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Effect of the long-term regular intake of virgin olive oil on the phenolic metabolites in human fasting plasma

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ABSTRACT

The effect of repeated consumption of virgin olive oil on endogenous phenolic metabolites of fasting plasma is unknown. For this reason, we hypothesized that regular long-term virgin olive oil intake could have an indirect protection effect on the endogenous phenols. Thus, the aim of the study was to determine the phenolic profile of human plasma in a fasting state of long-term regular virgin olive oil consumers, using the fasting plasma of non-consumers as a natural control. Forty participants living in the area of Reus (Catalonia, Spain) were selected, 20 life-long regular consumers of virgin olive oil and a natural control of 20 non-consumers, the latter being Rumanians who dislike the taste of olive oil. The diet was obtained from 3-day food records. The results showed similar phenolic composition of fasting plasmas of the two volunteer groups. Of special interest is that more of the compounds quantified showed higher concentration in fasting plasma from habitual virgin olive oil consumers. The compounds were semi-quantified using caffeic acid as the calibration standard. The quantification of fasting consumer's plasma showed higher concentration of a hydroxyflavanone type compound (2.90 ± 0.04 μM vs 1.5 ± 0.04 μM) and a catecholamine derivative (0.70 ± 0.03 μM vs 0.56 ± 0.03 μM) than the plasma of non-consumers ($P < 0.05$). The results suggest an indirect protective mechanism of long-term regular virgin olive oil consumption related to the protection of the endogenous antioxidant system.

1. Introduction

Epidemiologic studies have clearly shown that the habitual consumption of olive oil, as a principle fat source in the diet, is associated with a low incidence of coronary heart disease, certain types of cancers and modulation of immune and inflammatory responses. It seems that this protection is due to diverse combinations of biological effects including antioxidant [1], anti-inflammatory [2,3], vasodilatation [4] and anti-platelet aggregation properties [2], as well as the modulation of gene expression [5,6]. For years, the healthy properties of virgin olive oil (VOO) were exclusively attributed to its high monounsaturated fatty acid (MUFA) content, mostly in the form of oleic acid (18:1n-9). The recent results of the EUROLIVE study have provided evidence of the antioxidant in vivo role of phenolic compounds from VOO in humans [7]. The most important effects observed were related to the increase in high-density lipoprotein (HDL) cholesterol levels and the decrease in lipid oxidation damage, after high phenolic oil consumption.

The chemical composition of VOO is divided into major components, which include glycerols, representing more than 98% of total oil weight, and minor components. These components, which are present in very low amounts (about 2% of oil weight), include more than 230 chemical compounds, such as aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile compounds and antioxidants (carotenoids, and lipophilic and hydrophilic phenolic compounds) [8]. Some of these minor components of olive oil, mainly the hydrophilic phenols, are removed during the refining process. As a consequence, these compounds are specific to VOO.

The most abundant phenols in VOO are the secoiridoid derivatives formed during olive oil extraction by enzymatic removal of glucose from the polar parent compounds oleuropein and ligstroside, glycoside phenolic structures from the olive fruit. The final products are the polar compounds hydroxytyrosol and tyrosol respectively [9], that are in minor concentration in VOO. However, after VOO consumption, the phenol compounds are extensively

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metabolized which are subsequently absorbed into the systemic circulation and detected in plasma in their conjugated forms. As a consequence, some of these polyphenol metabolites have been used as VOO consumption markers in the most recent interventional studies [10,11], that have demonstrated the dose-dependent absorption of olive oil phenols in humans and their urinary excretion as glucuronide conjugates.

However, the effect of repeated consumption of VOO on endogenous phenolic metabolites of fasting plasma is unknown. For this reason, we hypothesized that regular long-term VOO intake could have an indirect protection effect on the endogenous phenols. Thus, the aim of the study was to determine the phenolic profile of human plasma in a fasting state of long-term regular VOO consumers, using the fasting plasma of non-consumers as a natural control.

2. Experimental

2.1. Reagents and chemicals

Reference standards of ascorbic acid, 3,4-dihydroxyphenylacetic acid (DOPAC), 3,4-dihydroxymandelic acid, 3,4-dihydroxyphenylalanine (DOPA), L-noradrenaline, L-norepinephrine, L-epinephrine, 2′-hydroxyflavanone and 4′-hydroxyflavanone were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Taxifoline (as internal standard), and reference standards of hydroxytyrosol, caffeic acid and luteolin were purchased from Extrasynthese (Genay, France). Tryptophan, homovanillic acid, dopamine and tyrosine were purchased from Fluka Co. (Buchs, Switzerland). Methanol (HPLC grade) was provided by Scharlau Chemie (Barcelona, Spain). Water was of Milli-Q quality (Millipore Corp, Bedford, MA, USA). All other reagents were of analytical grade.

2.2. Subjects

Forty healthy participants of whom 20 (10 women and 10 men) were regular long-term VOO consumers and 20 (10 women and 10 men) were non-consumers, the latter being Rumanian people, who never ingested any types of olive oil, specially VOO because they disliked its taste. The criteria for the group of consumers were long-term consumption of VOO (more than 23 g/day over a period of 10 years), and for the group of non-consumer, the total absence of VOO in the diet during the previous 10 years. The criteria for exclusion, derived from medical history and a complete physical examination, were diabetes mellitus, congestive heart failure, renal or hepatic insufficiency, thyroid or other endocrine disease, blood pressure >140/90 mmHg and the current use of drugs or vitamins.

The human study design was approved by the Ethical Committee of Clinical Research at Sant Joan University Hospital, Reus, Spain (Reference 08-04-24/4 proj5). The study protocol was fully explained to the patients and they gave their written consent on enrolment.

2.3. Preparation of the plasma samples

Human plasma samples were obtained by venipuncture from the forty healthy volunteers (both consumers and non-consumers). Blood samples (50 ml) were collected after a 12 h overnight fasting period. The samples were stored in Vacutainer™ tubes containing ethylenediamine tetraacetic acid (EDTA) as an anticoagulant. They were protected from the light with aluminium foil, and centrifuged for 15 min at 1500 × *g* and at 4 °C (Kokusan, H-103RS, Japan), and the plasma was immediately separated from the cells and kept at −80 °C until analysis.

2.4. Laboratory measurements

Total plasma cholesterol, very low-density lipoprotein (VLDL) and total triacylglycerol concentrations were measured with enzymatic kits (Boehringer Mannheim, Mannheim, Germany). HDL cholesterol was measured by a direct method (Immuno AG, Vienna, Austria) and low-density lipoprotein (LDL) cholesterol determined by the Friedewald algorithm [LDL-cholesterol = total cholesterol − (triacylglycerols/2.2 + HDL-cholesterol)] [12]. Inter-assay coefficients of variation ranged from 2.5 to 3.0% for total plasma cholesterol, 2.4 to 3.1% for HDL-cholesterol, and 2.6 to 4.8% for total plasma triacylglycerols.

2.5. Solid-phase extraction (SPE) of phenols in human plasma

The phenolic extracts of fasting human plasma were obtained by a solid-phase extraction procedure using the method in Ruiz-Gutierrez et al. [13] with modifications. Just before extraction, a standard solution (10 μg/ml) of taxifolin as internal standard and 30 μl of phosphoric acid (85% v/v) was added to 3 ml aliquots of defrosted plasma and mixed in a vortex for 1 min. SPE of phenols from plasma samples was carried out on an Oasis HLB extraction cartridge (200 mg, 6 ml; WAT 106202) purchased from Waters (Milford, USA). The sorbent of the OASIS HLB SPE column is a copolymer of *N*-vinylpyrrolidone and divinylbenzene. The hydrophilic nature of this sorbent increases the wettability of the copolymer necessary to trapping the hydrophilic analytes as phenols or similar chemical structures. Prior to use, the cartridge was conditioned with 3 ml of methanol followed by equilibration with 3 ml of water. A 3 ml aliquot of spiked acidified plasma was slowly loaded into the cartridge, followed by 1.5 ml of water Milli-Q, and then 3 ml of 5% (v/v) methanol/water. The phenolic fraction was eluted under vacuum with 6 ml of methanol and collected in a tube containing 30 μl of 1% ascorbic acid solution. The phenolic fraction was dried under a nitrogen stream. Before the high-performance liquid chromatography (HPLC) analysis the residue was resolved with 200 μl of methanol and 100 μl of water/acetic acid 2‰.

2.6. Semi-quantification of phenols in fasting plasma samples

The phenolic fraction obtained from the fasting plasma samples was analysed and quantified by HPLC on Waters Inc. (Milford, USA) liquid chromatography equipment with a Waters 600 E pump, Waters 717 plus auto sampler (loop 20 μl) and Waters 996 diode-array detector (DAD). The column was an Inertsil ODS-3 (5 μm, 15 cm × 4.6 mm i.d.) from GL Sciences Inc. (Tokyo, Japan) equipped with a Spherisorb S5 ODS-2 pre-column (5 μm, 1 cm × 4.6 mm i.d.) from Teknokroma (Barcelona, Spain). Empower Software 2002 from Waters Corporation (Milford, USA) was used to manage the system and to process the information. The phenolic extract was filtered through the 0.20 μm filter before injection. Water/acetic acid 2‰ was used as solvent A, and methanol as solvent B. Solvent A was held isocratically at 95% for 2 min, then decreased to 75% at 10 min, followed by further linear reduction to 60% at 20 min, then decreased to 50% at 30 min, and reduced to 0% at 40 min with 5 min isocratic time, followed by strong linear ramping to 95% at 55 min and then held constant for 5 min. For each chromatographic peak the spectral data were acquired with the diode-array detector (DAD) between 200 and 600 nm wavelengths. The chromatograms were obtained at 278 and 339 nm. The phenols from the fasting plasma samples were semi-quantified by the external standard method using caffeic acid and the results are expressed as μM caffeic acid of plasma.

2.7. Mass spectrometry analysis

For the tentative identification of the phenol plasma metabolites, the mass spectrum characteristics of the chromatographic peaks were obtained with a TQD (triple quadrupole)^{TM} detector (Waters, Milford, MA, USA) using the electrospray ionisation (ESI) source Z-spray^{TM}. The source working conditions were as follows: capillary voltage of 3.0 kV; source temperature, 150 °C; desolvation temperature, 400 °C; cone gas flow rate, 80 l/h; and desolvation gas flow rate, 800 l/h. Nitrogen (99.9% purity) was used as a nebulising and desolvation gas and was provided by a high-purity nitrogen generator N_2LCMS (Claind, Como, Italy), and argon (99.9999% purity, Air Liquide, Barcelona, Spain) was used as the collision gas. The MassLynx 4.1 software was used to collect and analyse the obtained data. Full-scan data were obtained by scanning from m/z 80 to 1200 in both negative and positive modes. In the product ion scan experiments, tandem mass spectrometry (MS/MS) was used, and the product ions were produced by collision-induced fragmentation of selected precursor ions in the collision cell of the triple quadrupole mass spectrometer (MS) and their mass was analysed with the instrument's second analyser.

2.8. Validation procedure

The SPE of phenols from human plasma followed by HPLC-DAD analysis was validated according to the Food and Drug Administration [14]. In assessing the quality of the method, we examined the following characteristics: repeatability, intra-day precision and inter-day precision, linearity, accuracy, detection power with the limit of detection (LOD) and limit of quantification (LOQ), and recovery.

For validation purposes, a homogeneous plasma matrix (without phenols) or "blank plasma" was used. It was spiked with pool of hydroxytyrosol, caffeic acid and luteolin as standards, representative compounds of simple phenols, phenolic acid family and flavonoid family, respectively, covering the expected concentration range in the plasma matrix (0.05–50.0 μM). Calibration curves were constructed with six concentration points by spiking known quantities of target compounds into "blank plasma" (plasma treated with SPE). The peak areas at 278 nm for hydroxytyrosol and caffeic acid, and 339 nm for luteolin, were plotted against the concentration for each target compound. The linearity of the data was checked by performing linear least-squares regression analysis.

The percentage of SPE recovery of all standard compounds was assessed at all the concentration levels of the calibration curve. Four replicates ($n = 4$) were done for each concentration level. The chromatographic peak areas obtained from spiked "blank plasma" samples with known concentrations were compared with the peak areas obtained from the pure methanol solutions.

Table 1
Characteristics of the human volunteers' and plasma biochemical parameters after a 12 h overnight fasting period.

	Consumer	Non-consumer
Age (year)	45.5 ± 9.5	35.0 ± 6.5
Weight (kg)	76.2 ± 16.6	86.0 ± 16.1
Height (cm)	164.6 ± 7.9	166.8 ± 5.7
BMI (kg/m^2)	28.1 ± 5.6	30.8 ± 4.9
TCh (mM)	5.1 ± 1.0	5.0 ± 0.8
HDL Ch (mM)	1.3 ± 0.4	1.3 ± 0.5
VLDL (mM)	0.5 ± 0.2	0.6 ± 0.4
LDL (mM)	3.5 ± 0.9	3.2 ± 0.7
TG (mM)	0.9 ± 0.5	1.2 ± 1.0

BMI: body mass index; TCh: total cholesterol; HDL Ch: HDL cholesterol; VLDL cholesterol; LDL cholesterol; TG: triacylglycerols.
Values are the means ± SEM, n = 20.

The repeatability study (system precision) was performed by injecting the standards (hydroxytyrosol, caffeic acid and luteolin) in six replicates ($n = 6$). The repeatability was expressed as relative standard deviation (RSD) (coefficient of variation). The inter-day precision assay was carried out by performing the overall assay, SPE and chromatographic methods. The peak areas were considered for calculating the concentration and establishing the precision that was expressed as the relative standard deviation. The measurement was repeated on a second day for the inter-day precision.

The LOD was defined as the analyte concentration that gave a signal-to-noise ratio (S/N) of 3 [14]. The LOQ referred to the lowest concentration of the analyte ($n = 5$) that could be determined with 20% of accuracy and precision.

2.9. Statistical analysis

Data are expressed as the mean ± SEMs (standard error of the means). Consumer and non-consumer groups were assumed to be random samples drawn from a normally distributed population. In order to compare the concentration of plasma phenols among consumers and non-consumers, an analysis of variance was performed using the general linear models (GLM) procedure ($\alpha = 0.05$).

The variances of the two groups were equal. The factors of age, sex and olive oil consumption and their interactions were tested on the possible phenol compound concentrations. If two factors interacted, an LSMEANS analysis was carried out. TUKEY's multiple range tests were conducted to determine significant differences among treatments. The statistical analyses were performed with the Statistical Package for Social Science software package (SPSS version 15.0).

3. Results

3.1. Characteristics and dietary composition of the participants

Recruitment for the study was carried out between September and November 2008. The characteristics, anthropometrics and lipid profile of the human plasma volunteers are shown in Table 1. No significant differences were observed between the two groups, except for age, which was lower in the group of non-consumers (mean ± SEM; 35.0 ± 6.5 year) than among consumers (45.5 ± 9.5 year) ($P < 0.05$).

The diet composition of the participants is shown in Table 2. This was similar except for a higher intake of polyunsaturated fatty acids (PUFA) in the non-consumer group ($P < 0.05$) and higher MUFA consumption in the virgin olive oil consumer group ($P < 0.001$).

3.2. Optimization and validation of the analytical method off-line SPE-HPLC-DAD

The proposed method includes a clean-up SPE procedure using an Oasis HLB extraction system (200 mg) with good recovery efficiency and a volume of 3 ml of the plasma sample. During the optimization of the phenolic extraction procedure, three different plasma volumes (1–3 ml) were assessed. In parallel, three packed cartridges, of 30, 60 and 200 mg, were evaluated. The maximal resolution of the chromatographic peaks was obtained when 3 ml of plasma was extracted using a 200 mg packed cartridge. The recovery experiments were conducted using phenol-free plasma spiked with hydroxytyrosol, caffeic acid and luteolin. The results showed that the recovery by SPE extraction was found to be 104–97% for hydroxytyrosol, 93–89% for caffeic acid and 75–70% for luteolin.

The method validation reveals good repeatability, linearity and detection power for the phenol standards selected by using a plasmatic matrix free of phenols. The intra- and inter-day accuracy and precision were assessed and the results are shown in Table 3. Good

Table 2
Composition of diets of the volunteers in the study.

Parameter	Consumer	Non-consumer
Energy (kcal/day)	2099 ± 846	1547 ± 883
Protein (% of energy)	16.4 ± 2.8	17.9 ± 6.9
Total carbohydrates (% of energy)	38.8 ± 9.4	43.6 ± 5.3
Total fat (% of energy)	41.4 ± 7.5	35.1 ± 4.4
PUFA (% of energy)	5.1 ± 1.5	11.4 ± 4.8
MUFA (% of energy)	20.6 ± 4.1	10.0 ± 2.1
SFA (% of energy)	12.4 ± 3.6	10.1 ± 2.1
Fiber (g/day)	20.8 ± 10.2	15.0 ± 8.8
Alcohol (g/day)	10.6 ± 9.3	13.1 ± 9.4
Cholesterol (mg/day)	362 ± 208	222 ± 151

PUFA: polyunsaturated fatty acid; MUFA: saturated fatty acid; SFA: saturated fatty acid. Values are the means ± SEM, $n = 20$.

linearity was achieved for all the analytes with correlation coefficients (R^2) of 0.999, 0.993 and 0.996 for hydroxytyrosol, caffeic acid and luteolin respectively.

The detection power of the method, presented as its LOD, was 2.4 nmol/ml for hydroxytyrosol, 3 nmol/ml for caffeic acid and 0.5 nmol/ml for luteolin. The LOQ was 5 nmol/ml for hydroxytyrosol and caffeic acid, and 8 nmol/ml for luteolin.

3.3. Phenolic compounds profile of the fasting plasma of regular VOO consumers and non-consumers

The chromatograms of the fasting plasma samples of the two groups, regular VOO consumer and non-consumers, are presented in Fig. 1. A total of 17 chromatographic peaks with the characteristic ultraviolet (UV) spectrum of the phenolic structures were selected for semi-quantification based on the calibration curve of caffeic acid.

The comparative analysis of the chromatographic profiles of the phenolic extracts of plasma samples from the two volunteer groups showed no qualitative differences. The compounds selected were confirmed to be the same compound in all the plasma samples from each group by HPLC–MS/MS. Thus, no specific compound was detected in the plasma samples from regular consumers of VOO that could be used as a biomarker of long-term VOO consumption.

In order to obtain structural information about the phenolic compounds detected in the fasting plasma samples, the deprotonated molecule by negative mode $[M–H]^-$ and the protonated molecule by positive mode $[M+H]^+$ in full-scan mode was firstly studied. The mass spectral characteristics of the results obtained by negative and positive modes are shown in Table 4. ESI in negative and in full-scan mode of the peaks 2, 6, 10 and 11 of the fasting plasma chromatogram (Fig. 1) showed a molecular loss of (m/z 44) that could be tentatively assigned to the loss of a carbon dioxide group; and this product ion $[M–H–CO_2]^-$ was the most intense fragment ion for these peaks. For peak 1, the product ion m/z 115 was generated by the expulsion of secondary and tertiary alcohols $[M–H–C_2H_5O_2]^-$ that corresponded to the loss of 60 m/z, and this compound was identified as ascorbic acid comparing with the spectral characteristics of the corresponding standard. This was added to the plasma samples before phenolic extraction to improve the stability of the phenols during the extraction phase. In addition, ascorbic acid forms part of the plasma endogenous antioxidant defence system as an electron donor, $^1O_2$ quencher or by regeneration of the α-tocopheryl radical [15]. Additionally the analysis of the spectral characteristics of peaks 4 and 11 permitted their identification as tryptophan and homovanillic acid, respectively comparing with the spectral characteristics of the respective standards. Peak 17 could be assigned to a flavonoid structure, based on its UV spectrum with its maximum at 339 nm (Fig. 2A). The precursor ion of P17 was m/z 239 similarly with the precursor ion of 2′-hydroxyflavanone and 4′-hydroxyflavanone standards [16].

Afterwards, ESI in positive and in full-scan mode (Table 4) showed the precursor ions with m/z ranging from 154 to 242. This corresponded to the peaks 3, 5, 7–9, 12, 14–16 of the fasting plasma chromatogram (Fig. 1). By comparing the precursor ion and product ions with those reported in the literature, peaks 3 and 5 were identified as dopamine and tyrosine respectively [17,18]. The identity of these compounds was also confirmed by comparing their retention times and spectral characteristics with those of their commercial standards.

In order to confirm whether the other compounds quantified in the fasting plasma corresponded to the catecholamines, their metabolites or their precursors, the plasma samples were spiked with commercial standards of 3,4-

Table 3
Accuracy and precision of the analysis of hydroxytyrosol, caffeic acid and luteolin in plasma blanks spiked with 5 μM.

	Intra-day RSD (%)	Inter-day RSD (%)	Accuracy RE (%)
Hydroxytyrosol	6.8	8.3	−10.6
Caffeic acid	3.6	8.9	7.3
Luteolin	9.6	6.8	−1.8

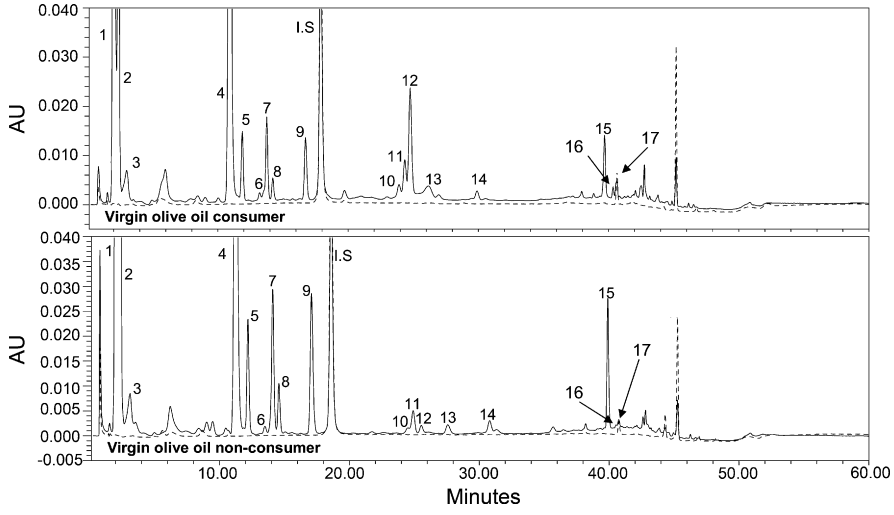

Fig. 1. Chromatographic profile (HPLC-DAD) extracted at 278 nm (—) and 339 nm (– – –) of phenolic metabolites in the fasting human plasma of regular VOO consumers and non-consumers. Peak numbers refer to Table 4.

Table 4
Retention time, mass spectral characteristics and proposed fragmentation of chromatographic peaks (Fig. 1) of the fasting plasma phenolic extracts by HPLC-ESI-MS/MS in negative ESI(−) and positive ESI(+) modes, respectively. MW: molecular weight.

Peak	RT (min)	Precursor ion (*m*/*z*)	Product ions and proposed fragmentation (*m*/*z*)	MW	Compound
ESI(−)					
P1	1.9	175 $[M-H]^-$	115 $[M-H-C_2H_5O_2]^-$	176	Ascorbic acid
P2	2.1	344 $[M-H]^-$	300 $[M-H-CO_2]^-$	345	Unknown
P4	10.9	203 $[M-H]^-$	175 $[M-H-28]^-$ 116 $[M-H-CO_2CH_3]^-$	204	Tryptophan
P6	13.2	129 $[M-H]^-$	85 $[M-H-CO_2]^-$	130	Unknown
P10	23.4	255 $[M-H]^-$	211 $[M-H-CO_2]^-$	256	Unknown
P11	24.4	181 $[M-H]^-$	137 $[M-H-CO_2]^-$	182	Homovanillic acid
P13	29.5	255 $[M-H]^-$	211 $[M-H-CO_2]^-$ 195 $[M-H-CO_2CH_3]^-$ 167 $[M-H-2CO_2]^-$	256	Unknown
P17	42.6	239 $[M-H]^-$	195 $[M-H-CO_2]^-$ 151 $[M-H-2CO_2]^-$	240	Hydroxyflavanone
ESI(+)					
P3	2.8	154 $[M+H]^+$	137 $[M+H-NH_3]^+$ 119 $[M+H-NH_3-H_2O]^+$	153	Dopamine
P5	11.6	182 $[M+H]^+$	165 $[M+H-NH_3]^+$ 136 $[M+H-CO_2H]^+$	181	Tyrosine
P7	13.8	181 $[M+H]^+$	163 $[M+H-H_2O]^+$	180	Unknown
P8	14.2	181 $[M+H]^+$	124 $[M+H-57]^+$	180	Unknown
P9	16.7	195 $[M+H]^+$	177 $[M+H-H_2O]^+$	194	Unknown
P12	24.8	223 $[M+H]^+$	177 $[M+H-CO_2H]^+$ 159 $[M+H-CO_2H-H_2O]^+$	222	Unknown
P14	29.9	188 $[M+H]^+$	156 $[M+H-32]^+$	189	Catecholamine (tentatively)
P15	39.6	224 $[M+H]^+$	159 $[M+H-74]^+$	225	Catecholamine (tentatively)
P16	40.6	242 $[M+H]^+$	130 $[M+H-112]^+$	243	Catecholamine (tentatively)

dihydroxyphenilacetic acid (DOPAC), 3,4-dihydroxymandelic acid, 3,4-dihydroxyphenylalanine (DOPA), L-noradrenaline, L-norepinephrine, L-epinephrine. However, it was not possible to show a complete correspondence between the molecular weights and the chromatographic and spectral characteristics of the compounds quantified in plasma and the catecholamine standards.

After analysing the spectral characteristics of each chromatographic peak, it was possible to confirm that no qualitative differences were observed between the plasma samples of VOO consumers and non-consumers. The semi-quantification of these 17 chromatographic peaks (Table 5) showed a high inter-individual variability in both groups. The general linear model (GLM) analysis reflected that there were significant quantitative differences in two compounds, P17 and P15. Fasting consumer's plasma showed higher concentration of a proposed hydroxyflavanone type compound (P17) (2.90 ± 0.04 μM vs 1.5 ± 0.04 μM) and a proposed catecholamine derivative (P15) (0.70 ± 0.03 μM vs 0.56 ± 0.03 μM) than the plasma of non-consumers ($P < 0.05$).

Moreover, the results of the GLM procedure showed that the concentration of some peaks was affected by the sex factor, besides the effect of olive oil consumption. Concerning the interaction of oil consumption and sex, the concentration of some peaks (P4 and P10) corresponding to tryptophan and a carboxylic acid structure respectively, was significantly higher ($P < 0.05$) in the fasting plasma of women. The concentration of the compounds

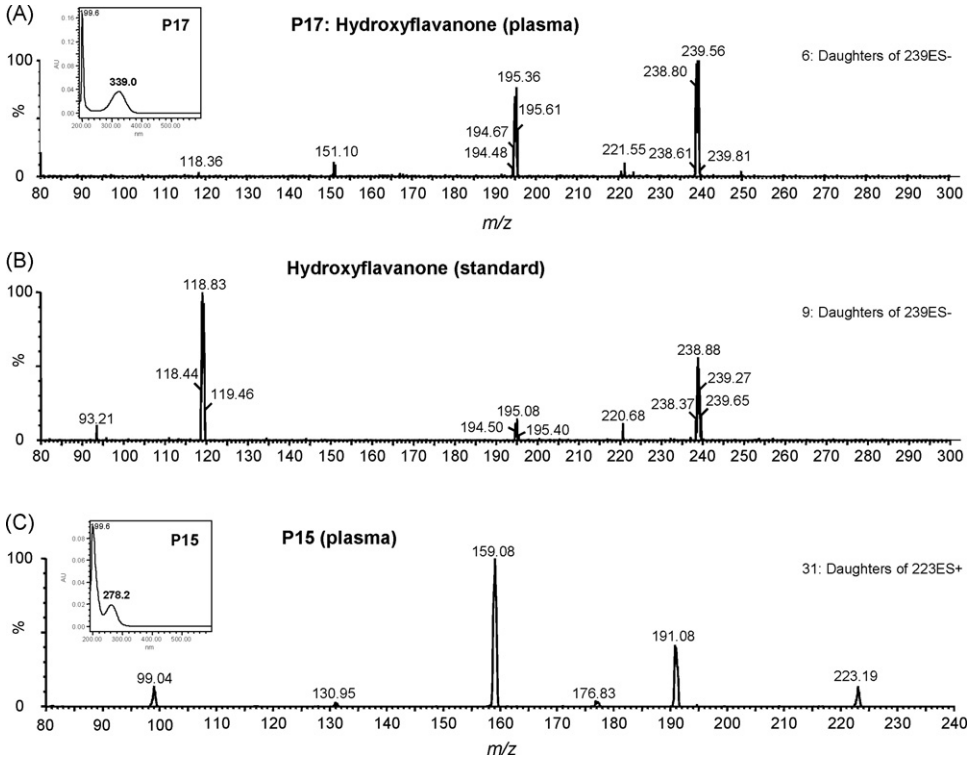

Fig. 2. Product ion scan chromatograms and the UV spectrum for the peaks: (A) P17 plasma; (B) hydroxyflavanone standard; (C) P15 plasma.

Table 5
Concentration of phenols in human plasma of the consumers and non-consumers of virgin olive oil.

Peak	RT (min)	Concentration in plasma (μM cafeic acid)	
		Consumer	Non-consumer
P1	1.9	18.5 ± 3.5	23.8 ± 5.1
P2	2.1	26.4 ± 5.0	25.4 ± 4.6
P3	2.8	4.9 ± 0.8	4.3 ± 0.8
P4	10.9	36.6 ± 2.3	35.5 ± 2.1
P5	11.6	6.9 ± 1.5	5.6 ± 1.4
P6	13.2	0.65 ± 0.17	0.50 ± 0.15
P7	13.8	3.4 ± 0.7	2.7 ± 0.6
P8	14.2	1.02 ± 0.18	0.92 ± 0.16
P9	16.7	3.3 ± 1.3	4.2 ± 1.2
P10	23.4	0.58 ± 0.09	0.52 ± 0.08
P11	24.4	0.97 ± 0.23	0.45 ± 0.22
P12	24.8	2.2 ± 0.3	1.7 ± 0.3
P13	29.5	0.71 ± 0.11	0.55 ± 0.10
P14	29.9	0.81 ± 0.12	1.09 ± 0.11
P15	39.6	0.70 ± 0.03*	0.56 ± 0.03*
P16	40.6	0.81 ± 0.14	0.76 ± 0.13
P17	42.6	2.90 ± 0.04*	1.50 ± 0.04*

Values are the means ± SEM (standard error of the mean), $n = 20$.
* Means differ significantly, $P < 0.05$.

semi-quantified in fasting plasmas were in the reference intervals described in the literature for plasma catecholamines, showing a wide range from 0.01 to 3.58 μM [19].

4. Discussion

Our hypothesis, that the long-term consumption of VOO phenols could increase the concentration of endogenous simple phenols, with a potential antioxidant function, in fasting plasma from healthy individuals, is confirmed. There is limited data regarding the effect of the repeat dosing of dietary phenols on the concentration of endogenous (i.e., non-dietary) simple phenols in the plasma from healthy individuals. Because of the low physiologic concentrations of endogenous metabolites in human plasma under fasting conditions, and the potential interference from a large number of other endogenous and exogenous metabolites that are chemically, physically, and physiologically quite similar, analysing and identifying the plasma phenol metabolites is complex [18]. To minimize these interferences and to preconcentrate of the sample, before the chromatographic analysis, a solid phase clean-up of the plasma samples was applied. To evaluate the efficiency in the phenol retention, the eluted fraction corresponding to the load sample and the water and 5% methanol wash phases were analysed by HPLC-DAD method described in Material and methods section. Results showed that neither compound with spectral characteristics of phenols was detected in the collected fractions, load and wash phases.

The comparative analysis between fasting plasmas of habitual VOO consumers and non-consumers showed a similar phenolic profile (Fig. 1). As a general rule, the metabolites of polyphenols are rapidly eliminated from the plasma, which indicates that the consumption of plant products on a daily basis is necessary to maintain high concentrations of metabolites in the blood [20]. The pharmacokinetic studies of tyrosol, hydroxytyrosol, and 3-*O*-methyl-hydroxytyrosol show that the time taken to reach peak concentrations in plasma are around 1 h after the ingestion of VOO with medium and high phenol content [11]. In parallel, their estimated elimination half-life is lower than 3.5 h, so the concentration of these phenols in plasma 8 or 10 h after VOO consumption is negligible. This rapid elimination of the phenols could explain why no specific biomarker of long-term regular VOO consumption was detected in our study in the plasma from the consumers under the fasting conditions.

The inclusion criterion of the volunteers in our study was based on the long-term consumption of VOO, or by total absence of VOO in the diet. As a result, the dietary phenols in both groups were provided by fruits, vegetables, coffee, wine, etc. Not having detected some specific food phenols in fasting plasma samples makes us think that the structures quantified in our study could correspond to endogenous metabolites, such as catecholamines or their metabolites with a similar catecholic structures.

If no qualitative differences were observed between the plasmas from the two volunteers groups, the semi-quantification of the 17 compounds with the characteristic UV spectrum of phenolic structures revealed quantitative differences. The concentration of most of the compounds was higher in plasma from VOO consumers (Table 5). As a consequence of the interindividual variation in each group of volunteers, statistically significant differences, without interaction with other factors such as age and sex, were only observed in the concentration of two compounds, a proposed hydroxyflavanone-type compound (P17) with a UV spectrum with maximum at 339 nm, and a proposed catecholamine structure (P15) with a UV spectrum with maximum at 278 nm (Fig. 2). Peak 17 showed the same precursor ion as 2′- and 4′-hydroxyflavanone, 239 m/z. Then, the generated product ions of peak 17 were compared with those generated by the authentic standards, 2′- and 4′-hydroxyflavanones. The compared compounds showed the same product ion, 195 m/z. Although the retention time of peak 17 did not match those of the above mentioned standards, peak 17 can be characterized as having hydroxyflavanone structure.

In addition, the more concentrated compounds, corresponding to peak 2, tryptophan (P4) and tyrosine (P5), showed higher concentration in plasma of olive oil consumers. Both tyrosine and tryptophan are metabolic precursors of essential central and peripheral neurotransmitters: catecholamines, e.g. dopamine, and hydroxyindoleamines, e.g. serotonin.

Catecholamine neurotransmitters and tryptophan derivatives, including serotonin, N-acetylserotonin, 5-HIAA, and melatonin, possess free radical scavenging and neuroprotective powers [21–23]. This fact could confirm our hypothesis that the long-term consumption of VOO phenols could increase the concentration of simple endogenous phenols with a potential antioxidant function, in plasma of healthy individuals. In this sense, an interesting review by Clifford and Brown [24] concluded that it is difficult to envisage how diet-derived polyphenol metabolites can make a major contribution to radical scavenging in plasma, compared with the contribution to be expected from the endogenous antioxidants in healthy individuals.

Catechol-*O*-methyl transferase (COMT; EC 2.1.1.6) catalyzes the transfer of a methyl group from *S*-adenosyl-L-methionine to polyphenols having an *o*-diphenolic (catechol) moiety and plays a crucial role in the metabolism of dopamine [25]. This way, the habitual ingestion of dietetic phenols from VOO could mean a competitive inhibition of COMT that may explain the higher concentration of catecholamines and their metabolites in the plasma. Thus, the potential competitive inhibition of the COMT-catalyzed *O*-methylation of endogenous catecholamines and catechol estrogens by polyphenols with catechol groups may have a beneficial effect on cardiovascular pathologies. Another reason explaining the increased catecholamine derivatives in fasting plasma with regular VOO consumption observed in our study, could be related to the results of the study by Oi-Kano et al. [26] who reported that the phenolic fraction of VOO enhances the noradrenaline and adrenaline secretions in plasma which explain the enhanced triglyceride catabolism and thermogenesis. In that study, the average total amount of phenols consumed in rats fed with the VOO diet containing 30% virgin olive oil (in the case of diet intake of 17 g per day) were about 20 mg per rat during the 28-day experimental period. In our study, the diet of the regular consumers of VOO con-

tained around 50 ml of VOO per day, which supposes about 7 mg of olive oil phenols. The stimulation of noradrenaline and adrenaline secretions by olive oil phenols could be related to the greater concentration of these compounds quantified in the plasma of habitual consumers of VOO.

In summary, no specific phenolic structure related to VOO ingestion (hydroxytyrosol, tyrosol and their conjugate forms) was detected in the fasting plasma from the habitual VOO consumers, which suggests that the phenols are completely metabolised within 8 h after ingestion. The fact of not detecting food phenols in the plasma samples makes us believe that the structures quantified in the study could correspond to endogenous (i.e., non-dietary) metabolites, probably catecholamines or their metabolites, with similar chemical structures to phenols. Of special interest is that more of the compounds quantified showed a higher concentration in the fasting plasma from habitual VOO consumers. This suggests an indirect protective mechanism of long-term regular VOO consumption related to the protection of the endogenous antioxidant system.

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