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RESEARCH ARTICLE

Distribution of olive oil phenolic compounds in rat tissues after administration of a phenolic extract from olive cake

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Scope: The distribution and accumulation of olive oil phenolic compounds in the body are topics lacked of information. The aim of this study was to evaluate the bioavailability, metabolism and distribution of phenolic compounds from olive cake.

Methods and results: The metabolism and distribution of phenolic compounds were examined by UPLC-MS/MS after an acute intake of a phenolic extract from olive cake, analyzing plasma and tissues (heart, brain, liver, kidney, spleen, testicle and thymus) 1, 2 and 4 h after ingestion using Wistar rats as the in vivo model. The results showed a wide distribution of phenolic compounds and their metabolites in the tissues, with a main detoxification route through the kidneys. Highlighting the quantification of the free forms of some phenolic compounds, such as oleuropein derivative in plasma (Cmax 4 h: 24 nmol/L) and brain (Cmax 2 h: 2.8 nmol/g), luteolin in kidney (Cmax 1 h: 0.04 nmol/g), testicle (Cmax 2 h: 0.07 nmol/g) and heart (Cmax 1 h: 0.47 nmol/g); and hydroxytyrosol in plasma (Cmax 2 h: 5.2 nmol/L), kidney (Cmax 4 h: 3.8 nmol/g) and testicle (Cmax 2 h: 2.7 nmol/g).

Conclusion: After a single ingestion of olive oil phenolic compounds, these were absorbed, metabolized and distributed through the blood stream to practically all parts of the body, even across the blood-brain barrier.

Keywords:
Distribution / Olive oil / Phenolic compounds / Tissues

1 Introduction

One of the most characteristic elements of the Mediterranean diet is undoubtedly olive oil. It is well known that through its well-balanced composition of virgin olive oil may have significant positive effects in the body [1–3]. However, it is not only its high content in monounsaturated fatty acids that is important, but also the minor compounds it contains, such as phenolics. The phenolic fraction of virgin olive oil is formed by a wide range of groups, including phenolic alcohols (hydroxytyrosol and tyrosol), secoiridoid derivatives (the dialdehydic form of elenolic acid linked to hydroxytyrosol or 3,4-DHPEA-EDA, and the dialdehydic form of elenolic acid linked to tyrosol or *p*-HPEA-EDA), phenolic acids (vanillic and *p*-coumaric acids), lignans (pinoresinol and acetoxypinoresinol) and flavonoids (luteolin and apigenin) [1–7].

The antioxidant capacity of olive oil has been widely studied and it is known that after ingestion, the phenolic compounds of olive oil may display local antioxidant capacity in the gastrointestinal tract as affirmed by Owen et al. [8]. Moreover, after absorption and metabolism of olive oil, the antioxidant actives may display their activity at the

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Abbreviations: PEOC, phenolic extract from olive cake; **μSPE,** microelution solid-phase extraction

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cellular level [3]. On the other hand, not all the beneficial effects attributed to the intake of olive oil or its phenolic compounds, such as oleuropein or hydroxytyrosol, are related to its antioxidant capacity; other beneficial properties, such as a neuroprotective effect [3, 9], cardioprotective effect [3, 10–15], anti-inflamatory effect [16–18] or anti-hypertensive effect [19, 20] are being studied.

Some clinical trials have provided evidence that phenolic compounds are absorbed in a dose-dependent manner [21, 22] and around 98% are metabolized. This leads to a wide range of metabolites that are found in biological fluids, mainly glucuronide and sulphate conjugates [1, 23]. Nevertheless, the phenolic compounds of olive oil and their metabolites are present in biological fluids at very low concentrations and some authors are cautious about attributed a direct relationship between ingestion and the beneficial effects of this minor fraction of virgin olive oil [1, 4].

The key to the relationship between the ingestion of virgin olive oil and its beneficial effect is probably not to be found in such widely analyzed biological fluids as plasma and urine, but must be sought in those tissues in which the phenolic compounds and their metabolites and other minor olive oil compounds contribute positively to the normal cell metabolic process. However, the distribution and accumulation of phenolic compounds of olive oil are still not well understood, because only one method has been developed to determine the phenolic compounds of olive oil in various rat tissues (liver, kidney, heart, muscle, testicles, fat and brain) spiked with standards of 3,4-dihydroxyphenylglycol, hydroxytyrosol and tyrosol [24].

To provide a new perspective for understanding the mechanism through olive oil acts in the body, the present work aims to describe the distribution of olive oil phenolic compounds and their metabolites in plasma and rat tissues after the ingestion of a phenolic extract from olive cake (PEOC).

2 Materials and methods

2.1 Chemicals and reagents

The standards of apigenin, luteolin, hydroxytyrosol, tyrosol and *p*-coumaric acid were purchased from Extrasynthese (Genay, France); Caffeic and homovanillic acids were purchased from Fluka (Buchs, 125 Switzerland). (+)-Pinoresinol was acquired from Arbo Nova (Turku, Finland), and catechol from Sigma-Aldrich (Germany). The secoiridoid derivatives 3,4-DHPEA-EDA and *p*-HPEA-EDA, and the lignan acetoxypinoresinol are not available commercially and were isolated from virgin olive by semi-preparative HPLC as described in Suarez et al. [25]. A stock solution of each standard compound was dissolved in methanol, and all the solutions were stored in a dark flask at 4°C. Catechol was used as an internal standard (IS) prepared in 4% phosphoric acid.

ACN (HPLC-grade), methanol (HPLC-grade), glaciar acetic acid (≥99.8%), formic acid and L(+)-ascorbic acid (reagent grade) were all provided by ScharlauChemie (Barcelona, Spain). Pure hydrochloric acid (37%) was from Prolabo (Badalona, Spain). Ortho-phosphoric acid (85%) was purchased from Mont Plet& Esteban S.A. (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2 PEOC

Olive cake is the main byproduct of the olive oil extraction process. PEOC was administrated to the rats in order to study the distribution of the metabolites distributed in the different body tissues. The composition of PEOC and how it was obtained is shown in our previous report [26]. Briefly, an accelerated solvent extractor ASE100 (Dionex, Sunnyvale, CA, USA) was used to extract the phenolic compounds from the olive cake using solvents (ethanol/water, 80:20, v/v) at high temperature (80°C) and pressure. The resulting PEOC was rotary evaporated until all the ethanol had been eliminated. Then, it was freeze-dried and stored at −80°C in N_2 atmosphere. The phenolic composition was analysed according to the method in Suarez et al. [26].

2.3 Treatment of rats and plasma and tissue collection

The Animal Ethics Committee of the University of Lleida approved the study (CEEA 03-02/09, 9th November 2009). Three-month-old male Wistar rats were obtained from Charles River Laboratories (Barcelona, Spain). The rats were housed in cages on a 12-h light–12-h dark cycle at controlled temperature (22°C). They were given a commercial feed, PanLab A04 (Panlab, Barcelona, Spain), and water ad libitum. The rats were later kept under fasting conditions for between 16 and 17 h with access to tap water and after this time, a single dose of 3 g of PEOC/kg of body weight dispersed in water was administered by intragastric gavage (Fig. 1). The rats were anesthetized with isoflurane (IsoFlo, VeterinariaEsteve, Bologna, Italy) and euthanized by exsanguinations 1 ($n = 4$), 2 ($n = 4$) and 4 ($n = 4$) hour after the ingestion of the PEOC. Additionally, a control group of rats ($n = 4$) was maintained under fasting conditions without PEOC ingestion and then similarly euthanized.

Blood samples were collected from the abdominal aorta with heparin-moistened syringes. The plasma samples were obtained by centrifuging ($2000 \times g$ for 30 min at 4°C) and stored at −80°C until the chromatographic analysis. Different tissues (liver, kidney, testicle, brain, spleen, heart and thymus) were excised from the rats, stored at −80°C and freeze-dried for phenolic extraction and chromatographic analysis.

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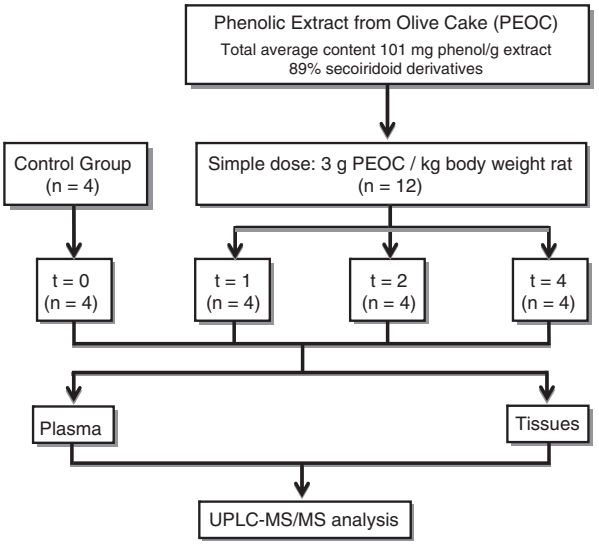

Figure 1. Schema of the treatment of rats and plasma and tissues collection

2.4 Phenolic extraction of plasma and tissues

In order to clean-up the biological matrix and preconcentrate the phenolic compounds, the plasma samples were pretreated by microelution solid-phase extraction (μSPE) [27], and the tissues were sequentially pretreated by a combination of liquid–solid extraction (LSE) and μSPE. To develop the tissue extraction method, a pool of each tissue obtained from rats from the control group was spiked with the phenolic standards (hydroxytyrosol, tyrosol, *p*-coumaric acid, caffeic acid, homovanillic acid, pinoresinol, apigenin and luteolin) dissolved in phosphoric acid (4%). For the analysis of different tissues by LSE, 60 mg of freeze-dried tissue or 30 mg of freeze-dried heart were weighted, and then 50 μL of ascorbic acid 1% and 100 μL of phosphoric acid (4%) were added. The sample was treated four times with 400 μL of water/methanol/phosphoric acid 4% (94:4.5:1.5, v/v/v). In each extraction, 400 μL of extraction solution was added; the sample was sonicated (S-150D Digital SonifierR Cell Disruptor, Branson, Ultrasonidos S.A.E., Barcelona, Spain) for 30 s maintaining the sample in a freeze water bath to avoid heating and then centrifuged for 15 min at 9000 rpm at 20°C. The supernatants were collected, and then an aliquot of the extract was treated by μSPE to clean up the sample before the chromatographic analysis. The offline μSPE was based on the methodology described in a previous report where olive oil phenolics were analyzed in plasma samples [27]. Briefly, OASIS HLB μElution Plates 30 μm (Waters, Milford, MA, USA) were used and two methodologies were applied, one to determine hydroxytyrosol, and the second to determine the other studied phenolic compounds. For the analysis of hydroxytyrosol, the micro-cartridges were conditioned sequentially with 250 μL of methanol and 250 μL of Milli-Q at pH 2 with hydrochloric acid. Nearly, 200 μL of phosphoric acid (4%) was added to 200 μL of tissue extract, and then this mixture was loaded onto the plate. The loaded plates were washed with 75 μL of Milli-Q water and 75 μL of Milli-Q water at pH 2. The retained hydroxytyrosol was then eluted with 2×25 μL of acetonitrile/Milli-Q water solution (50:50, v/v). On the other hand, for the analysis of the rest of the studied phenolic compounds, the micro-cartridges were conditioned sequentially with 250 μL of methanol and 250 μL of Milli-Q at pH 2 with hydrochloric acid. Totally, 350 μL of phosphoric acid 4% was added to 350 μL of tissue extract, and then this mixture was loaded onto the plate. The loaded plates were washed with 200 μL of Milli-Q water and 200 μL of Milli-Q water at pH 2. The retained phenolic compounds were then eluted with 2×50 μL of methanol. The eluted solutions (phenolic extracts) were directly injected into the UPLC-MS/MS, and the injection volume was 2.5 μL.

2.5 Quantitative analysis of plasma and tissue metabolites

Phenolic compounds were analyzed by Acquity Ultra-Performance^{TM} liquid chromatography and tandem MS from Waters (Milford MA, USA), as reported in our previous studies [27–30]. Briefly, the column was an Acquity UPLCTM BEH C18 (100 mm × 2.1 mm id, 1.7 μm particle size), also from Waters. The mobile phase was 0.2% acetic acid as eluent A and ACN as eluent B, and the flow rate was 0.4 mL/min. The elution started at 5% of eluent B for 2.1 min, then was linearly increased to 40% of eluent B in 20 min, further increased to 100% of eluent B in 0.1 min and kept isocratic for 1.9 min. Then, back to initial conditions in 0.1 min, and the reequilibration time was 2.9 min.

The UPLC system was coupled to a PDA detector AcQuity UPLCTM and a triple quadrupole detector (TQDTM) mass spectrometer (Waters). The software used was MassLynx 4.1. Ionization was done by electrospray (ESI) in the negative mode and the data were collected in the selected reaction monitoring (SRM) mode. The ionization source parameters and the transition acquired for each compound (one for quantification and a second one for confirmation purposes) as reported in our previous study [27].

2.6 Statistical analysis

The data on polyphenol metabolite levels are presented as mean values ± standard error ($n = 4$). In order to simplify the results related to the metabolite distribution in tissues, the standard error was omitted because all values were lower than 10%. The data were analysed by a multifactor ANOVA test to determine significant differences between post-

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Table 1. Phenolic composition of the olive extract

Compound	mg/g extract
Tyrosol	0.08
Hydroxytyrosol	3.45
Total phenyl alcohols	3.51
Vanillin	0.02
p-Coumaric acid	0.05
Vanillic acid	0.03
Caffeic acid	0.04
Total phenolic acids	0.10
Elenolic acid	25.9
3,4-DHPEA-AC	0.34
p-HPEA-EA	0.11
p-HPEA-EDA	0.19
3,4-DHPEA-EDA	60.2
3,4-DHPEA-EA	1.43
Methyl 3,4-DHPEA-EA	0.12
Ligstroside derivative	0.30
Oleuropein derivative	0.08
Total secoiridoid derivatives	89
Pinoresinol	0.07
Acetoxypinoresinol	0.14
Total lignans	0.21
Apigenin	0.40
Luteolin	1.82
Apigenin-7-*O*-glucoside	0.21
Luteolin-7-*O*-glucoside	3.07
Rutin	1.71
Total flavonoids	7.2
Verbascoside	0.61
Total average content	101

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

ingesta times (1, 2 and 4 h) and to compare these with the control group. A significant difference was considered at a level of $p \leq 0.05$. All the statistical analysis was carried out using STATGRAPHICS Plus 5.1.

3 Results

The composition of PEOC used in this study is shown in Table 1. The extract contained the most representative phenolic compounds of virgin olive oil, including phenyl alcohols, phenolic acids, secoiridoid derivatives, lignans and flavonoids. Hydroxytyrosol showed a high concentration with 3.45 mg/g PEOC, and the main phenolic compound quantified in PEOC was 3,4-DHPEA-EDA with 60.2 mg/g PEOC.

In relationship to the bioavailability (Table 2), the main phenolic family detected in the plasma at different times after the PEOC ingestion were the phenolic alcohols. Hydroxytyrosol-sulphate was the main plasma metabolite with approximately 55% of the total phenolic alcohols quantified in all tested times (1, 2 and 4 h), followed by tyrosol-sulphate. Of the other conjugated metabolites, hydroxytyrosol-glucuronide and tyrosol-glucuronide were detected in all the plasma samples, hydroxytyrosol-glucuronide being the most abundant. Moreover, the free form of hydroxytyrosol was detected at concentrations between 4.3 and 5.2 nmol/L after the ingestion of PEOC. In relationship to the secoiridoids, a metabolite identified as oleuropein derivative was detected and quantified in the plasma samples reaching the maximum concentration (24 nmol/L) 4 h after ingestion. The second main group of phenolic metabolites quantified in plasma was the phenolic acids, homovanillic-sulphate acid being the main phenolic acid quantified. Vanillic-sulphate, elenolic, hydroxybenzoic and hydroxyphenylacetic acids were also quantified but at lower concentrations. Nevertheless, hydroxybenzoic and hydroxyphenyl acetic acids were detected in the plasma from the control group at trace level (data not shown). Luteolin in its free form was detected at trace levels in all the samples, with no significant differences between the times after the ingestion of PEOC and the control group (data not shown). However, its glucuronide conjugate was quantified in the plasma samples at low levels (<1 nmol/L) only after the ingestion of the PEOC (Table 2). Analyzing the importance in percentage of the phenolic families (phenyl alcohols, phenolic acids and secoiridoid derivatives) quantified in the phenolic extract (PEOC) and in rat plasma 1, 2 and 4 h post-ingestion of 3 g of PEOC/kg of rat body weight (Fig. 2), the high percentage of total phenyl alcohols (mainly metabolites of hydroxytyrosol and tyrosol) in the plasma was noteworthy in relationship to their low concentration in the extract. The increase in hydroxytyrosol metabolites in plasma after the PEOC ingestion may be related to the high content of secoiridoids in the extract acting as a source of hydroxytyrosol.

After the absorption of phenols from the PEOC, a wide range of phenolic compounds was quantified practically in all the tissues analyzed, showing a wide distribution thoughout the body. Nevertheless, oral bioavailability was low, with values lower than 5%. For example, the main phenolic compound of PEOC, hydroxytyrosol, showed a low oral bioavailability with 2.35% (1 h), 3.75% (2 h) and 2.00% (4 h) including hydroxytyrosol and its derivatives and taking into account all analyzed tissues. The liver and kidney were the organs with the greatest presence of phenolic metabolites, followed by the testes (Table 3). The heart, brain, spleen and thymus (Table 4) showed a lower number of metabolites with phenolic acids being the main metabolites quantified. Oleuropein derivative was present in all the tissues analysed 1 h after the PEOC ingestion, except in the thymus. In general, the maximum tissue concentration of

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Table 2. Plasma concentration of phenol metabolites following the acute intake of 3 g/kg of body weight of olive extract[a)]

	Time (hours) after extract intake[b)]		
Metabolite (nmol/L)	1 h	2 h	4 h
Hydroxytyrosol	4.3±0.4[b]	5.2±0.5[a]	5.1±0.6[ab]
Hydroxytyrosol-sulphate	51±4[b]	89±7[a]	82±8[a]
Hydroxytyrosol-glucuronide	1.5±0.2[b]	1.8±1.4[a]	1.8±1.6[a]
Tyrosol-glucuronide	1.0±0.2[a]	1.2±0.2[a]	0.44±0.07[b]
Tyrosol-sulphate	36±3.1[b]	53±6.0[a]	50±4.3[a]
Total Phenolic Alcohols	94±7.9[b]	150±15[a]	139±14[a]
Vanillin-sulphate	1.4±0.1[b]	2.5±0.2[a]	1.1±0.2[b]
Vanillic-sulphate acid	2.9±0.1[b]	4.5±0.4[a]	4.8±0.5[a]
Homovanillic-sulphate acid	16±1.3[b]	36±3.8[a]	13±1.5[b]
Hydroxybenzoic acid	1.2±0.1[c]	2.3±0.2[a]	1.6±0.2[b]
Hydroxyphenylacetic acid	1.5±0.1[ab]	1.6±0.1[a]	1.2±0.2[b]
Elenolic acid	1.1±0.3[a]	1.1±0.2[a]	0.89±0.09[b]
Total phenolic acids	24±3.9[b]	48±6.3[a]	23±4.2[b]
Oleuropein derivate	13±1.2[b]	21±2.3[a]	24±2.3[a]
Total secoiridoid derivatives	13±1.2[b]	21±2.3[a]	24±2.3[a]
Luteolin-glucuronide	0.68±0.07[a]	0.58±0.03[ab]	0.54±0.2[b]
Total flavonoids	0.68±0.07[a]	0.58±0.03[ab]	0.54±0.2[b]
Total phenolic metabolites	131±13[b]	198±23[a]	186±20[a]

Results are expressed as nmol phenolic metabolite/L plasma.
a) Data expressed as mean values±standard error ($n = 4$).
b) Different letters within the same row indicate a significant difference ($p < 0.05$).

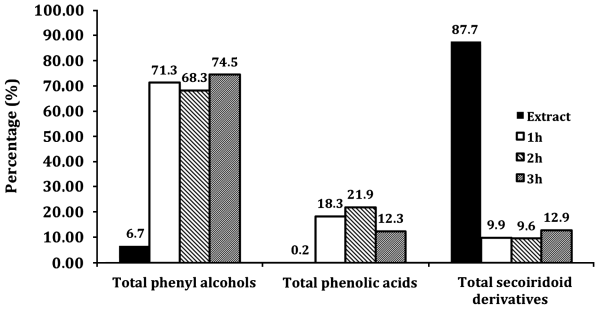

Figure 2. Importance in percentatge of phenolic families (phenyl alcohols, phenolic acids and secoiridoid derivatives) quantified in the phenolic extract (PEOC) and in rat plasma at 1, 2 and 4 h post-ingestion of 3 g of PEOC/kg of rat body weight.

this compound (C_{max}) was reached in all the tissues at 2 h (t_{max}) (Table 3). However, the maximum concentration was observed in the kidney, 71 nmol/g tissue 4 h after the PEOC ingestion. Phenyl alcohols were the main metabolites quantified, with hydroxytyrosol-sulphate as the main metabolite detected in all the analyzed tissues. Hydroxytyrosol-sulphate was also present in the kidneys from the control rats (0 h), but at very low concentration (16 nmol/g tissue). Nevertheless, its concentration increased rapidly, reaching values of 3163 nmol/g tissue 1 h (t_{max}) after the PEOC ingestion, and then decreased slowly until 1979 nmol/g tissue 4 h after ingestion. The testes also presented a high concentration of hydroxytyrosol-sulphate, but the accumulation started later in that tissue, with 1005 nmol/g tissue 2 h (t_{max}) after the PEOC ingestion. Its concentration in the liver was not as high as in the testicle and kidney ($C_{max} = 237$ nmol/g tissue, $t_{max} = 2$ h). On the other hand, hydroxytyrosol-sulphate was detected in the heart, thymus, brain and spleen at lower concentrations, under 17 nmol/g tissue (Table 4). Hydroxytyrosol-glucuronide was another quantified conjugated form of hydroxytyrosol (kidney: $C_{max} = 15$ nmol/g tissue, $t_{max} = 1$ h; liver: $C_{max} = 2.1$ nmol/g tissue, $t_{max} = 2$ h; testicle: $C_{max} = 3.18$ nmol/g tissue, $t_{max} = 2$ h) and also the free form of hydroxytyrosol was detected in the kidney ($C_{max} = 3.8$ nmol/g tissue, $t_{max} = 4$ h) and testicle ($C_{max} = 2.68$ nmol/g tissue, $t_{max} = 2$ h).

Tyrosol-sulphate was quantified in all the tissues except in brain, and as with the hydroxytyrosol conjugate, the maximum concentration was detected in the kidney ($C_{max} = 684$ nmol/g tissue, $t_{max} = 1$ h) but no significant differences were observed between 1, 2 and 4 h and a lower concentration (24 nmol/g tissue) was quantified in the kidney from rats of control group (0 h). The testes showed the C_{max} (410 nmol/g tissue) 2 h after the ingestion of the extract. Lower concentrations of tyrosol-sulphate were detected in the heart, thymus and spleen (<23 nmol/g tissue). Besides, the glucuronidated form of tyrosol was present in the kidney and testes. The free or, in some cases, conjugated form (glucuronidated or sulphated) of some characteristic phenolic acids of virgin olive oil, such as caffeic acid or vanillic acid were present in tissues. Specifically, vanillic-sulphate acid was quantified in all the tissues.

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Table 3. Quantities of phenolic metabolites in liver, kidney and testicle from control rats (0 h) and rats after an acute intake of olive oil extract (1, 2 and 4 h)[a)]

Metabolite (nmol/g tissue)	Liver[b)]				Kidney[b)]				Testicle[b)]			
	0 h	1 h	2 h	4 h	0 h	1 h	2 h	4 h	0 h	1 h	2 h	4 h
Hydroxytyrosol	n.d.	n.d.	n.d.	n.d.	n.d.	1.9^{b}	1.3^{c}	3.8^{a}	n.d.	n.d.	2.68^{a}	1.40^{b}
Hydroxytyrosol-sulphate	n.d.	122^{c}	237^{a}	184^{b}	16^{a}	3163^{a}	3100^{a}	1979^{b}	n.d.	n.q.	1005^{b}	201^{c}
Hydroxytyrosol-glucuronide	n.d.	1.7^{a}	2.1^{a}	1.0^{b}	n.d.	15^{b}	12^{b}	2.6^{c}	n.d.	n.d.	3.18^{a}	1.70^{b}
Tyrosol-sulphate	1.3^{b}	68^{a}	73^{a}	70^{a}	24^{b}	684^{a}	599^{a}	605^{a}	n.d.	100^{b}	410^{a}	129^{b}
Tyrosol-glucuronide	n.d.	n.d.	n.d.	n.d.	n.d.	1.9^{a}	2.5^{a}	n.q.	n.d.	n.q.	6.9	n.q.
Oleuropein derivate	n.d.	5.3^{a}	20^{b}	14^{b}	n.d.	52^{b}	60.8^{b}	71^{b}	n.d.	3.88^{b}	16.63^{a}	7.73^{b}
Vanillin-sulphate	n.d.	0.4^{b}	1.0^{a}	0.4^{b}	0.15^{d}	2.7^{b}	3.0^{a}	1.4^{c}	n.d.	0.46^{b}	2.0^{a}	0.54^{b}
Cumaric-sulphate acid	n.d.	0.5^{a}	0.6^{a}	0.8^{a}	2.2^{d}	22^{a}	12^{c}	18^{b}	n.d.	0.36^{b}	1.5^{a}	0.43^{b}
Cumaric-glucuronide acid	n.d.	n.d.	n.d.	n.d.	n.d.	0.19^{a}	0.3^{a}	n.q.	n.d.	n.d.	n.d.	n.d.
4-Hydroxy-3-metoxyphenylacetaldehid	n.d.	12^{b}	16^{a}	11^{b}	93^{b}	191^{a}	99^{b}	74^{c}	39^{a}	27.39^{a}	86.20^{b}	n.d.
Vanillic acid	0.74^{b}	2.7^{a}	2.7^{a}	2.4^{a}	2.8^{a}	3.6^{b}	$3.3^{a,b}$	3.8^{b}	n.d.	n.d.	n.d.	0.06
Vanillic-sulphate acid	n.d.	2.4^{b}	4.5^{a}	3.2^{b}	1.3^{a}	38^{c}	$34^{b,c}$	32^{b}	n.d.	0.41^{b}	4.56^{a}	0.52^{b}
Cafeic acid	n.d.	0.28^{b}	$0.14^{a,b}$	0.10^{a}	n.d.	1.5^{b}	2.1^{a}	1.2^{b}	n.d.	n.d.	n.d.	n.d.
Cafeic-sulphate acid	n.d.	n.d.	n.d.	$n.d.^{b}$	n.d.	2.1^{a}	1.2^{b}	1.1^{b}	n.d.	n.d.	n.d.	n.d.
Homovanillic acid	n.d.	0.11^{a}	0.19^{b}	n.d.	n.d.	2.5^{a}	2.6^{a}	1.3^{b}	n.d.	n.q.	6.37^{a}	1.68^{b}
Homovanillic-sulphate acid	n.d.	2.2^{b}	7.4^{a}	3.2^{b}	4.3^{a}	25^{c}	24^{c}	12^{b}	3^{a}	6.86^{b}	24.96	8.54^{b}
Ferulic-sulphate acid	$n.d.^{a}$	n.d.	n.d.	n.d.	6.3^{a}	4.4^{c}	3.5^{d}	5.5^{b}	n.d.	0.12^{a}	0.72^{b}	0.21^{a}
Hydroxyphenylacetic acid	0.01	0.5^{b}	0.63^{b}	0.52^{b}	1.5^{b}	6.9^{b}	6.7^{a}	2.2	n.d.	n.d.	n.d.	n.d.
Enterolactone	1.44^{a}	6.4^{a}	5.2^{b}	4.0^{b}	n.d.	2.7^{b}	2.3^{a}	0.67	n.d.	n.d.	n.d.	n.d.
Enterolactone-sulphate	2.0^{a}	5.2^{b}	5.6^{b}	4.2^{b}	n.d.	5.8^{b}	5.0^{a}	10	n.d.	n.d.	n.d.	n.d.
Enterolactone-glucuronide	0.01^{a}	$0.4^{b,c}$	0.5^{c}	0.3^{b}	1.9^{a}	$3.3^{b,c}$	5.4^{d}	3.6	n.d.	n.d.	n.d.	n.d.
Luteolin	n.d.	n.d.	n.d.	n.d.	n.q.	0.04	n.q.	n.q.	n.d.	n.q.	0.07^{a}	0.02^{a}

n.d.: not detected; n.q.: not quantified. Results are expressed as nmol/g tissue.

a) Standard error was omitted because all values were lower than 10%.

b) For each tissue, different letters within the same row indicate a significant difference ($p<0.05$).

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Table 4. Quantities of metabolites in brain, spleen, heart and thymus from control rats (0 h) and rats after an acute intake of olive oil extract (1, 2 and 4 h)[a)]

	Brain[b)]				Spleen[b)]			
	0 h	1 h	2 h	4 h	0 h	1 h	2 h	4 h
Metabolite (nmol/g tissue)								
Hydroxytyrosol sulfate	n.d.	28.4[b]	50[a]	27[b]	n.d.	0.09	12	34
Tyrosol sulphate	n.d.	6.81[b]	10.14[a]	10.49[a]	n.d.	13	21	23
Oleuropein derivate	n.d.	0.44[c]	2.8[a]	1.3[b]	n.d.	3.1 [b]	4.8	3.3[a,b]
Vanillin sulphate	n.d.	0.02[a]	0.02	0.03	n.d.	n.d.	n.d.	n.d.
Vanillic acid	0.86[a]	1.8[b]	1.7	2.0	1.1	1.6[,b]	1.9	1.1
Vanillic sulphate acid	n.d.	0.38[b]	0.73	0.29	n.d.	2.8	5.6	2.6
Cafeic acid	n.d.	n.d.	n.d.	n.d.	n.d.	0.16	0.25	3.4
Homovanillic sulphate acid	0.77[a]	2.0[b]	1.8	2.1	n.d.	n.d.	0.12	n.q.
Hydroxyphenylacetic acid	n.d.	n.d.	n.d.	n.d.	n.d.	0.37	0.45	n.q.
	Heart[b)]				Thymus[b)]			
Metabolite (nmol/g tissue)								
Hydroxytyrosol sulphate	n.d.	157[b]	263[a]	128[b]	n.d.	n.d.	n.d.	n.d.
Tyrosol sulphate	n.d.	13[b]	17[a]	6.9[c]	n.d.	n.d	4.80[b]	9.99[a]
Oleuropein derivate	n.d.	2.6[c]	8.4[a]	4.9[b]	n.d.	n.d.	n.d.	n.d.
Vanillin sulphate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.q.	
Cumaric sulphate acid	n.d.	n.d.	n.d.	n.d.	n.d.	0.08[b]	0.36[a]	0.06[b]
Vanillic sulphate acid	n.d.	0.13[a]	0.74[b]	0.35[c]	n.d.	1.31[a]	1.80[a]	n.q.
Homovanillic sulphate acid	n.d.	1.4	n.q.	n.q.	n.d.	n.d.	n.d.	n.d.
Hydroxyphenylacetic acid	n.q.	0.14[b]	0.25[a]	n.q.	n.q.	n.q.	n.q.	1.80
Luteolin	n.d.	0.47[a]	0.16[b]	0.27[b]	n.d.	n.d.	n.d.	n.d.

n.d.: not detected; n.q.: not quantified. Results are expressed as nmol/g tissue.

a) Standard error was omitted because all values were lower than 10%.

b) For each tissue, different letters within the same row indicate a significant difference ($p<0.05$).

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However, 4-hydroxy-3-methoxyphenylacetaldehid was the most abundant phenolic acid present in the kidney ($C_{max} = 191$, $t_{max} = 1$ h), liver ($C_{max} = 16$, $t_{max} = 2$ h) and testes ($C_{max} = 86$, $t_{max} = 2$ h) but this compound was also detected in the kidney from the control rats (0 h) at 93 nmol/g tissue. Among the flavonoids, only luteolin was quantified in some tissues (kidney, testicle and heart) at low concentrations, < 0.47 nmol/g tissue, and no lignans were quantified in their native form in the tissues. In addition, enterolactone and its glucuronide and sulphate conjugated metabolites were quantified in the liver and kidney (Table 3).

4 Discussion

Because of the short life of polyphenols in plasma, to observe its potential benefit the studies should be carried out during the postprandial state, immediately after its intake [28, 29]. Thus, the acute intake of olive oil phenols may be useful in a future repeated low-dose experiments obtaining a concentration of phenolic metabolites in the tissues that allow their detection and quantification.

After ingestion, the secoiridoid derivatives may be partially modified in the acidic environment of the stomach, suffering a non-enzymatic metabolism that considerably decreases their concentration and increases moderately the total phenyl alcohols and the total phenolic acids [30, 31]. A rapid absorption was observed in the current study, with phenolic compounds being detected in the plasma 1 h after ingestion of PEOC, although the t_{max} was at 2 h for most of the quantified compounds. Similarly, different studies have reported the C_{max} of the phenolic compounds in the plasma between 1 and 2 h after ingestion of virgin olive oil [22, 32].

As expected, most of the metabolites that were identified in the plasma appeared in their conjugated form, mainly sulphated followed by glucuronidated forms [22], indicating that rat hepatocytes were capable of sulphating these phenols [33–35]. Additionally, the large amount of sulpho-conjugated may reflect a high-sulphotransferase activity of platelets [36]. On the contrary, Miro-Casas et al. [23] reported that 98% of hydroxytyrosol in human plasma and urine was mainly glucurono-conjugated. D'Angelo et al. [35] proposed a metabolic pathway for hydroxytyrosol that could be similarly applied to tyrosol, in which the action of cathecol-*O*-methyltransferases promoted the appearance of homovanillic acid and its conjugated forms. Related with this metabolism, hydroxytyrosol, hydroxytyrosol-sulphate, homovanillic acid, homovanillic-sulphate acid and the mono-hydroxylated form of phenylacetic acid were quantified in rat plasma samples at all the times tested. As previously reported for human plasma [22], this suggests that all the intermediate forms were rapidly subjected to the UDP-glucuronosyltransferase and sulphotransferase enzymatic activities resulting in conjugated forms.

Instead of this first-step metabolism in the gut and liver, the free form of hydroxytyrosol was detected in plasma (Table 2), in contrast to the results of previous studies in which only the conjugated forms of hydroxytyrosol were quantified in human plasma after the ingestion of virgin olive oil [1, 23, 31]. Similarly, a phenolic structure, identified as an oleuropein derivative, was the only secoiridoid derivative detected as an unconjugated form in the plasma. Nevertheless, this could be explained because the parental form of the secoiridoids are not the major bioavailable form in vivo [37], which explains the absence of other secoiridoids in the plasma after the ingestion of a secoiridoid-rich extract like PEOC. Probably, the first step of the intestine/hepatic metabolism could be related to the ingested dose. High doses could saturate the conjugation metabolism of the olive phenolic compounds and this may allow the detection of free forms in the plasma in the present study, these probably being absorbed by passive diffusion. Of special interest is the presence of these free aglycones circulating in blood, probably with biological effects different from those of conjugated metabolites.

Less data are available in the literature on the distribution and accumulation of olive phenolic compounds in tissues. Our results showed that the phenolic metabolites were uniformly distributed in all the tested tissues with a high concentration and number of metabolites in the liver and kidney, showing a preferential renal uptake of the metabolites, as observed by D'Angelo et al. [35] using intravenously injected [^{14}C] hydroxytyrosol. The presence of phenolic metabolites in the liver (Table 3) could be explained by the functionality of this organ in the metabolism of phenolic compounds. Linked to this functionality, the presence of free and conjugated (sulphated and glucuronidated) forms of the phenolic acids and phenolic alcohols suggest the importance of the liver in the conjugation metabolism and suggest an active metabolism during the experimental time. This metabolic detoxification process, common to many xenobiotics, facilitates the urinary elimination of phenolics [38]. In relationship to this, the high concentration of sulphateconjugated forms of hydroxytyrosol and tyrosol quantified in the kidney could indicate the main excretion path for olive phenolic compounds [39]. In contrast, glucuronidated forms have been described mainly as metabolites in human urine [21, 39, 40].

A wide range of metabolites were detected in testes (Table 3). Despite the extensive presence of metabolites in this tissue, there are no studies about the function of olive phenolic compounds in testes. Nevertheless, procyanidins were widely distributed in lymphoid tissues, as Urpi-Sarda et al. [41] reported in their study, and possibly the lymphoid nature could be related to the accumulation of phenolic metabolites. Moreover, Wang et al. [42] reported a protective effect of phenolic compounds from *Ginkgo biloba* on an injury produced by an inducing stress agent in testicles.

It is of interest the detection in heart of conjugated forms of hydroxytyrosol, mainly hydroxytyrosol-sulphate (Table 4),

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and the free forms of oleuropein derivative and luteolin. These phenolic metabolites could exert a direct protective effect related to oxidative process in the arterial wall. In this sense some studies [43–47] have postulated that LDL oxidation does not take place in the circulation, and occur in the arterial wall because serum lipoprotein lipids are well protected in circulation from oxidation by the robust antioxidant defenses.

The increase in some phenolic metabolites in the brain as a consequence of the ingestion of the phenolic extract is of special interest too. The presence of phenolic compounds from PEOC in the brain could exert a protective effect against stress of different nature, such as oxidative stress in dopaminergic neurons [48] or nitrosative stress [49]. Additionally, the interest of the results of this study could be reinforced by the absence of free homovanillic and 3,4-dihydroxyphenylacetic acids, these being the main oxidized metabolites deriving from dopamine metabolism in the central nervous system [35].

The spleen is an important cross-point for antigenic information transported by the blood and immune system, and a major antibody-producing organ [50]. The close relationship between the spleen and blood could explain the presence of some metabolites in this tissue, although the functionality of these metabolites in the spleen is not clear due to a lack of information. However, other phenolic compounds, such as protocatechuic acid, showed a strong ability to attenuate the ageing alterations of the antioxidative defense systems in the spleen and liver [51]. So, given their antioxidant nature, olive phenolic compounds could also affect it.

The quantification of metabolites of the phenolic compounds in the plasma and multiple tissues indicated that after an acute ingestion of olive phenolic compounds, they were absorbed, metabolized and distributed though the blood stream to practically all parts of the body, even across the blood–brain barrier. Sulphate conjugates of phenyl alcohols (mainly hydroxytyrosol and tyrosol) were the main metabolites quantified in the plasma and tissues and free forms of some phenolic compounds, such as oleuropein derivative in the plasma and brain, luteolin in the kidney, testicle, brain and heart, or hydroxytyrosol in the plasma, kidney and testicle were quantified. That work may suppose a breakthrough in the research about how olive oil acts positively in the prevention and improvement of some diseases, such as cardiovascular disease; or how olive oil exert positive effects in specific parts of the body, such as neuroprotective effect. Nevertheless, further experiments are required to investigate the effect of olive phenolic compounds on the functions of such organs as the spleen, liver, brain or heart.

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