reported the presence of homovanillic alcohol in urine, a compound that is derived from hydroxytyrosol by the action of the enzyme catechol-*O*-methyltransferase<sup>(10)</sup>. On the other hand, the study by Miró-Casas *et al.*<sup>(11)</sup> reported the presence of conjugated forms of hydroxytyrosol in plasma. The importance of these compounds has been reinforced by the demonstration that they can aid in preventing the oxidation of LDL<sup>(12)</sup>. Recently, an association between

**Abbreviations:** 3,4-DHPEA-EDA, dialdehydic form of elenolic acid linked to hydroxytyrosol; EVOO, enriched virgin olive oil; MOPET, 3-methoxy-4-hydroxy-phenylethanol; UPLC, ultra performance liquid chromatography; VOO, virgin olive oil.

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hydroxytyrosol and LDL in plasma has been described, a fact that could have important physiological implications<sup>(13)</sup>. All these studies demonstrated the importance of hydroxytyrosol and tyrosol; however, little has been done about the other components of the phenolic fraction of VOO, which have also been shown to have high biological activity such as the dialdehydic form of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA)<sup>(14)</sup>.

More recent studies have indicated that the beneficial effects of the phenolic compounds from VOO are not only due to their anti-oxidant and related activities, but they also have a nutrigenomic effect, which means that their intake has a direct impact on the human genome. Thus, a study carried out by Konstantinidou *et al.*<sup>(15)</sup> showed that the phenols from VOO have a significant role in the down-regulation of proatherogenic genes such as *IFN-γ*, *ARHGAP15* and *IL7R*. This reinforces the importance of including VOO in the regular diet (the Food and Drug Administration recommends a daily intake of 23 g of olive oil).

Nevertheless, the amount of phenolic metabolites from VOO detected in human plasma samples is highly variable and depends on both extraction and analytical procedure. Thus, Vissers *et al.*<sup>(16)</sup> reported that the bioavailability of these compounds in ileostomized volunteers was up to 66% of the total ingested. Considering a mean intake of 50 g of VOO/d, these authors estimated that the total amount of phenolic compounds in the plasma samples could be at most 0.06 μmol of hydroxytyrosol equivalents/l plasma. However, some studies revealed that a higher concentration is required to protect LDL from oxidation. For example, Leenen *et al.*<sup>(17)</sup> concluded that at least 50–100 μmol of phenolic compounds from VOO/l plasma are required to have a protective effect on LDL. Therefore, according to these results, enough phenols are unlikely to be obtained from VOO to have a direct protective effect on LDL. In addition, the concentration of phenolic compounds in commercial VOO is widely dependent on agronomical and technical factors<sup>(18)</sup>. This decreases the ingestion of phenols from VOO compared with the doses ingested from other sources and makes it difficult to ensure a regular dose of phenols through the daily intake of VOO. For this reason, the enrichment of VOO with their own phenols, thus giving a high and standardised content of phenolic compounds, could be desirable in order to increase the daily intake of phenols without the drawback of a higher energy content.

The aim of the present study was to evaluate the effect of the enrichment of VOO with their own phenolic compounds in their bioavailability in human subjects. To fulfil this objective, the phenol metabolites were identified and quantified in human plasma samples obtained after an acute ingestion of 30 ml of a phenol-enriched VOO (EVOO). Plasma samples were obtained at 0, 60, 120, 240 and 300 min in order to study the pharmacokinetic behaviour of these metabolites. The results were compared with those obtained after the ingestion of the VOO (without phenol addition).

## Experimental methods

### Chemicals and reagents

Standard phenolic compounds were used to obtain calibration curves to quantify the compounds in the olive oils and in the plasma samples. Apigenin, luteolin, hydroxytyrosol, tyrosol and *p*-coumaric acid were purchased from Extrasynthese (Genay, France). Caffeic and homovanillic acids were purchased from Fluka Company (Buchs, Switzerland). (+)-Pinoresinol was acquired from Arbo Nova (Turku, Finland), and catechol was from Sigma-Aldrich (Schnellendorf, Germany). The secoiridoid derivatives 3,4-DHPEA-EDA, the dialdehydic form of elenolic acid linked to tyrosol and the lignan acetoxypinoresinol are not available commercially, and were isolated from virgin olive by semi-preparative HPLC, as described by Artajo *et al.*<sup>(19)</sup>. A stock solution of each standard compound was dissolved in methanol, and all the solutions were stored in a dark flask at  $-40^{\circ}\text{C}$ .

Methanol (HPLC grade), acetonitrile (HPLC grade) and acetic acid were provided by Scharlau Chemie (Barcelona, Spain). *Ortho*-phosphoric acid (85%) was purchased from Panreac (Barcelona, Spain). Water was MilliQ quality (Millipore Corporation, Bedford, MA, USA).

### Virgin olive oil

The VOO used as control and as matrix for phenolic enrichment was from the Siurana Protected Denomination of Origin (Catalonia, Spain), which is produced exclusively from the Arbequina cultivar. On the other hand, the samples of the olive cake used to obtain the phenolic extract for phenol enrichment were taken from a commercial olive oil mill from the olive-growing area of Les Garrigues (Catalonia, Spain), which works by the two-phase centrifugation system. These samples were taken at the decanter outlet, and liquid N<sub>2</sub> was immediately added to avoid oxidative damage. The samples were stored at  $-40^{\circ}\text{C}$  until the extraction of the phenolic compounds.

### Preparation of the phenol-enriched olive oil

The EVOO was prepared by the addition of an extract rich in the main phenolic compounds of VOO. This extract was obtained from the olive cake following the method described by Suárez *et al.*<sup>(20)</sup> and basically included secoiridoid derivatives (89.4%), phenyl alcohols (3.5%) and flavonoids (6.0%).

To carry out phenolic enrichment of the oil, 7 mg of olive cake phenolic extract/ml oil and 0.3% (p/v) of lecithin (Emulpur; Cargill, Barcelona, Spain) were dissolved in ethanol–water (50:50, v/v) and added to the oil using a Polytron (Kinematica, Littau, Switzerland) until completely homogenised. Finally, the EVOO was filtered through a paper filter Ahlstrom (Ahlstrom S.A., Barcelona, Spain) and bottled in dark bottles. The VOO used as control (without added phenolic compounds) was also submitted to the process of mixing and filtering to ensure equal conditions in all the olive oils under study. The phenolic compounds of both the VOO and the EVOO were extracted and characterised with the method described in our previous study<sup>(21)</sup>.

### Volunteers and experimental design

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Ethics Committee of Clinical Research of Sant Joan University Hospital, Reus, Spain (Trial Registration clinicaltrials.gov Identifier: ISRCTN03450153). The subjects gave written informed consent. A total of sixteen healthy volunteers (eight women and eight men) were recruited to carry out the study. However, at the moment of ingestion, three of the volunteers (two women and one man) gave up the study, thus reducing the final number of volunteers to thirteen. The volunteers, aged between 25 and 69 years, were considered healthy according to the results obtained after physical examination and routine laboratory tests. The men had a mean weight of 78.3 (SD 13.0) kg and the women had a mean weight of 64.5 (SD 5.7) kg, while the BMI was 26.7 (SD 1.9) kg/m<sup>2</sup> for men and 24.9 (SD 1.5) kg/m<sup>2</sup> for women.

A randomised, controlled, cross-over trial was designed using VOO (without phenol addition) and EVOO. Thus, the volunteers who consumed VOO in the first intervention ingested the EVOO in the second intervention after a 2-week washout period (Fig. 1) and *vice versa*, volunteers who consumed the EVOO in the first intervention ingested the VOO in the second intervention. The participants were instructed to follow a stabilisation diet with 10% of SFA during the week before the postprandial test and the day before a polyphenol free diet was prescribed. Between the tests, the volunteers followed the stabilisation diet. The recommendations were to avoid VOO, olives, fresh fruits or juices, wine, chocolate, coffee, tea, soya, legumes and beer. The participants were instructed to perform intense physical exercise 3 d before the postprandial test.

Acute ingestion of the oil was done at 08.00 hours after an overnight fast. The VOO was included in the study to enable the comparison of the resulting data and to extract conclusions.

### Plasma samples

Plasma samples were obtained by venepuncture from volunteers after the ingestion of 30 ml of either VOO or EVOO. The blood samples were collected under basal conditions

(after a 12 h overnight fasting period) and 60, 120, 240 and 300 min after the consumption of 30 ml of either enriched or control VOO. To obtain plasma samples at every measuring point, blood (50 ml) was collected into Vacutainer™ tubes containing EDTA as an anti-coagulant. They were protected from light with Al foil and centrifuged for 15 min at 1500 *g* and 4°C (H-103RS; Kokusan, Tokyo, Japan). After that, the plasma was immediately separated from the cells and kept at -80°C until analysis.

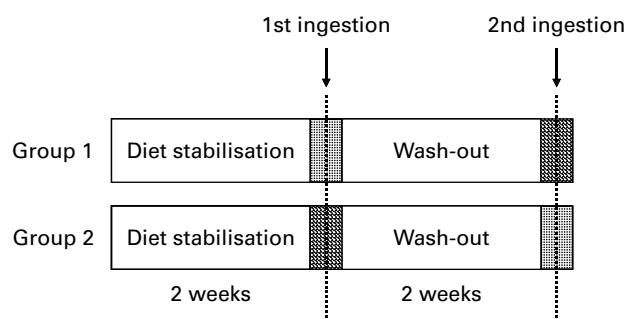
The extraction of the phenolic compounds from the plasma samples was carried out following the method developed and validated in our previous study<sup>(22)</sup> using microelution plates (Waters, Milford, MA, USA) packed with 2 mg of OASIS HLB sorbent (Waters).

### Analysis of phenolic compounds in olive oils and plasma samples

The analysis of the phenolic compounds from both oils (VOO and EVOO) and plasma samples was carried out by ultra performance liquid chromatography (UPLC) coupled to tandem MS. The UPLC system consisted of an AcQuity™ UPLC equipped with a Waters binary pump system using an AcQuity UPLC™ BEH C<sub>18</sub> column (1.7 μm, 100 mm × 2.1 mm inner diameter). During the analysis, the column was kept at 30°C and the flow rate was 0.4 ml/min, using MilliQ water-acetic acid (100:0.2, v/v) as solvent A and acetonitrile as solvent B. The elution gradient was the one reported in our previous study<sup>(21)</sup>.

Full-scan mode MS and MS/MS, based on neutral loss scan and product ion scan, were used to identify and quantify the phenolic compounds and their metabolites from plasma samples. These techniques are very effective in verifying the structural information of the compounds when no commercially standards are available. To start with, the analyses were carried out in full-scan mode (from 80 to 800 *m/z*) by applying different cone voltages from 20 to 60 V. The MS spectrum obtained when low cone voltages were used gave information about the precursor ion or the [M - H]<sup>-</sup>. On the other hand, when high cone voltages were applied, specific fragment ions were generated and the MS spectrum gave information about their structure. The structural information was also verified by using the product ion scan and neutral loss scan in the MS/MS mode. In the product ion scan experiments, the product ions are produced by collision-activated dissociation of the selected precursor ion in the collision cell. Neutral loss scans of 80 and 176 units were used to characterise the sulphate and glucuronide forms, respectively. The detection and quantification of the phenolic compounds and their metabolites were then performed based on their ion fragmentation in the MS/MS mode using selected reaction monitoring (SRM) and double SRM.

Due to the lack of standards for these metabolites, they were quantified with the calibration curves corresponding to their phenolic precursors. Hydroxytyrosol and tyrosol metabolites were quantified using the calibration curves of hydroxytyrosol and tyrosol, respectively; homovanillic acid, vanillic acid and vanillin metabolites were quantified using the



**Fig. 1.** Study design of the acute ingestion study of control and phenol-enriched olive oil. □, 30 ml of control olive oil; ▨, 30 ml of phenol-enriched olive oil.

calibration curve of homovanillic acid; *p*-coumaric acid, *p*-hydroxybenzoic acid and ferulic acid metabolites were quantified by means of the *p*-coumaric acid calibration curve. Apigenin metabolites were quantified with the apigenin calibration curve.

### Statistical analysis and pharmacokinetic parameters

Values are presented as means and standard deviations. Data analysis was performed using Statgraphics plus v.5.1 software (Manugistics, Inc., Rockville, MA, USA). The normality of the data was evaluated by means of the values of the coefficients of asymmetry and kurtosis. Significant differences among the results from the two olive oils were evaluated by the Kruskal–Wallis ANOVA ( $P < 0.05$ ).

On the other hand, kinetic parameters of the different metabolites of the phenolic compounds were calculated by means of pharmacokinetic (PK) functions (for Microsoft Excel). This software allowed us to determine the maximum concentration of the metabolites ( $C_{max}$ ) and their area under the curve, which can be used to compare the effect of the ingestion of the different oils (control or phenol-enriched).

## Results and discussion

### Characterisation of the virgin olive oils

The phenolic profiles of both VOO and EVOO were analysed by UPLC-electrospray ionisation-MS/MS. As can be seen in Table 1, the addition of the olive cake extract significantly

**Table 1.** Phenolic composition of the control and phenol-enriched olive oil determined by ultra performance liquid chromatography (UPLC)-electrospray ionisation (ESI)-MS/MS

(Mean values and standard deviations)

Phenolic compounds	Control virgin olive oil (mg/kg oil)		Phenol-enriched olive oil (mg/kg oil)	
	Mean	SD	Mean	SD
Hydroxytyrosol	0.37	0.06	6.64	0.64
Tyrosol	1.03	0.09	8.70	2.00
Total phenyl alcohols	1.40		15.3	
Vanillic acid	0.37	0.02	3.94	0.18
<i>p</i> -Coumaric acid	0.08	0.02	0.84	0.11
Vanillin	0.16	0.01	1.44	0.15
Total phenolic acids	0.61		6.21	
3,4-DHPEA-EDA	45.4	9.29	527.8	30.55
3,4-DHPEA-EA	15.1	0.97	26.2	5.58
3,4-DHPEA-AC	52.8	11.28	102.5	18.26
<i>p</i> -HPEA-EDA	9.72	1.03	24.1	4.14
Total secoiridoid derivatives	123.1		680.6	
Pinoresinol	115.8	10.19	173.1	14.18
Acetoxypinoresinol	46.3	5.91	78.8	9.39
Total lignans	162.1		251.9	
Luteolin	1.44	0.26	6.28	0.25
Apigenin	0.27	0.01	0.80	0.04
Total flavonoids	1.71		7.08	
Total phenolic compounds	288.9		961.2	

3,4-DHPEA-EDA, dialdehydic form of elenolic acid linked to hydroxytyrosol; 3,4-DHPEA-EA, oleuropein aglycone; 3,4-DHPEA-AC, 4-(acetoxylethyl)-1,2-dihydroxybenzene; *p*-HPEA-EDA, dialdehydic form of elenolic acid linked to tyrosol.

increased the concentration of all the phenolic compounds in the oils. The 10.95-fold increase experimented by the phenyl alcohol fraction, made up of hydroxytyrosol and tyrosol, was especially remarkable. These compounds are biologically active, so it is very interesting to have them at a higher and optimal concentration in the olive oil available to be absorbed by the organism after ingestion.

It is also important to highlight the increase observed in the secoiridoid derivatives and the flavonoid group. The importance of the secoiridoid derivatives is based on their chemical structure. These compounds are precursors of phenyl alcohols, which, as was stated earlier, are very active compounds. In addition, recent studies have demonstrated that some secoiridoid derivatives, such as 3,4-DHPEA-EDA, are even better at protecting erythrocytes from oxidative damages than hydroxytyrosol<sup>(23)</sup>. Therefore, the secoiridoid derivatives are valuable compounds that could have a beneficial effect on the organism. On the other hand, flavonoids are the most widespread natural phenolic compounds whose important activity is due to their characteristic triple aromatic ring chemical structure. However, their concentration in VOO is very low compared with that obtained from other phenolic sources. Thus increasing the amount of flavonoids in olive oils is of interest to increase their intake from this source.

Therefore, the greater concentration of phenolic compounds in the EVOO is expected to increase the level of their corresponding metabolites in human plasma after the consumption of the oils due to the higher amount available to be absorbed by the organism.

### Phenol metabolite identification and quantification in human plasma

Phenolic compounds and their corresponding metabolites in the plasma samples after ingestion of either VOO or EVOO were identified and quantified following the procedure described by Suárez *et al.*<sup>(22)</sup>. Table 2 shows the main chromatographic peaks that appeared in the chromatograms. On the basis of their retention time, corresponding molecular weight and ion transition, and the information obtained from the literature, we proposed which phenolic compounds and metabolites could match these peaks. Some of the compounds had more than one chromatographic peak, appearing at different retention times but maintaining the molecular weight and the mass spectrometric fragments. This suggests the existence of several isomeric forms of these compounds in the plasma samples.

As expected, most of the metabolites identified in the plasma were derived from the secoiridoids and phenyl alcohol groups and appeared in their conjugated form, especially sulphated and glucuronidated. This could suggest that basically the phenolic compounds from olive oil follow two different pathways: one mediated by the action of the sulphotransferase enzymes and the other governed by the glucurotransferases. This agrees with the results obtained by other authors, namely De la Torre-Carbot *et al.*<sup>(24)</sup> who identified hydroxytyrosol and tyrosol in their glucuronidated and sulphated forms in human LDL after the intake of

**Table 2.** Chromatographic parameters and proposed compound of the main chromatographic peaks observed in the plasma samples after ingestion of the olive oils (control and phenol-enriched)

Peak	Retention time (min)	[M – H] <sup>–</sup>	Fragments MS/MS	Proposed compound
1	3.62; 10.65	137	93	Hydroxybenzoic acid
2	4.63; 6.52	151	107	Hydroxyphenylacetic acid
3	6.55	151	136	Vanillin
4	4.85	167	123	Vanillic acid
5	5.17	179	135	Caffeic acid
6	17.1	181	137	Homovanillic acid derivative
7	10.14	197	153	3,4-DHPEA-EA derivative
8	8.55; 19.15	217	137	Tyrosol sulphate
9	5.06; 7.98; 12.45	230	150	4-Methoxy-phenylethanol sulphate
10	9.32; 14.11	231	151, 136	Vanillin sulphate
11	6.15	233	153, 123	Hydroxytyrosol sulphate
12	6.67; 13.5; 21.75	243	163, 119	Coumaric acid sulphate
13	11.77; 16.86; 19.3	245	165	4-Methoxy-phenylacetic acid sulphate
14	10.05	247	167, 123	Vanillic acid sulphate
15	19.15; 23.83	259	179, 135	Caffeic acid sulphate
16	9.48	261	181	Homovanillic acid sulphate
17	11.27	273	193, 134	Ferulic acid sulphate
18	4.56; 6.74	327	150	4-Methoxy-phenylethanol glucuronide
19	8.77; 18.97	327	151, 136	Vanillin glucuronide
20	7.00	369	193, 134	Ferulic acid glucuronide
21	10.57	373	197	3,4-DHPEA-EA derivative glucuronide
22	15.15	445	269	Apigenin glucuronide
23	10.69; 13.24	495	319, 153	3,4-DHPEA-EDA glucuronide

3,4-DHPEA-EA, oleuropein aglycone; 3,4-DHPEA-EDA, dialdehydic form of elenolic acid linked to hydroxytyrosol.

VOO. Thus, these conjugated forms are good biomarkers of phenolic compounds of VOO.

The chromatographic analysis of the plasma samples also revealed the existence of other compounds (either conjugated or in their free form), including phenolic acids (hydroxybenzoic, hydroxyphenylacetic, ferulic and caffeic acids and their derivatives) and flavonoids (apigenin glucuronide).

With regard to the metabolites derived from hydroxytyrosol, the appearance of compounds structurally related to homovanillic acid suggests the existence of a methylation process in the organism, probably due to the activity of the enzyme catechol-*O*-methyltransferase. D'Angelo *et al.*<sup>(25)</sup> proposed a metabolic pathway for hydroxytyrosol in which the action of this enzyme promoted the appearance of homovanillic acid and its conjugated forms. In the case of the plasma samples obtained after the ingestion of both VOO and EVOO, we were able to identify the metabolites that appear at the end of this chain of reactions: hydroxytyrosol sulphate, homovanillic acid derivative and homovanillic acid sulphate. Homovanillic acid derivative was a compound that presented the same precursor and product ions as homovanillic acid but eluted at a different retention time from the commercial standard. The non-identification of free hydroxytyrosol and the other intermediate forms may suggest that these compounds are rapidly subjected to the activity of the enzymes, originating in the sulphated or methyl-sulphated forms, which are more likely to be absorbed by the organism. Fig. 2 shows the metabolic pathway of hydroxytyrosol proposed by D'Angelo *et al.*<sup>(25)</sup> with some variations, indicating in bold the metabolites identified in the plasma samples after the ingestion of olive oil.

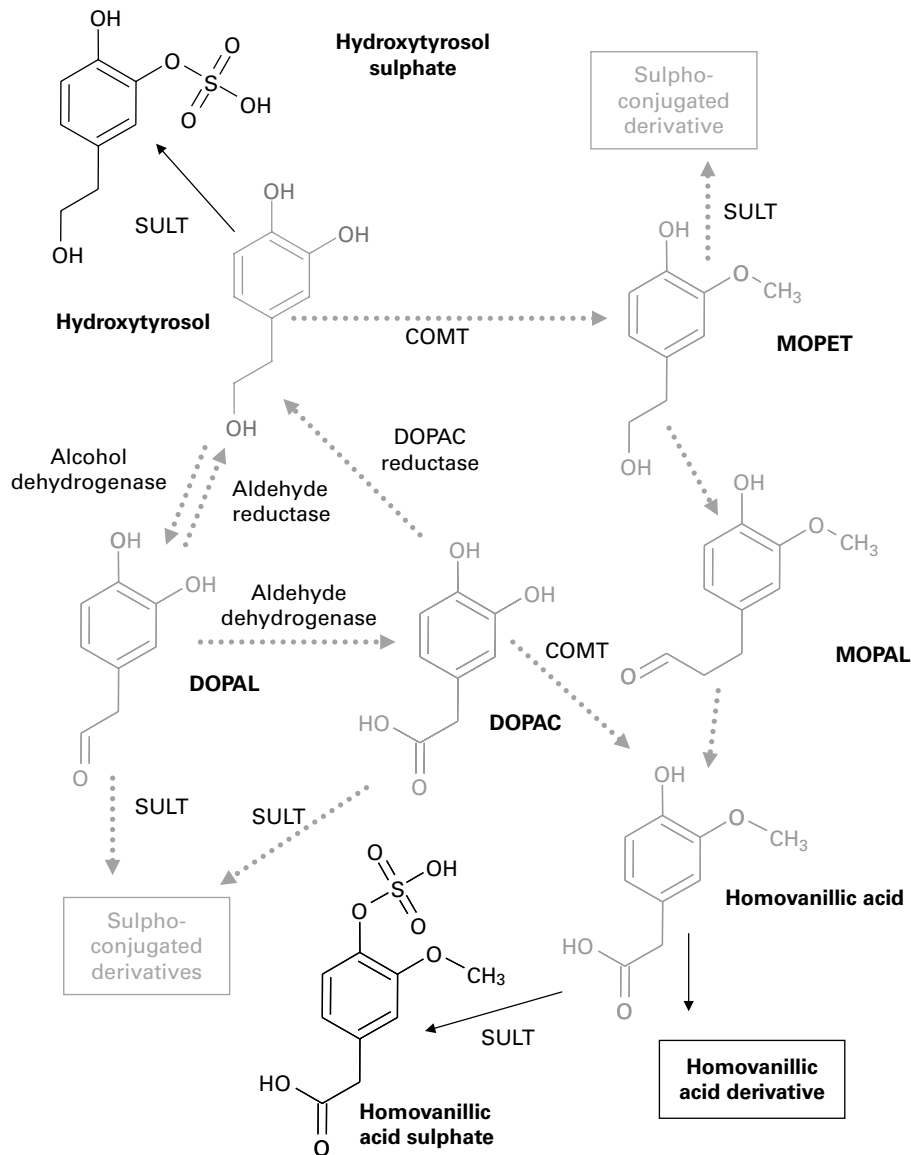
Similar to the metabolic pathway described in hydroxytyrosol, the analysis of the plasma samples revealed the existence

of some methylated metabolites that probably originated from tyrosol. Thus, peaks 9, 13 and 18 given in Table 2 could be formed from tyrosol, with an analogous chemical structure to the one obtained as a result of the activity of catechol-*O*-methyltransferase on hydroxytyrosol. Peak 9 is equivalent to 3-methoxy-4-hydroxy-phenylethanol (MOPET) sulphate, peak 13 is equivalent to the homovanillic sulphate and peak 18 was the result of the glucuronidation of the analogue structure of MOPET in tyrosol.

These results contrast with those obtained by García-Villalba *et al.*<sup>(26)</sup> who did not identify methyl conjugate metabolites from tyrosol in urine samples after the ingestion of 50 ml of VOO. They attributed this to the lack of activity of catechol-*O*-methyltransferase on this compound due to the need for an ortho-diphenolic structure of this enzyme. The appearance of the methylated compounds in the plasma samples in the present study could suggest the presence of enzymatic activity able to methylate tyrosol. Thus, Fig. 3 shows the proposed metabolic pathways that tyrosol could undergo in the organism, highlighting in bold the metabolites identified in the plasma samples after the consumption of the VOO. Based on the pathway described by D'Angelo *et al.*<sup>(25)</sup>, we hypothesised that tyrosol could also be affected by the activity of alcohol dehydrogenase and aldehyde reductase, although the metabolites produced by the activity of these enzymes were not found. This could be due to the high instability of these compounds and the possible preference for methylation.

#### Pharmacokinetic parameters of phenol metabolites

The concentration of the phenolic compounds and their metabolites in the plasma samples was monitored 0, 60, 120,



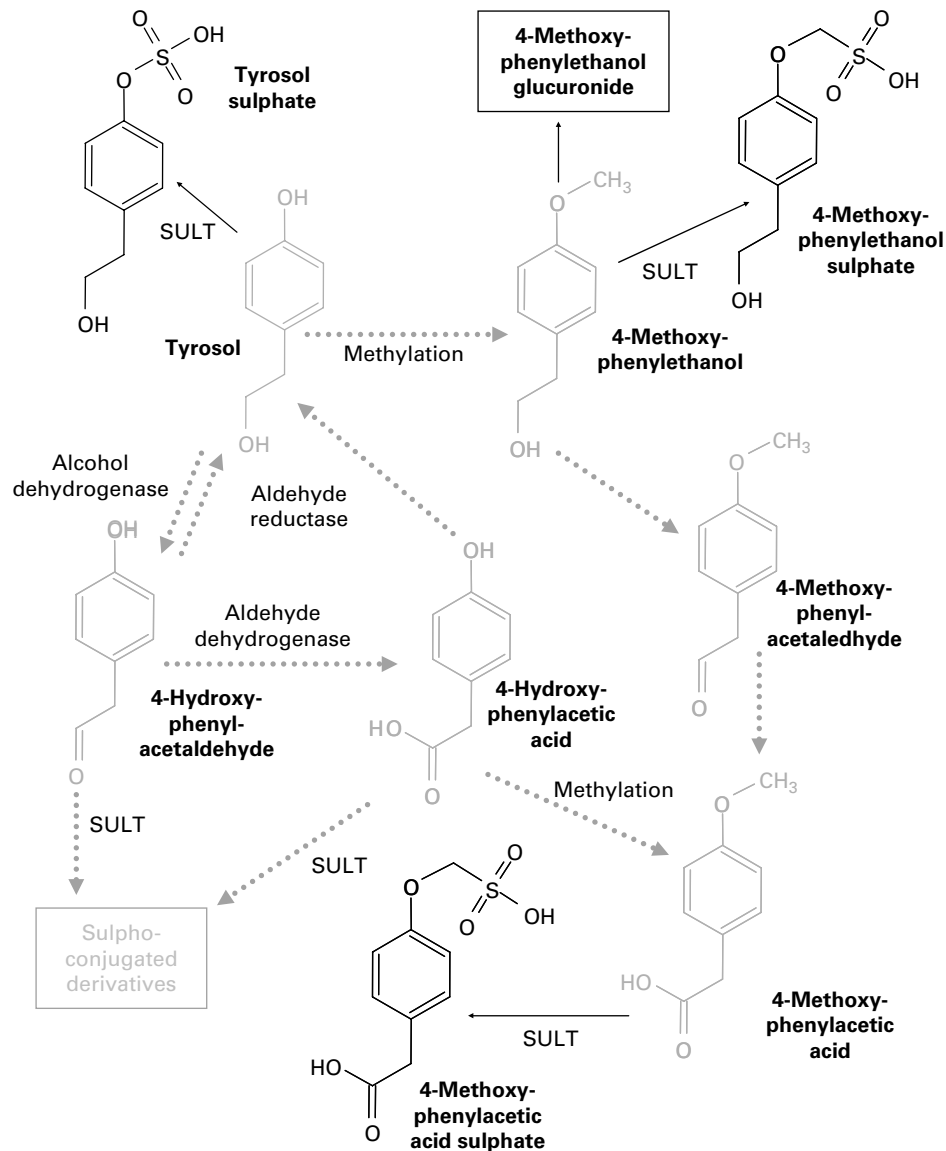
**Fig. 2.** Metabolic pathways of hydroxytyrosol (3,4-dihydroxy-phenylethanol; adapted from D'Angelo *et al.*<sup>(25)</sup>). The chemical structures in bold were identified in the plasma samples after olive oil ingestion. MOPET, 3-methoxy-4-hydroxy-phenylethanol; SULT, sulphotransferase; COMT, catechol-O-methyltransferase; DOPAL, 3,4-dihydroxy-phenylacetaldehyde; DOPAC, 3,4-dihydroxy-phenylacetic acid; MOPAL, 3-methoxy-4-hydroxy-phenylacetaldehyde; homovanillic acid, 4-hydroxy-3-methoxy-phenylacetic acid.

240 and 300 min after the ingestion of either VOO or EVOO, and the difference between their bioavailability depending on the olive oil consumed was evaluated.

In general, the data revealed that the amount of phenolic metabolites in the plasma samples obtained after the ingestion of the EVOO was higher than that obtained after the intake of the VOO. Tables 3 and 4 show the pharmacokinetic parameters obtained after analysing the chromatographic results with the PK functions software. This software allows us to tentatively determine the value of the maximum concentration of the compounds in the plasma ( $C_{max}$ ) and the area under the curve in the interval of study.

As can be seen in Table 3, almost all the identified compounds that are involved in the hydroxytyrosol pathway reached higher concentrations in plasma samples obtained

after the ingestion of EVOO compared with the values obtained after the intake of VOO. This suggests that, effectively, the enrichment of the olive oil with the phenol extract successfully produced an increase in the concentration of these compounds in plasma after oil consumption. This is desirable as a way to achieve a concentration of phenol metabolites in plasma able to produce a real effect in the organism. However, the variability of the results among the different volunteers was very high, as can be observed in the area under the curve range. This is because bioavailability of phenolic compounds not only depends on their concentration in the oil, but is also affected by each individual's characteristic parameters, such as their enzymatic activity and habitual diet. Thus, despite the mean values being higher after the consuming EVOO, only the concentration of hydroxytyrosol sulphate and vanillin sulphate



**Fig. 3.** Proposed metabolic pathways of tyrosol. The chemical structures in bold were identified in the plasma samples after olive oil ingestion. SULT, sulphotransferase.

was statistically higher than in the VOO. This inter-individual variability was also reported by other authors. D'Archivio *et al.*<sup>(27)</sup> pointed out in their review about the bioavailability of phenolic compounds in human subjects, suggesting that one important factor to be considered in these studies is the different genomic profile of the individuals. This influence of the genomic profile in the absorption and effectiveness of the polyphenols suggests that in the future further studies should be carried out to evaluate the suitability of the introduction of a personalised factor in the design of functional foods.

On the other hand, although in all the cases an increase in the concentration of the peaks for the tyrosol metabolites was detected after consumption of both VOO and EVOO, no significant differences between these oils were observed. In addition, the mean values were very similar, suggesting that the bioavailability of these compounds was not affected by phenol enrichment. It is possible that complete absorption

of the phenols ingested with EVOO could be limited by the membrane carriers. However, the intestinal mechanism of the gastrointestinal absorption of polyphenols remains poorly understood, and the membrane carriers that could be involved in polyphenol absorption have not been identified. The major components of the VOO phenolic fraction, the secoiridoid derivatives (oleuropein and ligstroside aglycones), are hydrolysed in the gastrointestinal tract<sup>(28)</sup>, after which the transport of the resulting polar phenols, tyrosol and hydroxytyrosol, might occur via passive diffusion. In a previous study, we have shown that there was extensive transport of tyrosol and hydroxytyrosol and their conjugates to the basolateral side using a Caco-2/TC7 model of the small intestine<sup>(29)</sup>. In general, apical loading of individual phenols resulted in time-dependent efflux of different conjugates. However, future studies are necessary to establish the optimal dose:bioavailability ratio.

**Table 3.** Pharmacokinetic parameters of the metabolites derived from the secoiridoids in plasma samples after the intake of either control or phenol-enriched olive oil (*n* 13)

(Mean values, standard deviations and ranges)

Phenolic compound	Oil	$C_{max}$ ( $\mu\text{mol/l}$ )		AUC ( $\mu\text{mol} \times \text{min/l}$ )		AUC range ( $\mu\text{mol} \times \text{min/l}$ )	<i>P</i>
		Mean	SD	Mean	SD		
Hydroxytyrosol sulphate	Control	0.53	0.30	103	65	50–238	< 0.05
	Enriched	0.86	0.24	159	49	93–287	
Homovanillic acid derivative	Control	0.53	0.29	132	59	80–246	NS
	Enriched	0.34	0.10	80	20	63–102	
Homovanillic acid sulphate	Control	0.78	0.46	177	125	83–330	NS
	Enriched	0.96	0.88	169	83	55–282	
3,4-DHPEA-EDA glucuronide	Control	0.24	0.21	58	59	26–127	NS
	Enriched	0.20	0.18	54	49	26–111	
3,4-DHPEA-EA derivative	Control	0.36	0.27	75	62	5–235	NS
	Enriched	0.60	0.59	98	78	7–245	
3,4-DHPEA-EA derivative glucuronide	Control	0.04	0.03	6	6	2–14	NS
	Enriched	0.22	0.34	34	54	3–96	
Tyrosol sulphate	Control	1.16	1.07	228	189	59–601	NS
	Enriched	0.95	0.64	240	169	73–538	
4-Methoxy-phenylacetic acid sulphate	Control	3.63	2.20	840	482	249–1891	NS
	Enriched	2.38	2.05	565	519	46–2024	
4-Methoxy-phenylethanol sulphate	Control	2.85	1.74	617	378	119–1256	NS
	Enriched	2.44	2.59	611	645	106–2054	
4-Methoxy-phenylethanol glucuronide	Control	0.03	0.01	6	5	2–9	NS
	Enriched	0.03	0.01	6	5	3–9	

$C_{max}$ , maximum concentration of the metabolites; AUC, area under the curve; 3,4-DHPEA-EDA, dialdehydic form of elenolic acid linked to hydroxytyrosol; 3,4-DHPEA-EA, oleuropein aglycone.

**Table 4.** Pharmacokinetic parameters of the metabolites derived from the phenolic acids and the lignans in plasma samples after the intake of either control or phenol-enriched olive oil (*n* 13)

(Mean values, standard deviations and ranges)

Phenolic compound	Oil	$C_{max}$ ( $\mu\text{mol/l}$ )		AUC ( $\mu\text{mol} \times \text{min/l}$ )		AUC range ( $\mu\text{mol} \times \text{min/l}$ )	<i>P</i>
		Mean	SD	Mean	SD		
Apigenin glucuronide	Control	0.09	0.05	16	7	8–35	NS
	Enriched	0.08	0.03	16	9	8–36	
Hydroxybenzoic acid	Control	2.23	1.65	439	285	128–933	NS
	Enriched	2.42	1.71	471	257	72–923	
Hydroxyphenylacetic acid	Control	4.95	2.63	1,135	723	185–2454	NS
	Enriched	5.79	2.67	1,317	742	172–2507	
Vanillic acid	Control	6.70	2.22	1,254	512	824–1821	NS
	Enriched	3.19	0.45	538	166	368–700	
Vanillic acid sulphate	Control	0.64	0.75	137	161	32–416	NS
	Enriched	0.75	0.39	174	87	75–232	
Caffeic acid	Control	4.06	2.19	798	530	284–1885	NS
	Enriched	4.98	3.09	959	621	355–2094	
Caffeic acid sulphate	Control	1.20	0.87	226	181	19–477	NS
	Enriched	2.58	4.98	357	583	32–1818	
Coumaric acid sulphate	Control	1.10	0.60	222	129	63–533	NS
	Enriched	1.19	0.88	271	225	89–750	
Vanillin	Control	1.42	1.19	126	113	19–245	NS
	Enriched	2.42	3.41	302	475	42–1014	
Vanillin sulphate	Control	12.96	10.78	2693	1970	718–6563	< 0.05
	Enriched	23.55	11.73	5161	2852	1711–10 800	
Vanillin glucuronide	Control	0.59	0.71	140	161	46–327	NS
	Enriched	0.36	0.39	81	79	31–172	
Ferulic acid derivative	Control	0.27	0.03	70	6	49–131	NS
	Enriched	0.23	0.05	51	12	39–71	
Ferulic acid derivative sulphate	Control	5.56	6.54	919	901	154–3245	NS
	Enriched	6.74	2.90	1,257	687	454–2575	
Ferulic acid glucuronide	Control	0.53	0.51	119	106	19–214	NS
	Enriched	0.77	0.73	189	166	23–413	

$C_{max}$ , maximum concentration of the metabolites; AUC, area under the curve.



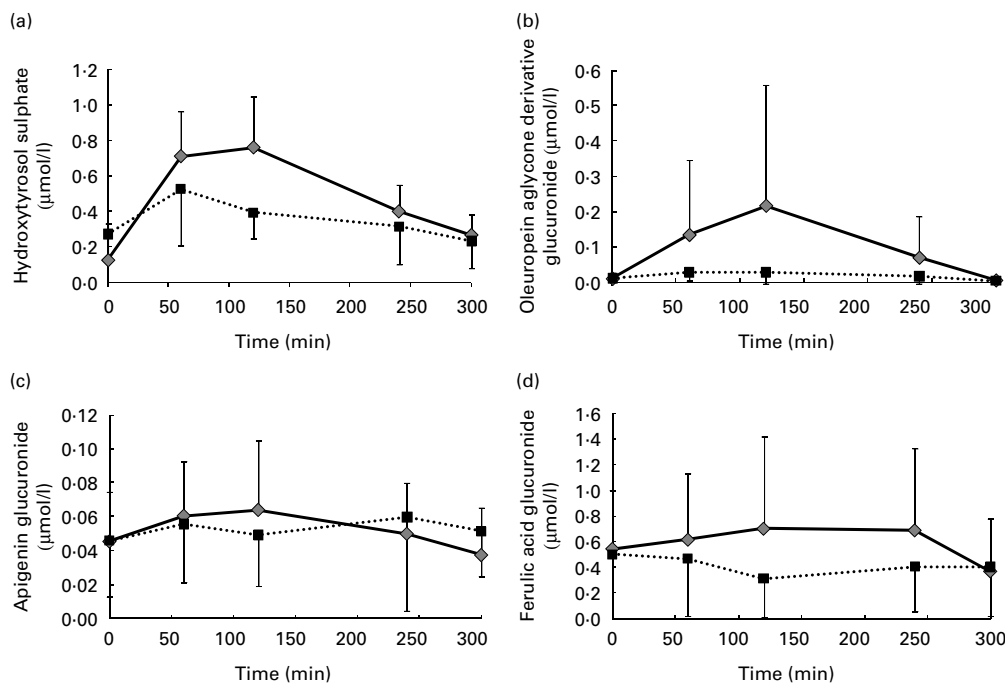
Regarding the metabolites originating from phenolic acids, it could be considered that the compounds that appeared in the basal conditions are fermentation products, generated through the effect of the human gut microbiota on the dietary fibre and some phenolic compounds, such as flavonoids, in the habitual diet of the volunteers<sup>(30)</sup>. Once the VOO has been ingested, the increase in the concentration of the phenolic acid metabolites in the plasma over time could be partly attributed to the absorption of this fraction included in the olive oil. However, the enrichment of the olive oil with the phenolic extract also increases the concentration of the flavonoid group, and it is possible that this could generate a further increase in the concentration of the phenolic acid metabolites once the human gut microbiota act on them. Therefore, in addition to the direct effect on the content of the phenolic acid metabolites observed in the plasma during the period of study, it is possible that an indirect increase occurs through the fermentation of the flavonoids in the colon. Nevertheless, further studies would be needed to confirm this.

It is also remarkable that a certain shift in the time when the maximum concentration of some of the identified metabolites was reached was observed as a consequence of the phenol enrichment of the oil. As an example, Fig. 4 shows the graphical evaluation of the concentration of some metabolites from the different phenolic groups identified in the plasma after the intake of both the VOO and the EVOO: hydroxytyrosol sulphate (phenyl alcohol), oleuropein aglycone derivative glucuronide (secoiridoid), apigenin glucuronide (flavonoid) and ferulic acid glucuronide (phenolic acid). After the consumption of the VOO, the maximum concentration of these peaks was reached at 60 min. However, consumption of the EVOO shifted this maximum to 120 min. This could be due to a higher concentration leading to a certain saturation of

the mechanisms involved in the absorption and transport of these compounds in the human organism. Therefore, the phenol-enriched oil needs more time to reach the maximum concentration in the plasma.

In addition to this change in the time to reach the highest concentration, there was a general quantitative increment in the value of these metabolites in the plasma after the ingestion of the EVOO compared with the one obtained after consumption of the VOO. This is of great importance and points to the suitability of phenol enrichment of the oil as a means of increasing the amount of these desirable compounds in the organism, which is necessary to produce a real effect on the development of some types of cancers, such as colon cancer<sup>(31)</sup>.

Analysing the pharmacokinetic parameters of the different groups of phenolic compounds under study, their different pattern of absorption could be evaluated. To start with, the time when the maximum concentration is reached suggests that their absorption occurs approximately in the small intestine after being subjected to the strong metabolism of the organism. Thus, hydroxytyrosol sulphate (and by extension the other phenyl alcohols metabolites) could come from the high spectra of secoiridoid derivatives in the oil by hydrolysis in the stomach (phase I metabolism) and later conjugation by the action of the sulphotransferases (phase II). As was reported in our previous study<sup>(28)</sup>, after simulated *in vitro* digestion of VOO, there was a significant increment in hydroxytyrosol in the stomach (rising by approximately 70%) due to the acidic conditions of the media (pH 2). This can be related to the high proportion of phenyl alcohols conjugated as their different metabolites that appeared in the plasma. The absorption of phenolic acid metabolites seems to follow the same pattern.



**Fig. 4.** Pharmacokinetics of (a) hydroxytyrosol sulphate, (b) oleuropein aglycone derivative glucuronide, (c) apigenin glucuronide and (d) ferulic acid glucuronide in human plasma after the intake of control (■) and phenol-enriched olive oil (◆). Data are expressed as  $\mu\text{mol}$  phenolic metabolite per litre plasma.

On the other hand, the appearance of oleuropein aglycone derivative and its glucuronide form seems to suggest that some of the secoiridoids in the oil are able to resist the strong conditions of the digestion process and reach the blood stream in a chemical form higher than hydroxytyrosol. This is of great importance due to the healthy effects that these compounds could exert<sup>(23)</sup>.

The non-identification of any metabolic form derived from lignans in the plasma could be due to their degradation by the colonic flora of the gut and further transformation into enterodiol and enterolactone. This process is believed to take place in the period of 8–10 h after ingestion<sup>(32)</sup>.

### Concluding remarks

The enrichment of olive oil with an olive cake extract increased the amount of phenolic compounds in the resulting oil and had a direct influence on their bioavailability. The *in vivo* study showed that the concentration of fourteen of twenty-four compounds detected was higher in the plasma samples from the EVOO than after ingestion of VOO. Among these, two of them, hydroxytyrosol sulphate and vanillin sulphate, were statistically significant in attending their pharmacokinetic parameters, demonstrating the suitability of enrichment. In general, a displacement of the time to reach the maximum concentration is observed in the samples, which indicates that more time is needed to absorb the higher phenolic content. However, inter-individual variability in the concentration of the plasma phenol metabolites shows that it is difficult to show statistically significant differences between the VOO and the EVOO. This could be due to the high number of variables that interfere in the absorption of these compounds and suggests that the metabolism of phenols is affected first by the individual. These preliminary results suggest the need for a major interventional study with a larger number of volunteers to confirm the effectiveness of the enrichment of olive oil in increasing the plasma concentration of the phenol metabolites.

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R. S. and R. M. V. drafted the manuscript. M.-J. M. and R. S. participated in the critical revision of the manuscript for important intellectual content. M. G. and S. F. provided administrative, technical and logistic support.

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