UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA

DEPARTAMENT DE NUTRICIÓ I BROMATOLOGÍA

Efecto del consumo del aceite de oliva sobre la composición de las lipoproteínas de baja densidad en individuos de diferentes países europeos.

Karina de la Torre Carbot, 2007

IV. PUBLICACIONES

Publicación 1

"Characterization and quantification of phenolic compounds in olive oils by Solid-Phase Extraction, HPLC-DAD and HPLC-MS/MS" K. de la Torre-Carbot, O. Jauregui, E. Gimeno, A.I. Castellote, R.M. Lamuela-Raventós and M.C. López-Sabater. J ***Agric Food Chem***, 53 (11),2005

Resumen

Los compuestos fenólicos del aceite de oliva virgen, han sido estudiados ampliamente en estos últimos años porque son esenciales para su calidad y propiedades nutricionales.

Por la complejidad de los compuestos fenólicos que se encuentran en el aceite de oliva, muchos de estos continúan sin haber sido identificados y es difícil comparar los datos de la bibliografía existente actualmente, siendo a menudo incompleta y contradictoria.

Este trabajo fue realizado para desarrollar y validar un método reproducible para determinar cualitativa y cuantitativamente los compuestos fenólicos existente en aceite de oliva. Otro objetivo fue la recopilación de información existente sobre los secoiridoides contenidos en el aceite de oliva virgen. Para este objetivo, se desarrolló un método de extracción en fase sólida de los compuestos fenólicos con la combinación de tres disolventes: agua, metanol y acetonitrilo para su análisis posterior por HPLC/DAD-MS/MS. La detección y cuantificación fue llevada a cabo con DAD a 280, 240 y 320 nm, mientras que la MS se utilizó exclusivamente para la identificación de los compuestos.

En este trabajo, se evaluaron las fases hidrosolubles de tres aceite de oliva virgen distintos. Fueron cuantificados un total de 23 compuestos, 20 de ellos fueron caracterizados.

Entre la mayoría de los compuestos fenólicos presentes en los aceite de oliva virgen estudiados, se encuentran diversos secoiridoides derivados de ligstrósido

como de oleuropeína. Estos secoiridoides comprenden del 77 al 88% del total de compuestos fenólicos presentes. El hidroxitirosol y tirosol se encontraron también en concentraciones importantes.

Durante la molienda y el batido en el proceso para la elaboración del aceite de oliva, se produce la hidrólisis del enlace glucosídico formando los aglicones de los fenoles. Sin embargo también ocasiona modificaciones en el fragmento elenólico generando una gran variedad de derivados que conservan el anillo fenólico, y entre algunos de ellos constituyen isoformas.

En un menor porcentaje se encontraron los compuestos fenólicos simples ácido vainíllico, vainillina y ácido *p*-coumárico y los flavonoides: luteolina, apigenina y metoxiluteolina.

El método mostró tener una linealidad, recuperación y precisión satisfactorias y unos límites de detección y cuantificación apropiados.

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Characterization and Quantification of Phenolic Compounds in Olive Oils by Solid-Phase Extraction, HPLC-DAD, and HPLC-MS/MS

KARINA DE LA TORRE-CARBOT,[†] OLGA JAUREGUI,[‡] EVA GIMENO,[†] ANA I. CASTELLOTE,[†] ROSA M. LAMUELA-RAVENTÓS,*[,†] AND M. CARMEN LÓPEZ-SABATER[†]

Departament de Nutrició i Bromatologia, Centre de Referència en Tecnologia dels Aliments (CeRTA), Facultat de Farmàcia, Universitat de Barcelona, Avda. Joan XXIII s/n 08028 Barcelona, Spain, and Unitat de Tècniques Separatives, Serveis de Suport a la Recerca, Universitat de Barcelona, Josep Samitier 1-5 08028 Barcelona, Spain

A simple and reproducible method for qualitative and quantitative analysis of phenolic compounds in virgin olive oils by solid-phase extraction (SPE), high performance liquid chromatography with diode array detector (HPLC-DAD), and HPLC-mass spectrometry (MS) in tandem mode was developed. The polar fraction was obtained from samples of three different virgin olive oils. Detection and quantification were performed at 280, 240, and 320 nm. For identification purposes, HPLC-MS/MS was equipped with turbo ion spray source in the negative-ion mode. Twenty compounds of twenty-three detected and quantified were characterized. The method showed satisfactory linearity ($r > 0.99$), good recovery, satisfactory precision, and appropriate limits of detection (LOD) and quantification (LOQ).

KEYWORDS: Phenolic compounds; olive oil; secoiridoids; liquid chromatography; mass spectrometry

INTRODUCTION

Phenolic compounds are a complex but important group of naturally occurring compounds in plants (*1*). Although the main phenolic compounds in olive fruit are secoiridoid derivatives, olives also contain phenyl acids, phenyl alcohols, and flavonoids (*2*). The secoiridoids is a very specific group that are abundant in *Oleaceas* and many other plants that are produced from the secondary metabolism of terpenes as precursors of various indole alkaloids, and are usually derived from the oleoside type of glucoside oleosides, which are characterized by a combination of elenolic acid and a glucosidic residue. It could be stated that these compounds proceed from the acetate/mevalonate pathway (*3*, *4*).

Because virgin olive oil is a natural product, its chemical composition varies. Thus, the phenolic content of virgin olive oil is affected by the variety, location, environmental conditions, degree of ripeness, and the type of oil extraction (*2*−*6*). However, phenolic compounds are removed when the oil is refined.

Phenolic compounds present in olive oil have received considerable attention in recent years because they are essential to its quality and nutritional properties. They affect its shelf life because they retard oxidation (*4*, *7*, *8*) and its sensorial properties: color, astringency, bitterness, and flavor (*4*, *9*−*12*). In addition, some studies have been performed on the protective effects of olive oil phenolic compounds on health, including the protection on risk factors for cardiovascular disease (*13*−*17*).

To identify and quantify these compounds in olive oil, several extraction procedures and analytical methods have been developed. Traditionally, the phenolic fraction of olive oil has been isolated by liquid−liquid extraction (*5*, *18*−*20*). However, as extraction with these methods is very laborious, more time and higher quantities of dissolvent are required. Some attempts to isolate these compounds by solid-phase extraction have been made (*21*−*24*), but good recovery is not regularly achieved (*25*).

Total phenolic compounds in oil are quantified mainly by the Folin-Ciocalteau method, based on the reduction properties of phenols in alkaline medium. However, this is a nonspecific colorimetric method. Thus, some authors have tried to separate and quantify specific phenols by gas chromatography (*21*, *26*), by HPLC-DAD (*22*−*24*, *27*, *28*), or by HPLC-MS (*29*−*31*), but the phenolic concentrations reported in the literature are often not comparable. The formality of expression and the spectrophotometric features of the reference phenol dramatically affect the calculation of phenolic concentration in the same oil (*24*).

However, among the methods used for the determination of phenols, the coupling of HPLC-MS with atmospheric pressure

* Author to whom correspondence should be addressed [telephone +34-93-403 48 43; fax + 34-93-403 59 31; e-mail lamuela@.ub.edu].
[†] Departament de Nutrició i Bromatologia, Universitat de Barcelona.
[‡] Unitat de Tècniques Separatives, Universitat de Barcelona.

10.1021/jf0501948 CCC: $30.25
Published on Web 05/10/2005

ionization techniques, that is, electrospray ionization (ESI) (*32*) or atmospheric pressure chemical ionization (APCI) (*29*), is a powerful tool for identification of natural products in crude plant extracts because of their soft ionization. Its main advantage over the gas chromatography coupled to MS methods described in the literature is that no derivatization is needed.

Many studies have provided good information, and some of them have helped to clarify the structures of some phenolic compounds in oil. Nevertheless, because of the complexity of the wide group of secoiridoids, many of these phenolic compounds in olive oils remain unidentified (*27*). It is difficult to compare data within the literature, because of the lack of consistency: information is not only incomplete but sometimes contradictory as well.

The aims of this study were to develop a simple and reproducible method for the qualitative and quantitative analysis of phenolic compounds in virgin olive oils by SPE, HPLC-DAD, and HPLC-MS, and to summarize the information compiled on olive oil secoiridoids information.

MATERIAL AND METHODS

Chemicals. Phenolic standards: tyrosol, oleuropein, luteolin, methoxyluteolin, and apigenin were purchased from Extrasynthèse (Genay, France), *p*-coumaric acid and vanillic acid were purchased from Fluka (Buchs, Switzerland), and vanillin was purchased from Panreac (Barcelona, Spain). All solvents (methanol, acetonitrile, and *n*-hexane) were of HPLC grade and were purchased from SDS (Peypin, France). Formic acid was purchased from Sigma Chemical Co. (St. Louis, MO). Ultrapure water generated by the MilliQ system (Millipore, Bedford, MA) was used. MilliQ water:methanol (90:10 v:v) was used as the most suitable solvent for the standards.

Samples. The analyses were run on three virgin olive oils: oil A, from Arbequina olives (Catalonia Spain); oil B, from Picual olives (Andalucía Spain); and oil C, a commercial virgin olive oil. Samples were stored in dark-brown glass bottles at 4 °C until analysis. The oil was extracted from high-quality olives and met the standards set by the European Commission (*33*) for extra-virgin quality.

Instruments. Samples were extracted by an SPE-Vacuum Manifold from Tecknokroma (Barcelona, Spain).

HPLC-DAD Instrument. The phenolic compounds were analyzed in a Hewlett-Packard-1050 Series liquid chromatograph with an automatic injector and DAD 1050 series instrument and with a HP Chemstation (Waldbronn, Germany). A 5-μm particle size C_{18} Luna column, 15 cm × 2 mm i.d., was used (Phenomenex, UK).

HPLC-MS/MS Instrument. An Agilent 1100 HPLC (Waldbronn, Germany) equipped with an autosampler and coupled to an API3000 triple-quadrupole mass spectrometer (PE Sciex, Concord, Ontario, Canada) with a turbo ion spray source was used with the same column.

Extraction of Phenolic Compounds. To clean and concentrate the sample, the polar fraction was obtained from 3 g of oil sample using an SPE diol cartridge (Vac RC 500 mg, Waters, Milford, MA). The cartridge was activated with 6 mL of *n*-hexane, 6 mL of methanol:water (80:20), and 3 mL of acetonitrile. The oil was dissolved in 6 mL of *n*-hexane and percolated into the cartridge. To remove the nonpolar fraction, the oil was washed under vacuum with 10 mL of *n*-hexane. Afterward, phenolic compounds were eluted with 8 mL of methanol:water and 4 mL of acetonitrile. During the entire process, the vacuum was maintained at less than 30 kPa. The eluent was evaporated to 2 mL by a rotary evaporator, and the temperature was always controlled (<40 °C), to avoid the deterioration of phenols. The samples were then filtered through a 13-mm PTFE 0.45 μm membrane filter from Waters. According to our previous experience (*34*), filters were checked after various retention assays of the phenolic compounds with olive oil extracted samples. After this, 20 μL was injected into the liquid chromatograph. The entire process was performed in conditions of darkness and with brown glass material.

HPLC-DAD Conditions and Quantification. The column was kept at 40 °C. The mobile phase consisted of a binary solvent system using water acidified with 0.1% formic acid (solvent A) and 100% acetonitrile (solvent B), kept at a flow rate of 0.5 mL/min. The gradient program started with 90% eluent A and 10% eluent B, which ramped linearly to 25% in 12 min. This percentage was maintained for 7 min, and eluent B was ramped again linearly to 40% at 30 min and to 60% at 40 min. Initial conditions were reached in 5 min, so the total run time was 45 min. Detection and quantification were performed at 280, 240, and 320 nm.

Each phenolic compound was expressed with its standard when it was available. Due to the absence of standard for all of the compounds detected, both phenolic alcohols 3,4-dihydroxyphenyl and 4-hydroxyphenyl were expressed as tyrosol, secoiridoids and elenolic acid were expressed as oleuropein, and unknown flavonoids were expressed as luteolin.

HPLC-MS Conditions. The HPLC conditions were as described for the HPLC-DAD system. All of the analyses used the turbo ionspray source in negative mode with the following settings: capillary voltage −3500 V, nebulizer gas (N_2) 10 (arbitrary units), curtain gas (N_2) 12 (arbitrary units), collision gas (N_2) 4 (arbitrary units), declustering potential (DP) −30 V, focusing potential −250 V, entrance potential 10 V, and collision energy (CE) −30 V. Drying gas was heated to 300 °C and introduced at a flow-rate of 5000 $cm^3\ min^{-1}$. Full-scan data were acquired by scanning from *m*/*z* 100 to 800 in profile mode using a cycle time of 2 s with a step size of 0.1 u and a pause between each scan of 2 ms. In product ion scan experiments, MS/MS product ions were produced by collision-activated dissociation (CAD) of selected precursor ions in the collision cell of the triple quadrupole mass spectrometer and mass-analyzed with the second analyzer of the instrument.

Log α. All peaks in the three chromatograms were compared and identified using logarithms of selectivity values (log α). The reference peaks were selected because they appear in the three studied samples and present a good stability. Log α was calculated on the basis of the retention time of each phenolic compound, relative to peak 23 (see **Table 1**), in the case of compounds observed at 280 nm, and peak 11 (luteolin), in the case of compounds observed at 320 nm, considering in both cases, the peak corresponding to the first eluted peak as t_0.

RESULTS AND DISCUSSION

Sample Preparation. Traditionally, the phenolic fraction of olive oils is laboriously isolated by extraction of an oil solution in hexane with several portions of water:methanol, followed by solvent evaporation of the hydro alcoholic extract. SPE extraction is quicker than liquid−liquid extraction and reduces the amount of solvents used. However, previous research using C_{18} (*21*, *35*), C_8 (*23*), and diol cartridges has shown low recoveries (*24*, *25*). In our research, diol cartridge was selected because of its negligible activity on labile esters (*22*) in comparison with reversed phases. Washing and elution conditions were studied to improve the recovery of the extraction. Initially, a mixture of *n*-hexane and ethyl acetate was tried as washing solvent. However, ethyl acetate also elutes some phenolic compounds, because it is a solvent used to extract phenolic compounds, except anthocianins (*36*). In fact, some investigators use it to elute phenolic compounds of non-oily plant extract (*37*, *38*), then it was decided to use *n*-hexane alone. Afterward, elution solvents were also studied: assays were done with methanol, but some polar phenols did not completely elute, so water had to be added to the elution solvents. Nevertheless, when acetonitrile was also added, better recovery was observed. An average increment of 36% was observed when ethyl acetate was eliminated from the washing process and the two solvents were used for the elution: methanol:water (80:20) and acetonitrile. Moreover, the product was concentrated up to 2 mL, instead of evaporated to dryness prior to its final extraction with solvent: methanol, water, acetonitrile, or a combination of these. This evaporation to dryness causes a decrease in recovery of

Table 1. Phenolic Compounds Found in Olive Oils

peak	RT[a]	log α[b]	compound	*m*/*z*[c]	MS/MS ions	standard
1	2.23	−1.4765	hydroxytyrosol	153	123(10)	no
2	3.48	−1.1110	tyrosol	137		yes
3	4.50	−0.9481	vanillic acid	167		yes
4	7.00	−0.4373	vanillin	151	123(60)	yes
5	8.11	−0.3567	*p*-coumaric acid	163		yes
6	9.63	−0.5327	ligstroside derivative	553	137(100), 257(98), 181(70), 109(38), 341(32)	no
7	12.89	−0.3895	ligstroside derivative	335	199(100), 111(40), 155(27)	no
8	13.38	−0.3712	oleuropein derivative	377	307(100), 275(90), 139(16), 111(7)	no
9	14.20	−0.3428	oleuropein derivative	377	275(100), 307(90), 139(18), 111(5)	no
10	15.77	−0.2925	oleuropein derivative	319	199(100), 111(37), 153(5)	no
11	15.79	0.0000	luteolin	285	133(35), 199(13), 107(10), 217(10), 175(8)	yes
12	16.39	−0.2750	ligstroside derivative	361	291(100), 259(30), 101(25), 127(7), 138(8)	no
			oleuropein derivative	365	229(100), 153(25), 138(18)	no
13	16.92	0.0332	apigenin	269	117(30), 107(35), 151(15)	yes
14	17.28	−0.2500	ligstroside derivative	361	291(100), 101(27), 259(25), 127(5), 139(3)	no
			ligstroside derivative	393	317(30), 257(15), 349(5)	no
15	18.07	−0.2291	ligstroside derivative	393	317(55), 349(10), 257(6)	no
16	18.74	−0.2122	ligstroside derivative	393	317(97), 349(15), 257(8)	no
17	19.92	−0.1964	oleuropein derivative	377	275(100), 307(90), 139(18), 111(5)	no
18	20.66	0.1262	methoxyluteolin	299	199(25), 191(20), 227(10)	yes
19	22.60	−0.1254	oleuropein derivative	377	275(100), 307(80), 333(20), 301(7), 181(5)	no
			ligstroside derivative	361	291(100), 101(25), 259(23), 127(7), 139(3)	no
20	25.03	−0.0254	unknown			no
21	27.23	0.2145	ligstroside derivative	361	291(100), 101(27), 259(25), 127(8), 139(3)	no
22	28.97	0.2529	unknown			no
23	29.74	0.000	ligstroside derivative	361	291(100), 101(35), 259(30), 127(8), 139(3)	no

[a] Retention time (min). [b] Logarithms of selectivity values. [c] Mass charge value.

phenolic compounds. Thus, recovery of phenolic compounds was 20% higher on average than when the product was evaporated to dryness.

In summary, olive oil was washed with *n*-hexane to eliminate apolar compounds. Subsequently, the polar fraction was eluted with 8 mL of methanol:water (80:20) and 4 mL of acetonitrile and was later concentrated to 2 mL, prior to its HPLC injection.

HPLC Conditions. The composition of the HPLC mobile phase was optimized to achieve a good resolution between peaks. Different gradients between formic acid (0.1%) in water and acetonitrile, methanol, or a mixture acetonitrile:methanol (1:1) were assayed. In the case of the use of methanol as phase B, the appearance of the final chromatogram was better, but there was more coelution. The best resolution and peak shapes were obtained of a gradient formic acid 0.1% as phase A and acetonitrile as phase B. Detection was performed at 280, 320, and 240 nm. **Figure 1** shows the LC-DAD chromatograms of the phenolic compounds present in the virgin olive oils studied.

Identification of Phenolic Compounds in Olive Oil Samples. MS/MS Experiments. The deprotonated molecule $[M - H]^-$ in full-scan mode and the main product ion of each compound are reported in **Table 1**. HPLC-DAD gives rise to complex profiles of the phenolic fraction due to overlapping of various compounds (peaks 7, 8, and 9). MS offers the possibility of excluding the presence of interference, mainly when high complex matrixes such as olive oil polar compounds are analyzed. MS modes (such as full scan) and MS/MS modes (such as product ion scan) verify structural information of the compounds present in a virgin olive oil extract. The combined use of HPLC-MS/MS systems identifies olive oil phenols further.

The examination of the chromatograms in full-scan mode revealed the presence of several compounds that were positively identified by means of comparison with available standards. When these standards were not available and thus this comparison was not possible (the case of secoiridoids), MS/MS experiments had to be used.

In full-scan mode, several compounds with the same *m*/*z* were observed. To identify differences between them, samples were injected in the product ion scan mode of 393 and 361, both of which were ligstroside derivatives, and 377 an oleuropein derivative. These MS/MS experiments split the derivatives into a number of fragments according to strict pathways, which may enable their differences to be identified.

Olive oil extracts were injected in product ion scan mode of *m*/*z* 241, 285, 269, and 299 (corresponding to elenolic acid, luteolin, apigenin, and metoxyluteolin), to confirm the presence of these compounds.

The product ion scan modes of *m*/*z* 335 and 319 (for ligstroside and oleuropein derivatives) were evaluated to clarify where the different models came from.

Nine basic models of ligstroside and oleuropein aglycons were found in the bibliography. Each model shares the same elenolic acid derivative ring structure. In **Table 2**, these possible forms of derivatives of aglycons of oleuropein and ligstroside in virgin olive oil are shown.

Phenolic Alcohols. Hydroxytyrosol (peak 1) was identified by examination of the chromatogram belonging to the different sub-fractions in full-scan mode. The spectra generated for this compound in negative ion mode gave the deprotonated molecule $[M - H]^-$ at *m*/*z* 153. The ion fragment at *m*/*z* 123 is due to the loss of the CH_2OH group.

Tyrosol (peak 2) was identified by comparison with standard even when its mass spectrum was hidden by background.

Phenolic Acids. Peaks 3 and 5 are minor constituents (vanillic acid and *p*-coumaric acid) and were confirmed by using standard, spectral data, and log α. The mass spectrum of vanillic acid was hidden by background, but the deprotonated molecule $[M - H]^-$ of *p*-coumaric (163) was clearly visible.

Vanillin. The spectra generated for peak 4, vanillin, in negative ion mode gave the deprotonated molecule $[M - H]^-$ at *m*/*z* 151. The ion fragment at *m*/*z* 123 present is due to the loss of the CHO. This compound was confirmed by using standard, spectral data, and log α.

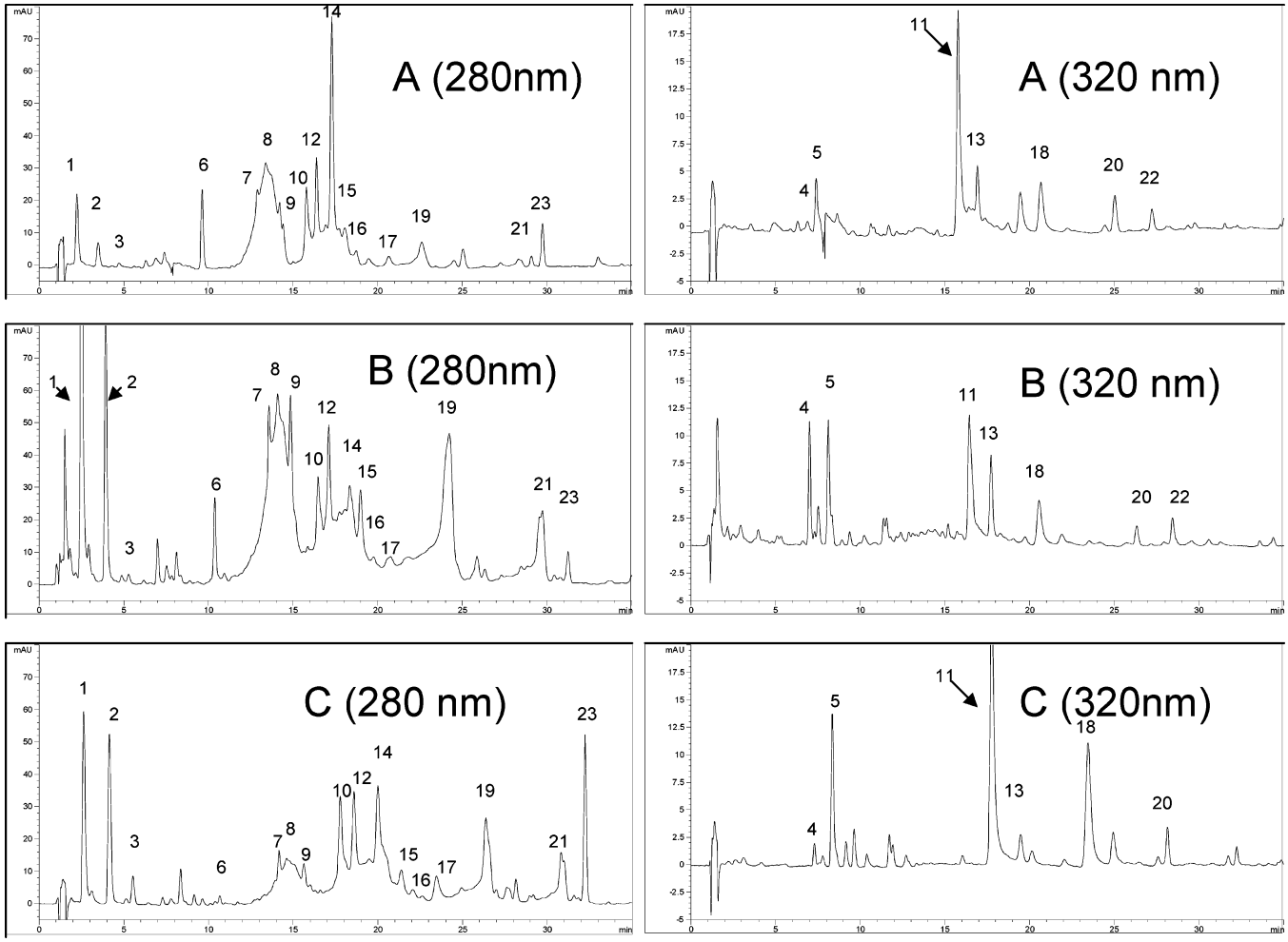


Figure 1. Phenolic compounds in virgin olive oil studied. Oil A, from Arbequina olives, Catalonia, Spain; oil B, from Picual olives, Jaén, Andalucía Spain; and oil C, a commercial virgin olive oil. Phenolic compounds: (1) hydroxytyrosol, (2) tyrosol, (3) vanillic acid, (4) vanillin, (5) *p*-coumaric acid, (6) ligstroside derivative *m*/*z* 553, (7) ligstroside derivative *m*/*z* 335, (8) oleuropein derivative *m*/*z* 377, (9) oleuropein derivative *m*/*z* 377, (10) oleuropein derivative *m*/*z* 319, (11) luteolin, (12) ligstroside derivative *m*/*z* 361, oleuropein derivative *m*/*z* 365, (13) apigenin, (14) ligstroside derivative *m*/*z* 361, ligstroside derivative *m*/*z* 393, (15) ligstroside derivative *m*/*z* 393, (16) ligstroside derivative *m*/*z* 393, (17) oleuropein derivative *m*/*z* 377, (18) methoxyluteolin, (19) oleuropein derivative *m*/*z* 377, ligstroside derivative *m*/*z* 361, (20) unknown, (21) ligstroside derivative *m*/*z* 361, (22) unknown, (23) ligstroside derivative *m*/*z* 361. HPLC-DAD conditions are as described in text.

Ligstroside Derivatives. Peak 7 had a deprotonated molecule at *m*/*z* 335. The product ion scan spectrum showed the *m*/*z* 199 ion (loss of tyrosol unit). The *m*/*z* 111 ion can be explained by the loss of 104 mass units of molecular mass of the elenolic derivative fragment (*m*/*z* 215) formed by an aldehyde and the COOH group loss. The *m*/*z* 155 ion can be explained by the loss of the $CH_2OH-OCH_3$ group of the elenolic derivative fragment too. This compound was assigned to a ligstroside derivative, which for the purpose of these tests corresponded to model 8 present in **Table 2**.

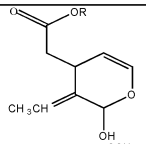
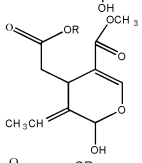
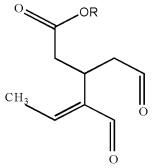
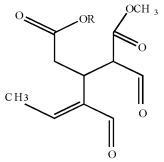
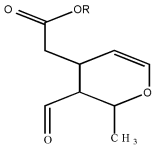
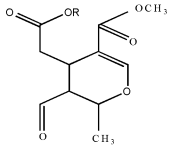
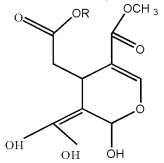
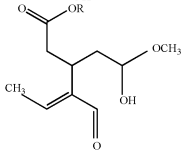
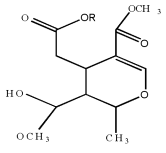
Peak 12 showed *m*/*z* 361 in full-scan mode. Trace chromatogram of this *m*/*z* ratio gave four more peaks (14, 19, 21, and 23). According to the literature, these *m*/*z* 361 compounds may correspond to models 2, 4, and 6 (*12*, *23*, *24*, *26*, *29*, *30*, *39*−*44*). Injection of the extract in product ion scan mode of *m*/*z* 361 gave no differences for the five peaks even when they were injected at different collision energies (up to −50 V) (see **Figure 2**). For all of the compounds, *m*/*z* 291 had a relative abundance of 100% in product ion spectra. This ion at *m*/*z* 291 is probably derived from the C_4H_6O loss fragment, in the case of models 2 and 6 as described by Caruso et al. (*29*). In the case of model 4, it could be justified by the two dialdehydes and a methyl group loss. The 259 and 101 ions may be due to rearrangement fragments. The ion at *m*/*z* 127 is justified by the loss of the upper C_7H_7O fragment of the molecule, the same C_4H_6O group mentioned above, and carboxylic group loss in the case of models 2 and 6 (see **Figure 3**). In model 4, it could be justified by a loss of 114 units of molecular mass of the elenolic fragment (*m*/*z* 241) caused by the liberation of the COOH, both aldehyde and a methyl group, or for the two dialdehyde groups and the CH_2-COOH loss of the same elenolic fragment. In models 2 and 6, it can be justified by the loss of the COOH group and the C_4H_6O group loss of the elenolic fragment. The loss of 103 units of mass of the elenolic fragment caused by carboxylic liberation and the COOH group gave a molecule at *m*/*z* 139 justified in the three models.

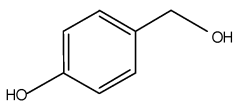
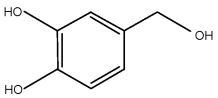
Peak 14 showed a deprotonated molecule at *m*/*z* 393 that could be attributed to a ligstroside derivative because its product ion scan spectra gave the ion for a loss of the tyrosol unit (*m*/*z* 257). Two other peaks (15 and 16) were also present in the trace chromatogram. No differences were observed in their product ion scan spectra.

The literature shows two models of these ligstroside derivatives (models 7 and 9) (*29*, *40*, *41*).

Oleuropein Derivatives. The spectra generated for peaks 8, 9, 17, and 19 gave the deprotonated molecule at *m*/*z* 377, which

Table 2. Possible Models of Derived Secoiridoids

Model	Característics	Base	*m/z*			Bibliography
			Acid Elenolic Derivative[1]	Ligstroside Derivative[2]	Oleuropein Derivative[3]	
1	Closed ring Decarboxilade form		183	303	319	*(12, 19, 26, 29, 39, 44, 46)*
2	Closed ring Carboxilade form		241	361	377	*(19, 23, 26, 30, 42, 44, 46, 50)*
3	Open ring Decarboxilade Dialdehidic form		183	303	319	*(22, 24, 26, 35, 39, 40, 42-44)*
4	Open ring Carboxilade Dialdehidic form		241	361	377	*(26, 29, 43, 44)*
5	Closed ring Decarboxilade Aldehidic form		183	303	319	*(12, 22, 23, 39, 41, 44)*
6	Closed ring Carboxilade Aldehidic form		241	361	377	*(12, 23, 24, 29, 39-41, 43, 44, 51)*
7	Closed ring Carboxilade Hidroxilade form		273	393	409	*(29, 41)*
8	Open ring Decarboxilade Aldehidic form		215	335	351	*(40)*
9	Closed ring Carboxilade form		272	393	409	*(40)*

[1]R = H [2]R=  [3]R= 

demonstrates the presence of oleuropein derivative in carboxylic form (models 2, 4, and 6) (*23*, *24*, *26*, *29*, *30*, *39*−*45*). Product ion scan spectra of *m*/*z* 377 for the four peaks revealed no differences at different collision energies (see **Table 1**), except for peak 19, which shows characteristic product ions at *m*/*z* 333, 301, and 181, which may derive from rearranged fragments. The product ion at *m*/*z* 307 is justified in models 2 and 6 by the loss of a C_4H_6O fragment (*29*). The product ions at *m*/*z* 275 may derive from rearranged fragments. These results are corroborated by Caruso et al. (*29*). The loss of the COOH and

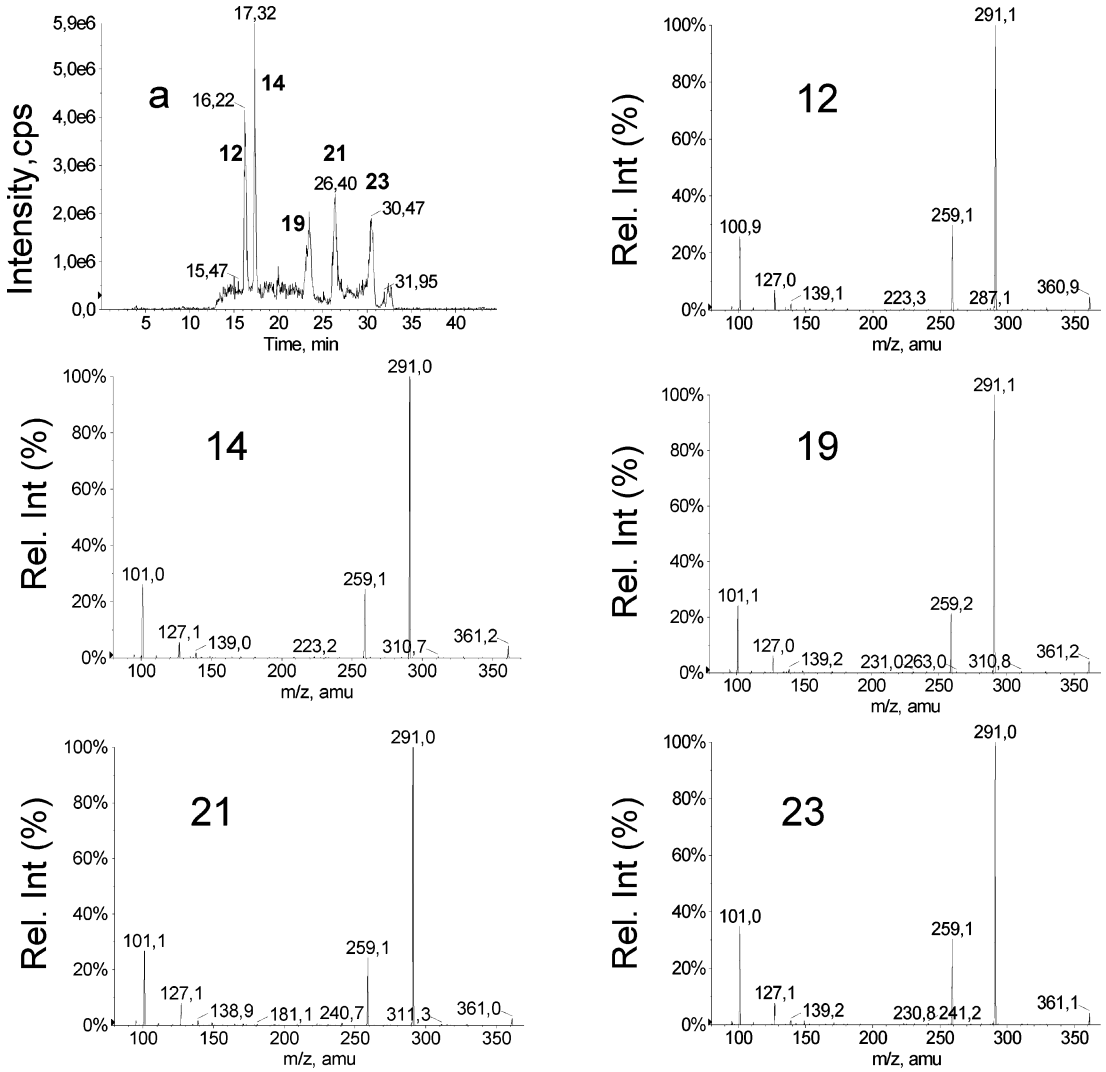


Figure 2. (**a**) Trace chromatogram of *m*/*z* 361 in full-scan mode for the olive oil extract. Mass spectra of five ligstroside derivatives in product ion scan mode of *m*/*z* 361 (12, 14, 19, 21, and 23). HPLC-MS/MS conditions are as described in the text.

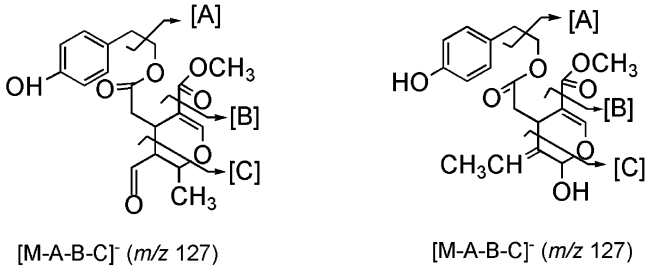


Figure 3. Possible rupture in the 2 and 6 ligstroside model to give the ion molecule at *m*/*z* 127.

the $COOCH_3$ unit of elenolic fragment derivative (*m*/*z* 241) produces a fragment ion at *m*/*z* 139. The ion at *m*/*z* 111 can be justified by $COOCH_3$, COOH, and the aldehyde group loss of the elenolic acid fragment in model 6.

Peak 10 is an oleuropein derivative in decarboxylade form shown by its deprotonated molecule $[M - H]^-$ at *m*/*z* 319 (*12*, *19*, *22*−*24*, *26*, *29*, *39*−*44*, *46*), and by the fact that the product ion scan of *m*/*z* 319 produces an ion at *m*/*z* 153, which demonstrates the existence of the hydroxytyrosol molecule. The bibliography gives three different models of oleuropein derivatives with this mass molecule (models 1, 3, and 5), but in our experience only one derivative showing this *m*/*z* was observed. The molecular ion at *m*/*z* 111 can be explained by the loss of 72 units of mass of the elenolic derivative fragment (*m*/*z* 183) caused by CHO and COOH liberation in aldehyde forms in models 3 and 5.

A compound with a deprotonated molecule at *m*/*z* 365 is present in peak 12. Its spectrum shows other fragment ions: *m*/*z* at 153 indicates the hydroxytyrosol molecule liberation, which means it is probably an oleuropein derivative. To our knowledge, an oleuropein derivative present in olive oil with this *m*/*z* is here described for the first time in this work (**Figure 4**).

Although different collision energies were proved, it was very difficult to differentiate the isoforms, due to their identical spectrum profiles and the identical fractions present.

The presence or absence of aldehyde, carboxyl, and/or methyl groups and the open or closed form of the elenolic acid ring structure indicate the differences between aglycons. Oleuropein and ligstroside aglycons differ from each other, in the existence of a mono or ortho-dihydroxy structure on the phenol ring.

Flavonoids. Peaks 11, 13, and 18, luteolin, apigenin, and methoxyluteolin, respectively, can easily be identified by their full-scan spectrum. They were also corroborated by the spectrum reference and the retention time of the standard and log α.

Elenolic Acid. This compound cannot be considered a phenolic compound, which only corresponds to the secoiridoid

Table 3. Content of Phenolic Compounds

peak	compound	*m*/*z*	content (μg/mL) oil A	oil B	oil C
1	hydroxytyrosol	153	7.03	63.47	17.87
2	tyrosol	137	2.94	24.38	16.45
3	vanillic acid	167	0.15	0.22	0.85
4	vanillin	151	0.05	0.47	0.04
5	*p*-coumaric acid	193	0.10	0.29	0.34
6	ligstroside derivative	553	18.45	19.84	1.89
7	ligstroside derivative	335	48.80	89.20	23.55
8	oleuropein derivative	377	117.29	202.70	45.92
9	oleuropein derivative	377	17.59	46.74	9.94
10	oleuropein derivative	319	12.22	18.84	30.47
11	luteolin	285	3.97	3.10	6.80
12	ligstroside derivative	361	18.36	28.49	23.77
	oleuropein derivative	365			
13	apigenin	269	0.49	0.73	0.41
14	ligstroside derivative	361	50.00	14.58	20.63
	ligstroside derivative	393			
15	ligstroside derivative	393	4.56	18.74	7.62
16	ligstroside derivative	393	2.01	3.87	2.24
17	oleuropein derivative	377	4.57	5.77	16.89
18	methoxyluteolin	299	0.61	0.61	1.59
19	oleuropein derivative	377	12.98	53.16	27.51
	ligstroside derivative	361			
20	unknown		0.86	0.48	0.72
21	ligstroside derivative	361	4.29	23.45	20.49
22	unknown		0.47	0.63	0.00
23	ligstroside derivative	361	9.81	9.86	41.81
	total		338	630	318

part of oleuropein (*2*). It can only be observed at 240 nm in HPLC-DAD. A very intense peak at minute 10 is observed at this wavelength, and its characteristic ion at *m*/*z* 241 corresponding to the deprotonated molecule of elenolic acid and *m*/*z* 139 due to the $COOCH_3$ group loss is clearly present.

Quantification. The quantification of phenolic compounds using HPLC-DAD expressed as caffeic acid, gallic acid, syringic acid, or tyrosol, oleuropein equivalents, or other phenolic compounds has been reported in many papers (*5*, *19*, *21*, *22*, *24*). However, a variation of 18−80% in the total concentration values of phenolic compounds was demonstrated by Pirisi et al. (*24*) using these quantification methods. Here, each phenolic compound was expressed with the respective standard when it was available. When this was not possible, the phenolic compounds were divided and expressed with a representative and available standard compound of each group, on the basis that responses of each group are significantly different from each other. Secoiridoids were expressed as oleuropein; the phenolic alcohols, 3,4-dihydroxyphenyl and 4-hydroxyphenyl were expressed as tyrosol; and unknown flavonoids were expressed as luteolin. Quantification was carried out at the wavelength for the higher absorbency for each selected compound (**Table 3**).

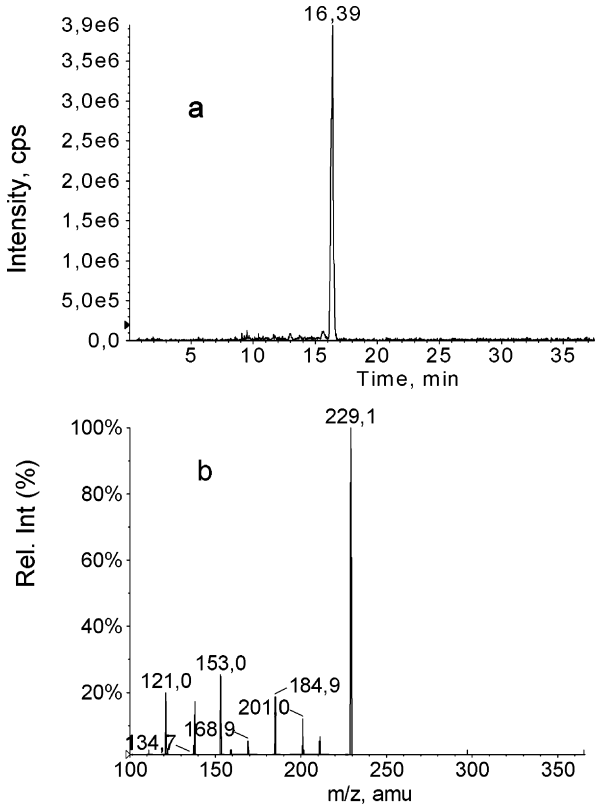


Figure 4. (**a**) Trace chromatogram of *m*/*z* 365 in full-scan mode for the olive oil extract. (**b**) Mass spectra in product ion scan mode of *m*/*z* 365. HPLC-MS/MS conditions are as described in the text.

Elenolic acid, a nonphenolic compound, but a derivative, was expressed as oleuropein, and quantification showed it at 287, 502, and 194 μg/mL in oils A, B, and C, respectively.

As reported in previous studies, we found that virgin olive oil contains low amounts of phenyl acids and phenyl alcohols and high concentrations of secoiridoid derivatives such as oleuropein and ligstroside aglycons, which originate from the oleureuropein, dimethyloleuropein, and ligstroside glycosides found in olives (*26*, *27*, *35*, *39*, *47*).

In the sample studied, secoiridoids comprised 77−88% of total phenolic compounds. If hydroxytyrosol and tyrosol are considered, the oleuropein and ligstroside derivatives comprised 87−92% of the total phenolic content in the samples studied.

During the crushing and malaxing processes, hydrolysis of the glycosidic bond occurs and the aglycons pass into the oil. The process also causes partial modification of oleuropein and ligstroside aglycons, which generates isoforms in the elenolic structure, although they conserve the phenol ring (*23*). Some

Table 4. Linearity of Phenolic Compounds Standards and Sensitivity of the HPLC-DAD Method

compound	concentration range (μg/mL)	linear regression	r[a]	LOD[b] (μg/mL)	LOQ[c] (μg/mL)
tyrosol	2–40	$y = 22.01x - 5.08$	0.999	0.28	0.39
vanillic acid	0.05–1.5	$y = 67.61x + 0.68$	0.999	5×10^{-3}	0.04
vanillin	0.5–1	$y = 122.94x + 0.29$	0.999	6×10^{-3}	0.03
p-coumaric acid	0.05–1	$y = 269.41x - 0.17$	0.999	7×10^{-3}	0.02
oleuropein	2–1000	$y = 8.07x - 1.28$	0.999	0.29	0.60
luteolin	0.05–20	$y = 43.73x - 8.89$	0.999	0.25	0.35
apigenin	0.3–15	$y = 101.59x - 28.9$	0.999	0.30	0.35
methoxyluteolin	0.3–15	$y = 110.21x - 14.44$	0.999	0.15	0.19

[a] Correlation coefficients of the regression equation. [b] LOD = limit of detection. [c] LOQ = limit of quantification.

Table 5. Recovery Studies of Phenols[a]

compound	recovery mean (%)	SD[b]
tyrosol	115	3.19
p-coumaric	105	0.99
oleuropein	76	8.64
luteolin	103	4.04

[a] $n = 3$. [b] Standard deviation of recovery studies.

of them are reversible to equilibrium sustenance (*23*, *39*, *40*, *44*).

Method Validation. To assess the validity of the method, validation tests were run. All test parameters were carefully chosen to cover the range of samples and concentrations involved.

Linearity. The linearity of standard curves was expressed in terms of the determination coefficient from plots of the integrated peak area versus concentration of the same standard (μg/mL). These equations were obtained over a wide concentration range in accordance with the levels of these compounds found in the olive samples. Details are given in **Table 4**. The system was linear in all cases ($r > 0.99$).

Precision. The method's precision was satisfactory, with acceptable values under the Horwitz criteria (*48*). Six replicate determinations on the same day and twelve replicate determinations on different days with the same sample were carried out. Relative standard deviations were calculated, with results of coefficients of variation less than 7% in repeatability (intralaboratory precision) and less than 8% in reproducibility (interlaboratory precision).

Sensitivity. LOD and LOQ were studied to check the sensitivity of the methods under the working conditions proposed. Both followed USP criteria (*49*) (**Table 4**). These limits, referring to the concentrations in olive oil needed if they were to be detected and quantified, were of the order of nanograms. The method has excellent sensitivity.

Recovery. To assess the recovery of the proposed method, three samples of refined olive oil without phenolic compounds were spiked with different amounts of tyrosol, *p*-coumaric acid, luteolin, and oleuropein. The samples were submitted to the complete proposed procedure. As is shown in **Table 5**, the mean recoveries were between 76% and 115%.

Conclusions. A simple and reproducible SPE-HPLC-DAD and HPLC-MS/MS method was developed to characterize and quantify the phenolic compounds present in virgin olive oil. In this study, 20 out of 23 compounds detected were characterized through a combination of the HPLC-DAD and HPLC-MS/MS systems. The method proposed is faster, with a very good recovery (76−115%), and low solvent and sample consumption is required. As the method shows good precision, recovery, linearity, and sensitivity, it is suitable for routine analyses of various kinds of olive oil. Compounds such as acids, alcohols, flavonoids, and the various secoiridoids can be detected and quantified. There are a high variety of secoiridoid compounds derived from oleuropein and ligstroside in virgin olive oil, and these secoiridoids make up a high percentage of all phenolic compounds in virgin olive oil. Besides this, in comparison with other investigations, this work recognizes the existence of diverse isomers belonging to the secoiridoids group. As many of them share the mass weight for generated isoforms, further studies are required to look into these keto-elenolic tautomeric forms.

ABBREVIATIONS USED

SPE, solid-phase extraction; HPLC-DAD, high performance liquid chromatography with diode array detector; HPLC-MS/MS, high performance liquid chromatography with double mass spectrometer; MS, mass spectrometry; LOD, limits of detection; LOQ, limits of quantification; ESI, electrospray ionization; APCI, atmospheric pressure chemical ionization; DP, declusterin potential; CE, collision energy; CAD, collision-activated dissociation.

ACKNOWLEDGMENT

We thank Robin Rycroft for revising this manuscript.

Supporting Information Available: S-1, hydroxytyrosol fragment; S-2, ligstroside derivative fragments; S-3, oleuropein derivative fragments. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Ryan, D.; Robards, K.; Prenzler, P.; Antolovich, M. Applications of mass spectrometry to plant phenols. *TrAC, Trends Anal. Chem.* **1999**, *18*, 362−372.

(2) Esti, M.; Cinquanta, L.; La Notte, E. Phenolic Compounds in Different Olive Varieties. *J. Agric. Food Chem.* **1998**, *46*, 32−35.

(3) Tovar, M. J.; Motilva, M. J.; Romero, M. P. Changes in the phenolic composition of virgin olive oil from young trees (Olea europaea L. cv. Arbequina) grown under linear irrigation strategies. *J. Agric. Food Chem.* **2001**, *49*, 5502−5508.

(4) Soler-Rivas, C.; Espin, J. C.; Wichers, H. J. Oleuropein and related compounds. *J. Sci. Food Agric.* **2000**, *80*, 1013−1023.

(5) Gimeno, E.; Castellote, A. I.; Lamuela-Raventos, R. M.; De la Torre, M. C.; Lopez-Sabater, M. C. The effects of harvest and extraction methods on the antioxidant content (phenolics, alpha-tocopherol, and beta-carotene) in virgin olive oil. *Food Chem.* **2002**, *78*, 207−211.

(6) Romero, M. P.; Tovar, M. J.; Girona, J.; Motilva, M. J. Changes in the HPLC phenolic profile of virgin olive oil from young trees (Olea europaea L. cv. Arbequina) grown under different deficit irrigation strategies. *J. Agric. Food Chem.* **2002**, *50*, 5349−5354.

(7) Keceli, T.; Gordon, M. H. The antioxidant activity and stability of the phenolic fraction of green olives and extra virgin olive oil. *J. Sci. Food Agric.* **2001**, *81*, 1391−1396.

(8) Okogeri, O.; Tasioula-Margari, M. Changes occurring in phenolic compounds and alpha-tocopherol of virgin olive oil during storage. *J. Agric. Food Chem.* **2002**, *50*, 1077−1080.

(9) Angerosa, F.; Mostallino, R.; Basti, C.; Vito, R. Virgin olive oil odour notes: their relationships with volatile compounds from the lipoxygenase pathway and secoiridoid compounds. *Food Chem.* **2000**, *68*, 283−287.

(10) Mateos, R.; Cert, A.; Perez-Camino, M. C.; Garcia, J. M. Evaluation of virgin olive oil bitterness by quantification of secoiridoid derivatives. *J. Am. Oil Chem. Soc.* **2004**, *81*, 71−75.

(11) Ranalli, A.; Modesti, G.; Patumi, M.; Fontanazza, G. The compositional quality and sensory properties of virgin olive oil from a new olive cultivar -- I-77. *Food Chem.* **2000**, *69*, 37−46.

(12) Servili, M.; Montedoro, G. Contribution of phenolic compounds to virgin olive oil quality. *Eur. J. Lipid Sci. Technol.* **2002**, *104*, 602−613.

(13) Covas, M. I.; Fito, M.; Marrugat, J.; Miro, E.; Farre, M.; de la Torre, R.; Gimeno, E.; Lopez-Sabater, M. C.; Lamuela-Raventos, R.; Torre-Boronat, M. C. Coronary heart disease protective

factors: antioxidant effect of olive oil. *Therapie* **2001**, *56*, 607−611.
(14) Covas, M. I.; Fito, M.; Lamuela-Raventos, R. M.; Sebastia, N.; Torre-Boronat, C.; Marrugat, J. Virgin olive oil phenolic compounds: Binding to human low-density lipoprotein (LDL) and effect on LDL oxidation. *Int. J. Clin. Pharmacol. Res.* **2000**, *20*, 49−54.
(15) Bravo, L. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev.* **1998**, *56*, 317−333.
(16) Leenen, R.; Roodenburg, A. J. C.; Vissers, M. N.; Schuurbiers, J. A. E.; van Putte, K. P. A. M.; Wiseman, S. A.; van de Put, F. H. M. M. Supplementation of plasma with olive oil phenols and extracts: Influence on LDL oxidation. *J. Agric. Food Chem.* **2002**, *50*, 1290−1297.
(17) Visioli, F.; Galli, C. Olive oil phenols and their potential effects on human health. *J. Agric. Food Chem.* **1998**, *46*, 4292−4296.
(18) Akasbi, M.; Shoeman, D. W.; Csallany, A. S. High-Performance Liquid-Chromatography of Selected Phenolic-Compounds in Olive Oils. *J. Am. Oil Chem. Soc.* **1993**, *70*, 367−370.
(19) Montedoro, G.; Servili, M.; Baldioli, M.; Miniati, E. Simple and Hydrolyzable Phenolic-Compounds in Virgin Olive Oil. 1. Their Extraction, Separation, and Quantitative and Semiquantitative Evaluation by Hplc. *J. Agric. Food Chem.* **1992**, *40*, 1571−1576.
(20) Tsimidou, M.; Lytridou, M.; Boskou, D.; PappaLouisi, A.; Kotsifaki, F.; Petrakis, C. On the determination of minor phenolic acids of virgin olive oil by RP-HPLC. *Grasas Aceites* **1996**, *47*, 151−157.
(21) Liberatore, L.; Procida, G.; d'Alessandro, N.; Cichelli, A. Solid-phase extraction and gas chromatographic analysis of phenolic compounds in virgin olive oil. *Food Chem.* **2001**, *73*, 119−124.
(22) Mateos, R.; Espartero, J. L.; Trujillo, M.; Rios, J. J.; Leon-Camacho, M.; Alcudia, F.; Cert, A. Determination of phenols, flavones, and lignans in virgin olive oils by solid-phase extraction and high-performance liquid chromatography with diode array ultraviolet detection. *J. Agric. Food Chem.* **2001**, *49*, 2185−2192.
(23) Pirisi, F. M.; Angioni, A.; Cabras, P.; Garau, V. L.; diTeulada, M. T. S.; dosSantos, M. K.; Bandino, G. Phenolic compounds in virgin olive oils 0.1. Low-wavelength quantitative determination of complex phenols by high-performance liquid chromatography under isocratic elution. *J. Chromatogr., A* **1997**, *768*, 207−213.
(24) Pirisi, F. M.; Cabras, P.; Cao, C. F.; Migliorini, M.; Muggelli, M. Phenolic compounds in virgin olive oil. 2. Reappraisal of the extraction, HPLC separation, and quantification procedures. *J. Agric. Food Chem.* **2000**, *48*, 1191−1196.
(25) Bendini, A.; Bonoli, M.; Cerretani, L.; Biguzzi, B.; Lercker, G.; Toschi, T. G. Liquid−liquid and solid-phase extractions of phenols from virgin olive oil and their separation by chromatographic and electrophoretic methods. *J. Chromatogr., A* **2003**, *985*, 425−433.
(26) Saitta, M.; Lo Curto, S.; Salvo, F.; Di Bella, G.; Dugo, G. Gas chromatographic-tandem mass spectrometric identification of phenolic compounds in Sicilian olive oils. *Anal. Chim. Acta* **2002**, *466*, 335−344.
(27) Tasioula-Margari, M.; Okogeri, O. Isolation and characterization of virgin olive oil phenolic compounds by HPLC/UV and GC-MS. *J. Food Sci.* **2001**, *66*, 530−534.
(28) Tasioula-Margari, M.; Okogeri, O. Simultaneous determination of phenolic compounds and tocopherols in virgin olive oil using HPLC and UV detection. *Food Chem.* **2001**, *74*, 377−383.
(29) Caruso, D.; Colombo, R.; Patelli, R.; Giavarini, F.; Galli, G. Rapid evaluation of phenolic component profile and analysis of oleuropein aglycon in olive oil by atmospheric pressure chemical ionization-mass spectrometry (APCI-MS). *J. Agric. Food Chem.* **2000**, *48*, 1182−1185.
(30) Perri, E.; Raffaelli, A.; Sindona, G. Quantitation of oleuropein in virgin olive oil by ionspray mass spectrometry − Selected reaction monitoring. *J. Agric. Food Chem.* **1999**, *47*, 4156−4160.
(31) Bianco, A.; Buiarelli, F.; Cartoni, G.; Coccioli, F.; Jasionowska, R.; Margherita, P. Analysis by liquid chromatography-tandem mass spectrometry of biophenolic compounds in virgin olive oil, part II. *J. Sep. Sci.* **2003**, *26*, 417−424.
(32) Sanchez-Rabaneda, F.; Jauregui, O.; Casals, I.; Andres-Lacueva, C.; Izquierdo-Pulido, M.; Lamuela-Raventos, R. M. Liquid chromatographic/electrospray ionization tandem mass spectrometric study of the phenolic composition of cocoa (Theobroma cacao). *J. Mass Spectrom.* **2003**, *38*, 35−42.
(33) Commission Regulation (EEC) n 2568/91 of July 1991 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis, 1991.
(34) Ibern-Gomez, M.; Andres-Lacueva, C.; Lamuela-Raventos, R. M.; Buxaderas, S.; Singleton, V. L.; de la Torre-Boronat, M. C. Browning of cava (sparkling wine) during aging in contact with lees due to the phenolic composition. *Am. J. Enol. Vitic.* **2000**, *51*, 29−36.
(35) Servili, M.; Baldioli, M.; Selvaggini, R.; Miniati, E.; Macchioni, A.; Montedoro, G. High-performance liquid chromatography evaluation of phenols in olive fruit, virgin olive oil, vegetation waters, and pomace and 1D-and 2D-nuclear magnetic resonance characterization. *J. Am. Oil Chem. Soc.* **1999**, *76*, 873−882.
(36) Macheix, J.-J.; Fleuriet, A.; Billot, J. *Fruit Phenolics*; CRC Press: Florida, 2000.
(37) Sanchez-Rabaneda, F.; Jauregui, O.; Lamuela-Raventos, R. M.; Viladomat, F.; Bastida, J.; Codina, C. Qualitative analysis of phenolic compounds in apple pomace using liquid chromatography coupled to mass spectrometry in tandem mode. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 553−563.
(38) Sanchez-Rabaneda, F.; Jauregui, O.; Lamuela-Raventos, R. M.; Bastida, J.; Viladomat, F.; Codina, C. Identification of phenolic compounds in artichoke waste by high-performance liquid chromatography-tandem mass spectrometry. *J. Chromatogr., A* **2003**, *1008*, 57−72.
(39) Angerosa, F.; Dalessandro, N.; Konstantinou, P.; Digiacinto, L. Gc-Ris Evaluation of Phenolic-Compounds in Virgin Olive Oil. *J. Agric. Food Chem.* **1995**, *43*, 1802−1807.
(40) Montedoro, G.; Servili, M.; Baldioli, M.; Selvaggini, R.; Miniati, E.; Macchioni, A. Simple and Hydrolyzable Compounds in Virgin Olive Oil. 3. Spectroscopic Characterizations of the Secoiridoid Derivatives. *J. Agric. Food Chem.* **1993**, *41*, 2228−2234.
(41) Cortesi, N.; Azzolini, M.; Rovellini, P.; Fedeli, E. Minor Polar Components of Virgin Olive Oils: a Hypothetical Structure by LC-MS. *La Rivista Italiana delle Sostanze Grasse* **1995**, *LXXII*, 241−251.
(42) Owen, R. W.; Mier, W.; Giacosa, A.; Hull, W. E.; Spiegelhalder, B.; Bartsch, H. Identification of lignans as major components in the phenolic fraction of olive oil. *Clin. Chem.* **2000**, *46*, 976−988.
(43) Tsimidou, M. Polyphenols and quality of virgin olive oil in retrospect. *Ital. J. Food Sci.* **1998**, *10*, 99−116.
(44) Angerosa, F.; Dalessandro, N.; Corana, F.; Mellerio, G. Characterization of phenolic and secoiridoid aglycons present in virgin olive oil by gas chromatography chemical ionization mass spectrometry. *J. Chromatogr., A* **1996**, *736*, 195−203.
(45) Kaur, C.; Kapoor, H. C. Antioxidants in fruits and vegetables − the millennium's health. *Int. J. Food Sci. Technol.* **2001**, *36*, 703−725.
(46) Montedoro, G.; Servili, M.; Baldioli, M.; Miniati, E. Simple and Hydrolyzable Phenolic-Compounds in Virgin Olive Oil. 2. Initial Characterization of the Hydrolyzable Fraction. *J. Agric. Food Chem.* **1992**, *40*, 1577−1580.
(47) Gomez-Alonso, S.; Salvador, M. D.; Fregapane, G. Phenolic compounds profile of Cornicabra virgin olive oil. *J. Agric. Food Chem.* **2002**, *50*, 6812−6817.
(48) Horwitz, W.; Albert, R.; Deutsch, M.; Thompson, J. N. Precision Parameters of Methods of Analysis Required for Nutrition Labeling. 2. Macro Elements − Calcium, Magnesium, Phosphorus, Potassium, Sodium, and Sulfur. *J. AOAC Int.* **1992**, *75*, 227−239.

(49) The United States Pharmacopeia (USPXXIII), Mack Printing, Easton, 1989.
(50) Psomiadou, E.; Tsimidou, M. Simultaneous HPLC determination of tocopherols, carotenoids, and chlorophylls for monitoring their effect on virgin olive oil oxidation. *J. Agric. Food Chem.* **1998**, *46*, 5132−5138.
(51) Perrin, J. L. Les composés mineurs et les antioxygènes naturels de l'olive et de son huile. *Etude et Recherche* **1992**, *4*, 25−32.

Received for review January 27, 2005. Revised manuscript received March 17, 2005. Accepted March 18, 2005. We thank the "Ministerio de Ciencia y Tecnología" (Spain) (proyect "AGL2000-0525-LO2-02"), the European Union (EUROLIVE project n° QCK1-CT-2001-00287) for their financial support, and the University of Barcelona for the grant received by K.T.-C.

JF0501948

Publicación 2

"Postprandial LDL phenolic content and LDL oxidation are molulated by olive oil phenolic compounds in human" .M.I. Covas, K. de la Torre, M. Farré-Albaladejo, J. Kaikkonnen, M. Fitó, C. López-Sabater, M. A.Pujadas-Bastardes, J.Oglar, T. Weinbrenner, R. Lamuela-Raventós, and R. de la Torre. ***Free Radic Biol Med***. 40, 2006.

Resumen

Los compuestos fenólicos del aceite de oliva son potentes antioxidantes *in vitro*, sin embargo la evidencia de la acción antioxidante *in vivo* es controvertida.

El objetivo de este trabajo fue demostrar la acción antioxidante de los compuestos fenólicos provenientes del aceite de oliva *in vivo*, por lo que se examinó entonces el papel de los compuestos fenólicos en relación al estrés postprandial oxidativo.

Para ello, se realizó un ensayo cruzado con 12 voluntarios varones sanos, los cuales consumieron 40 mL en ayunas de tres tipos de aceite de oliva: aceite de oliva virgen, aceite de oliva y aceite de oliva refinado, los tres de composición similar, pero con diferente concentración de compuestos fenólicos. El consumo fue en una dosis única, después de 10 días de blanqueo con restricción en el consumo de compuestos fenólicos y otros antioxidantes de otras fuentes por cada intervención.

El hidroxitirosol y tirosol se absorbieron de una forma dosis-dependiente de la concentración de compuestos fenólicos en el aceite y la concentración de compuestos fenólicos totales en LDL incrementó en el periodo postprandial de acuerdo al contenido de compuestos fenólicos ingeridos. Dicha concentración total de compuestos fenólicos en LDL tuvo una correlación directa con las concentraciones de plasma del hidroxitirosol, tirosol y alcohol homovainíllico después del consumo de aceite rico en compuestos fenólicos, mientras que el estrés oxidativo postprandial y la oxidación de la LDL fueron reducidos en

relación con el contenido de compuestos fenólicos del aceite administrado. La ingesta de 40 mL de aceite de oliva promovió el estrés oxidativo, sin embargo, el grado de oxidación de la LDL fue inversamente proporcional a la concentración de compuestos fenólicos en el aceite.

Los compuestos fenólicos del aceite de oliva parecen modular el contenido de compuestos fenólicos en LDL y el estrés oxidativo postprandial tras su ingesta aguda.

Available online at www.sciencedirect.com
SCIENCE DIRECT
Free Radical Biology & Medicine 40 (2006) 608 – 616
www.elsevier.com/locate/freeradbiomed

Original Contribution

Postprandial LDL phenolic content and LDL oxidation are modulated by olive oil phenolic compounds in humans

María-Isabel Covas [a,*], Karina de la Torre [c], Magí Farré-Albaladejo [b], Jari Kaikkonen [d], Montserrat Fitó [a], Carmen López-Sabater [c], María A. Pujadas-Bastardes [b], Jesus Joglar [e], Tanja Weinbrenner [a], Rosa M. Lamuela-Raventós [c], Rafael de la Torre [b]

[a] *Lipids and Cardiovascular Epidemiology Unit, Institut Municipal d'Investigació Mèdica (IMIM), 08003 Barcelona, Spain*
[b] *Pharmacology Research Unit, Institut Municipal d'Investigació Mèdica (IMIM), Barcelona, Spain*
[c] *Department of Nutrition and Bromatology, University of Barcelona, Spain*
[d] *Oy Jurilab, Kuopio, Finland*
[e] *Department of Biological Organic Chemistry, IIQAB-CSIC, Barcelona, Spain*

Received 17 August 2005; accepted 9 September 2005
Available online 18 October 2005

Abstract

Olive oil phenolic compounds are potent antioxidants in vitro, but evidence for antioxidant action in vivo is controversial. We examined the role of the phenolic compounds from olive oil on postprandial oxidative stress and LDL antioxidant content. Oral fat loads of 40 mL of similar olive oils, but with high (366 mg/kg), moderate (164 mg/kg), and low (2.7 mg/kg) phenolic content, were administered to 12 healthy male volunteers in a cross-over study design after a washout period in which a strict antioxidant diet was followed. Tyrosol and hydroxytyrosol, phenolic compounds of olive oil, were dose-dependently absorbed ($p < 0.001$). Total phenolic compounds in LDL increased at postprandial state in a direct relationship with the phenolic compounds content of the olive oil ingested ($p < 0.05$). Plasma concentrations of tyrosol, hydroxytyrosol, and 3-O-methyl-hydroxytyrosol directly correlated with changes in the total phenolic compounds content of the LDL after the high phenolic compounds content olive oil ingestion. A 40 mL dose of olive oil promoted a postprandial oxidative stress, the degree of LDL oxidation being lower as the phenolic content of the olive oil administered increases. In conclusion, olive oil phenolic content seems to modulate the LDL phenolic content and the postprandial oxidative stress promoted by 40 mL olive oil ingestion in humans.

Keywords: Olive oil; Tyrosol; Hydroxytyrosol; LDL phenolic content; LDL oxidation; Postprandial; Free radical

Introduction

There is increasing evidence that oxidative modification of low density lipoprotein (LDL) plays a key role in the development of atherosclerosis [1]. Elevated levels of oxidized LDL showed a positive correlation with the severity of acute coronary events [2] and have been considered a biochemical marker for coronary heart disease [3]. The process of LDL oxidation led to the modification of the protein moiety of LDL, directly, i.e. via myeloperoxidase-derived HOCl [4], or indirectly, i.e. via the peroxidation of the polyunsaturated fatty acids [5]. The modified apo B has immunogenic properties prompting the generation of autoantibodies against oxidized LDL [5]. Linoleic acid accounts for 90% of the polyunsaturated fatty acids (PUFA) present in LDL and is the major substrate for its oxidation [6]. Therefore, diets rich in PUFA may increase the risk of LDL oxidation. On the other hand, diets rich in oleic acid generate particles that appear to be more resistant to this process [7,8]. However, apart from its fatty acid profile, the formation of oxidized LDL depends upon its antioxidant content, such as vitamin E and phenolic compounds, present in LDL [8–10].

Besides containing high quantities of oleic acid, virgin olive oil, obtained exclusively by physical procedures, is rich in phenolic compounds. In animal and in in vitro studies, olive oil

* Corresponding author. Fax: +34 932213237.
E-mail address: mcovas@imim.es (M.-I. Covas).

0891-5849/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2005.09.027

phenolic compounds have been shown to be strong antioxidants protecting lipids from oxidation [11–14]. The biological activities of olive oil phenolic compounds have prompted several studies on their potential activity in the prevention of cardiovascular diseases and cancer. However, controversial results have been obtained in the randomized, cross-over, controlled human in vivo studies performed on the olive oil phenolic compounds antioxidant capacity [15–18]. Tyrosol (T) and hydroxytyrosol (HT) are the main olive oil phenolic compounds which are present as free or conjugate forms as secoroids or aglycones [19].

Postprandial lipemia has been recognized as a risk factor for atherosclerosis development as it is associated with oxidative changes [20,21]. After a high-fat meal an oxidative stress occurs impairing endothelial function [22]. However, the consumption of fatty meals with suitable sources of antioxidants, such as red wine [23], vitamin C [24], or antioxidant drugs such as simvastatin [22], minimizes this postprandial oxidative stress. In this report, through a randomized, cross-over, controlled study, we describe the impact of three similar types of olive oils, but with differences in their phenolic content, on the postprandial oxidative stress and the antioxidant content of the low density lipoproteins (LDL).

Subjects and methods

Subjects and study design

Twelve healthy male volunteers were recruited, with a mean age of 21.2 years (range 20–22 years), and a mean body mass index of 22.9 (range 20.8–31.6 kg/m^2). Subjects were considered healthy on the basis of physical examination and routine biochemical and hematological laboratory determinations. The protocol was approved by the CEIC-IMAS Ethic Committee. The protocol was fully explained to the participants before they gave their written informed consent.

Each subject was tested three times in a randomized cross-over, double-blind manner with regard to the administration of 40 mL of olive oil with low (LPC, 2.7 mg/kg), medium (MPC, 164 mg/kg), and high (HPC, 366 mg/kg) phenolic compounds content. The amount of phenolic compounds administered with the 40 mL olive oil dose was: 0.097 mg, 5.92 mg, and 13.2 mg, for LPC, MPC, and HPC olive oil, respectively. Two Latin squares of 3 × 3 for the three treatments were used to randomize participants into six orders of olive oil administration. Prior to each intervention volunteers followed a 10-day washout period. During the first 7 days of the washout period participants were asked to avoid excessive antioxidant intake. During the last 3 days before the day of the intervention (days 8–10 of the washout period) they followed a strict phenolic compound-low diet (Fig. 1). A nutritionist instructed them on excluding several foods, rich in phenolic compounds, from their diet (vegetables, legumes, fruit, juice, wine, coffee, tea, caffeine-containing soft drinks, beer, cacao, marmalade, and olives). LPC olive oil was given to the participants for raw and cooking purposes (including supplies for the family) during washout periods, and for cooking purposes in the intervention day. Daily dietary records were obtained from each volunteer. At 8 a.m., after an overnight fast, volunteers were provided with 40 mL of one of the three olive oils, which was administered as a single dose accompanied by a standard piece of bread. The 40 mL olive oil dose was the sole source of olive oil or antioxidants during the intervention day. Venous blood was collected in tubes containing 1g/L EDTA at baseline of each intervention period (0 h) and at several periods after olive oil administration. Plasma was obtained by centrifugation of blood at 1500 × g at 4°C for 20 min. Aliquots of the plasma samples were mixed with 3,5-di-tert-butyl-4-hydroxytoluene 100 μM to avoid auto-oxidation and stored at −80°C until analyzed. All biochemical and analytical determinations were performed in duplicate.

Nutrient intakes were calculated from the daily dietary records of each intervention period and the three previous days of the washout periods by a nutritionist using the software MediSystem 2000 (Conaycyte S.A, Madrid, Spain).

Measurement of tyrosol, hydroxytyrosol, and 3-O-methyl-hydroxytyrosol, in plasma

Tyrosol (T), hydroxytyrosol (HT), and 3-O-methyl-hydroxytyrosol (MHT) a biological metabolite of HT, were

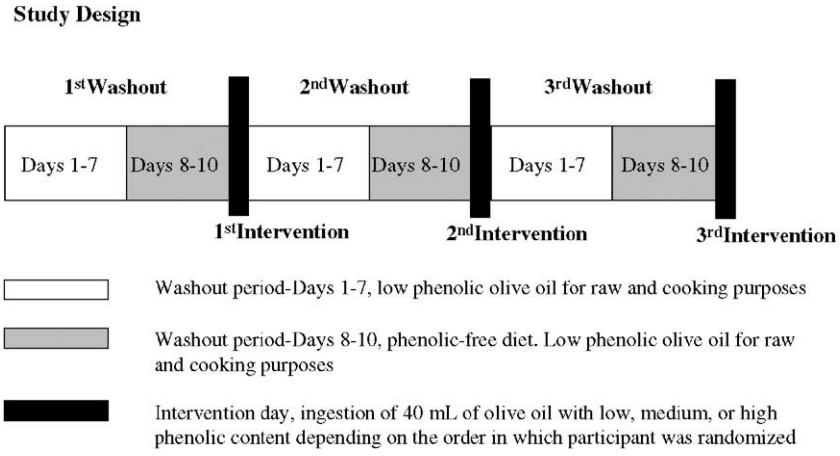


Fig. 1. Time-line showing the study design.

determined by gas chromatography-mass spectrometry (GC/MS). Analyses were carried out on a Hewlett-Packard (Palo Alto, CA) gas chromatograph coupled to a mass spectrometer detector system consisting of an HP5980 gas chromatograph, a HP5973 mass-selective detector, and a HP7683 series injector. Separation of hydroxytyrosol and tyrosol was carried out using a HP Ultra 2 (12.5 m × 0.2-mm i.d. and 0.33-μm film thickness) cross-linked 5% phenylmethyl silicone capillary column (Hewlett-Packard). Instrumental, hydrolytic and extraction conditions of samples were previously described [25]. All chemicals and organic solvents used were of analytical grade. Pharmacokinetic parameters were calculated using specific functions in a spreadsheet (PK Functions for Microsoft Excel).

Measurement of the fatty acid profile, α-tocopherol, and total phenolic content of the LDL

The antioxidant content of the LDL was measured at several times (0–1.5 h) around the maximum peak of plasma T, HT, and MHT concentrations. α-Tocopherol in LDL was determined by HPLC [26]. Phenolic compounds in LDL were determined by HPLC-DAD [27]. The fatty acid composition of LDL was also determined following the method described by Bondía et al. [28] in which fatty acids are transformed into methyl esters and analyzed by gas chromatography (GC-FID).

Measurement of plasma and lipoproteins oxidative stress markers

Oxidized LDL (oxLDL) in plasma was measured by a sandwich ELISA procedure using the murine monoclonal antibody, mAb-4E6, as capture antibody bound to microtitration wells, and a peroxidase conjugated anti-apolipoprotein B antibody recognizing oxLDL bound to the solid phase (oxLDL, Mercodia AB, Uppsala, Sweden). Antibodies against oxidized LDL (Ab-oxLDL) were measured by ELISA using copper-oxidized LDL as antigen and a specific peroxidase conjugated with anti-human IgG antibodies (OLAB, Biomedica, Vienna, Austria) [29]. Plasma total $F_{2\alpha}$-isoprostanes (8-iso-$PGF_{2\alpha}$) were determined using HPLC-ESI-MS-MS and stable isotope dilution mass spectrometry [30]. 3-chlorotyrosine (3-CT) was measured in a mixture of Apo B containing lipoproteins. Very low density lipoproteins (VLDL) and LDL were isolated from plasma by ultracentrifugation. 3-CT and its C13 analogue (3-(13C6)-chlorotyrosine) analogue (as internal standard) were synthesized as previously described [31]. Samples containing VLDL+LDL and internal standard were submitted to a basic hydrolysis with NaOH 4M for 16 hours at 120°C. After hydrolysis, samples were adjusted to pH 3–4 with trifluoroacetic acid (TFA), and the mixture was passed over a solid/liquid phase C18 extraction column (Bond-Elut-C18, 500 mg, Varian, Palo Alto, California, USA) equilibrated with 2 mL of 0.1% TFA. The column was washed with 2 mL of the same buffer, eluted with 2mL of 20% methanol in 0.1% TFA, and the recovered amino acids were dried under vacuum. Aminoacids were derivatized with a mixture of MSTFA:NH4I:2-mercaptoethanol (1000:2:6, v/w/v) at 60°C for 1 hour and analyzed by GC/MS in the electron impact ionization mode and in the single ion monitoring acquisition mode [31]. Concentration of 3-CT was very low in plasma Apo B containing lipoproteins of participants as has been previously described for healthy individuals [4]. Thus, VLDL+LDL samples from 2 individuals were pooled on the basis of similar concentrations of Apo B in lipoproteins.

Measurement of serum glucose and lipid profile

Plasma glucose, total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), and triglyceride (TG) levels were determined using enzymatic kits (Hofmann-La Roche Diagnostic, Basel, Switzerland) adapted to a Cobas Mira Plus autoanalyzer (Hoffmann-La Roche, Basel, Switzerland). LDL cholesterol (LDL-C) was calculated by means of the Friedewald formulae.

Characteristics of the olive oils

A virgin olive oil with high phenolic content (366 mg/kg) was selected. Fatty acids and vitamin E composition were measured. Harvests of virgin olive oils from the same cultivar and soil, which were submitted to refinement, were tested in order to select one with a similar fatty acid and micronutrient profile to that of the virgin olive oil selected. An adjustment of vitamin E to similar values of that present in this virgin olive oil was performed. Phenolic compounds are lost in the refination process, due to this, the refined olive oil had a low phenolic content (2.7 mg/kg). By mixing virgin and refined olive oil, an olive oil with an intermediate phenolic content (164 mg/kg) was obtained. The characteristics of the three olive oils with high (HPC), medium (MPC), and low (LPC) phenolic content were similar to that of olive oils present in the market, and are shown in Table 1. Olive oils had similar fatty acid profile and minor components content, but, with differences in their phenolic content.

Statistical analyses

The normality of variables was assessed by the Kolmogorov-Smirnov test and by analyses of skewness and kurtosis. Spearmans correlation coefficients were used to assess the relationship between continuous variables. One-factor ANOVA and Kruskal-Wallis test were used to determine differences in basal characteristics and nutrient intake among the three olive oil interventions. A general linear model for repeated measurements was used with multiple paired comparisons, in order to assess the effect of each type of olive oil. The comparison of post-intervention changes in oxidative stress variables was carried out by a General Linear Mixed Model (GLMM) with the olive oil phenolic dose (high, medium, low) as a fixed factor; individual level of test subjects as random effect, and olive oil administration order

Table 1
Characteristics of the olive oils administered

	Type of olive oil		
	LPC	MPC	HPC
Quality parameters			
Free acidity (% oleic acid)	0.03	0.08	0.18
Peroxide value (mEq O_2/kg)	4.12	5.89	11.28
Fatty acids (%)			
C14:0	<0.1	<0.1	<0.1
C16:0	10.6	10.5	10.6
C16:1	0.9	0.9	0.9
C17:0	<0.1	<0.1	<0.1
C17:1	<0.1	<0.1	<0.1
C18:0	3.3	3.1	2.8
C18:1	79.1	79.8	80.6
C18:2	4.6	4.2	3.3
C20:0	0.4	0.4	0.4
C18:3	0.6	0.6	0.6
C20:1	0.3	0.3	0.3
C22:0	0.1	0.1	0.1
C24:0	<0.1	<0.1	<0.1
α-Tocopherol (ppm)	229	228	228
Phenolic compounds (mg/kg)	2.7	164	366
Squalene (mg/g)	3.0	3.2	3.4
β-sitosterol (mg/g)	1.4	1.5	1.5

LPC, MPC, and HPC, olive oils with low (2.7 mg/kg), medium (164 mg/kg), and high (366 mg/kg) phenolic content, respectively.

as covariates. Models were corrected by Tukey's method for multiple comparisons. Linearity of values across olive oil interventions was determined by these models as a test for the dose-response effect of phenolic compounds. Statistical significance was defined as $p < 0.05$ for a two-sided test. These statistical analyses were performed using the SPSS statistical software (SPSS Incorporated Co., Chicago, IL, version 11.1).

Results

Dietary intake

No significant differences were observed among groups for energy, protein, carbohydrate, and fat intake, or for the main antioxidant (i.e. β-carotenoid, vitamin C, α-tocopherol) or pro-oxidant (iron) intake during the study.

Pharmacokinetics of plasma tyrosol, hydroxytyrosol, and 3-O-methyl-hydroxytyrosol

Plasma concentration of T, HT, and MHT increased in a dose-dependent manner with the phenolic content of the olive oil administered ($p < 0.001$) (Fig. 2). The time to reach peak concentrations (Tmax) was (mean ± SD): 0.83 ± 0.58 h and 0.90 ± 0.81 h for T, 0.70 ± 0.51 h and 0.91 ± 0.84 h for HT, and 1.31 ± 1.02 h and 1.12 ± 0.74 h for MHT, for MPC and HPC olive oils, respectively. The increase in plasma phenolic compounds after LPC ingestion was negligible. The estimated elimination half-life was (mean ± SD): 3.41 ± 1.68 h and 2.89 ± 0.60 h for T, 3.01 ± 1.01h and 3.00 ± 1.46 h for HT, and 2.37 ± 1.29 and 2.96 ± 0.87 h for MHT, for MPC and HPC olive oils, respectively.

Effect on the LDL fatty acid, α-tocopherol, and total phenolic content

No changes were observed in the LDL fatty acid and vitamin E content after any type of olive oil ingestion. The phenolic content of the LDL, however, changed with the type of olive oil administered (Table 2). LDL total phenolic content decreased after 1.5 h of LPC ingestion ($p < 0.05$) whereas an increasing trend in the phenolic content of LDL was observed after HPC ingestion ($p < 0.05$). When changes in the amount of phenolic compounds in LDL from baseline among olive oils were compared, the phenolic content of LDL increased in a dose-dependent manner with the phenolic content of the olive oil administered at 1 h and 1.5 h after olive oils ingestion ($p < 0.05$) (Table 2).

Changes in the phenolic content of the LDL directly correlated with plasma T, HT, and MHT after HPC ingestion (Table 3). These relationships were significant at 30 min for HT and at 1 h and 1.5 h for T and MHT after HPC ingestion ($p < 0.05$), reaching in the remaining cases a borderline significance ($p < 0.1$). Concerning MPC, no significance was obtained in any case when the above mentioned relationships were

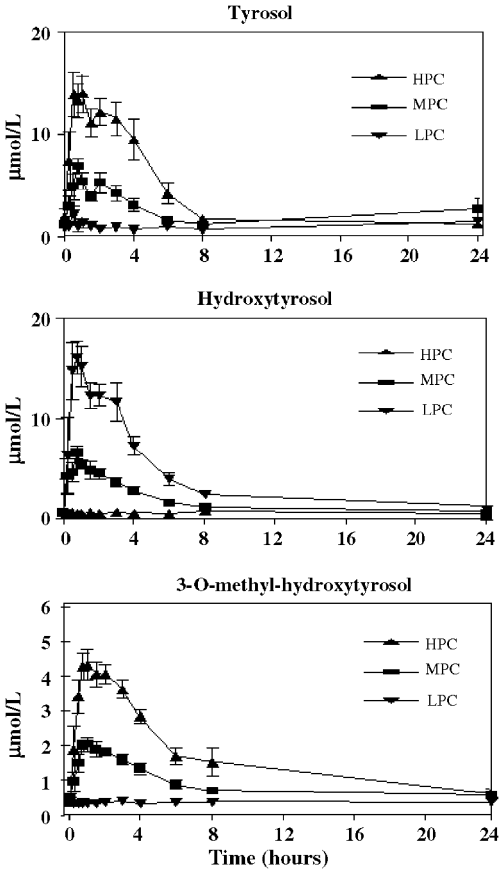


Fig. 2. Concentration versus Time curves for tyrosol, hydroxytyrosol, and 3-O-methyl-hydroxytyrosol after ingestion of 40 mL of olive oil with high (HPC), medium (MPC), and low (LPC) phenolic content.

Table 2
Percentage of changes[a] from baseline of phenolic compounds in LDL after a single dose of 40 mL of olive oil with low (LPC), medium (MPC), and high (HPC) phenolic content

Olive oil	Baseline (0 h)[b]	Changes (%) after olive oil ingestion			p[c]
		30 min	1 h	1.5 h	
LPC	0.54 (0.25)	−1.7 (15.0)	−9.3 (13.7)	−27 (5.4)[d]	0.081
MPC	0.48 (0.28)	−7.0 (17.3)	0.79 (9.3)	−9.7 (11.0)	0.442
HPC	0.43 (0.23)	14.3 (16.6)	44 (15.1)[e]	57 (14.2)[d,e]	0.046
p[f]	0.879	0.254	0.046	0.017	

[a] Data are expressed as the mean (SEM).
[b] ng/ g total fatty acids.
[c] p for linear trend across time for each olive oil evaluated, general linear model.
[d] $p < 0.05$ versus baseline, Tukey's test for multiple comparisons.
[e] $p < 0.05$ versus LPC olive oil at the evaluated time, Tukey's test for multiple comparisons.
[f] p for linear trend across olive oils for each evaluated time, general linear model.

examined, despite of a direct trend in the associations (data not shown).

Effect on the plasma and lipoprotein concentration of oxidative stress markers

After a 40 mL olive oil dose, hypertriglyceridemia together with oxidative stress occurred with all types of olive oils administered. Changes, expressed as percentage from baseline concentrations, of triglycerides, F2-isoprostanes, ox-LDL, and Ab-oxLDL are shown in Table 4 for 2, 4, and 6 h after the intake of HPC, MPC, and LPC. A significant increase in triglycerides at 2 and 4 h, and in F2-isoprostanes at 4 and 6 h, was observed after ingestion of the three olive oils ($p < 0.05$). Concerning the isoprostanes increases, the highest (21.3% and 29.9% at 4 h and 6 h postprandial, respectively) was observed after LPC ingestion, and the lowest (11.6% and 16.5% at 4 h and 6 h postprandial, respectively) after HPC ingestion. The increase in plasma oxidized LDL was only significant after LPC ingestion, Ab-ox LDL followed a different pattern depending on the type of olive oil ingested. A decreasing linear trend, a decreasing quadratic trend, and no significant changes, were observed after LPC, MPC, and HPC, respectively (Table 4). Fig. 3 shows the concentrations of 3-chlorotyrosine in Apo B containing lipoproteins after olive oils ingestion. An increase at 4 h after LPC ingestion was observed ($p < 0.05$). After 4 h of MPC ingestion concentrations of 3-chlorotirosine in VLDL+LDL increased, but significance was not reached, whereas no changes were observed after HPC ingestion. The increase (percentage of change) of 3-chlorotyrosine in Apo B containing lipoproteins at 4 h from baseline was significantly lower after HPC ingestion than after MPC ingestion ($p = 0.014$).

The data in Table 4 suggest a higher antioxidant capacity of the HPC olive oil versus the LPC one. The different effects of the olive oil phenolic dose are shown in Table 5. The decrease in ox-LDL changes from LPC to HPC observed in Table 4 was seen as a significant trend 4 h and 6 h after olive oil intake. The increase in Ab-oxLDL values from LPC to HPC was seen as a significant trend in all evaluated times. Paired comparisons showed significantly lower ox-LDL and higher Ab-oxLDL values after HPC versus LPC interventions in all evaluated times No differences were observed for F2-isoprostanes, although the decrease after HPC versus LPC at 4h reached a borderline significance at 4 h and 6 h after olive oil administration ($p = 0.062$ and $p = 0.070$, respectively).

Discussion

In this study, three similar types of olive oils, but with difference in their phenolic content, were used. Participants were submitted to a strict low-antioxidant diet 3 days before and during the intervention periods. The use of LPC olive oil during wash-out periods for raw and cooking purposes, and for cooking purposes the day of the study, avoided differences in the main fat ingestion, and permitted an homogenization of the LDL fatty acid composition. The type of fat ingested is a key factor concerning LDL oxidation. Oleate rich-LDL is less susceptible to oxidation than linoleate-rich LDL (8). With our design we avoided the interference of oleic acid when assessing the in vivo antioxidant capacity of phenolic compounds from olive oil. Phenolic compounds from olive oils were also the only differential source of antioxidants during the intervention day. Phenolic compounds were absorbed and had a pharmacokinetic profile which was dose-dependent on the phenolic content of the olive oil administered. These results agree with those obtained by Visioli et al [32] in which the absorption of T and HT from olive oils enriched with high levels of free forms of these phenolic compounds were also absorbed in a dose-dependent manner.

Dietary phenolic compounds can bind human LDL lipoprotein [33]. Phenolic compounds which can bind LDL are likely to exert their peroxyl scavenging activity in the arterial intima, where oxidation of LDL mainly occurs in microdomains sequestered from the richness of antioxidants present in plasma [1]. In ex vivo studies, we observed that plasma incubation with virgin olive oil extracts led to an increase of the phenolic compounds previously bound to LDL [34]. Here, we report an in vivo increase at postprandial time in the total phenolic content of LDL in a dose-dependent manner with the phenolic content of the olive oil administered. The decrease in the LDL phenolic content after LPC ingestion could be attributed to an oxidation of the LDL phenolics by the postprandial oxidative stress. Our data suggest that in the case of MPC and HPC ingestion, the

Table 3
Spearman's correlation coefficients (p for significance) between percentage of changes of phenolic compounds in LDL and plasma tyrosol, hydroxytyrosol, and 3-O-methyl-hydroxytyrosol after 40 mL of olive oil with high phenolic content

Time	Tyrosol	Hydroxytyrosol	3-O-methyl-hydroxytyrosol
30 min	0.510 (0.090)	0.780 (0.009)	0.524 (0.080)
1 h	0.608 (0.036)	0.517 (0.085)	0.636 (0.026)
1.5 h	0.699 (0.011)	0.507 (0.089)	0.629 (0.028)

Table 4
Percentage of changes[a] of triglycerides, F_2-isoprostanes, oxidized LDL, and antibodies against oxidized LDL (Ab-ox LDL), after ingestion of 40 mL of olive oil with low (LPC), medium (MPC), and high (HPC) phenolic content

Olive oil	Baseline (0 h)	Changes (%) after olive oil ingestion (time in hours)			p (trend)[b]
		2 h	4 h	6 h	
HPC					
Triglycerides (mmol/L)	1.25 (0.46)	39 (14)[c]	43 (13)[c]	12 (10)	0.012 (quadratic)
F_2-isoprostanes (pg/ml)	24 (5.5)	4.9 (3.5)	11.6 (3.3)[c]	16.5 (4.8)[c]	0.005 (linear)
Oxidized LDL (U/L)	51 (21)	−9.5 (9.2)	5.2 (8.6)	−15.2 (8.7)	n.s.
Ab-ox LDL (U/L)	251 (113)	20.8 (13.6)	22.5 (13.1)	22.1 (18.9)	n.s
MPC					
Triglycerides (mmol/L)	1.16 (0.33)	36 (12)[c]	33 (15)[c]	9.2 (10)	0.039 (quadratic)
F_2-isoprostanes (pg/ml)	26 (7.1)	11.9 (6.0)	14.2 (5.6)[c]	23.8 (5.4)[d]	0.002 (linear
Oxidized LDL (U/L)	45 (20)	5.6 (9.2)	10.0 (8.5)	8.9 (9.2)	n.s.
Ab-oxLDL (U/L)	304 (196)	−17.8 (9.1)	−14.25 (7.7)	−0.20 (13.5)	0.0 42 (quadratic)
LPC					
Triglycerides (mmol/L)	1.24 (0.50)	32 (11)[c]	50 (21)[c]	27 (20)	0.038 (quadratic)
F_2-isoprostanes (pg/ml)	26 (6.3)	5.6 (5.0)	21.3 (6.8)[d]	29.9 (5.1)[d]	0.001 (linear)
Oxidized LDL (U/L)	46 (20)	23.2 (8.2)[c]	24.7 (8.6)[c]	20.9 (8.9)	n.s.
Ab-ox LDL (U/L)	262 (130)	−19.2 (11.2)	−28.7 (12.6)[c]	−27.1 (8.2)[c]	0.011 (linear)

n.s., not significant.
[a] Data are expressed as the mean (SEM).
[b] p for trend from general linear model.
[c] $p < 0.05$ from baseline, general linear model with Tukey's correction for multiple comparisons.
[d] $p < 0.01$ from baseline, general linear model with Tukey's correction for multiple comparisons.

phenolic content of the olive oil protected the LDL phenolic content from degradation. The direct relationship observed among plasma T and HT with the changes in the phenolic content of LDL after HPC ingestion also supports the idea that the postprandial increase in the total phenolic content in LDL observed could be attributed to the olive oil phenolic compounds ingested with the olive oil. The fact that phenolic compounds from olive oil can protect the phenolic content of LDL reinforces their role as antioxidants in vivo.

In previous studies, the ingestion of a 25 mL dose of virgin olive oil did not promote postprandial oxidative stress [35], whereas a 50 mL dose did [36]. The balance of pro-oxidant and antioxidant reactions is well regulated in the organism. Thus, an intervention with an antioxidant-rich compound, without any additional impact of oxidative stress, may exert only a marginal effect, if any. The data presented in this report support the action of reactive oxygen species oxidizing TG-rich lipoproteins after 40 mL olive oil ingestion. These TG-rich lipoproteins are sequentially converted to LDL. Thus, a residence time of 2 to 4 hours (required for the conversion of TG-rich lipoproteins to LDL) may be adequate to propagate the oxidative process once the oxidation-initiating element has been transferred to the lipoprotein [21]. In the present study study the degree of postprandial oxidative stress, however, was lower depending

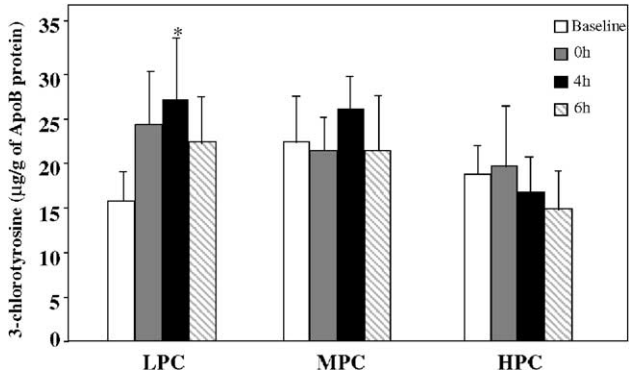


Fig. 3. Concentration of 3-chlorotyrosine (µmoL/g of ApoB protein) in pooled samples of VLDL+LDL after ingestion of 40 mL of olive oil with high (HPC), medium (MPC), and low (LPC) phenolic content ($n = 6$). *$p < 0.05$ from LPC baseline.

Table 5
p values obtained by ANOVA[a] and paired comparisons

Time	F2-isoprostanes	Oxidized LDL	Ab-oxLDL
2 hours			
p for trend	0.414	0.056	**0.016**
HPC versus LPC	0.227	**0.017**	**0.011**
HPC versus MPC	0.875	0.258	**0.014**
MPC versus LPC	0.290	0.190	0.782
4 hours			
p for trend	0.137	**0.043**	**0.003**
HPC versus LPC	0.062	**0.013**	**0.001**
HPC versus MPC	0.702	0.169	**0.010**
MPC versus LPC	0.128	0.229	0.413
6 hours			
p for trend	0.188	**0.028**	**0.031**
HPC versus LPC	0.070	**0.009**	**0.009**
HPC versus MPC	0.344	0.074	0.173
MPC versus LPC	0.356	0.362	0.190

Ab-oxLDL, antibodies against oxidized LDL.
[a] Data from Table 4 were analyzed for statistical significance for the intake of the sequence: high-phenolic (HPC), medium-phenolic (MPC), and low-phenolic (LPC) olive oil by a general lineal mixed model (GLMM) with Tukey's correction for multiple comparisons. Bold values indicate significance.

on the phenolic content of the olive oil. Oxidized LDL only increased with significance after LPC olive oil. Postprandial changes in in vivo oxidized LDL decreased in a dose-dependent manner with the phenolic content of the olive oil administered. Ab-oxLDL changes followed a different pattern depending on the olive oil ingested. No changes in Ab-oxLDL occurred after HPC ingestion, in concordance with the lack of significant changes in ox-LDL. The transient reduction in Ab-oxLDL after LPC and MPC olive oils could be explained by the excess generation of oxidized LDL at postprandial state. Although discrepancies exist in the interpretation of the clinical significance of Ab-oxLDL levels [37], these levels are lower in acute oxidative stress situations, such as immediately after smoking a cigarette [38] or acute myocardial infarction [39]. It has been hypothesized that the physiological role of Ab-oxLDL is to remove oxidized LDL by means of soluble antigen-antibody complexes, these may interfere with Ab-oxLDL determination [40]. In accordance with this hypothesis, an inverse correlation has been observed between Ab-oxLDL and oxLDL in healthy individuals [41] using the same antibodies and methods for analyses as in the present study. In one of our previous studies we observed low levels of Ab-oxLDL together with high levels of oxidized LDL in stable coronary heart disease patients [29]. Transient reduction in the level of circulating autoantibodies against oxidized LDL linked with postprandial lipemia in atherosclerotic patients, after the ingestion of a test meal that provided 26 g of polyunsaturated fatty acids (PUFA), had been previously reported [42]. Although the protective or pathogenic role of the ox-LDL-Ab-oxLDL antigen-antibody complexes remains to be elucidated [37], immunization of laboratory animals with oxLDL increased the complexes inhibiting the progression of atherosclerosis [43]. An inverse relationship between Ab-oxLDL and carotid artery intima-media thickness, an indicator of subclinical atherosclerosis [44], in a healthy population has also been reported using the same antibodies and method as in the present study [45].

The ingestion of olive oil with high phenolic compounds also avoided the postprandial rise in 3-chlorotyrosine concentrations in ApoB containing lipoproteins observed after low- and medium-phenolic content olive oil ingestion. 3-chlorotyrosine is reported to be a specific marker for direct LDL protein oxidation by the myeloperoxidase-H_2-O_2-Cl^- system [4]. The tyrosyl radical generated by myeloperoxidase is also a physiological catalyst for the initiation of lipid peroxidation in lipoproteins [46]. Olive oil phenolic compounds have been shown to counteract both metal- and radical-dependent LDL oxidation and to act as chain-breaking antioxidants for lipid peroxidation [11–13]. Differences in the postprandial degree of oxidative stress were reflected in markers directly associated with LDL oxidation; the dose-dependent increase in the LDL phenolic content observed after MPC and HPC ingestion could be an explanation for this fact. The effect of a high phenolic content in an olive oil dose versus a medium or low one on the LDL oxidation is in line with some long-term studies in which high phenolic content olive oil was more effective than low phenolic content olive oil in both decreasing circulating oxidized LDL levels and increasing the resistance of LDL oxidation (16–18). Despite the high sensitivity of F2-isoprostanes [47], derived from arachidonic acid and with a more broad spectra of sources in blood (i.e. cell membranes), perhaps a higher dose of phenolic compounds or a larger number of individuals in the study would be required to achieve significance in the differences observed among olive oil interventions. Recently, a decrease in postprandial F_2-isoprostanes after a high-flavonol cocoa drink providing 187 mg of flavonols, combined with physical exercise, has been described [48]. Visioli et al. [49] showed that the administration of 50 mL olive oil with high concentrations of phenolic compounds (≥975 mg/L) resulted in a dose-dependent reduction in the 24 h urinary excretion of F_2-isoprostanes in humans.

Concluding remarks

From this work, as well as from other recent reports, we may conclude that the phenolic compounds content of an olive oil can modulate the oxidative/antioxidative balance in plasma and LDL, in an oxidative stress situation. Further clinical studies are warranted with individuals such as diabetics, hypertensive, endurance sportsmen, and smokers, who are prone to oxidative stress.

From the comparison of high- and low-phenolic content olive oil it follows that the content of phenolic compounds in an olive oil is an important determinant for its nutritional value. The phenolic content of the olive oils depends on several factors, such as the crop, variety, ripeness, conservation of the olives, technological processes used for oil extraction, olive oil transport, and harvesting systems. If the beneficial effects of high phenolic content olive oil are substantiated by further human studies, then measures for phenolic compound enhancement in crops, and its conservation in manufacturing processes, may be required to enhance the nutritional properties of olive oil.

Acknowledgments

Supported by Grant QLK1-CT-2001-00287 from EU Commission, the EUROLIVE Study, and from Instituto de Salud Carlos III (PREDIMED Network of Excellence Groups, G03/140), Spain.

References

[1] Witzum, J. L. The oxidation hypothesis of atherosclerosis. *Lancet* **344:**793–795; 1994.

[2] Ehara, S.; Ueda, M.; Naruko, T.; Haze, K.; Itoh, A.; Otsuka, M.; Komatsu, R.; Matsuo, T.; Itabe, H.; Takano, T.; Tsukamoto, Y.; Yoshiyama, M.; Takeuchi, K.; Yoshikawa, J.; Becker, A. E. Elevated levels of oxidized low density lipoprotein show a positive relationship with the severity of acute coronary syndromes. *Circulation* **103:**1955–1960; 2001.

[3] Toshima, S.; Hasegawa, A.; Kurabayashi, M.; Itabe, H.; Takano, T.; Sugano, J.; Shimamura, K.; Kimura, J.; Michishita, I.; Suzuki, T.; Nagai, R. Circulating oxidized low density lipoprotein levels. A biochemical

risk marker for coronary heart disease. *Arterioscler. Thromb. Vasc. Biol.* **20:**2243–2247; 2000.

[4] Hazen, S. L.; Crowley, J. R.; Mueller, D. M.; Heinecke, J. M. Mass spectrometric quantification of 3-chlorotyrosine in human tissues with attomole sensitivity: a sensitive and specific marker for myeloperoxidase-catalyzed chlorination at sites of inflammation. *Free Radic. Biol. Med.* **23:**909–916; 1997.

[5] Steinberg, D.; Parthasarathy, S.; Carew, T. E.; Khoo, J. C.; Witztum, J. L. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **320:**915–924; 1989.

[6] Esterbauer, H.; Gebicki, J.; Puhl, H.; Jürgens, G. The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free. Rad. Biol. Med.* **13:**341–390; 1992.

[7] Reaven, P.; Parthasarathy, S.; Grasse, B. J.; Miller, E.; Steinberg, D.; Witzum, J. Effect of oleate-rich and linoleate-rich diets on the susceptibility of low density lipoprotein to oxidative modification in mildly hypercholesterolemic subjects. *J. Clin. Invest.* **91:**668–676; 1993.

[8] Reaven, P.; Grasse, B. J.; Tribble, D. L. Effects of linoleate enriched and oleate enriched diets in combination with α-tocopherol on the susceptibility of LDL and LDL subfractions to oxidative modification in humans. *Atheroscler. Thromb.* **14:**557–566; 1994.

[9] Esterbauer, H.; Puhl, H.; Dieber-Rotheneder, M.; Waeg, G.; Rabl, H. Effect of antioxidants on oxidative modification of LDL. *Ann. Med.* **23:**573–581; 1991.

[10] Fuller, C. J.; Jialal, I. Effects of antioxidants and fatty acids on low density lipoprotein oxidation. *Am. J. Clin. Nutr.* **60:**1010–1013; 1994.

[11] Visioli, F.; Bellomo, G.; Galli, C. Free radical-scavenging properties of olive oil polyphenols. *Biochem. Biophys. Res. Commun.* **247:**60–64; 1998.

[12] Visioli, F.; Galli, C.; Plasmati, E., et al. Olive phenol hydroxytyrosol prevents passive smoking-induced oxidative stress. *Circulation* **102:**2169–2171; 2000.

[13] Fitó, M.; Covas, M. I.; Lamuela-Raventos, R. M., et al. Protective effect of olive oil and its phenolic compounds against low density lipoprotein oxidation. *Lipids* **35:**633–638; 2000.

[14] De la Puerta, R.; Ruiz Gutierrez, V.; Hoult, J. R. Inhibition of leukocyte 5-lipoxygenase by phenolics from virgin olive oil. *Biochem. Pharmacol.* **57:**445–449; 1999.

[15] Vissiers, M. N.; Zock, P. L.; Katan, M. B. Bioavailability and antioxidant effects of olive oil in humans: a review. *Eur. J. Clin. Nutr.* **58:**955–965; 2004.

[16] Masella, R.; Giovannini, C.; Vari, R.; Di Benedetto, R.; Coni, E.; Volpe, R.; Fraone, N.; Bucci, A. Effects of dietary virgin olive oil phenols on low density lipoprotein oxidation in hyperlipidemic patients. *Lipids* **36:**1195–1202; 2001.

[17] Marrugat, J.; Covas, M. I.; Fitó, M., et al. Effects of differing phenolic content in dietary olive oils on lipids and LDL oxidation. A randomized controlled trial. *Eur. J. Nutr.* **43:**140–147; 2004.

[18] Fitó, M.; Cladellas, M.; De la Torre, R.; Martí, J.; Alcántara, M.; Pujadas-Bastardes, M.; Marrugat, J.; Bruguera, J.; López-Sabater, M. C.; Vila, J.; Covas, M. I.; the members of the SOLOS Investigators. Antioxidant effect of virgin olive oil in patients with stable coronary heart disease: a randomised, crossover, controlled, clinical trial. *Atherosclerosis* **181:**149–158.

[19] Owen, R. W.; Mier, W.; Giacosa, A.; Hull, W. E.; Spiegelhalder, B.; Bartsch, H. Phenolic compounds and squalene in olive oils: the concentration and antioxidant potential of total phenols, simple phenols, secoroids, lignans and squalene. *Food. Chem. Toxicol.* **38:**647–659; 2000.

[20] Roche, H. M.; Gibney, M. J. The impact of postprandial lipemia in accelerating atherothrombosis. *J. Cardiovasc. Risk.* **7:**317–324; 2000.

[21] Regnstrom, J.; Nilsson, J.; Tornvall, P.; Landou, C.; Hamsten, A. Susceptibility to low-density lipoprotein oxidation and coronary atherosclerosis in man. *Lancet* **339:**1183–1186; 1992.

[22] Ceriello, A.; Taboga, C.; Tonutti, L., et al. Evidence for an independent and cumulative effect of postprandial hypertriglyceridemia and hyperglycemia on endothelial dysfunction and oxidative stress generation: effects of short- and long-term simvastatin treatment. *Circulation* **106:**1211–1218; 2002.

[23] Natella, F.; Ghiselli, A.; Guidi, A.; Ursini, F.; Scaccini, C. Red wine mitigates the postprandial increase of LDL susceptibility to oxidation. *Free. Radic. Biol. Med.* **30:**1036–1044; 2001.

[24] Ling, L.; Zhao, S. P.; Gao, M.; Zhou, Q. C.; Li, Y. L.; Xia, B. Vitamin C preserves endothelial function in patients with coronary heart disease after a high-fat meal. *Clin. Cardiol.* **25:**219–222; 2002.

[25] Miró-Casas, E.; Covas, M. I.; Farré, M., et al. Hydroxytyrosol disposition in humans. *Clin. Chem.* **49:**945–952; 2003.

[26] Gimeno, E.; Castellote, A. I.; Lamuela-Raventós, R. M.; De la Torre, M. C.; López-Sabater, M. C. Rapid HPLC method for the simultaneous determination of retinol, α-tocopherol, β-carotene in human plasma and LDL. *J. Chromatogr. B.* **758:**315–322; 2001.

[27] Lamuela-Raventós, R. M.; Covas, M. I.; Fitó, M.; Marrugat, J.; De la Torre-Boronat, M. C. Detection of dietary antioxidant phenolic compounds in human low density lipoproteins. *Clin. Chem.* **45:**1870–1872; 1999.

[28] Bondía, E. M.; Castellote, A. I.; López-Sabater, M. C.; Rivero, M. Determination of plasma fatty acid composition in neonates by gas chromatography. *J. Chromatogr.* **658:**369–374; 1994.

[29] Weinbrenner, T.; Cladellas, M.; Covas, M. I., et al. High oxidative stress in patients with stable coronary heart disease. *Atherosclerosis* **168:**99–106; 2003.

[30] Morrow, J. D.; Roberts, L. J. Mass spectrometry of prostanoids: F2-isoprostanes produced by non-cyclooxygenase free radical-catalyzed mechanism. *Methods. Enzymol.* **233:**163–174; 1994.

[31] Hazen, S. L.; Heinecke, J. W. 3-Chlorotyrosine, a specific marker of myeloperoxidase-catalyzed oxidation is markedly elevated in low density lipoprotein isolated from human atherosclerotic intima. *J. Clin. Invest.* **99:**2075–2081; 1997.

[32] Visioli, F.; Galli, C.; Bornet, F., et al. Olive oil phenolics are dose-dependently absorbed in humans. *FEBS Lett.* **468:**159–160; 2000.

[33] Lamuela-Raventós, R. M.; Covas, M. I.; Fitó, M.; Marrugat, J.; De la Torre-Boronat, M. C. Detection of dietary antioxidant phenolic compounds in human LDL. *Clin. Chem.* **45:**1870–1872; 1999.

[34] Covas, M. I.; Fitó, M.; Lamuela-Raventós, R. M.; Sebastià, N.; De la Torre, M. C.; Marrugat, J. Virgin olive oil phenolic compounds: binding to human LDL and effect on LDL oxidation. *Int. J. Pharmacol. Res* **XX** (3/4)**:**49–54; 2000.

[35] Weinbrenner, M.; Fitó, T.; Farré-Albaladejo, M.; Sáez, G. T.; Rijken, P.; Tormos, C.; Coolen, S.; de laTorre, R.; Covas, M. I. Bioavailability of olive oil phenolic compounds from olive oil and oxidative/antioxidative status at postprandial state in humans. *Drugs Exp. Clin. Res.* **XXX** (5/6)**:**207–212; 2004.

[36] Fitó, M.; Gimeno, E.; Covas, M. I., et al. Postprandial and short-term effects of dietary virgin olive oil on oxidant/antioxidant Status. *Lipids* **37:**245–251; 2002.

[37] Shoenfield, Y.; Wu, R.; Dearing, L. L.; Matsuura, E. Are anti-oxidized low-density lipoprotein antibodies pathogenic or protective? *Circulation* **110:**2552–2558; 2004.

[38] Zaratin, A.; Gidlund, M.; Boschcov, P.; Castilho, L.; Cotta de Faria, E. Antibodies against oxidized low-density lipoprotein in normolipidemic smokers. *Am. J. Cardiol.* **90:**651–653; 2002.

[39] Schumacher, M.; Eber, B.; Tatzber, F.; Kaufmann, P.; Halwachs, G.; Fruhwald, F. M.; Zweiker, R.; Esterbauer, H.; Klein, W. Transient reduction of autoantibodies against oxidized LDL in patients with acute myocardial infarction. *Free. Radic. Biol. Med.* **18** (6)**:**1087–1091; 1995 (Jun).

[40] Lopes-Virella, M. F.; Virella, G.; Orchard, T. J., et al. Antibodies to oxidized LDL and LDL-containing immune complexes as risk factors for coronary artery disease in diabetes mellitus. *Clin. Immunol.* **90:**165–172; 1999.

[41] Shoji, T.; Nishizawa, Y.; Fukumoto, M., et al. Inverse relationship between circulating oxidized low density lipoprotein (oxLDL) and anti-oxLDL antibody levels in healthy subjects. *Atherosclerosis* **148:** 171–177; 2000.

[42] Le, N. A.; Li, X.; Kyung, S.; Virgil Brown, W. Evidence for the in vivo generation of oxidatively modified epitopes in patients with atherosclerotic endothelium. *Metabolism* **49:**1271–1277; 2000.

[43] Palinski, W.; Miller, E.; Witztum, J. L. Immunization of low density lipoprotein (LDL) receptor-deficient rabbits with homologous malondialdehyde-modified LDL reduces atherogenesis. *Proc. Natl. Acad. Sci.* **92:**821–825; 1995.

[44] Juonala, M.; Viikari, J. S.; Laitinen, T.; Marniemi, J.; Helenius, H.; Ronnemaa, T.; Raitakari, O. T. Interrelations between brachial endothelial function and carotid intima-media thickness in young adults: the cardiovascular risk in young Finns study. *Circulation* **110:**2918–2923; 2004.

[45] Fukumoto, M.; Shoji, T.; Emoto, M.; Kawagishi, T.; Okuno, Y.; Nishizawa, Y. Antibodies against oxidized LDL and carotid arteriy intima-media thickmess in a healthy population. *Arterioscler. Thromb. Vasc. Biol.* **20:**703–707; 2000.

[46] Savenkova, M. I.; Mueller, D. M.; Heinecke, J. W. Tyrosil radical generated by myeloperoxidase is a physiological catalyst for initiation of lipid peroxidation in low density lipoprotein. *J. Biol. Chem.* **269:** 20394–20400; 1994.

[47] Basu, S. Isoprostanes:novel bioactive products of lipid peroxidation. *Free Rad. Res.* **38:**105–122; 2004.

[48] Wiswedel, I.; Hirsch, D.; Kropf, S.; Gruening, M.; Pfister, E.; Schewe, T.; Sies, H. Flavanol-rich cocoa drink lowers plasma F_2-isoprostane concentrations in humans. *Free Rad. Biol. Med.* **37:**411–421; 2004.

[49] Visioli, F.; Caruso, D.; Galli, C.; Viappiani, S.; Galli, G.; Sala, A. Olive oils rich in natural catecholic phenols decrease isoprostane excretion in humans. *Biochem. Biophys. Res. Commun.* **278:**797–799; 2000.

Publicación 3

"Presence of virgin olive oil phenolic metabolites in human low density lipoprotein fraction: determination by high-performance liquid chromaotgraphy-electrospray ionization tandem mass spectrometry"
K. de la Torre-Carbot, J.L. Chávez-Servín, O. Jaúregui, A.I. Castellote, R.M. Lamuela Raventós, M. Fitó, M.I. Covas, D. Muñoz-Aguallo, and M.C. López-Sabater. ***Anal Chim Acta.*** 583, 2007.

Resumen

Los efectos biológicos del aceite de oliva virgen relacionados con la prevención de la oxidación de la LDL parecen estar ligados a su alto contenido de AGMI, pero también a su contenido de compuestos fenólicos.

Un requisito para valorar la significancia fisiológica *in vivo* de los compuestos fenólicos es determinar su presencia en la LDL humana tras la ingesta de aceite de oliva virgen.

El objetivo del presente estudio fue determinar si los metabolitos del tirosol e hidroxitirosol son capaces de unirse a la LDL humana.

Para este fin, siete voluntarias sanas con dietas no suplementadas participaron en el estudio. Las participantes ingirieron 50 mL de aceite de oliva extra virgen en ayunas. Las muestras de sangre fueron tomadas 60 minutos después de la ingesta del aceite para su posterior análisis. Las muestras fueron comparadas con muestras obtenidas antes de consumir el aceite.

Se detectaron y cuantificaron en las LDL cinco metabolitos de compuestos fenólicos provenientes de compuestos fenólicos del aceite de oliva que fueron:

tirosol glucurónido, tirosol sulfato, hidroxitirosol monoglucurónido, hidroxitirosol monosulfato y ácido homovainíllico sulfato.
Dos métodos de separación de LDL fueron comparados, de los cuales el método más corto mostró una mejor recuperación de los compuestos antioxidantes, sin afectar en la recuperación de ácidos grasos.

Se postula que el hidroxitirosol y tirosol sulfato y el hidroxitirosol y tirosol glucurónido provienen de la sulfatación y glucuronidación respectiva del hidroxitirosol y tirosol contenidos en el aceite de oliva en su forma libre o en su forma compuesta con derivados elenólicos, mientras que el ácido homovainíllico sulfatado puede provenir de la acción de la catecol-*O*-metiltransferasa y la posterior acción de la sulfotransferasa sobre el hidroxitirosol en el hígado.

El hecho de que estos metabolitos son capaces de encontrarse en LDL sugiere que estos compuestos pueden funcionar como antioxidantes *in vivo*.

Available online at www.sciencedirect.com
ScienceDirect
Analytica Chimica Acta 583 (2007) 402–410
ANALYTICA CHIMICA ACTA
www.elsevier.com/locate/aca

Presence of virgin olive oil phenolic metabolites in human low density lipoprotein fraction: Determination by high-performance liquid chromatography–electrospray ionization tandem mass spectrometry

Karina de la Torre-Carbot [a], Jorge L. Chávez-Servín [a], Olga Jaúregui [b], Ana I. Castellote [a], Rosa M. Lamuela-Raventós [a], Montserrat Fitó [c], María-Isabel Covas [c], Daniel Muñoz-Aguayo [c], M. Carmen López-Sabater [a,*]

[a] *Department of Nutrition and Food Science, Reference Center in Food Technology, Faculty of Pharmacy, University of Barcelona, Avda. Joan XXIII s/n, 08028 Barcelona, Spain*
[b] *Scientific and Technical Services, University of Barcelona, Josep Samitier 1-5, 08028 Barcelona, Spain*
[c] *Lipids and Cardiovascular Epidemiology Unit, Institut Municipal d' Investigació Mèdica (IMIM), Barcelona, Spain*

Received 3 August 2006; received in revised form 9 October 2006; accepted 9 October 2006
Available online 2 November 2006

Abstract

The biological benefits of olive oil in preventing the oxidation of low density lipoprotein (LDL) would seem to be linked to its high monounsaturated fatty acid contents, but also to its respective phenolic compounds contents. One prerequisite to assess the *in vivo* physiological significance of phenolic compounds is to determine their presence in human LDL following the ingestion of virgin olive oil.

In this work, olive oil phenolic metabolites were identified using high-performance liquid chromatography in tandem with electrospray mass spectrometry (HPLC–ESI-MS/MS) detection, after solid phase extraction (SPE). Quantitative methods were developed in carrying out linearity, precision, sensitivity and recovery tests. The results from two methods of LDL separation were compared and shorter LDL isolation procedure showed a better recovery for antioxidants compounds in LDL. The metabolites identified in LDL were: hydroxytyrosol monoglucuronide, hydroxytyrosol monosulfate, tyrosol glucuronide, tyrosol sulfate and homovanillic acid sulfate. The fact that olive oil phenolic metabolites are able to bind LDL strengthens claims that these compounds act as *in vivo* antioxidants.

Keywords: Olive oil; Low density lipoprotein; Hydroxytyrosol; Tyrosol; Homovanillic acid; Phenolic metabolites; High-performance liquid chromatography; Mass spectrometry; Solid phase extraction

1. Introduction

The protection afforded against cardiovascular disease in the Mediterranean area may be attributed to the high content of phenolic compounds in the region's diet [1–3]. It would appear that this protection is due to diverse combinations of biological effects including, antioxidant [1,4–9], anti-inflammatory [3,4], vasodilatation [4], and anti-platelet aggregation [3,8] properties, as well as the modulation of gene expression [3,4,8,2]. Recent claims suggest that the benefits to be derived from this diet are the result of the synergistic combination of phytochemicals and fatty acids [10–12].

Tyrosol and hydroxytyrosol, in simple forms or as conjugates, are the main phenolic compounds present in olive oil [13]. Both compounds are bioavailable in animals [14–16] and humans [17–29]. In the latter, olive oil phenolic compounds undergo extremely extensive first-pass intestinal/hepatic metabolism in the body [15,20,21,25,27]. Thus, it would appear that their biological activity is more likely to be linked to the biological

Abbreviations: HPLC–ESI-MS/MS, high-performance liquid chromatography in tandem with electrospray mass spectrometry; LDL, low density lipoprotein; SPE, solid phase extraction; DAD, diode array detector; IS, internal standard; DP, declustering potential; MRM, multiple reaction monitoring; CE, collision energy; CAD, collision-activated dissociation; LOD, limit of detection; LOQ, limit of quantification; Apo-B, Apolipoprotein-B; R.S.D., relative standard deviation; u, atomic mass units

* Corresponding author at: Departament de Nutrició i Bromatologia, Facultat de Farmàcia, Universitat de Barcelona, Av. Joan XXIII s/n, E-08028 Barcelona, Spain. Tel.: +34 93 402 45 12; fax: +34 93 403 59 31.
E-mail address: mclopez@ub.edu (M.C. López-Sabater).

0003-2670/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2006.10.029

metabolites of the phenolic compounds rather than to the primary species present in olive oil. *In vivo* glucoronide, sulfate and methyl conjugates of hydroxytyrosol and tyrosol in plasma and urine have been described [14–17,21,25,27,29–32].

Thus, olive oil protects LDL from oxidation, while virgin olive oil shows increased antioxidant activity because of its higher phenol content [33,34]. Numerous studies have shown the phenolic compounds of olive oil to be potent inhibitors of LDL oxidation *in vitro* and *ex vivo* [14,20,23,33–38], and *in vivo* with animals [14,16,39–44] and *in vivo* with humans [10,20,23,11,45–47]. Similarly, it has been demonstrated that these phenolic compounds can aid the inhibition of 5- and 12-lipoxygenases, and also aid the prevention of platelet aggregation [4,8].

Several studies demonstrate that the concentration of phenolic compounds in LDL and the capacity of these compounds to protect this fraction against oxidation increase with the consumption of such food types [18,48,11]. Covas et al. [48] show that tyrosol binds LDL *in vitro*.

A number of studies have reported the incorporation of components of virgin olive oil, including α-tocopherol, retinol, and β-carotene [49,11], into LDL, with α-tocopherol being the most prevalent antioxidant [49]. However, few studies have identified the presence of phenolic compounds from olive oil [20,50], or those from other food sources in LDL [51]. Furthermore, evidence supporting determinations of the phenolic metabolites of olive oil in LDL is scarce and the few bioavailabilty studies that have been conducted following enzymatic hydrolysis [20] do not report the profiles of these metabolites. Besides this, to our knowledge, it is the first time a new type of LDL separation has been developed in order to preserve antioxidants in this particle.

Given the very low levels of concentration (ng mL^{-1}) of these metabolites in human LDL, a highly sensitive detection technique is required, following an adequate extraction procedure so as to minimize matrix effects. Although the diode array detector (DAD) has also been used to determine phenolic compounds in biological fluids [52,53]. HPLC–MS/MS has emerged as one of the preferred bioanalysis techniques for the quantification of drugs, metabolites and endogenous biomolecules in biological matrices [31,51,54–56].

This study aims to determine whether tyrosol and hydroxytyrosol metabolites are able to bind the human LDL. Two methods of isolating LDL from plasma were compared in terms of their ability to reduce phenolic compounds in LDL degradation during the isolation procedure. Blood from seven volunteers was used to evaluate the precision of the method used and thus to demonstrate its significance to real sample. Pre- and post-olive oil consumption metabolite values were compared.

2. Material and methods

2.1. Chemical, reagents and solutions

Hydroxytyrosol, homovanillic acid and taxifoline (as internal standard, IS) were purchased from Extrasynthèse (Genay, France). HPLC grade methanol and acetonitrile were purchased from SDS (Peypin, France). Formic acid was purchased from Sigma (Saint Louis, MO, USA). Phosphoric acid was purchased from Panreac (Barcelona, Spain). Ultrapure (MilliQ) water Millipore (Bedford, MA, USA) was used. Ultrapure water: methanol (95/5, v/v) was found to be the most suitable solvent for the standards.

2.2. Subjects and study design

Seven healthy female volunteers on nonsupplemented diets, with an average weight of 61.5 ± 9.61 kg and a body mass index of 23.13 ± 3.37 kg m^{-2} took part in the study. Participants ingested 50 mL of extra virgin olive oil (produced from the Picual variety of *Olea europea* L. fruit) in the morning after fasting for 12 h. One hour after this meal, 30 mL of blood was drawn from each subject into vacutainer tubes. Control samples were obtained from volunteers after a 12-h fast. Before these blood samples were taken, controls were subjected to a 3-day washout period, during which phenol-rich food was excluded from their diet and only refined olive oil (with no phenolic compounds) was used for dressing raw foods and cooking. The protocol was approved by de CEIC-IMAS Ethic Committee. The volunteers gave written informed consent before their inclusion in the study.

2.3. Instrumentation

2.3.1. SPE-Vacuum

Samples were extracted using an SPE-Vacuum Manifold manufactured by Tecknokroma (Barcelona, Spain).

2.3.2. Concentrator

Organic solutions were evaporated with nitrogen in a Techne sample concentrator (Duxford, UK).

2.3.3. HPLC–DAD-MS/MS instrument

An Agilent 1100 HPLC (Waldbronn, Germany) equipped with an autosampler at 4 °C and coupled to an API3000 triple-quadrupole mass spectrometer PE Sciex (Concord, Canada) with a turbo ion spray source was used. A 5-μm particle size C_{18} Luna column, 15 cm × 2 mm ID with a C_{18}, 4 mm guard cartridge Phenomenex (Macclesfield, UK) was used.

2.4. LDL isolation methods

After centrifugation (1000 × *g*, 15 min), EDTA plasma was pooled and aliquots were stored at −80 °C. The first step in the two methods tested involved very low density lipoprotein isolation. This was performed as follows: 1 mL of isotonic saline containing EDTA, 1.091 mmol L^{-1}, and NaCl, 0.198 mol L^{-1}, was layered carefully on top of the plasma (2 mL), in a centrifuge tube. The tubes were centrifuged in a Beckman-Coulter XL-70, using the Fixed-Angle Type 50.4 rotor at 199,808 × *g* for 18 h at 4 °C. Two different procedures were used in the second step of ultracentrifugation. In Method 1 [57], the infranatant from the first step (3 mL) was placed in a centrifuge tube containing 0.075 g sucrose and 0.116 g potassium bromide (KBr). Three millilitres of isotonic saline, containing EDTA 1.091 mmol L^{-1}, NaCl 0.198 mol L^{-1}, and KBr 2.704 mol L^{-1}, were layered

carefully on the top of the infranatant. Tubes containing distilled water (5 mL) were centrifuged in the SW40Ti rotor at 202,048 × *g* for 20 h at 4 °C. The middle layer containing LDL was aspirated and kept frozen at −80 °C. In Method 2, the infranatant (3 mL) was put in a centrifuge tube containing 0.170 g KBr, stained with 70 μL of 18.16 mmol L^{-1} Coomassie Brilliant Blue R solution, and vortexed. Two millilitres of isotonic saline, containing KBr 0.524 mol L^{-1}, were carefully on the top of the infranatant up to the base of the tube vertex. The tubes were sealed before being introduced in the NVT100 rotor and centrifuged at 697,760 × *g* for 5 h at 4 °C. The LDL layer was aspirated and kept frozen at −80 °C. LDL apolipoprotein-B content was determined by immunoturbidimetry.

Fatty acid composition [58] and α-tocopherol [49] were measured as has been described elsewhere.

2.5. Extraction of phenolic compounds

Before each procedure, 20 μL of phosphoric acid 85% (v/v) and 40 μL of taxifolin solution (200 ng mL^{-1}) were added to 1 mL aliquots of LDL and mixed with a vortex for 1 min. An Oasis HLB (60 mg) cartridge from Waters (Mildford, MA, USA) was used for the SPE. The cartridge was activated with 1 mL of methanol and 1 mL of water. The LDL sample was percolated through the cartridge. To remove interfering components, the sample was washed with 9 mL of water and 1 mL of methanol (5% in water) under vacuum. The phenolic compounds were then eluted with 3 mL of methanol. The eluate was evaporated under nitrogen until dry and reconstituted with 150 μL of the mobile phase. The temperature was controlled (<30 °C) so as to avoid the deterioration of phenolic compounds. The samples were filtered through a 4 mm Politetrafluoroetilene (PTFE) 0.45 μm membrane from Waters (Mildford, MA, USA) into amber vials for HPLC–MS/MS analysis. Fifty microliters of filtered sample were then injected into the HPLC–ESI-MS/MS. The entire process was performed in the dark and/or with brown glass material.

2.6. HPLC conditions

The column was kept at 40 °C. The mobile phase consisted of a binary solvent system using water acidified with 0.1% formic acid (solvent A), and 100% acetonitrile (solvent B), at a flow-rate of 0.5 mL min. The gradient program began with 0.5% eluent B. This percentage was maintained for 10 min, then eluent B was ramped linearly to 5.0% in 2 min. Eluent B was ramped again linearly to 15.0% at minute 17 and ramped to 25.0% until minute 29. Next, eluent B was ramped linearly to 100% at minute 30 and maintained until minute 35. The column was re-equilibrated for 15 min between runs. In the latter, the flow was split after the column directing 1/3 toward the MS instrument.

2.7. MS conditions

Before use, the instrument was shown to meet the manufacturer's required specifications. The triple-quadrupole mass spectrometer was calibrated with the turbo ion spray, using a test solution of (propyleneglycol) obtained from Applied Biosystems. The mass spectrometer was calibrated to ensure that mass accuracy specifications and sensitivity readings could be obtained for the entire mass range. All analyses were conducted with the turbo ion spray source in negative mode and with the following settings: capillary voltage −3500 V, nebulizer gas (N_2) 10 (arbitrary units), curtain gas (N_2) 12 (arbitrary units), collision gas (N_2) 4 (arbitrary units), declustering potential (DP) −25 V for homovanillic acid, −30 V for taxifoline and −40 V for hydroxytyrosol. The DP for multiple reaction monitoring (MRM) of two transitions: −10 and −10 V for tyrosol sulfate −10 and −10 V for tyrosol glucuronide, −25 and −30 V for homovanillic acid sulfate, −25 and −30 V for hydroxytyrosol monosulfate and −25 and 30 V for hydroxytyrosol monoglucuronide. Focusing potential −250 V, entrance potential 10 V and collision energy (CE) −11 V for homovanillic acid, −20 V for hydroxytyrosol and −30 V for taxifoline. CE for MRM of two transitions: −20 and −15 V for homovanillic acid sulfate, −20 and −15 V for hydroxytyrosol sulfate, −20 and −15 V for hydroxytyrosol glucuronide, −25 and −25 V for tyrosol sulfate and −25 and 25 V for tyrosol glucuronide. Drying gas was heated to 300 °C and introduced at a flow-rate of 5000 cm^3 min^{-1}. Full-scan data were acquired by scanning from *m/z* 100 to 800 in profile mode, using a cycle time of 2 s, with a step size of 0.1 atomic mass units (u) and a pause of 2 ms between scans. In the product ion scan experiments, MS/MS product ions were produced by the collision-activated dissociation (CAD) of selected precursor ions in the collision cell of the triple-quadrupole mass spectrometer. These were then mass-analyzed with the instrument's second analyzer.

2.8. Metabolite characterization and quantification

The characterization of the metabolites in the LDL samples was based on their ion fragmentation in the MS/MS mode. Collision-induced dissociation-MS/MS (CID-MS/MS), neutral loss scan and MRM of two transitions analyses were used for confirmation.

MRM give the highest selectivity and sensitivity in HPLC–MS/MS [59]. This was carried out with a dwell time of 200 ms for each transition and a pause between mass ranges of 5 ms so as to be able to monitor five double transitions for each metabolite analysis: hydroxytyrosol monoglucuronide *m/z* 329 → 153 and 153 → 123; hydroxytyrosol monosulfate *m/z* 233 → 153 and 153 → 123; tyrosol glucoronide *m/z* 313 → 137 and 137 → 93, tyrosol sulfate *m/z* 217 → 137 and 137 → 93; and homovanillic acid sulfate *m/z* 261 → 181 and 181 → 137 carried out with a dwell time of 300 ms. As standards of these metabolites are not available, tyrosol and hydroxytyrosol metabolites were quantified using a hydroxytyrosol standard, while the homovanillic metabolite was quantified with a homovanillic acid standard. To achieve this, MRM of one transition for each metabolite was used, and three more transitions were monitored: IS taxifolin *m/z* 303 → 285; hydroxytyrosol *m/z* 153 → 123 and homovanillic acid *m/z* 181 → 137. The use of these phenol standards is justified by the similarity of their chemical properties to those of the metabolites studied.

2.9. Validation procedures

2.9.1. Linearity

To conduct the quantitative analysis, calibration curves were constructed by plotting the HPLC–MS/MS peak area ratio between hydroxytyrosol and homovanillic acid and IS taxifolin (at 50 ng mL^{-1}), against the analyte concentration. A standard curve was made of hydroxytyrosol (1–800 ng mL^{-1}) and homovanillic acid (5–800 ng mL^{-1}) with matrix (human LDL) of Sigma–Aldrich (Saint Quentin Fallavier, France), and with water.

2.9.2. Precision and recovery

Within-day and between-day precision was made by determination of the metabolites present in LDL samples following the ingestion of olive oil, then, to reduce systematic errors, a reproducibility and repeatability test was carried out for each metabolite studied, using blood from the seven volunteers. To test the recovery of the proposed method, samples of human LDL were spiked with varying amounts of hydroxytyrosol and homovanillic acid. The samples were then submitted to the full procedure as described above. The IS was added once the sample had been passed through the cartridge.

2.9.3. Sensitivity

The limits of detection (LOD) and quantification (LOQ) were calculated by measuring the analytical background response when running six blanks at the maximum sensitivity allowed by the system. LOD was considered to be three times the standard deviation of the six blank samples analyzed, while LOQ was considered to be 10 times the standard deviation of these same blank samples.

2.9.4. Statistical analysis

For statistical analysis, we used a one-way analysis of variance (ANOVA) or the Student's *t*-test for paired data for evaluates the differences between variables. Statistical significance was defined as $p < 0.05$ for a two-sided test. SPSS 11.0 statistical software was used.

3. Results

3.1. Phenolic content in used virgin olive oil

The phenolic content of olive oil used was determined by HPLC–DAD as described elsewhere [13]. It presented a concentration of 648 μg mL^{-1} of total phenolic compounds. Hydroxytyrosol and tyrosol were present in a concentration mean of 70.6 and 27.01 μg mL^{-1} and Oleuropein and ligstroside derivatives accounted for 84% of the total phenolic content.

3.2. LDL isolation procedures

The two methods used in isolating the LDL were carefully compared. The apolipoprotein-B (apo-B), total phenolic compounds, α-tocopherol and fatty acid values for the two methods are shown in Table 1. Although Method 2 (the method with the shorter second ultracentrifugation step) resulted in a more highly diluted LDL being recovered from the plasma, this procedure showed a better recovery for antioxidant compounds in LDL, with a significance level of $p < 0.05$. Given these findings, the procedures described below are those adopted for LDL isolation using Method 2. The colorant used in the LDL separation was analyzed by HPLC–ESI-MS/MS and was found not to interfere with the system.

3.3. Validation of the analytical method used

In assessing the quality of the method, we examined the following characteristics: linearity, precision, recovery and sensitivity.

3.3.1. Linearity

Although the behavior was linear for the curves constructed with water ($r > 0.999$) and those constructed with commercial LDL ($r > 0.99$) for both standards used (hydroxytyrosol and homovanillic acid), a number of differences were observed when using either water or the LDL fraction as the real matrix. Thus, below, the curves used to measure the phenolic compounds are those constructed in the matrix. In this way, any interference of

Table 1
Apolipoprotein, phenolic compounds, fatty acids and Vitamin E values in LDL obtained with the two LDL isolation methods evaluated

	Method 1[a]		Method 2[b]	
	Concentration	R.S.D.	Concentration	R.S.D.
Apolipoprotein-B (apo-B) (mg mL^{-1})	1.06[a]	8.32	0.48[b]	9.85
C16:0 (μg mg^{-1} apo-B)	384.05[a]	9.47	395.00[a]	5.00
C18:0 (μg mg^{-1} apo-B)	118.94[a]	9.39	123.87[a]	5.09
C18:1 (μg mg^{-1} apo-B)	466.67[a]	9.46	479.57[a]	5.45
C18:2 (μg mg^{-1} apo-B)	841.25[a]	9.18	874.95[a]	5.07
C20:4 (μg mg^{-1} apo-B)	155.33[a]	8.92	161.54[a]	4.62
Total phenolic compounds (ng mg^{-1} apo-B)	45.50[a]	7.21	104.00[b]	7.66
Vitamin E (μg mg^{-1} apo-B)	8.84[a]	5.01	11.40[b]	7.73

A difference in the letters in the same file indicates significant difference ($p < 0.05$).

[a] Method 1: second centrifugation step at 202,048 × *g* for 20 h at 4 °C.

[b] Method 2: second centrifugation step at 697,760 × *g* for 5 h at 4 °C.

Table 2
Metabolites found in LDL 60 min post-ingestion of 50 mL extra virgin olive oil by the HPLC–MS/MS method described in the text

Metabolite	MRM[a] transition	tR[b] (min)	Concentration mean (ng mg^{-1} apo-B)	Intra-day precision (R.S.D.)	Inter-day precision (R.S.D.)
Hydroxytyrosol monoglucuronide (peak 1)	329/153	15.4	2.45	8.65	9.63
Hydroxytyrosol monoglucuronide (peak 2)	329/153	15.7	2.55	9.92	9.99
Hydroxytyrosol monosulfate	233/153	19.52	34.22	7.72	8.16
Tyrosol glucuronide	313/137	14.4	0.96	6.06	9.92
Tyrosol sulfate	217/137	18.9	17.23	7.39	9.73
Homovanillic acid sulfate	261/181	23.1	48.02	7.50	9.06

[a] Multiple reaction monitored transition.
[b] Retention times.

the matrix effect on our measurements can be eliminated. Residual analysis for this range of concentrations was (mean (S.D.)): 95.9 (9.8) and 102.5 (9.9), for hydroxytyrosol and homovanillic acid, respectively.

3.3.2. Precision

Precision was expressed as relative standard deviation (R.S.D.). Within-day precision ($n = 10$) and between-day precision evaluated over a 3-day period ($n = 30$) was less than 10%. The concentration of phenolic compounds in the lipoprotein fractions is shown in Table 2.

3.3.3. Sensitivity and recovery

LOD and LOQ for hydroxytyrosol were 0.32 and 1.08 ng mL^{-1}, respectively, while LOD and LOQ for homovanillic acid were 0.89 and 2.9 ng mL^{-1}, respectively. These values are, in fact, lower than those reported in the literature for hydroxytyrosol and homovainillic acid quantified in biological fluids by MS [31,60]. Table 3 shows the mean (S.D.) recoveries for hydroxytyrosol and homovanillic acid from LDL for a range of concentrations from 50 to 500 ng mL^{-1}. Results did not present significant difference.

3.4. Tyrosol and hydroxytyrosol metabolites determination in LDL

3.4.1. Quantification

As shown in Table 2 and Fig. 1, the metabolites of olive oil phenolic compounds found following the ingestion of virgin olive oil were: hydroxytyrosol monoglucuronide (two peaks), hydroxytyrosol monosulfate, tyrosol glucuronide, tyrosol sulfate and homovanillic acid sulfate. The metabolites presenting the highest concentrations were hydroxytyrosol monosulfate and homovanillic acid sulfate. The overall concentration of phenolic compounds in LDL was approximately 105.43 ng mg^{-1} apo-B. In the control samples, tyrosol glucuronide and hydroxytyrosol monoglucuronide were not detectable. Similarly, hydroxytyrosol monosulfate, tyrosol sulfate and homovanillic acid sulfate were found in lower concentrations in controls (2.45, 3.06 and 8.42 ng mg^{-1} apo-B, respectively) ($p < 0.01$).

Table 3
Recovery studies of phenolic compounds in LDL %mean (S.D.)

	Concentration studied		
	50 ng mL^{-1}	100 ng mL^{-1}	500 ng mL^{-1}
Hydroxytyrosol	69.1 (3.65)	74.5 (2.4)	80.0 (0.89)
Homovanillic acid	71.6 (4.4)	95 (4.1)	89.2 (1.0)

3.4.2. Identification

Hydroxytyrosol monosulfate (Mw 234) was identified by monitoring two MRM transitions: 233 → 153 and 153 → 123. The latter was conducted at a higher DP so that CID-MS/MS could be performed. The first ion identified was that related to the loss of the sulfate group from the deprotonated molecule $[M-H]^-$, while the second was that related to the loss of the CH_2OH (30 u) from the hydroxytyrosol deprotonated molecule (*m/z* 153). The same procedure was adopted for identifying tyrosol sulfate (Mw 218) monitoring first for the loss of the sulfate group from the deprotonated molecule (217 → 137) and then the loss of the CH_2–CH_2OH group from the deprotonated tyrosol (137 → 93) (see Fig. 2). Homovanillic acid sulfate (Mw 262) was confirmed in the product ion scan of the transition *m/z* 261, monitoring for the loss of the sulfate unit (*m/z* 181) and the loss of the COOH group (see Fig. 2). Hydroxytyrosol and tyrosol glucuronide (*m/z* 329 and *m/z* 313, respectively) were confirmed by injection of the sample in a neutral loss scan of 176 u experiments. However, when Q1 and Q3 quadrupoles were operated at unit resolution, their presence could not be confirmed owing, it would seem, to their low levels of concentration. Thus, confirmation had to be obtained by setting Q3 at a low resolution. MRM 392 → 153 transition chromatogram presented two peaks at retention time 15.4 and 15.7 (peaks 1 and 2, Fig. 1).

4. Discussion

The Picual olive variety was chosen because it presents high levels of phenolic compounds [13]. The post-prandial time was selected because higher concentrations of phenolic compounds in plasma [27,47] and LDL [18,20] have been found in post-prandial analyses, and hydroxytyrosol and tyrosol metabolites are rapidly eliminated in urine [16,31].

When analyzing labile compounds, considerable care needs to be taken. Thus, with isolation Method 2 higher concentrations of total phenolic compounds and α-tocopherol were obtained. Phenolic compounds are labile and prone to deterioration when

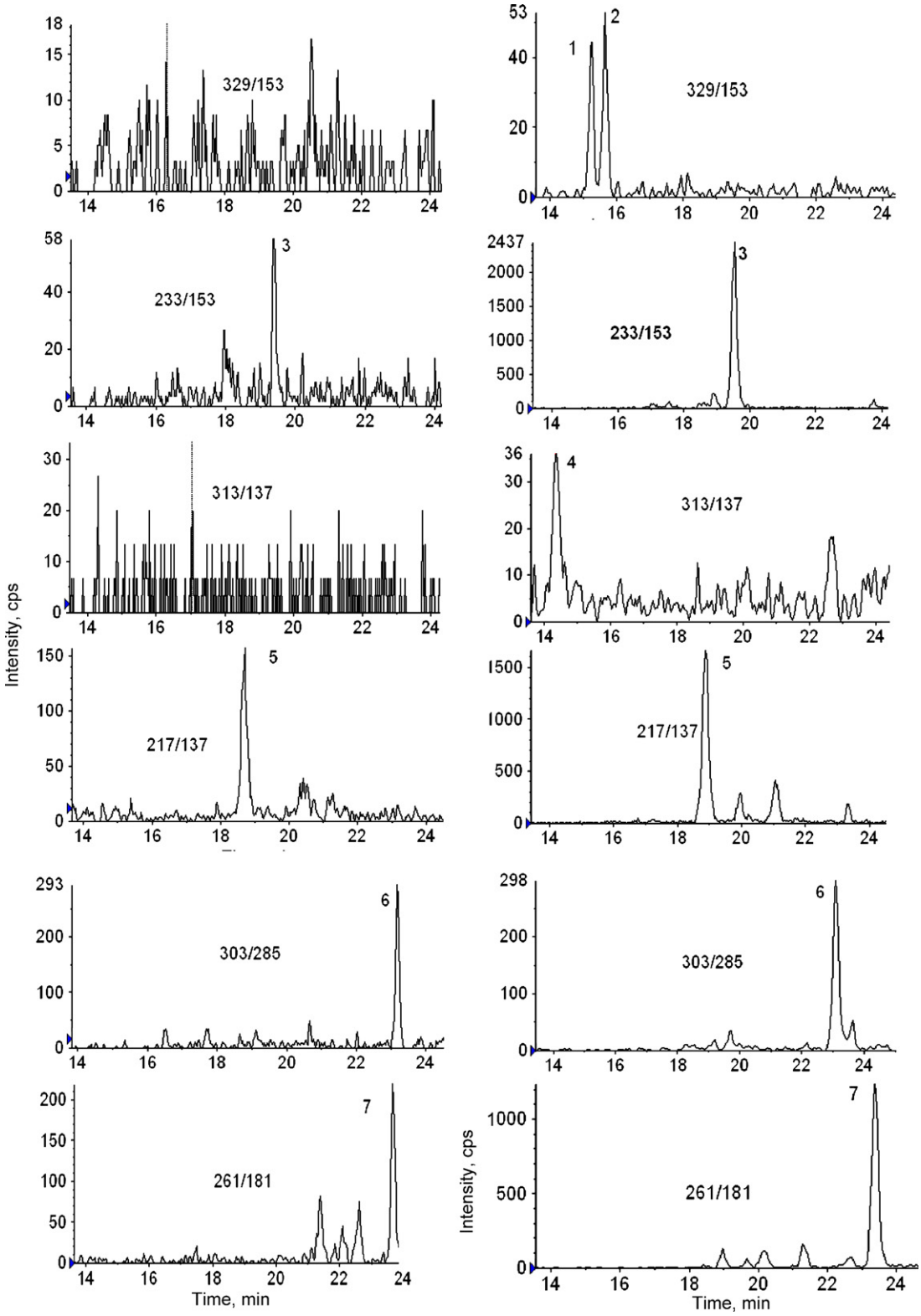


Fig. 1. Metabolites found in LDL, 60 min post-ingestion of 50 mL extra virgin olive oil, by HPLC–ESI-MS/MS detection: (1) hydroxytyrosol monoglucuronide peak 1; (2) hydroxytyrosol monoglucuronide peak 2; (3) tyrosol glucuronide; (4) hydroxytyrosol monosulfate; (5) tyrosol sulfate; (6) IS taxifoline; (7) homovanillic acid sulfate.

isolated from the whole biological system. The shorter LDL isolation time associated with Method 2 might therefore account for the better preservation of Vitamin E and phenolic compounds in LDL.

We studied complete metabolites as opposed to the compounds (resulting from hydrolysis) referred to in other works, as this would not have provided information about metabolic profiles. The biological samples we used contain the compounds

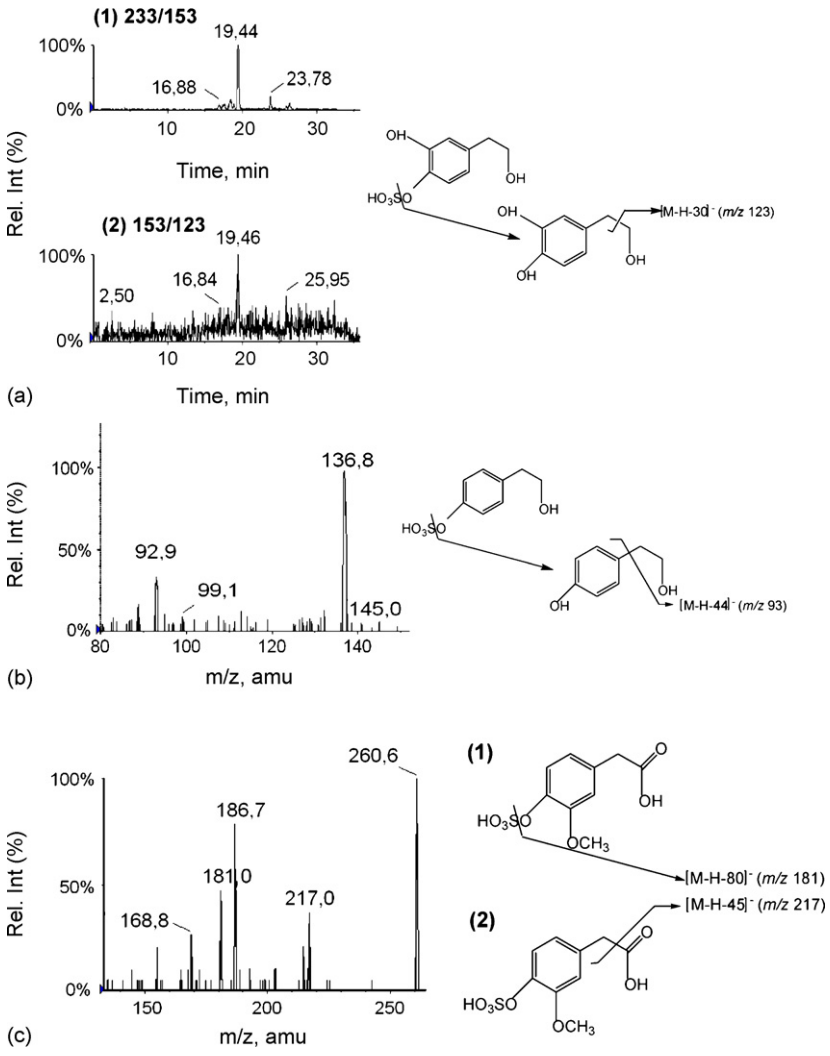


Fig. 2. (a) The two MRM transitions of hydroxytyrosol monosulfate: (1) 233 → 153 and (2) 153 → 123; (b) product ion scan spectra of *m/z* 217 (tyrosol sulfate); (c) homovanillic acid sulfate fragment: (1) loss of sulfate unit and (2) loss of the COOH group.

that arise from the natural biotransformation of the initial compound. The presence of this metabolite produces (via in-source fragmentation) an ion that is identical to the standard (the parent) for quantification.

The presence of these phenolic metabolites in LDL strengthens claims that these compounds could exert their antioxidant activity in this particle. The presence of hydroxytyrosol, tyrosol and homovanillic acid sulfate (albeit it lower concentrations) before olive oil consumption, may also be due to in addition to olive oil ingestion, hydroxytyrosol metabolites may result from endogenous dopamine metabolism [27–29].

The two peaks presented at MRM 392 → 153 transition chromatogram would seem to be attributable to the hydroxytyrosol molecule occupying a different glucoronidation position. In the previous work only one peak was detected for hydroxytyrosol monoglucuronide, possibly as a result of co-elution since a shorter column was used. The current work uses a longer column and there is a larger separation between the compounds detected. Although the previous method [50] offers a very rapid process that is useful in epidemiological studies, when many samples have to be processed in a short period of time, the present method offers better separation of the compounds as reflected in the case of hydroxytyrosol monoglucuronide.

In olive oil stored under acidic conditions, oleuropein and ligstroside give rise to the polar phenolic compounds of hydroxytyrosol and tyrosol. These two compounds may also result from the enzymatic hydrolysis of these secoiridoids. Thus, hydroxytyrosol and tyrosol are present in their own structure in the oil, or they are esterified with elenoic acid [13].

A potentially important factor in the metabolism of olive oil phenolic compounds in the body is that oleuropein and ligstroside aglycones are split into hydroxytyrosol or tyrosol and elenolic acid. These aglycones may be hydrolyzed in the gastrointestinal tract [19,21,31]. Once absorbed, phenolic compounds of dietary origin can be subjected to three main types of conjugation mechanism: methylation, sulfation, and glucoronidation [61]. Olive oil phenolic compounds are no different in this respect being extensively modified in the body [21,24,30,62]. In human liver microsomes, uridine diphosphate glucuronosyltransferases catalyze the transfer of glucuronic

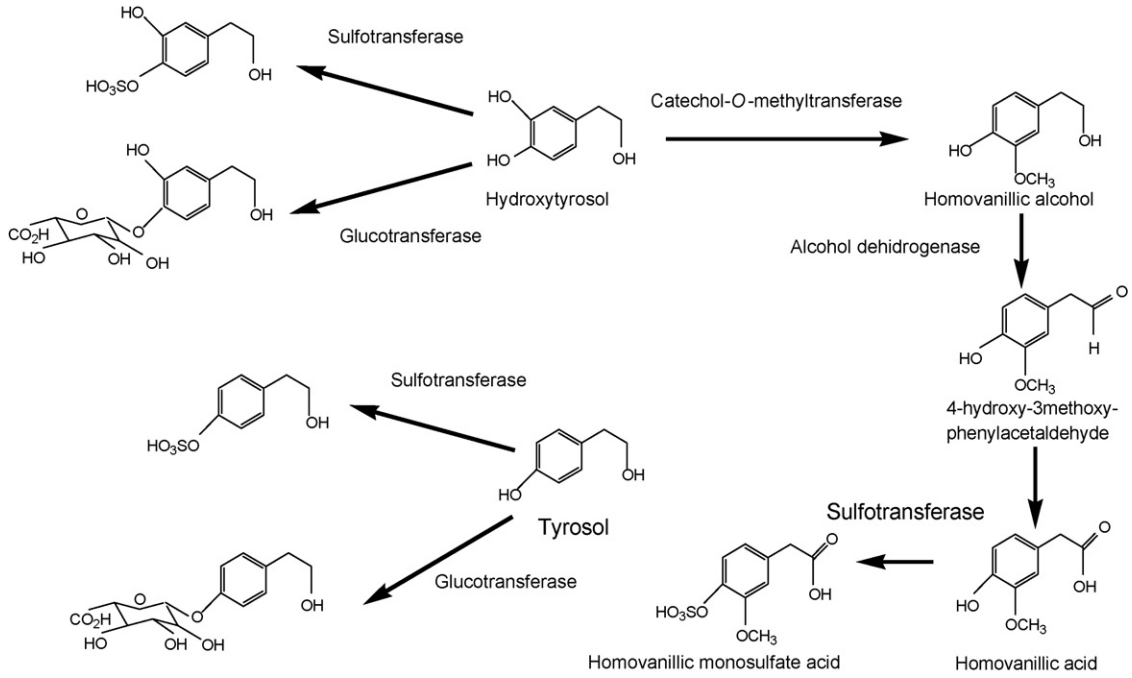


Fig. 3. Postulated enzymatic pathways for the hydroxytyrosol and tyrosol metabolites *in vivo*.

acid from the uridine diphosphate glucuronic acid. Sulfotransferase produces sulfo-conjugated derivatives mainly in the liver. Catechol-*O*-methyl transferase is present in a wide range of tissues, but its activity is greatest in the liver, kidneys and small intestine [15,61]. Fig. 3 shows possible biological enzymatic pathways for the metabolites identified in this study.

It has been hypothesized that the phenolic compounds can be bound to lipoproteins by ionic interactions with charged residues on the surface of the particles [61]. Were this to be the case, the action of phenolic compounds on LDL may be due not solely to their biological activity, but also to this ability to bind the LDL particle [63–65].

While diet can modify the fatty acid composition of the LDL profile, it can also affect the minor compounds and this can have a significant biological impact during the post-prandial phase [6,18]. These effects need to be studied further, while the roles and antioxidant capacity of specific metabolites such as hydroxytyrosol, tyrosol, and homovanillic acids sulfate and/or glucoronides should also be analyzed more closely. Phenolic compounds that can bind LDL can exert their antioxidant action *in vivo* in the arterial intima where most LDL oxidation occurs in microdomains sequestered from the richness of antioxidants present in plasma [66]. If these compounds can be determined in LDL they can serve as useful biomarkers for monitoring the adherence to virgin olive consumption in clinical and epidemiological studies.

5. Conclusions

Tyrosol and hydroxytyrosol metabolites are present in human LDL. Hydroxytyrosol monosulfate, hydroxytyrosol monoglucuronide, tyrosol sulfate, tyrosol glucuronide and homovanillic acid sulfate were found in this fraction. The methodology adopted in the determination of these phenolic compounds involved the use of a short second step ultracentrifugation for LDL isolation, and HPLC–ESI-MS/MS determination after SPE, and permits the detection and quantification of these phenolic compounds. The presence of these phenolic metabolites in LDL strengthens claims that these compounds act as *in vivo* antioxidants.

Acknowledgments

The authors would like to thank the “Ministerio de Ciencia y Tecnología” in Spain (project “SAF2004-08173-C03-03”) and the European Union (“QLKT-CT-2001-002872”) for their financial support; the University of Barcelona for the grant received by K. de la T.-C.; Marta Garrido for technical support; and Robin Rycroft and Sairica Rose for revising the English.

References

[1] M. Gerber, Clin. Chem. Lab. Med. 41 (2003) 999.
[2] J.C. Stoclet, T. Chataigneau, M. Ndiaye, M.H. Oak, B.J. El, M. Chataigneau, V.B. Schini-Kerth, Eur. J. Pharmacol. 500 (2004) 299.
[3] L.W. Morton, R.A. Caccetta, I.B. Puddey, K.D. Croft, Clin. Exp. Pharmacol. Physiol. 27 (2000) 152.
[4] F. Visioli, C. Galli, Crit. Rev. Food Sci. Nutr. 42 (2002) 209.
[5] P.L. Teissedre, A.L. Waterhouse, J. Agric. Food Chem. 48 (2000) 3801.
[6] J. Ruano, J. Lopez-Miranda, F. Fuentes, J.A. Moreno, C. Bellido, P. Perez-Martinez, A. Lozano, P. Gomez, Y. Jimenez, F. Perez Jimenez, J. Am. Coll. Cardiol. 46 (2005) 1864.
[7] C. Soler-Rivas, J.C. Espin, H.J. Wichers, J. Sci. Food Agric. 80 (2000) 1013.
[8] J.C. Espin, C. Soler-Rivas, E. Cantos, F.A. Tomas-Barberan, H.J. Wichers, J. Agric. Food Chem. 49 (2001) 1187.

[9] L. Bravo, Nutr. Rev. 56 (1998) 317.
[10] M. Fito, M. Cladellasc, R. De La Torre, J. Marti, M. Alcantara, M. Pujadas-Bastardes, J. Marrugat, J. Bruguera, M.C. Lopez-Sabater, J. Vila, M.I. Covas, Atherosclerosis 181 (2005) 149.
[11] E. Gimeno, M. Fito, R.M. Lamuela-Raventos, A.I. Castellote, M. Covas, M. Farre, M.C. de La Torre-Boronat, M.C. Lopez-Sabater, Eur. J. Clin. Nutr. 56 (2) (2002) 114–120.
[12] J.J. Moreno, M.T. Mitjavila, J. Nutr. Biochem. 14 (2003) 182.
[13] K. De La Torre-Carbot, O. Jauregui, E. Gimeno, A.I. Castellote, R.M. Lamuela-Raventos, M.C. Lopez-Sabater, J. Agric. Food Chem. 53 (2005) 4331.
[14] K.L. Tuck, P.J. Hayball, J. Nutr. Biochem. 13 (2002) 636.
[15] S. D'Angelo, C. Manna, V. Migliardi, O. Mazzoni, P. Morrica, G. Capasso, G. Pontoni, P. Galletti, V. Zappia, Drug Metab. Dispos. 29 (2001) 1492.
[16] K.L. Tuck, M.P. Freeman, P.J. Hayball, G.L. Stretch, I. Stupans, J. Nutr. 131 (2001) 1993.
[17] F. Visioli, C. Galli, F. Bornet, A. Mattei, R. Patelli, G. Galli, D. Caruso, FEBS Lett. 468 (2000) 159.
[18] M.I. Covas, K. de la Torre, M. Farré-Albaladejo, J. Kaikkonnen, M. Fitó, C. López-Sabater, M.A. Pujadas-Bastardes, J. Oglar, T. Weinbrenner, R.M. Lamuela-Raventós, R. de la Torre, Free Radic. Biol. Med. 40 (2006) 608.
[19] F. Visioli, C. Galli, S. Grande, K. Colonnelli, C. Patelli, G. Galli, D. Caruso, J. Nutr. 133 (2003) 2612.
[20] A. Bonanome, A. Pagnan, D. Caruso, A. Toia, A. Xamin, E. Fedeli, B. Berra, A. Zamburlini, F. Ursini, G. Galli, Nutr. Metab. Cardiovasc. Dis. 10 (2000) 111.
[21] M.N. Vissers, P.L. Zock, A.J.C. Roodenburg, R. Leenen, M.B. Katan, J. Nutr. 132 (2002) 409.
[22] T. Weinbrenner, M. Fito, A.M. Farre, G.T. Saez, P. Rijken, C. Tormos, S. Coolen, R. De La Torre, M.I. Covas, Drugs Exp. Clin. Res. 30 (2004) 207.
[23] J. Marrugat, M.I. Covas, M. Fito, H. Schroder, E. Miro-Casas, E. Gimeno, M.C. Lopez-Sabater, R. De La Torre, M. Farre, Eur. J. Nutr. 43 (2004) 140.
[24] E. Miró Casas, M. Farré Albadalejo, M.I. Covas, M. Fito, R. Lamuela-Raventos, R. de la Torre-Fornell, Clin. Chem. 47 (2001) 341.
[25] E. Miro-Casas, M.I. Covas, M. Fito, M. Farre-Albadalejo, J. Marrugat, R. De La Torre, Eur. J. Clin. Nutr. 57 (2003) 186.
[26] E. Miro-Casas, M.F. Albaladejo, M.I. Covas, J.O. Rodriguez, E.M. Colomer, R.M.L. Raventos, R. De La Torre, Anal. Biochem. 294 (2001) 63.
[27] E. Miro-Casas, M.I. Covas, M. Farre, M. Fito, J. Ortuno, T. Weinbrenner, P. Roset, R. De La Torre, Clin. Chem. 49 (2003) 945.
[28] D. Caruso, F. Visioli, R. Patelli, C. Galli, G. Galli, Metabolism 50 (2001) 1426.
[29] C. Manna, P. Galletti, G. Maisto, V. Cucciolla, S. D'Angelo, V. Zappia, FEBS Lett. 470 (2000) 341.
[30] K.L. Tuck, P.J. Hayball, I. Stupans, J. Agric. Food Chem. 50 (2002) 2404.
[31] P. Del Boccio, A. Di Deo, A. De Curtis, N. Celli, L. Iacoviello, D. Rotilio, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 785 (2003) 47.
[32] H.W. Tan, K.L. Tuck, I. Stupans, P.J. Hayball, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 785 (2003) 187.
[33] M. Fito, M.I. Covas, R.M. Lamuela-Raventos, J. Vila, J. Torrents, C. de la Torre, J. Marrugat, Lipids 35 (2000) 633.
[34] M. Fito, M.I. Covas, R.M. Lamuela-Raventos, J. Vila, C. de la Torre, J. Marrugat, Med. Clin. 115 (2000) 166.
[35] J.A. Moreno, J. Lopez-Miranda, P. Gomez, F. Benkhalti, E. El Boustani, F. Perez-Jimenez, Med. Clin. 120 (2003) 128.
[36] I. Stupans, A. Kirlich, K.L. Tuck, P.J. Hayball, J. Agric. Food Chem. 50 (2002) 2464.
[37] R. Masella, R. Vari, M. D'Archivio, R. Di Benedetto, P. Matarrese, W. Malorni, B. Scazzocchio, C. Giovannini, J. Nutr. 134 (2004) 785.
[38] R. Leenen, A.J.C. Roodenburg, M.N. Vissers, J.A.E. Schuurbiers, K.P.A.M. van Putte, S.A. Wiseman, F.H.M.M. van de Put, J. Agric. Food Chem. 50 (2002) 1290.
[39] E. Coni, R. Di Benedetto, M. Di Pasquale, R. Masella, D. Modesti, R. Mattei, E.A. Carlini, Lipids 35 (2000) 45.
[40] F. Visioli, C. Galli, E. Plasmati, S. Viappiani, A. Hernandez, C. Colombo, A. Sala, Circulation 102 (2000) 2169.
[41] F. Visioli, D. Caruso, E. Plasmati, R. Patelli, N. Mulinacci, A. Romani, G. Galli, C. Galli, Free Radic. Res. 34 (2001) 301.
[42] J.J. Ochoa, J.L. Quiles, M.C. Ramirez-Tortosa, J. Mataix, J.R. Huertas, Nutrition 18 (2002) 60.
[43] S. Gorinstein, O. Martin-Belloso, E. Katrich, A. Lojek, M. Ciz, N. Gligelmo-Miguel, R. Haruenkit, Y.S. Park, S.T. Jung, A. Trakhtenberg, J. Nutr. Biochem. 14 (2003) 154.
[44] S.A. Wiseman, L.B.M. Tijburg, F.H.M.M. van de Put, Lipids 37 (2002) 1053.
[45] F. Visioli, D. Caruso, C. Galli, S. Viappiani, G. Galli, A. Sala, Biochem. Biophys. Res. Commun. 278 (2000) 797.
[46] T. Weinbrenner, M. Fito, R. De La Torre, G.T. Saez, P. Rijken, C. Tormos, S. Coolen, M.F. Albaladejo, S. Abanades, H. Schroder, J. Marrugat, M.I. Covas, J. Nutr. 134 (2004) 2314.
[47] M. Fito, E. Gimeno, M.I. Covas, E. Miro, M.D. Lopez-Sabater, M. Farre, R. De La Torre, J. Marrugat, Lipids 37 (2002) 245.
[48] M.I. Covas, M. Fito, R.M. Lamuela-Raventos, N. Sebastia, C. De la Torre-Boronat, J. Marrugat, Int. J. Clin. Pharmacol. Res. 20 (2000) 49.
[49] E. Gimeno, A.I. Castellote, R.M. Lamuela-Raventos, M.C. de La Torre-Boronat, M.C. Lopez-Sabater, J. Chromatogr. B: Biomed. Sci. Appl. 758 (2) (2001) 315–322.
[50] K. de la Torre-Carbot, O. Jauregui, A.I. Castellote, R.M. Lamuela-Raventos, M.I. Covas, I. Casals, M.C. Lopez-Sabater, J. Chromatogr. A 1116 (2006) 69.
[51] M. Urpi-Sarda, O. Jauregui, R.M. Lamuela-Raventos, W. Jaeger, M. Miksits, M.I. Covas, M. Antolovich, C. Andres-Lacueva, Anal. Chem. 77 (2005) 3149.
[52] A. Tsarbopoulos, E. Gikas, N. Papadopoulos, N. Aligiannis, A. Kafatos, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 785 (2003) 157.
[53] V. Ruiz-Gutierrez, M.E. Juan, A. Cert, J.M. Planas, Anal. Chem. 72 (2000) 4458.
[54] B.L. Ackermann, M.J. Berna, A.T. Murphy, Curr. Top. Med. Chem. 2 (2002) 53.
[55] B.L. Ackermann, J.E. Hale, K.L. Duffin, Curr. Drug Metab. 7 (2006) 525.
[56] M. Jemal, Y.Q. Xia, Curr. Drug Metab. 7 (2006) 491.
[57] R.J. Havel, H.A. Eder, J.H. Bragdan, J. Clin. Invest. 34 (1995) 1345.
[58] I. Bondia-Pons, A.I. Castellote, M.C. Lopez-Sabater, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 809 (2) (2004) 339–344.
[59] D. Liu, Y. Xia, R. Barkhtiar, Rapid Commun. Mass Spectrom. 16 (2002) 1330.
[60] M.J. Magera, A.L. Stoor, J.K. Helgeson, D. Matern, P. Rinaldo, Clin. Chim. Acta 306 (2001) 35.
[61] C. Manach, A. Scalbert, C. Morand, C. Remesy, L. Jimenez, Am. J. Clin. Nutr. 79 (2004) 727.
[62] E. Schwedhelm, R. Maas, R. Troost, R.H. Boger, Clin. Pharmacokinet. 42 (2003) 437.
[63] R. de la Puerta, V.R. Gutierrez, J.R. Hoult, Biochem. Pharmacol. 57 (1999) 445.
[64] C. Giovannini, E. Straface, D. Modesti, E. Coni, A. Cantafora, M. De Vincenzi, W. Malorni, R. Masella, J. Nutr. 129 (1999) 1269.
[65] D. Caruso, B. Berra, F. Giavarini, N. Cortesi, E. Fedeli, G. Galli, Nutr. Metab. Cardiovas. Dis. 9 (1999) 102.
[66] J.L. Witztum, Lancet 344 (1994) 793.

Publicación 4

"Rapid high-performance liquid chromatography-electrospray ionization tandem mass spectrometry method for qualitative and quantitative analysis of virgin olive oil phenolic metabolites in human low-density lipoproteins". K. de la Torre-Carbot, O. Jauregui, A.I. Castellote, R.M. Lamuela-Raventos, R. M, M.I. Covas, I. Casals, I., and M.C. Lopez-Sabater, ***J Chromatogr A.*** 1116,2006.

Resumen

Algunos metabolitos de compuestos fenólicos del aceite de oliva han sido encontrados en plasma y orina en experimentos con animales y humanos, sin embargo, estudios sobre la caracterización de estos metabolitos en LDL son escasos.

En este trabajo, se desarrolló un método rápido para la detección y cuantificación de los 5 metabolitos anteriormente detectados en LDL. En el método se optimizó la extracción de estos compuestos por medio de extracción en fase sólida y utilizando HPLC/DAD-MS/MS para su determinación.

Para su extracción, la muestra fue lavada con agua y una solución metanólica del 5%. Posteriormente los compuestos fenólicos fueron eluidos con 1 mL de metanol. La fracción metabólica fue evaporada y la muestra fue reconstituida con una mezcla de agua y acetonitrilo. En el sistema cromatográfico, se utilizó una columna de 3μm de tamaño de partícula Luna C18, de 5 cm de largo y 2.0 mm de diámetro interno. La columna se calentó a 40°C. Se trabajó a un flujo de 0.6 mL/min con una fase móvil constituida por agua acidulada con ácido fórmico 0.1% y acetonitrilo, el gradiente que fue usado para la separación de los compuestos tuvo un perfil lineal. La elución de la muestra en el sistema cromatográfico es de 7 minutos.

El método mostró tener una linealidad, recuperación y precisión satisfactoria y unos límites de detección y cuantificación apropiados, por lo que el método descrito puede ser utilizado en análisis de rutina.

Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography A, 1116 (2006) 69–75
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Rapid high-performance liquid chromatography–electrospray ionization tandem mass spectrometry method for qualitative and quantitative analysis of virgin olive oil phenolic metabolites in human low-density lipoproteins ☆

Karina de la Torre-Carbot [a], Olga Jauregui [b], Ana I. Castellote [a], Rosa M. Lamuela-Raventós [a], María-Isabel Covas [c], Isidre Casals [b], M. Carmen López-Sabater [a,*]

[a] *Departamento de Nutrició i Bromatologia, Centre de Referència en Tecnologia dels Aliments (CeRTA), Facultat de Farmàcia, Universitat de Barcelona. Avda. Joan XXIII s/n, Barcelona 08028, Spain*
[b] *Unitat de Tècniques Separatives, Serveis Cientificotècnics, Universitat de Barcelona, Josep Samitier 1-5, Barcelona 08028, Spain*
[c] *Unitat de Lípids y Epidemiologia Cardiovascular de l'Institut Municipal d' Investigació Mèdica (IMIM), Barcelona, Spain*

Received 12 January 2006; received in revised form 3 March 2006; accepted 7 March 2006
Available online 17 April 2006

Abstract

A rapid method for detection and quantification of metabolites of specific olive oil phenolic compounds (hydroxytyrosol monoglucuronide, hydroxytyrosol monosulfate, tyrosol glucuronide, tyrosol sulfate and homovanillic acid sulfate) in low-density lipoprotein (LDL) fractions by solid-phase extraction (SPE) and high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry (HPLC/ESI-MS/MS) is described. A 3 μm particle size fast C_{18} Luna column, 5 cm × 2.0 mm I.D., was used at a flow rate of 0.6 mL/min with a mobile phase consisting of 0.1% (v/v) formic acid (A) and acetonitrile (B). A linear gradient profile was used for separation at column temperature 40 °C. The proposed chromatographic procedure is rapid without loosing its separation efficiency and sensitivity. Validation proofs were carried out for the method described, showing a linear system ($r > 0.99$) and a recovery of 81.9 and 101.3% for hydroxytyrosol and homovanillic acid, respectively. The results show that this method is effective and can be used in routine analysis.

Keywords: Phenolic metabolites; LDL; Olive oil; High-performance liquid chromatography; Mass spectrometry; Solid-phase extraction

1. Introduction

The healthy effects of a Mediterranean diet with regard to cardiovascular risks may derive, in part, from the enhancement of the body's antioxidant capacity. Olive oil is rich in Vitamin E and has a specific set of phenolic compounds, principally oleuropein and ligstroside derivatives [1,2]. Numerous studies have shown that these phenols are absorbed [3–14] and that they are potent inhibitors of DNA and LDL oxidation and damage [2,3,5,9,11,15–27]. Besides to this, olive phenolic compounds have different properties such prevention of platelet aggregation, inhibiting of 5- and 12-lipoxigenases [28] and modulating genes and protein expression [29].

Analytical methods suitable for measuring oleuropein, hydroxytyrosol and/or tyrosol from biological fluids have been mainly based on HPLC-diode array detection (DAD) [30,31], HPLC-fluorescence detection [32] HPLC-MS [6] and GC–MS [33,34] after several extraction procedures, such as liquid/liquid extraction [6,33], SPE [30,31], and protein precipitation with organic solvents [32].

Glucuronide, sulphate and methyl conjugates of hydroxytyrosol and tyrosol have been found in plasma and urine in both human and animal experiments [3–9,13,19,32,35,36]. However, characterizations of phenolic compounds metabolites in LDL remain scare [37]. Moreover, existing studies were carried out after enzymatic hydrolysis and fail to provide any information about metabolic profiles [9,17].

☆ Presented at the 11th Meeting on Instrumental Analysis, Barcelona, 15–17 November 2005.

* Corresponding author. Tel.: +34 93 402 45 12; fax: +34 93 403 59 31.
E-mail address: mclopez@ub.edu (M.C. López-Sabater).

0021-9673/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2006.03.022

Notwithstanding the antioxidant effects described in vitro, or the indirect relation found between the consumption of olive oil phenols and the antioxidant effects in LDL, the mechanisms underlying these metabolites, their bioavailability, and tissue distribution in humans still remain undefined. Such lack of information is due not only to the difficulty of developing sensitive methods for measuring these kinds of compounds, but also to the absence of commercially available pure standards. The lack of these products increases the risk in systematic inaccuracies. However, the isolation or synthesis of these conjugated compounds can involve strong complexities and for the moment there are not commercial laboratories that guarantee the stability quality of the product.

To better characterize the precise pharmacokinetic properties of phenolic metabolites, it is important to develop highly sensitive and simple analytical methods for their determination in LDL. In agreement with this goal, interest in much quicker separation techniques applying fast chromatographic columns has greatly increased over the last several years, especially for use in routine analyses.

The combination of a highly effective and selective isolation/purification procedure with an equally effective and sensitive separation method is essential for quantifying and identifying such metabolites. The low expected concentration (ng/mL) of these metabolites in human LDL requires a very sensitive and selective technique, such as liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS), following an adequate extraction procedure to minimize matrix effects.

To our knowledge, there are no reports describing a rapid analytical method for the identification and quantification of olive oil phenol metabolites in LDL.

The aims of this study were to optimize an SPE procedure for the isolation of five metabolites common to specific phenolic compounds in olive oil: hydroxytyrosol monoglucuronide, hydroxytyrosol monosulfate, tyrosol glucuronide, tyrosol sulfate, and homovanillic acid sulfate, and to optimize and validate the HPLC/ESI-MS/MS method using a fast column to characterize these compounds in low-density lipoprotein fraction samples.

2. Experimental

2.1. Chemicals

HPLC-grade acetonitrile and methanol were obtained from SDS (Peypin, France). Phenolic standards hydroxytyrosol, homovanillic acid and taxifolin (purity > 90%) were purchased from Extrasynthèse (Genay, France). Formic acid was purchased from Sigma (St. Louis, MO, USA). Phosphoric acid was purchased from Probus (Barcelona, Spain). Ultra pure water was generated by the MilliQ system (Millipore, Bedford, MA, USA). In MS experiments, HPLC-MS grade water (Sigma-Aldrich, Riedel-de Häen) has been used. MilliQ water:methanol (95:5, v/v) was used as the most suitable solvent for the standards.

2.2. Equipment

Samples were extracted using an SPE-Vacuum Manifold from Tecknokroma (Barcelona, Spain). Organic solutions were evaporated in a Techne sample concentrator (Duxford, Cambridge, UK)

An Agilent 1100 HPLC (Waldbronn, Germany) equipped with an autosampler and coupled to an API3000 triple-quadrupole mass spectrometer (PE Sciex, Concord, Canada) featuring a turbo ion spray source was used in tandem with a 3 μm particle size C_{18} Luna column, 5 cm × 2.0 mm I.D. with a C_{18}, 4 mm guard cartridge (Phenomenex, UK).

2.3. Samples treatment

2.3.1. LDL separation

Blood (45 mL) from healthy volunteers was collected. After centrifugation (1000 × *g*, 15 min) EDTA plasma was pooled. After this, 1 mL of isotonic saline containing EDTA 1.091 mmol/L and sodium chloride (NaCl) 0.198 mol/L, was layered carefully on top of plasma (2 mL) in a centrifuge tube. The tubes were centrifuged at 199,808 × *g* for 18 h at 4 °C. Infranatant from the first ultracentrifugation step (3 mL) was deposited in a centrifuge tube containing 0.075 g of sucrose and 0.116 g of potassium bromide (KBr). Three milliliters of isotonic saline, containing EDTA 1.091 mmol/L, NaCl 0.198 mol/L, and KBr 2.704 mol/L, was layered carefully on the top of the infranatant. Tubes were filled with distilled water (5 mL) and were centrifuged at 202,048 × *g* for 20 h at 4 °C. The LDL-containing middle layer was aspirated frozen at −80 °C [38]. All Samples were stored under −80 °C until analysis. LDL apolipoprotein B (Apo B) content was determined by immunoturbidimetry (Roche Diagnostics, Basel, Switzerland).

2.3.2. Extraction procedure

To determine the best conditions, optimization experiments were conducted. LDL samples were spiked with two concentrations of hydroxytyrosol and homovanillic acid: 0.15 and 1 μg/mL, respectively and processed. Finally, before each procedure, 20 μl of phosphoric acid 85% (v/v) and 100 μL of taxifolin solution (100 ng/mL) was added as internal standard (I.S.) for routine evaluation of SPE quality process and vigilance of possible lost purposes to 1 mL aliquots of LDL and mixed in a vortex for 1 min. An Oasis HLB (60 mg) cartridge from Waters (Milford, MA, USA) was used. The cartridge was activated with 1 mL of methanol and 1 mL of formic acid (5% in water). Acidified LDL was then percolated into the cartridge. To remove interfering components, the sample was washed under vacuum with 2 mL of water and 2 mL of 5% aqueous methanol. Afterwards, phenolic compounds were eluted with 1 mL of methanol divided in three volumes. The eluent was evaporated to dryness under nitrogen and the temperature was always controlled ($T < 30$ °C). The sample was dissolved with 150 μL water:acetonitrile (90:10). Samples were filtered through a 4 mm poly(vinylidene difluoride) (PVDF) 0.22 μm membrane Ultrafree-MC centrifugal filter unit from Millipore (Bedford, MA, USA) and transposed into an amber vial. Sub-

sequently, 20 μl was injected into the HPLC-MS/MS system. The entire process was performed under conditions of darkness using brown glass material.

2.4. Chromatography conditions

The column was maintained at 40 °C. The mobile phase consisted of a binary solvent system using water acidified with 0.1% formic acid (solvent A) and 100% acetonitrile (solvent B), kept at a flow rate of 0.6 mL/min. The gradient program started with 95% of eluent A and 5% of eluent B. Eluent B ramped linearly to 15% in 1 min, maintaining this level for 2 min before ramping again linearly to 100% at minute 5, which was maintained until minute 6. At minute 7, the gradient returned to the initial conditions and the column was re-equilibrated for 5 min between runs. LDL (20 μL) samples were injected at a constant flow rate of 0.6 mL/min, and the flow was split after the column directing 1/3 toward the MS instrument.

2.5. Mass spectrometry conditions

All the mass spectrometer parameters were manually fine-tuned to obtain the highest multiple reaction monitoring (MRM) signals. Prior to its use, the instrument was checked to meet the acceptance specifications defined by the manufacturer. The triple-quadrupole mass spectrometer was calibrated with the turbo ionspray using a test mixture solution of poly(propylene glycol) obtained from Applied Biosystems. The mass spectrometer was calibrated so that mass accuracy specifications and sensitivity were achieved over the entire mass range. Previously to validation and quantification, it was carried out a careful optimization process to achieve the best sensitivity detection in all compounds: standards and metabolites, with the most suitable declustering potential (DP) and energy collision (EC) for each one. All the analyses used the turbo ion-spray source in negative mode with the following settings: capillary voltage −3500 V, nebulizer gas (N_2) 10 (arbitrary units), curtain gas (N_2) 12 (arbitrary units), collision gas (N_2) 4 (arbitrary units), DP and CE for MRM and double MRM experiments are shown in Table 1. Focusing potential −250 V, entrance potential 10 V. Drying gas was heated to 300 °C and introduced at a flow-rate of 6000 mL/min. Full-scan data were acquired by scanning from *m/z* 100 to 800 in profile mode using a cycle time of 2 s with a step size of 0.1 u and a pause between each scan of 2 ms. In product ion scan (PIS) experiments, MS/MS product ions were produced by collision-activated dissociation (CAD) of selected precursor ions in the collision cell of the triple-quadrupole mass spectrometer, and then mass-analyzed with the instrument's second analyzer.

2.6. Metabolite characterization and quantification

Detection and quantification were performed using MRM and double MRM, in this last two sets of product and precursor masses that are known to be characteristic of certain target compound are specified for each compound. Absence of ion suppression was verified comparing results with those obtained with a 5 μm particle size C_{18} Luna column, 15 cm × 2.0 mm I.D. (Phenomenex, UK). Metabolite confirmation in LDL samples was based on their ion fragmentation in the MS/MS mode. MRM experiments were carried out with a dwell time for each transition of 200 ms and a pause between mass ranges of 5 ms. Showed transitions in Table 1 were monitored for each metabolite analysis.

Collision-induced dissociation-MS/MS (CID-MS/MS) was used in order to characterize the compound by MS/MS experiments at a relatively high DP potential (−60 V). Increasing the voltages beyond the optimal conditions can induce fragmentation before the ions enter the mass filters resulting in a decrease in sensitivity. In some instances this fragmentation can prove a valuable tool providing additional structural information. Moreover, neutral loss scan of 80 u and 176 were used to allow characterize sulfates and glucuronides. Neutral loss scan experiments look for all pairs of precursor ions and product ions that differ by a constant neutral loss. Hydroxytyrosol and tyrosol derivatives were expressed as hydroxytyrosol, while homovanillic acid metabolite was expressed as homovanillic acid. All calculations of concentration and regression parameters were performed using Analyst 1.4 software.

2.7. Validation assay

Commercial LDL (Sigma-Aldrich, Steinheim, Germany) with known concentrations of hydroxytyrosol and homovanil-

Table 1
Transitions, DP[a] and CE[b] in MRM and double MRM experiments

Analyte	Transitions	DP (V)	CE (V)
Hydroxytyrosol	153 → 123	−40	−20
Homovanillic acid	181 → 137	−25	−11
Taxifolin	303 → 281	−30	−30
Hydroxytyrosol monoglucuronide	329 → 153, 153 → 123	−25, −30	−20, −15
Hydroxytyrosol monosulfate	233 → 153, 153 → 123	−25, −30	−20, −15
Tyrosol glucuronide	313 → 137, 137 → 93	−10, −10	−25, −25
Tyrosol sulphate	217 → 137, 137 → 93	−10, −10	−25, −25
Homovanillic acid sulfate	261 → 181, 181 → 137	−25, −30	−20, −15

[a] Declustering potential.
[b] Collision energy.

lic acid standards was used to carry out method validation. The samples were spiked with five different concentrations: 1, 20, 100, 300 and 500 ng/mL of hydroxytyrosol and 15, 20, 100, 300 and 500 ng/mL of homovanillic acid for linearity assay. Three different concentrations per compound were used to evaluate the rest of the validation assay: 20, 100 and 500 ng/mL. Validation was carried out under USP, for linearity, sensitivity and recovery; [39], Horwitz, for precision [40] and Kiser and Dolan [41] criteria for accuracy.

2.7.1. Linearity

Linearity of standard curves was expressed in terms of the correlation coefficient, plotting the HPLC-MS/MS peak area of hydroxytyrosol or homovanillic acid against the concentration of the same standard (ng/mL). Standard curves of hydroxytyrosol and homovanillic acid were made with a matrix (human LDL) containing the increasing concentrations of these compounds.

2.7.2. Precision and accuracy

Precision and accuracy assay has been carried out with hydroxytyrosol and homovanillic acid standards. The intra-day precision of the procedure was determined by analysing three solutions at low (20 ng/mL) ($n = 10$), middle (100 ng/mL) ($n = 10$) and high (500 ng/mL) ($n = 10$) concentrations. The inter-day precision was determined by analyzing 10 samples of these standards at each level prepared on 3 different days. Precision was calculated as relative standard deviation (RSD) of the analyte peak areas obtained from the replicates. Accuracy is expressed as the relative percentage error defined as (assayed concentration − nominal concentration)/(nominal concentration) × 100.

2.7.3. Sensitivity

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated by measuring the analytical background response, running six blanks using the maximum sensitivity allowed by the system. LOD was considered to be three times the standard deviation of the six blank samples analyzed while LOQ was considered to be 10 times the standard deviation of the six blank samples analyzed.

2.7.4. Recovery

To assess the recovery of the proposed method, LDLs were spiked with different amounts of hydroxytyrosol and homovanillic acid. The samples were subjected to the complete procedure described herein. Areas generated for these standards after pass for the complete described process were compared with areas generated from diluted standards at expected final concentrations.

2.8. Application of the method to healthy volunteers

To verify the method, the process was also carried out with blood samples that from a pool of five healthy female volunteers who were not on supplemented diets. They consumed 50 g of virgin olive oil after a 12 h fast. The olive oil administered was produced with the Picual variety of *Olea europea* L. fruit due to its high level of phenolic compounds [1]

3. Results and discussion

3.1. Sample treatment

Various preliminary experiments were carried out to achieve effective recovery, minimize costs and reactive expenditure, create an easier and faster methodology, and to obtain a final sample clean enough and suitable to introduce the sample into the MS system. Each experiment was made in duplicate at a minimum and all were monitored by the HPLC-DAD and/or HPLC-DAD-MS/MS system.

As a first step, sample acidification was taken into account not only to disrupt possible phenol–protein binding, but also to enhance recovery [31].

Although taxifolin was not used for quantification, was chosen as qualitative internal standard because it does not coelute with the analytes studied, for its similarity in chemical properties with the studied compounds.

Cleanliness of the sample remained a critical point. A sample preparation was still required to remove proteins and non-volatile endogenous substances from biological samples. The presence of such interferences can overload the HPLC system, contaminate the MS source, and lead to suppression/enhancement of the MS signal.

Trying to clean samples, however, can sometimes endanger the recovery of the analytes of interest, and cause overcoating should the analytes have similar affinities with the clean solvents employed. The optimal amount of water and methanol:water (5:95) sufficient to wash the sample without eluting phenolic compounds was carefully evaluated due to the high polarity present in this type of molecule. Two different methodologies to clean the sample were tested: (1) washing with acidulated water (formic acid 5%) and a methanol solution (5%), and (2) washing only with acidulated water (formic acid to 2 and 5%). A third proof was made without cleaning. Results showed that optimum recovery occurred when the cleaning process was omitted, followed by the sample being cleaned only with acidulated water. When samples were run in the HPLC-MS/MS system, however, we observed a very high matrix effect, resulting in a worsening of sensitivity. Considering that cleaning the sample is a critical and indispensable step, washing with acidulated water and methanol solution (5%) was chosen on the basis that this was the optimum solution for reducing the degree of interference.

In addition, 0.22 μm filters were tested, rather than the 0.45 μm filter used previously. The use of durapore centrifugal filters (Millipore) proved the best choice based on the best final recovery and the most practical option for managing the filtering step.

Elution profile proofs were also carried out to determine the quantity necessary for eluting the analytes of interest. Samples were eluted with 3, 2, 1 and 0.5 mL of methanol. We subsequently determined that 1 mL was sufficient to run the phenolic compounds. We again carried out proofs in triplicate using 1 and 2 mL, corroborating that only 1 mL can be used to elute the ana-

lytes. In addition, better results were obtained when elution was carried out in three steps. Then, to elute the compounds in the final process with 1 mL of methanol divided in three volumes was decided.

In basis that polarity is an essential factor for elution in SPE and HPLC system, and the elution order in HPLC system is: hydroxytyrosol (standard) as first compound, then, tyrosol glucuronide, hydroxytyrosol monoglucuronide, tyrosol sulfate, hydroxytyrosol monosulfate, homovanillic acid (standard), taxifolin (I.S., with a retention time of 4.85 min and recovery mean of 89.95%), and finally homovanillic acid sulfate, it can be said with some evidence that in the SPE and the chromatographic process all studied compounds are satisfactory covered by the polar "range" in which eluted process is carried out.

Final reconstitution solvents were prooved as follows: acidulated water only; water:methanol (90:10); water:methanol: acetonitrile (90:5:5); water:acetonitrile (90:10), and water: methanol (60:40). The best results and optimal chromatogram appearance were obtained when water:acetonitrole (90:10) was used.

3.2. HPLC-MS/MS optimization and identification

Adapting the elution gradient is another necessary task when a new column is used. These proofs were processed in HPLC-MS/MS with samples used to verify the relevant method.

The gradient markedly influences the running of a chromatographic separation and an analytical detection of studied compounds. We studied the influence of the different mobile phase gradients and flow rates on the chromatographic separation and detection of metabolites. Proofs were carried out until we achieved the optimum values for the peak heights and symmetries over a short time span. To shorten phenolic compound analysis time, we selected a flow rate of 0.6 mL/min, with an overall analysis time of about 7 min. This characteristic can prove advantageous in studying a great number of samples in a short time. The selected flow rate was a compromise between the speed of analyses and the most effective values for the peak heights and symmetries. The separation efficiency was still very good, and we obtained nearly baseline separation for all metabolites.

The coupling of HPLC with MS is a powerful tool for identifying natural products and metabolites. Optimization of the method was achieved by selecting the best ionization mode and mass spectrometer parameters. Infusion experiments were performed in order to study MS and MS/MS behaviour of hydroxytyrosol and homovanillic acid. The MRM method was chosen as it exhibited the highest selectivity and sensitivity in HPLC-MS/MS [42]. Standards were satisfactorily identified when the transitions described in Section 2 were monitored. Fig. 1 shows a representative chromatogram of spiked LDL containing standards at 250 ng/mL concentration.

In addition, MS techniques as full scan, CID-MS/MS and PIS experiments are more sensitive and they offer an excellent tool when no available standards are possible since MS modes verify structural information of the compounds. This method has been successfully applied in the monitoring of virgin olive oil phenolic metabolites in LDL samples 60 min after consumption. The metabolites found included hydroxytyrosol monoglucuronide, hydroxytyrosol monosulfate, tyrosol glucuronide, tyrosol sulfate, and homovanillic acid sulfate (Table 2 and Fig. 2).

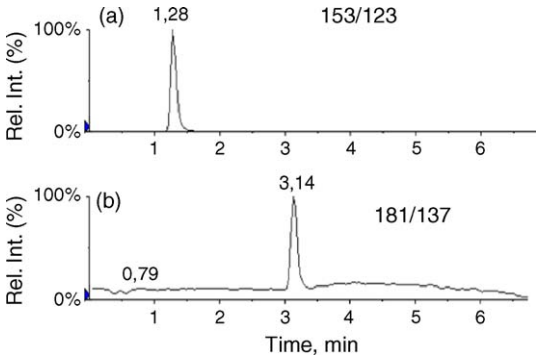


Fig. 1. (a) Hydroxytyrosol (250 ng/mL) and (b) homovanillic acid (250 ng/mL) standards in LDL.

Hydroxytyrosol monosulfate was confirmed by double MRM transition (233 → 153 and 153 → 123), related to the loss of the sulfate group and the hydroxytyrosol rupture. The ion fragment at *m/z* 123 is due to the loss of the CH_2OH group. This compound was confirmed by PIS mode. The ion fragment was present at 233 and at 153 as well.

Tyrosol sulfate was confirmed by MS/MS analysis as well. Preliminary examination, increasing DP in CID-MS/MS revealed the presence of tyrosol, and its presence was confirmed with PIS experiments in which it was present the ion fragment at 93 due to the loss of the CH_2–CH_2OH group of the tyrosol molecule.

Homovanillic acid sulfate were confirmed by PIS mode. The ion fragment present at 217 is possibly due to the loss of the COOH group loss, while glucoronides were confirmed by neutral loss scan of 176 mass units.

3.3. Quantification

Although it is preferable to use the same analyte standard to properly quantify, this is not always possible. Thus, we

Table 2
Metabolites found in LDL 60 min post-consumption of 50 mL extra virgin olive oil

Metabolite	Q1/Q3[a]	t_R[b] (min)	LDL concentration (ng/mg ApoB)
Hydroxytyrosol monoglucuronide	329/153	1.59	2.11
Hydroxytyrosol monosulfate	233/153	3.03	24.27
Tyrosol glucuronide	313/137	1.32	1.6
Tyrosol sulfate	217/137	2.91	14.87
Homovanillic acid sulfate	261/181	5.77	27.16

[a] Monitored transition for each metabolite analysis (Q1/Q3) in quantification process.
[b] Retention time.

Table 3
Precision and accuracy of the method

Compound	Intra-day precision RSD (%) ($n = 10$)			Inter-day precision RSD (%) ($n = 30$)			Accuracy (%)		
	20 ng/mL	100 ng/mL	500 ng/mL	20 ng/mL	100 ng/mL	500 ng/mL	20 ng/mL	100 ng/mL	500 ng/mL
Hydroxytyrosol	3.72	1.49	1.78	5.75	5.48	3.89	98.0	99.5	100.0
Homovanillic acid	7.51	4.31	2.46	9.87	4.75	4.59	88.7	98.1	99.2

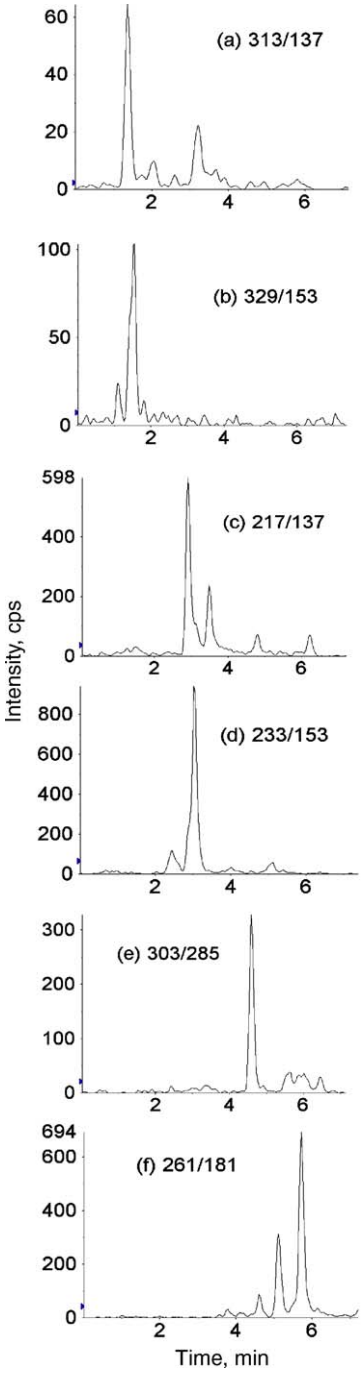


Fig. 2. Metabolites found in LDL 60 min post-consumption of 50 mL extra virgin olive oil. (a) Tyrosol glucuronide, (b) hydroxytyrosol monoglucuronide, (c) tyrosol sulfate, (d) hydroxytyrosol monosulfate, (e) taxifolin (I.S.) and (f) homovanillic acid sulfate.

Table 4
Sensitivity and recovery of the method

Compound	LOD[a] (ng/mL)	LOQ[b] (ng/mL)	Recovery (%)
Hydroxytyrosol	0.24	0.81	81.9
Homovanillic acid	3.05	10.18	101.3

[a] LOD, limit of detection.
[b] LOQ, limit of quantification.

attempted to quantify with standards exhibiting the greatest possible similarity to the analytes of interest in the basis of response will be alike because the similarity in chemical properties of each compound with the standard chosen for its quantification.

Tyrosol and hydroxytyrosol metabolites were quantified with a hydroxytyrosol standard, while the homovanillic metabolite was quantified with a homovanillic acid standard.

3.4. Validation assay

3.4.1. Linearity, precision and accuracy

The system was linear in all cases: $r > 0.99$. Intra-day precision expressed as RSD% were less than 4 and 8% for hydroxytyrosol and homovanillic acid values, respectively, while the inter-day results were less than 6 and 10%, respectively. Table 3 shows the method validation accuracy and precision results for both compounds. The residuals analysis for this range of concentration was [mean (SD)]: 99.9 (7.3) and 98.7 (7.1) for hydroxytyrosol and homovanillic acid, respectively.

3.4.2. Sensitivity and recovery

The system shows acceptable LOD and LOQ to encompass the quantification of metabolites. The estimated recoveries using the proposed method are within acceptable levels. Results are shown in Table 4. The recoveries for the metabolites are unknown due to the lack of metabolite reference material.

4. Conclusions

A rapid method for detection and quantification of metabolites of olive oil phenolic compounds (glucuronide metabolites of hydroxytyrosol and tyrosol and sulphate metabolites of hydroxytyrosol, tyrosol and homovanillic acid) in LDL by SPE and HPLC/ESI-MS/MS has been developed. Validation proves have demonstrated that the simultaneous quantification method using HPLC/ESI-MS/MS is specific, sensitive, and accurate. The lack of metabolites standards increases the risk in systematic inaccuracies. However, we have studied the metabolites in real samples in the basis of response will be alike because the similar-

ity in chemical properties of each compound with the standard chosen for its quantification, and this method can be used for determine these components and to evaluate their bioavailability and metabolism, in light of their roles antioxidant agents in LDL and other potential biological activities. Such a rapid assay would potentially allow for daily analysis of LDL samples without compromising quality or validation criteria. The proposed chromatographic procedure length is 7 min without any loss in efficiency of separation. This method is effective and can be used in further epidemiological studies or for investigations involving a great number of samples.

Acknowledgments

The authors thank the "Ministerio de Ciencia y Tecnología" (Spain) (project "SAF2004-08173-C03-03"), the European Union ("QLKT-CT-2001-002872") for their financial support, the University of Barcelona for the grant received by K.T.-C, Marta Poch for technical support, and Robin Rycroft for revising the English usage in the manuscript.

References

[1] K. De La Torre-Carbot, O. Jauregui, E. Gimeno, A.I. Castellote, R.M. Lamuela-Raventos, M.C. Lopez-Sabater, J. Agric. Food Chem. 53 (2005) 4331.
[2] C. Soler-Rivas, J.C. Espin, H.J. Wichers, J. Sci. Food Agric. 80 (2000) 1013.
[3] C. Manna, P. Galletti, G. Maisto, V. Cucciolla, S. D'Angelo, V. Zappia, FEBS Lett. 470 (2000) 341.
[4] M.N. Vissers, P.L. Zock, A.J.C. Roodenburg, R. Leenen, M.B. Katan, J. Nutr. 132 (2002) 409.
[5] K.L. Tuck, M.P. Freeman, P.J. Hayball, G.L. Stretch, I. Stupans, J. Nutr. 131 (2001) 1993.
[6] P. Del Boccio, A. Di Deo, A. De Curtis, N. Celli, L. Iacoviello, D. Rotilio, J. Chromatogr. B 785 (2003) 47.
[7] S. D'Angelo, C. Manna, V. Migliardi, O. Mazzoni, P. Morrica, G. Capasso, G. Pontoni, P. Galletti, V. Zappia, Drug Metab. Dispos. 29 (2001) 1492.
[8] F. Visioli, C. Galli, F. Bornet, A. Mattei, R. Patelli, G. Galli, D. Caruso, FEBS Lett. 468 (2000) 159.
[9] A. Bonanome, A. Pagnan, D. Caruso, A. Toia, A. Xamin, E. Fedeli, B. Berra, A. Zamburlini, F. Ursini, G. Galli, Nutr. Metab. Cardiovas. Dis. 10 (2000) 111.
[10] T. Weinbrenner, M. Fito, A.M. Farre, G.T. Saez, P. Rijken, C. Tormos, S. Coolen, R. De La Torre, M.I. Covas, Drug Exp. Clin. Res. 30 (2004) 207.
[11] J. Marrugat, M.I. Covas, M. Fito, H. Schroder, E. Miro-Casas, E. Gimeno, M.C. Lopez-Sabater, R. De La Torre, M. Farre, Eur. J. Clin. Nutr. 43 (2004) 140.
[12] E. Miro-Casas, M.F. Albaladejo, M.I. Covas, J.O. Rodriguez, E.M. Colomer, R.M.L. Raventos, R. De La Torre, Anal. Biochem. 294 (2001) 63.
[13] E. Miro-Casas, M.I. Covas, M. Fito, M. Farre-Albadalejo, J. Marrugat, R. De La Torre, Eur. J. Clin. Nutr. 57 (2003) 186.
[14] D. Caruso, F. Visioli, R. Patelli, C. Galli, G. Galli, Metab.-Clin. Exp. 50 (2001) 1426.
[15] E. Gimeno, M. Fito, R.M. Lamuela-Raventos, A.I. Castellote, M. Covas, M. Farre, M.C. de La Torre-Boronat, M.C. Lopez-Sabater, Eur. J. Clin. Nutr. 56 (2002) 114.
[16] M. Fito, M. Cladellasc, R. De La Torre, J. Marti, M. Alcantara, M. Pujadas-Bastardes, J. Marrugat, J. Bruguera, M.C. Lopez-Sabater, J. Vila, M.I. Covas, Atherosclerosis 181 (2005) 149.
[17] M.I. Covas, M. Fito, R.M. Lamuela-Raventos, N. Sebastia, C. De la Torre-Boronat, J. Marrugat, J. Clin. Pharmacol. Res. 20 (2000) 49.
[18] M.-I. Covas, K. de la Torre, M. Farré-Albaladejo, J. Kaikkonnen, M. Fitó, C. López-Sabater, M.A. Pujadas-Bastardes, J. Oglar, T. Weinbrenner, R.M. Lamuela-Raventós, R. de la Torre, Free Radic. Biol. Med. 40 (2006) 608.
[19] K.L. Tuck, P.J. Hayball, J. Nutr. Biochem. 13 (2002) 636.
[20] M. Fito, M.I. Covas, R.M. Lamuela-Raventos, J. Vila, J. Torrents, C. de la Torre, J. Marrugat, Lipids 35 (2000) 633.
[21] M. Fito, M.I. Covas, R.M. Lamuela-Raventos, J. Vila, C. de la Torre, J. Marrugat, Med. Clin. 115 (2000) 166.
[22] R. Leenen, A.J.C. Roodenburg, M.N. Vissers, J.A.E. Schuurbiers, K.P.A.M. van Putte, S.A. Wiseman, F.H.M.M. van de Put, J. Agric. Food Chem. 50 (2002) 1290.
[23] E. Coni, R. Di Benedetto, M. Di Pasquale, R. Masella, D. Modesti, R. Mattei, E.A. Carlini, Lipids 35 (2000) 45.
[24] F. Visioli, D. Caruso, E. Plasmati, R. Patelli, N. Mulinacci, A. Romani, G. Galli, C. Galli, Free Rad. Res. 34 (2001) 301.
[25] J.J. Ochoa, J.L. Quiles, M.C. Ramirez-Tortosa, J. Mataix, J.R. Huertas, Nutrition 18 (2002) 60.
[26] S.A. Wiseman, L.B.M. Tijburg, F.H.M.M. van de Put, Lipids 37 (2002) 1053.
[27] J. Ruano, J. Lopez-Miranda, F. Fuentes, J.A. Moreno, C. Bellido, P. Perez-Martinez, A. Lozano, P. Gomez, Y. Jimenez, F. Perez Jimenez, J. Am. Coll. Cardiol. 46 (2005) 1864.
[28] J.C. Espin, C. Soler-Rivas, E. Cantos, F.A. Tomas-Barberan, H.J. Wichers, J. Agric. Food Chem. 49 (2001) 1187.
[29] F. Visioli, C. Galli, Crit. Rev. Food Sci. Nutr. 42 (2002) 209.
[30] A. Tsarbopoulos, E. Gikas, N. Papadopoulos, N. Aligiannis, A. Kafatos, J. Chromatogr. B 785 (2003) 157.
[31] V. Ruiz-Gutierrez, M.E. Juan, A. Cert, J.M. Planas, Anal. Chem. 72 (2000) 4458.
[32] H.W. Tan, K.L. Tuck, I. Stupans, P.J. Hayball, J. Chromatogr. B 785 (2003) 187.
[33] M. Casas, M.F. Albadalejo, M.I. Covas, M. Fito, R. Lamuela-Raventos, R. de la Torre-Fornell, Clin. Chem. 47 (2001) 341.
[34] C. Bai, X.J. Yan, M. Takenaka, K. Sekiya, T. Nagata, J. Agric. Food Chem. 46 (1998) 3998.
[35] E. Miro-Casas, M.I. Covas, M. Farre, M. Fito, J. Ortuno, T. Weinbrenner, P. Roset, R. De La Torre, Clin. Chem. 49 (2003) 945.
[36] K.L. Tuck, P.J. Hayball, I. Stupans, J. Agric. Food Chem. 50 (2002) 2404.
[37] M. Urpi-Sarda, O. Jauregui, R.M. Lamuela-Raventos, W. Jaeger, M. Miksits, M.I. Covas, M. Antolovich, C. Andres-Lacueva, Anal. Chem. 77 (2005) 3149.
[38] R.J. Havel, H.A. Eder, J.H. Bragdan, Clin. Invest. 34 (2005) 1345.
[39] The United States Pharmacopeia (USPXXIII), Easton, 1989.
[40] W. Horwitz, J. Assoc. Off. Anal. Chem. 65 (1982) 525.
[41] M.M. Kiser, J.W. Dolan, LC–GC Eur. 17 (2006) 138.
[42] D. Liu, Y. Xia, R. Barkhtiar, Rapid Commun. Mass Spectrom. 16 (2002) 1330.

Publicación 5

"Changes of olive oil phenolic metabolites in human low density lipoprotein fraction after a long-term consumption of two different types of olive oil". K. de la Torre-Carbot, J.L. Chávez-Servín, O. Jaúregui, A.I. Castellote, R.M. Lamuela-Raventós, M. Fitó, M.I. Covas, D. Muñoz-Aguallo and M.C. López-Sabater. ***Atherosclerosis***. Enviada.

Resumen

Evidencia científica reciente muestra que las propiedades relacionadas con la salud del aceite de oliva virgen no es debida exclusivamente al contenido de AGMI, sino también al contenido de compuestos fenólicos.

Si los compuestos fenólicos que pueden unirse a la LDL ejercen su acción antioxidante, esta funcionalidad queda determinada por la disposición y cinética de los compuestos fenólicos en esta partícula después del consumo del aceite de oliva.

El objetivo del presente estudio fue evaluar el efecto del aceite de oliva virgen y aceite de oliva refinado con similar concentración pero con diferencia en la concentración de compuestos fenólicos en los cambios en la concentraciones de los compuestos fenólicos de la LDL después de una sostenida administración de 25 mL de aceite de oliva durante 3 semanas, ambos periodos seguidos de un periodo de restricción de otras fuentes ricas en antioxidantes.

Para este fin, fue diseñado un ensayo controlado, aleatorizado y de tipo cruzado en el que participaron 40 voluntarios varones sanos, reclutados en 6 centros de 5 países europeos.

Fueron encontrados en este estudio hidroxitirosol, tirosol y ácido homovainíllico sulfato, aunque se observa que los metabolitos encontrados tienen una tendencia a aumentar después del consumo de ambos tipos de aceite de oliva, en el caso del aceite de oliva virgen el incremento es mayor y

significativo en el caso ácido homovainíllico sulfato, hidroxitirosol sulfato y la suma de los tres fenoles.

Cuando el porcentaje de cambio es estudiado, hay una diferencia significativa comparando estos valores antes y después del consumo del aceite de oliva refinado con los valores del aceite de oliva virgen en el caso de los tres metabolitos encontrados.

El contendio de estos metabolitos en la LDL ha mostrado ser modulado por el contenido de compuestos fenólicos en el aceite administrado después de un consumo sostenido de tres semanas. Este incremento sugiere que estos compuestos pueden actuar como antioxidantes y que ellos pueden tener una acción importante en esta partícula.

Changes of olive oil phenolic metabolites in human low density lipoprotein fraction after a long-term consumption of two different types of olive oil.

Karina de la Torre-Carbot[1], Jorge L. Chávez-Servín[1], Olga Jaúregui[2], Ana I. Castellote[1], Rosa M. Lamuela-Raventós[1], Montserrat Fitó[3], María-Isabel Covas[3], Daniel Muñoz-Aguallo[3], *M.Carmen López-Sabater[1].

[1]Department of Nutrition and Food Science, Reference Center in Food Technology, Faculty of Pharmacy, University of Barcelona. Avda. Joan XXIII s/n, 08028 Barcelona, Spain.

[2] Scientific and Technical Services, University of Barcelona, Josep Samitier 1-5 08028, Barcelona, Spain

[3] Lipids and Cardiovascular Epidemiology Unit, Institut Municipal d' Investigació Mèdica (IMIM), Barcelona, Spain.

Corresponding autor

M. Carmen López Sabater

Departament de Nutrició i Bromatologia, Facultat de Farmàcia, Universitat de Barcelona, Av. Joan XXIII s/n, E-08028 Barcelona (Spain)

Telephone number: +34-93 402 45 12 Fax number: +34-93 403 59 31

e-mail: mclopez@ub.edu

Abstract

Recent evidence shows that the healthful properties of olive oil are not exclusively due for the oleic acid content. Olive oil contains a range of micronutrients, such as phenolic compounds, and some beneficial effects are attributed to them. If phenolic compounds that can bind LDL (Low density lipoprotein) exert their antioxidant action, this functionality is determined by the disposition and kinetic of phenolic compounds in this particle after the consumption of olive oil. The aim of the present study was to evaluate the effect of virgin olive oil and refined olive oil with differences in their phenolic compounds concentration on changes in olive oil phenolic metabolites concentrations in LDL after a sustained daily dose of olive oil. A controlled, cross-over, randomized trial was designed using two similar olive oils with different phenolic compound concentrations. Forty healthy males recruited in 6 Centers of 5 European Countries participated in the study, in which virgin olive oil and refined olive oil were sequentially administered over two periods of 3 weeks. Hydroxytyrosol, Tyrosol and Homovanillic acid sulfates were found. These metabolites increased after virgin olive oil consumption. Then, olive oil phenolic contents seems to modulate the LDL content of these metabolites and their metabolic activities could be determined for the capacity of these compounds to keep joined to LDL.

Keywords: Low density lipoprotein, olive oil, hydroxytyrosol, tyrosol homovanillic acid, phenolic compounds metabolites.

1.Introducction

The traditional dietary habits of the Mediterranean area have been consistently associated with lower incidence of cardiovascular disease [1] and it has been demonstrated a significant inverse correlation between phenolic compounds consumption and cardiovascular risk factors [2-6]. Olive oil is the predominant (often exclusive) and most typical source of fat of the Mediterranean diet. The healthful properties of olive oil have been often attributed to its high mono-unsatured fatty acid content, but recent evidence show that it is therefore unlikely that oleic acid is exclusively accountable for these properties of olive oil, In addition to oleic acid, olive oil contains a range of micronutrients, such as phenolic compounds, and many of their beneficial effects are attributed to them [4, 7-12]

The concentration of phenolic compounds in olive oil may range between 50 and 1000 mg/kg and hydroxytyrosol and tyrosol are the most abundant phenolic alcohols in the olive oil in simple form or as conjugates [13, 14].

Absorption and metabolism of some olive oil phenolic compounds have been documented in animal and human models [2, 15-26]. Besides to this, olive oil phenolic compounds have a wide metabolic activity and have shown to possess different functions related to cardiovascular protection for the antioxidant capacity *in vitro* [4, 10, 12, 27-31] and *in vivo* [2, 11, 32-37]. This antioxidand capacity of phenolic compounds are carried out for the free radical scavenging properties [4, 10, 12, 29]; or and related to hydrogen-donation and their ability to improve radical stability [4]; means the strong metal-chelation capacity [4]; for the NO liberation [38, 39]; decreasing free radical generation and liberation [10, 29]; stimulating antioxidant transcription and detoxification defence systems [11, 32, 40] and modulating other enzymatic systems

related with oxidation process (ciclooxygenases, lipooxigenases and NAD(P)H oxidase [10] .

Also they inhibit pro-inflammatory molecules [41, 42]; inhibit platelet aggregation [4, 38, 39, 41, 43]; they have a vasorelaxant effect [7, 38, 44] Inhibit the induced aggregation and adhesion molecules [9, 12, 45] and inhibit apoptosis of human cultured endothelial cells [46].

Oxidized LDL is currently thought to be more damaging to the arterial wall than native LDL cholesterol , because of the toxicity of the oxidised particle that is a pro-oxidant agent that cause tissue injury an it is a hall marker for atherosclerosis and cardiovascular heart disease development [6, 47].

Then, if phenolic compounds that can bind LDL exert their antioxidant action, this functionality it is determined by the availability and kinetic of phenolic compounds in this particle after the consumption of olive oil.

Papers related with this topic examine the impact in total concentration of phenolic compounds in LDL after olive oil consumption [2, 28, 48], without focus the attention to the phenolic composition that came from olive oil, or some phenols are determined after acid hydrolysis treatment [49], without consider the specific metabolites of these phenols. To our knowledge, it is the first time that specific metabolites of phenolic compounds of olive oil are studied in LDL after a long-term consumption.

The aim of the present study was to evaluate the effect of both virgin olive oil and refined olive oil with similar composition but with differences in their phenolic compounds concentration on changes in olive oil phenolic metabolites concentrations in LDL after three weeks of consumption of each olive oil in healthy human volunteers. A randomized, crossover, clinical and controlled intervention trial study was designed.

2. Materials and methods

2.1Olive oil characteristics

Used olive oils were specially prepared for the study. Both olive oils were obtained from the same cultivar (one of them submitted to refined process) and soil to find an olive oil with similar quantities of fatty acids and similar micronutrient profile, but with different concentrations of phenolic compounds: phenol-free for the refined and 445μg/mL (caffeic acid equivalents) for the virgin olive oil. An adjustment of vitamin E to similar values of that present in virgin olive oil was performed.

2.2 Participants.

The study population consisted of 40 healthy non-smoker males, 20 to 60 years of age, recruited in 6 Centers of 5 European Countries (Copenhagen, Denmark; Kuopio, Finland; Postdam, Germany; Berlin, Germany; Bolona, Italy and Barcelona, Spain). Exclusion criteria were: smoking, intake of antioxidant supplements, aspirin, or any other drug with established anti-oxidative properties, hyperlipidemia, obesity (body mass index > 30 kg/m2), diabetes, hypertension, celiac or other intestinal disease, any condition limiting mobility, life-threatening diseases, or any other disease or condition that would impair compliance. The subject pool at randomization consisted of 36 subjects. The protocol was fully explained to the participants before they gave their written informed consent and the local institutional ethics committees approved this protocol.

2.3 Study design

A controlled, cross-over, randomized trial was designed using two similar olive oils with different phenolic compound concentrations. A Latin square for the two treatments was used in the trial to randomize participants into the orders of olive oil administration. Virgin olive oil and refined olive oil were sequentially administered over two periods of 3 weeks preceded by two-week washout periods in which participants were requested to avoid olive oil consumption. During olive oil intervention periods, participants were requested to ingest a raw daily dose of 25 mL (22g) of olive oil distributed over three meals in replacement of other raw fats. Daily doses of 25 mL olive oil were blindly prepared in special containers with the two types of olive oil labelled with a code number. Containers with the corresponding 25 mL of raw olive oil were delivered daily to the participants. The participants were instructed to return the containers every morning when collecting the next daily dose for the amount of unconsumed olive oil to be registered. Participants were requested to avoid a high intake of foods listed as containing antioxidants (vegetables, legumes, fruits, tea, coffee, chocolate, wine, and beer). A nutritionist also personally advised participants to replace all types of habitually consumed raw fats with the olive oils. Laboratory determinations were carried out in samples obtained in fasting state drawn by venipuncture, before and at the end of the virgin olive oil and refined olive oil administration.

2.4. Laboratory assays

2.4.1. Used olive oil

Fatty acid composition of olive oil was measured by conventional gas chromatography (EEC/2568/91). Values of α-tocopherol were measured by HPLC

(high performance liquid chromatography) as previously described [50]. Total phenolic content of olive oils was measured by the Folin Ciocalteau method. Peroxide index (mEq O_2/kg) and Free acidity (% oleic acid), were determined following the analytical methods described in CEE/2568/91 of the European Commission.

2.4.2. LDL isolation

For the isolation of LDL [19], briefly after centrifugation, plasma, with 1 mL of isotonic saline containing EDTA and NaCl , was layered carefully on top of the plasma in a centrifuge tube. The tubes were centrifuged in a Beckman-Coulter XL-70, using the Fixed-Angle Type 50.4 rotor at 199,808 x*g* for 18h at 4°C. The infranatant (3 mL) was put in a centrifuge tube containing KBr, stained with Coomassie Brilliant Blue R solution and vortexed. Two mL of isotonic saline, containing KBr was layered carefully on the top of the infranatant up to the base of the tube vertex. The tubes were sealed before being introduced in the NVT100 rotor and centrifuged at 697,760 x*g* for 5h at 4°C. The LDL layer was aspirated and kept frozen at -80ºC. LDL cholesterol and triglicerides were determined by standard enzymatic methods. Apolipoprotein B content was determined by immunoturbidimetry. [19]

2.4.3. Phenolic metabolites determination

For determination of phenolic compounds [51], briefly was added 100 µL of protocatecol solution (200 ng/mL) as internal standard to acidified LDL. An Oasis HLB (60 mg) cartridge from Waters (Mildford, MA, USA) was activated with methanol and formic acid (5% in water) and acidified LDL was then percolated into the cartridge. The sample was washed with water and 5% aqueous methanol. Phenolic compounds were

eluted with methanol. The eluent was evaporated to dryness under nitrogen. The sample was dissolved with 150 μL water:acetonitrile (90:10). Samples were filtered and transposed into an amber vial. Subsequently, 20 μl was injected into the HPLC-MS/MS. The entire process was performed under conditions of darkness using brown glass material. A 3-μm particle size C_{18} Luna column, 5 cm x 2.0 mm I.D. with a C_{18}, 4 mm guard cartridge (Phenomenex, UK) was used. The mobile phase consisted of a binary solvent system using water acidified with 0.1% formic acid (solvent A) and 100% acetonitrile (solvent B), kept at a flow rate of 0.6 mL/min. LDL (20 μL) samples were injected at a constant flow rate of 0.6 mL/min. All the analyses used the turbo ion-spray source in negative mode. Quantification was performed using MRM. Hydroxytyrosol and tyrosol derivatives were expressed as hydroxytyrosol, while homovanillic acid metabolite was expressed as homovanillic acid. All calculations of concentration and regression parameters were performed using Analyst 1.4 software and all chemicals and organic solvents used were of analytical grade.

2.5. Statistical Analysis.

Statistical evaluation was performed with Student´s t-test for paired data to determine differences. Statistical significance was defined as a *P* value less than 0.050 for a 2-side test. Version 12 SPSS statistical software was used.

3. Results and Discussions.

Used olive oils, came from the same cultivar and harvest, with the objective of use two olive oils with similar composition, but with different phenolic compounds concentration. For this propose, one of them was submitted to refined process. An adjustment of vitamin E to similar values of that present in virgin olive oil was

performed because during refined process, olive oil loose the original antioxidants. Mono-unsatured percentage was 80.4 and 81.82%, satured fatty acid percentage was 14.47 and 13.99, and poli-unsatured percentage was 5.22 and 3.93% for refined and virgin olive oil respectively, α-tocopherol 229 μg/mL for both olive oils. The free acidity was of 0.02 and 0.18 % of oleic acid and peroxide values of 4.0 and 13.3 mEq O_2/kg in refined and virgin olive oil respectively. Finally, for phenolic compounds concentration values were phenol-free for the refined and 445μg/mL for the virgin olive oil.

It was requested to the participants to avoid all possible sources of phenolic compounds with the objective to avoid possible interferences whit phenolic compounds from other sources. Since the aim of the present study was to evaluate the effect of both olive oils in the phenolic metabolites in LDL after a long period of intervention, blood samples were obtained before and after three weeks for each intervention. The 2-week of washout period before both interventions were decided to reach the equilibrium and homogenization in the LDL profile.

Some phenolic metabolites previously found in LDL after virgin olive oil consumption were studied [19]. Since glucoronid compounds were not found in the current study, then three phenolic metabolites were quantified: hydroxytyrosol, tyrosol and homovanillic acid in sulfated form before and after one week of each kind of olive oil consumption. Glucoronid compounds were not found in the current study probably because samples in fast conditions were obtained. Glucoronid compounds were found exclusively after 60 minutes of olive oil consumption in the previous work.

In figure 1 it can be appreciated that these metabolites have a tendency to increase after the consumption of both olive oils. However, in the case of virgin olive oil consumption, the increase is higher. While there is not significant difference in the

case of before and after refined olive oil values, there is a significant difference between before and after virgin olive oil consumption in the case of homovanillic acid sulfate, hydroxytyrosol monosulfate and the sum of the three phenols. See results in table 1. Apolipoprotein B, cholesterol and triglicerids values are present also in this table.

The significant differences observed in the case of homovanillic acid sulfate, hydroxytyrosol monosulfate and the sum of the tree phenols, show how the phenolic composition can be determined by diet and phenols consumed. In this case it is related with the kind of olive oil administered, differentiated by concentration of phenolic compounds.

In the case of tyrosol there are not significant differences, although it is observed a tendency to increase. However, when results are divided by groups, the group that correspond to Postdam, Germany, (n=7), present a significant difference in the case of comparison of pre- refined olive oil and post- refined olive oil consumption ($p<0.05$). These values correspond to 5.23 and 2.34 ng/mg apo B respectively. Then, there is a clear reduction of tyrosol values after refined olive oil consumption with significant difference. This significant decrease after a consumption of refined olive oil may be caused by the strict phenolic compounds-low diet.

Also, when percentage of change are studied, there is a significant differences comparing the percentage of change before and after refined olive oil consumption and percentage of change before and after virgin olive oil consumption in the case of the three phenolic compounds. This percentage of change were for homovanillic acid 6.10 and 23.39% for hydroxytyrosol 19.47 and 58.05%, for tyrosol 5.25 and 19.94 for refined and virgin olive oil respectively. See figure 2.

It is important to know the concentrations of these phenolic compounds in LDL and their changes in postprandial states and after consumption of specific products

because they could exert their antioxidant capacity in this particle. It has been demonstrated the toxicity of Oxidized LDL [46, 52]. Oxidized LDL is a pro-oxidant agent that cause tissue injury and it has been demonstrated that it can be a determinant factor for cardiovascular disease development [47].

Phenolic compounds that can bind LDL could exert their antioxidant action *in vivo* in the arterial intima, in fact, the olive oil phenolic content modulated the oxidative/antioxidative status. Plasma antioxidant capacity has been evaluated in relation with olive oil consumption and it is well known an improvement in antioxidant capacity [2, 6-8, 11, 22, 32, 36, 37, 53, 54]

It has been demonstrated also the capacity of virgin olive oil phenolic compounds to protect other phenolic compounds previously bound to LDL. These results provide further evidence that phenolic compounds bound to LDL are likely to protect LDL from oxidation and that phenolic content of olive oil protect the LDL phenolic content from degradation [2, 28, 48].

In fact, others health benefits as an improve endothelium-dependent microvasuclar vasodilatation have been adjudicated to the antioxidant capacity [7].

Since olive oil phenolic compounds undergo extremely extensive first-pass intestinal/hepatic metabolism in the body, and it would appear that their biological activity is more likely to be linked to the biological metabolites of the phenolic compounds rather than to the primary species present in olive oil, it is necessary further investigation with this specific metabolites.

In this study, the concentrations of hydroxytyrosol, tyrosol and homovanillic acid found differ in concentrations found in a previous study (34.22, 17.23 and 34.22 ng/mg apo B respectively), however, the values found in the present work are in fast station after a long term of olive oil consumption, while in the past study, this values

correspond to 60 minutes after olive oil consumption. These results reflect that most phenolic compounds are eliminated rapidly. Then in studies after some minutes of virgin olive oil consumption it can be appreciated a marked increase of these metabolites. However, one prerequisite to asses the *in vivo* physiological significance of phenolic compounds is to determine and to keep watch their presence and kinetic evolution, liberation, union and availability in human LDL following both, acute and long-term ingestion of virgin olive oil.

In this paper, it is demonstrated that dietary phenolic compounds can have a specific kinetic profile which is dose-dependent of the phenolic content of olive oil administered. The increase of these phenols after virgin olive oil consumption claims that these compounds act as *in vivo* antioxidants and that they could have an important action in LDL. Although refined olive oil present a tendency to increase the values of hydroxytyrosol, tyrosol and homovanillic acid sulphate, maybe because the mediums rich in mono-unsatured fatty acids are less susceptible to oxidation and this can help to preserve phenolic compounds in LDL, virgin olive oil increase in a significant manner these metabolites. This reinforce that, although both olive oils grant benefits for health, this fact is reinforced in the case of virgin olive oil consumption.

Phenolic compounds which are able to bind LDL are good candidates for the effective prevention of lipid peroxidation and atherosclerotic processes and in a future, the presence and concentrations in phenolic compounds could be potential hall markers for atherosclerosis and cardiovascular heart disease development if retained their antioxidant properties *in vivo,* and, apart from the fatty acid composition, exogenous phenolic compound could be used to prevent the disease.

4. Conclusions:

Olive oil phenolic content seems to modulate the LDL content of three phenolic metabolites: hydroxytyrosol, tyrosol and homovanillic acid sulphate after a long term of consumption. These compounds increased in a dose dependent manner with the phenolic content of the olive oil consumed, and their metabolic activities could be determined for the capacity of these compounds to keep joined to LDL. In this study, phenolic compounds had a kinetic profile with a dose-dependent on the phenolic content of the olive oil administered during a term of three weeks. Further investigations to keep watching the kinetic of these phenolic compounds in LDL are needed.

Acknowledgment

The authors would like to thank the "Ministerio de Ciencia y Tecnología" in Spain (project "SAF2004-08173-C03-03"), the European Union ("QLKT-CT-2001-002872") and the grant support Spain Minister of Health (CIBER: CB06/02/0079) for their financial support; the University of Barcelona for the grant received by K. de la T-C; and Robin Rycroft for revising the English.

Figure legends

Figure 1. Values of homovanillic acid sulfate, tyrosol sulphate, hydroxytyrosol sulfate and total of phenolic metabolites determined before and after three weeks of refined and virgin olive oil consumption.

Figure 2. Percentage of changes in phenolic compounds metabolites between post- and pre-refined olive oil consumption.

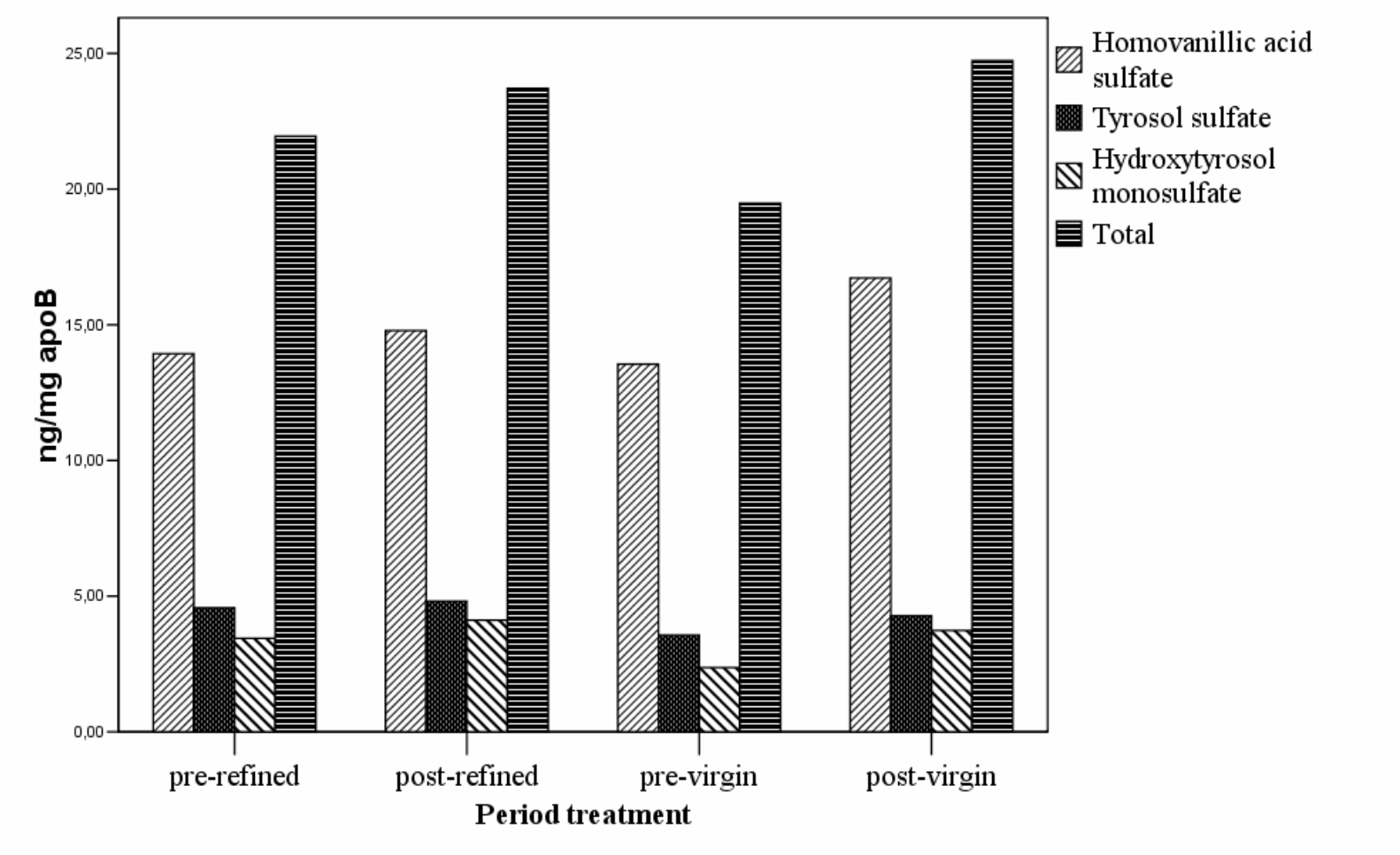
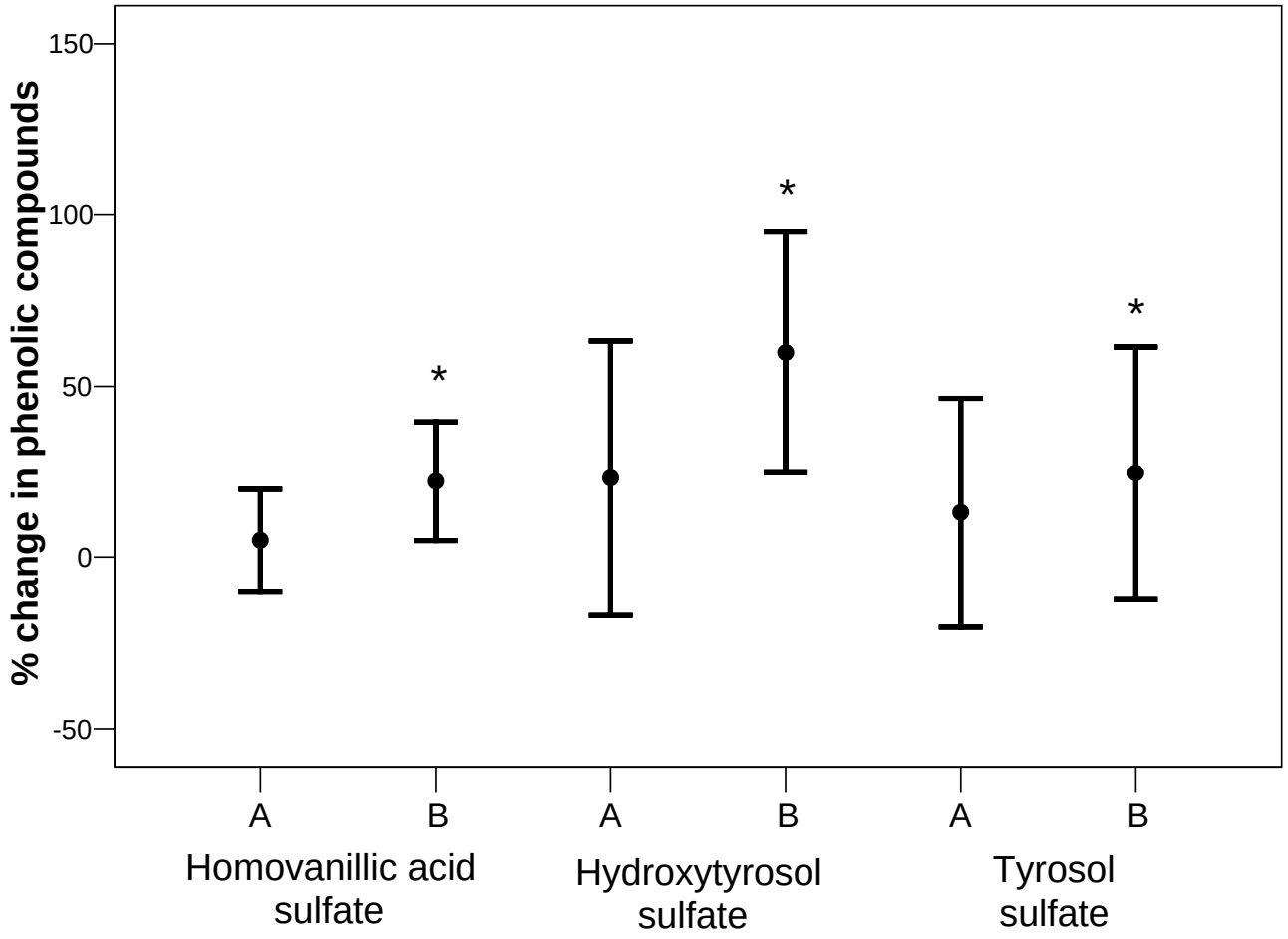

25,00
20,00
15,00
10,00
5,00
0,00
ng/mg apoB
pre-refined
post-refined
pre-virgin
post-virgin
Period treatment
Homovanillic acid sulfate
Tyrosol sulfate
Hydroxytyrosol monosulfate
Total

Figure 1



Changes betwen post- and pre-refined olive oil consumption (A)
Changes between post- and pre-virgin olive oil consumption (B)
*Denotes significand diference ($p<0.05$) between A and B of each metabolite

Figure 2

Reference List

[1] F.Visioli, P.Bogani, S.Grande, C.Galli, J Physiol Pharmacol 56 Suppl 1 (2005) 37.

[2] María-Isabel Covas, Karina de la Torre, Magí Farré-Albaladejo, Jari Kaikkonnen, Montserrat Fitó, Carmen López-Sabater, María A.Pujadas-Bastardes, J.Oglar, Tanja Weinbrenner, Rosa M.Lamuela-Raventós, Rafael de la Torre, Free Radical Biology and Medicine In Press (2005).

[3] P.M.Kris-Etherton, K.D.Hecker, A.Bonanome, S.M.Coval, A.E.Binkoski, K.F.Hilpert, A.E.Griel, T.D.Etherton, Am J Med 113 Suppl 9B (2002) 71S.

[4] F.Visioli, A.Poli, C.Gall, Med Res. Rev. 22 (2002) 65.

[5] J.C.Stoclet, T.Chataigneau, M.Ndiaye, M.H.Oak, B.J.El, M.Chataigneau, V.B.Schini-Kerth, Eur J Pharmacol 500 (2004) 299.

[6] M.I.Covas, K.Nyyssonen, H.E.Poulsen, J.Kaikkonen, H.J.Zunft, H.Kiesewetter, A.Gaddi, T.R.de la, J.Mursu, H.Baumler, S.Nascetti, J.T.Salonen, M.Fito, J.Virtanen, J.Marrugat, EUROLIVE Study Group, Ann. Intern. Med 145 (2006) 333.

[7] J.Ruano, J.Lopez-Miranda, F.Fuentes, J.A.Moreno, C.Bellido, P.Perez-Martinez, A.Lozano, P.Gomez, Y.Jimenez, F.Perez Jimenez, Journal of the American College of Cardiology 46 (2005) 1864.

[8] F.Visioli, D.Caruso, S.Grande, R.Bosisio, M.Villa, G.Galli, C.Sirtori, C.Galli, Eur. J. Nutr. 44 (2005) 121.

[9] M.A.Carluccio, L.Siculella, M.A.Ancora, M.Massaro, E.Scoditti, C.Storelli, F.Visioli, A.Distante, C.R.De, Arterioscler Thromb Vasc Biol 23 (2003) 622.

[10] J.A.Moreno, J.Lopez-Miranda, P.Gomez, F.Benkhalti, E.S.el Boustani, F.Perez-Jimenez, Med. Clin. (Barc.) 120 (2003) 128.

[11] M.Fito, M.Cladellasc, R.De La Torre, J.Marti, M.Alcantara, M.Pujadas-Bastardes, J.Marrugat, J.Bruguera, M.C.Lopez-Sabater, J.Vila, M.I.Covas, Atherosclerosis 181 (2005) 149.

[12] R.Turner, N.Etienne, M.G.Alonso, S.de Pascual-Teresa, A.M.Minihane, P.D.Weinberg, G.Rimbach, International Journal for Vitamin and Nutrition Research 75 (2005) 61.

[13] K.De La Torre-Carbot, O.Jauregui, E.Gimeno, A.I.Castellote, R.M.Lamuela-Raventos, M.C.Lopez-Sabater, Journal of Agricultural and Food Chemistry 53 (2005) 4331.

[14] M.Servili, R.Selvaggini, S.Esposto, A.Taticchi, G.Montedoro, G.Morozzi, J Chromatogr A 1054 (2004) 113.

[15] K.L.Tuck, P.J.Hayball, I.Stupans, Journal of Agricultural and Food Chemistry 50 (2002) 2404.

[16] S.D'Angelo, C.Manna, V.Migliardi, O.Mazzoni, P.Morrica, G.Capasso, G.Pontoni, P.Galletti, V.Zappia, Drug Metabolism and Disposition 29 (2001) 1492.

[17] P.Del Boccio, A.Di Deo, A.De Curtis, N.Celli, L.Iacoviello, D.Rotilio, Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences 785 (2003) 47.

[18] C.Bai, X.J.Yan, M.Takenaka, K.Sekiya, T.Nagata, Journal of Agricultural and Food Chemistry 46 (1998) 3998.

[19] K.de la Torre-Carbot, J.L.Chávez-Servín, O.Jaúregui, A.I.Castellote, R.M.Lamuela Raventós, M.Fitó, M.I.Covas, D.Muñoz-Aguallo, M.C.López-Sabater, Anal. Chim. Acta In press (2006).

[20] F.Visioli, C.Galli, F.Bornet, A.Mattei, R.Patelli, G.Galli, D.Caruso, Febs Letters 468 (2000) 159.

[21] M.N.Vissers, P.L.Zock, A.J.C.Roodenburg, R.Leenen, M.B.Katan, J. Nutr. 132 (2002) 409.

[22] T.Weinbrenner, M.Fito, A.M.Farre, G.T.Saez, P.Rijken, C.Tormos, S.Coolen, R.De La Torre, M.I.Covas, Drugs Under Experimental and Clinical Research 30 (2004) 207.

[23] E.Miro-Casas, M.I.Covas, M.Fito, M.Farre-Albadalejo, J.Marrugat, R.De La Torre, Eur. J. Clin. Nutr. 57 (2003) 186.

[24] E.Miro-Casas, M.I.Covas, M.Farre, M.Fito, J.Ortuno, T.Weinbrenner, P.Roset, R.De La Torre, Clinical Chemistry 49 (2003) 945.

[25] D.Caruso, F.Visioli, R.Patelli, C.Galli, G.Galli, Metabolism-Clinical and Experimental 50 (2001) 1426.

[26] C.Manna, P.Galletti, G.Maisto, V.Cucciolla, S.D'Angelo, V.Zappia, Febs Letters 470 (2000) 341.

[27] R.Leenen, A.J.C.Roodenburg, M.N.Vissers, J.A.E.Schuurbiers, K.P.A.M.van Putte, S.A.Wiseman, F.H.M.M.van de Put, Journal of Agricultural and Food Chemistry 50 (2002) 1290.

[28] M.I.Covas, M.Fito, R.M.Lamuela-Raventos, N.Sebastia, C.De la Torre-Boronat, J.Marrugat, International Journal of Clinical Pharmacology Research 20 (2000) 49.

[29] I.Stupans, A.Kirlich, K.L.Tuck, P.J.Hayball, Journal of Agricultural and Food Chemistry 50 (2002) 2464.

[30] F.Franconi, R.Coinu, S.Carta, P.P.Urgeghe, F.Ieri, N.Mulinacci, A.Romani, J Agric Food Chem 54 (2006) 3121.

[31] R.Masella, R.Vari, M.D'Archivio, R.Di Benedetto, P.Matarrese, W.Malorni, B.Scazzocchio, C.Giovannini, J. Nutr. 134 (2004) 785.

[32] T.Weinbrenner, M.Fito, R.De La Torre, G.T.Saez, P.Rijken, C.Tormos, S.Coolen, M.F.Albaladejo, S.Abanades, H.Schroder, J.Marrugat, M.I.Covas, J. Nutr. 134 (2004) 2314.

[33] R.Masella, C.Giovannini, R.Vari, R.Di Benedetto, E.Coni, R.Volpe, N.Fraone, A.Bucci, Lipids 36 (2001) 1195.

[34] M.Fito, E.Gimeno, M.I.Covas, E.Miro, M.D.Lopez-Sabater, M.Farre, R.De La Torre, J.Marrugat, Lipids 37 (2002) 245.

[35] J.J.Ochoa, J.L.Quiles, M.C.Ramirez-Tortosa, J.Mataix, J.R.Huertas, Nutrition 18 (2002) 60.

[36] J.Marrugat, M.I.Covas, M.Fito, H.Schroder, E.Miro-Casas, E.Gimeno, M.C.Lopez-Sabater, R.De La Torre, M.Farre, European Journal of Nutrition 43 (2004) 140.

[37] M.C.Ramirez-Tortosa, G.Urbano, M.Lopez-Jurado, T.Nestares, M.C.Gomez, A.Mir, E.Ros, J.Mataix, A.Gil, J Nutr 129 (1999) 2177.

[38] C.A.Palmerini, E.Carlini, C.Saccardi, M.Servili, G.Montedoro, G.Arienti, Mol Cell Biochem 280 (2005) 181.

[39] F.Visioli, C.Galli, Journal of Agricultural and Food Chemistry 46 (1998) 4292.

[40] R.Masella, R.Di Benedetto, Var&#x00ec, R., C.Filesi, C.Giovannini, The Journal of Nutritional Biochemistry 16 (2005) 577.

[41] J.J.Moreno, M.T.Mitjavila, The Journal of Nutritional Biochemistry 14 (2003) 182.

[42] E.A.Miles, P.Zoubouli, P.C.Calder, Nutrition 21 (2005) 389.

[43] G.I.Togna, A.R.Togna, M.Franconi, C.Marra, M.Guiso, J. Nutr. 133 (2003) 2532.

[44] F.Benkhalti, A.Legssyer, P.Gomez, E.Paz, J.Lopez-Miranda, F.Perez-Jimenez, E.S.el Boustani, Therapie 58 (2003) 133.

[45] M.Dell'Agli, R.Fagnani, N.Mitro, S.Scurati, M.Masciadri, L.Mussoni, G.V.Galli, E.Bosisio, M.Crestani, F.E.De, E.Tremoli, D.Caruso, J Agric Food Chem 54 (2006) 3259.

[46] O.Vieira, I.Escargueil-Blanc, O.Meilhac, J.Pierre Basile, J.Laranjinha, L.Almeida, R.Salvayre, A.Nègre-Salvayre, British Journal of Pharmacy 123 (1998) 565.

[47] J.L.Witztum, The Lancet 344 (1994) 793.

[48] E.Gimeno, M.Fito, R.M.Lamuela-Raventos, A.I.Castellote, M.Covas, M.Farre, M.C.Torre-Boronat, M.C.Lopez-Sabater, Eur. J. Clin. Nutr. 56 (2002) 114.

[49] A.Bonanome, A.Pagnan, D.Caruso, A.Toia, A.Xamin, E.Fedeli, B.Berra, A.Zamburlini, F.Ursini, G.Galli, Nutrition Metabolism and Cardiovascular Diseases 10 (2000) 111.

[50] E.Gimeno, A.I.Castellote, R.M.Lamuela-Raventos, M.C.de la Torre, M.C.Lopez-Sabater, J Chromatogr. A 881 (2000) 251.

[51] K.de la Torre-Carbot, O.Jauregui, A.I.Castellote, R.M.Lamuela-Raventos, M.I.Covas, I.Casals, M.C.Lopez-Sabater, J. Chromatogr. A 1116 (2006) 69.

[52] C.Giovannini, E.Straface, D.Modesti, E.Coni, A.Cantafora, M.De Vincenzi, W.Malorni, R.Masella, J. Nutr. 129 (1999) 1269.

[53] J.Marrugat, M.I.Covas, M.Fito, E.Miro-Casas, H.Schroeder, M.Farre, R.De La Torre, C.D.Boronat, European Heart Journal 23 (2002) 17.

[54] J.Moschandreas, M.N.Vissers, S.Wiseman, K.P.van Putte, A.Kafatos, Eur. J. Clin. Nutr. 56 (2002) 1024.

Publicación 6

"Changes in LDL fatty acid composition as a response to olive oil treatment are inversely related with the lipid oxidative damage: *the EUROLIVE Study*" S Nascetti, AFG Cicero, MC López-Sabater, R Elosua, JT. Salonen , K Nyyssönen, HE. Poulsen, HJF Zunft, H Kiesewetter, K de la Torre, MI Covas, J Kaikkonen, J Mursu, C Koenbick, H Bäumler, AV Gaddi, for the EUROLIVE Study Group. ***Nutrition.*** Enviada.

Resumen

El consumo del aceite de oliva está relacionado con la reducción del riesgo cardiovascular, por parte debido al alto contenido de AGMI. Han sido propuestos diferentes mecanismos por medio de los cuales el aceite de oliva previene el riesgo cardiovascuar y algunos de ellos se han centrado en la composición de la LDL.

El objetivo de este estudio fue evaluar los cambios en la composición de ácidos grasos de la LDL después de un consumo sostenido de aceite de oliva sostenido y moderado (25 mL/día) y su relación con el daño oxidativo.

El estudio fue realizado dentro de un ensayo aleatorizado y de tipo cruzado con tres aceite de oliva similares pero con diferente concentración de compuestos fenólicos. En este estudio participaron 200 voluntarios sanos de 6 diferentes centros Europeos. El periodo de intervención fue de tres semanas, separadas cada una por períodos de blanqueo. El contenido de ácidos grasos fue medido en las muestras al inicio y después del último período de intervención.

Después del consumo de aceite de oliva, las concentraciones de ácido graso oleico incrementaron en un 1.9%. El radio AGMI/AGPI y el radio oleico/linoleico también incrementaron significativamente.

Del mismo modo, se observó una relación inversa entre el radio de oleico/linoleico y los biomarcadores del estrés oxidativo y esto se debe a que el ácido graso oleico es menos susceptible a la oxidación.

El consumo del aceite de oliva en dosis habituales, modula la composición de ácidos grasos en LDL, mejora el perfil lipídico y reduce el daño oxidativo en los lípidos y la LDL.

Changes in LDL fatty acid composition as a response to olive oil treatment are inversely related with the lipid oxidative damage: *the EUROLIVE Study*

S Nascetti*, AFG Cicero*, MC López-Sabater[†], R Elosua[‡], JT. Salonen[¥], K Nyyssönen[‖], HE. Poulsen[§], HJF Zunft[¶], H Kiesewetter[#], K de la Torre[†], MI Covas[‡], J Kaikkonen[¥], J Mursu[‖¥], C Koenbick[¶], H Bäumler[#], AV Gaddi* for the EUROLIVE Study Group.

***Centro per lo Studio dell'Arteriosclerosi e delle Malattie Dismetaboliche "GC Descovich", Dipartimento di Medicina Clinica e Biotecnologia Applicata, Policlinico S. Orsola-Malpighi, Bologna, Italy: [†]Department of Nutrition and Bromatology, Barcelona University, Spain. [‡]Lipids and Cardiovascular Epidemiology Unit and the Pharmacology Research Unit, Municipal Institute for Medical Research (IMIM), Barcelona, Spain; [¥] Oy Jurilab, Kuopio, Finland; [‖] Research Institute of Public Health, University of Kuopio, Finland; [§]Department of Clinical Pharmacology, Rigshospitalet, University Hospital Copenhagen, Denmark; ¶ German Institute of Human Nutrition, Postdam-Rehbruecke, Germany; #Charité - Universitätsmedizin Berlin, Germany.**

Running title: Fatty acid profile of LDL and olive oil consumption

Financial support

This work was supported by Grant QLK1-CT-2001-00287 from EU Commission.

Corresponding author:
Simona Nascetti, MD, PhD
"GC Descovich" Atherosclerosis and Metabolic disease Research Unit
"D. Campanacci" Clinical medicine and Applied biotechnology Dept.
Via Massarenti, 9
40138 Bologna – Italy
Tel. ++39 0516363938 – Fax: ++39 051390646
E-mail: snats@med.unibo.it

Abstract

Background: Olive oil is claimed to be able to reduce cardiovascular disease risk. Objective: The aim of our study was to assess the changes in the fatty acid composition of low density lipoproteins (LDL) after sustained consumption of olive oil at real-life doses (25 mL/day) and their relationship with the lipid oxidative damage. Design: A multi-center randomized, cross-over, clinical trial with 3similar type of olive oils, but with differences in the phenolic content, was conducted on 200 European subjects. Intervention periods were of 3 weeks separated by 2-week washout periods. The LDL fatty acid content was measured in samples drawn at baseline and after the last intervention period. Results: After olive oil ingestion oleic acid concentration in LDL increased (1.9%) and those of linoleic (1.1%) and arachidonic acid (0.5%) decreased. Monounsaturated/polyunsaturated fatty acid and oleic/linoleic acid ratios in LDL increased after olive oil consumption. An inverse relationship between the oleic/linoleic acid ratio and biomarkers of oxidative stress was observed. One unit increase in the oleic/linoleic acid ratio was associated with a decrease of 15.9 U/L in plasma oxidized LDL and 4.2 μg/L in plasma isoprostanes. Conclusion: Consumption of olive oil at real-life doses improved the fatty acid profile in LDL, the changes being associated to a reduction of the oxidative damage to lipids and LDL.

Keywords: composition, fatty acids, LDL, nutrition, olive oil, poliphenols, oxidative markers.

Introduction

The Mediterranean diet is considered to be a protective factor in the primary and secondary prevention of coronary heart disease [1, 2]**,** and against oxidative stress associated processes [3, 4]. This protection has been related to the relatively high content in this diet of non hydrogenated, monounsaturated fatty acids (MUFA) [5, 6], since olive oil is the main source of fats in the Mediterranean diet [1]. The predominant fatty acid in olive oil is in fact the MUFA oleic acid (18:1, n-9) with percentages ranging from 56% to 84%, while the polyunsaturated fatty acid (PUFA) linoleic acid (18:2, n-6) is usually found at percentages between 3% and 21% [7]. However, olive oil also contains several minor components with potentially healthy biological properties [8]. Several mechanisms have been proposed to explain the preventive effects of olive oil on atherosclerosis development. Among them **a** reduction of the low-density lipoprotein (LDL) susceptibility to oxidation, and its consequences on cellular oxidative stress, thrombogenicity, and atheroma plaque formation [9, 10] **,** has been one of the main mechanisms addressed.

In a previous study we observed an increase in plasma fatty acids in the EUROLIVE population after olive oil consumption (29). However, contradictory data have been reported about the effects of olive oil (and its main components) on lipoprotein metabolism: some authors report that olive oil supplementation did not modify LDL fatty acid composition [11, 12]. In contrast, an increase in the oleic acid incorporation to LDL after olive oil consumption has been reported in humans [13,14] and in animal studies [15-17]. In some of these studies (13) a decrease in the LDL oxidability was observed together with the increase in oleic acid in LDL. Data from these studies are difficult to interpret because, in the most part of them, olive oil was ingested as a dietary supplement, nor in replacement of other fats (18-20) To the best of our knowledge, there

are no-studies with a large sample size in which the effect of olive oil ingestion on fatty acid LDL composition has been examined. In this context, the aim of the present study, based on data from the Eurolive Study (5^{th} EU-Framework Program) [21], is to assess the effect of olive oil on the LDL fatty acids content and its relationship with plasma oxidation markers in a large sample of healthy European male adults.

Materials and methods

Study design and Study population

The EUROLIVE study [21] was a randomized, crossover trial with three intervention periods of three weeks and two wash-out periods of 2 weeks. Three types of olive oils with high (HPC, 366 mg/Kg), medium (MPC, 164 mg/Kg)**,** and low (LPC, 2.7 mg/Kg) phenolic content were used. Olive oils were specially prepared for the trial from an extra virgin olive oil (produced from Picual olives, Spain). They had similar fat and micronutrient (i.e. vitamin E, triterpenes, sitosterols) composition, but with differences in their phenolic content (Table 1) [14]. Fatty acid composition was determined by gas chromatography [22].

We enrolled 200 healthy European males (mean age: 33.1±10.6 years) recruited from September 2002 through June 2003 in 6 Centers of 5 European Countries (Denmark, Finland, Germany, Italy, and Spain). Eligibility criteria were a willingness to provide written, informed consent and to agree to adhere to the protocol. Exclusion criteria were: smoking, intake of antioxidant supplements, aspirin, or drug**s** with established antioxidant properties, hyperlipidemia, obesity (body mass index >30 kg/m^2), diabetes, hypertension, celiac or other intestinal disease, any condition limiting mobility, life-threatening diseases, or any other disease or condition that could impair compliance. Subjects were considered healthy on the basis of physical examination and routine

biochemical and hematological laboratory determinations. The protocol was approved by the Ethics Committee of each Clinical Trial Center involved.

Olive oils were sequentially administered over three periods of 3 weeks preceded by two-week wash-out periods in which participants were requested to avoid olive oil and olives consumption. In intervention periods, subjects were provided with 25 mL/day of olive oil, administered among meals. Participants were requested to avoid a high intake of foods listed as containing antioxidants. Participants recorded their habitual diet on diet records during three consecutive days at baseline and the end of the study period. Participants were personally advised by a nutritionist on how to record food consumption and follow the above mentioned dietary recommendations. Food consumption was converted into the corresponding nutrient intake by means of a validated nutrition software from each country. Physical activity was recorded at baseline and at the end of the study (Taylor HL, Jacobs DR, Schucker B, Knudsen J, Leon AS, Debacker G. A questionnaire for the assessment of leisure time physical activities. J Chronic Dis. 1978; 31:741-55)The full protocol has been previously and fully described elsewhere [21].

For the present study we used data of the baseline and the endpoint (last intervention). Serum glucose, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and triacylglycerols (TG) were determined by standardized enzymatic methods. LDL cholesterol was calculated by the Friedewald formula. Plasma circulating oxidized LDL (oxLDL) was measured by enzimo-immunoassay. Plasma total $F_{2\alpha}$-isoprostanes were determined using high performance liquid chromatography and stable isotope dilution mass spectrometry. Serum LDL uninduced conjugated dienes (CD) were measured by spectrophotometry at 234 nm and 300 nm. CD concentration was adjusted for the cholesterol concentration in LDL. LDL isolation was performed by sequential flotation

ultracentrifugation from plasma EDTA samples. The fatty acid composition of the isolated LDL was determined following the method described by Bondía et al. [24] in which fatty acids are transformed into methyl esters and analyzed by gas chromatography. Apolipoprotein B in LDL was measured by immunoturbidimetry. Fatty acid were expressed as mg/g of LDL-apolipoprotein B100. All the same determinations were centralized in reference laboratories.

Statistical analyses

Baseline data are shown as mean±SD and 5% trimmed mean values. Kolmogorov Smirnov test and normal probability plots were used to assess normal distribution. One-factor ANOVA and Kruskal-Wallis test were used to determine differences in basal characteristics and nutrient intake among the three olive oil interventions. A Student´s t test for paired samples was used to compare LDL fatty acid composition at baseline and at the end of the intervention.

Multiple regression model**s** were fitted in order to evaluate the association between oxidative markers and fatty acids in LDL. These models used the oxidative markers postintervention values, adjusted by basal values, as dependent variable and the difference in the oleic/linoleic ratio in LDL as independent variable. For plasma isoprostanes model, arachidonic acid in LDL was also included as adjusting variable. Because there were no interactions with olive oil administration order, it was not included in the models)

Statistical significance was defined as $P<0.05$ for a two-sided test. All tests were performed using the SPSS System for Windows release 11.0.

Results

Eighteen participants (9%) did not completed the study. No one relevant side effect has been were registered during the study. At the beginning of the study, 193 (96.5%) participants submitted food records. Mean total energy intake was 2261 calories daily, with 48.6%, 33.8%, and 15.8% of calories derived from carbohydrate, fat, and protein, respectively. Table 2 shows the mean nutrient intake, at the beginning and at the end of the study. Mean total energy intake was unchanged. There was a significant increase in fat intake from the beginning to the end of the study (mean values, 86.4 g/day versus 95.1 g/day, $p<0.005$), mainly linked to the increase of MUFA (mean values, 30.9 g/day versus 39.8 g/day, $p<0.001$) as oleic acid (mean values, 27.5 g/day versus 34.8 g/day). Polyunsaturated (PUFA) and saturated fat (SFA) intake remained constant. Consumption of carbohydrates decreased (3.3% as average, $p<0.001$), while alcohol and protein intake did not change.

Plasma total and LDL cholesterol did not change. HDL cholesterol ($P<0.001$) and glucose ($p<0.05$) increased, whereas $F_{2\alpha}$-isoprostanes decreased ($p<0.01$). Lipid values and oxidative stress markers before and after interventions are shown in Table 3.

The fatty acid composition of LDL expressed as an absolute value in mg/g of LDL-apolipoprotein B100 and as a percentage of fatty acids is shown in Table 4. The Kolmogorov-Smirnov test demonstrates that the main LDL fatty acids have a baseline distribution that is not normal: the Q-Q plots between expected and observed values shows that the overlapping area between expected "normal" distribution and observed one is very high, but in two tails some values are markedly higher or lower than expected. In the hypothesis that these outliers represent subjects with peculiar characteristics, we also repeated comparative analyses with 5% trimmed means.

A significant enrichment of oleic acid (C18:1 n-9) in LDL was observed ($p<0.001$). MUFA/PUFA and oleic/linoleic acid ratios in LDL increased. When considering the LDL percent content of fatty acids, oleic acid increased ($p<0.001$), and linoleic and arachidonic acid decreased ($p<0.005$). No changes were observed in the percentage of palmitic acid and stearic acids. When paired comparisons in absolute values obtained at the end of the study were examined by the GLM, olive oil administration increased significantly oleic acid in LDL ($p<0.001$). When differences were also adjusted for energy intake and order of olive oil administration, the comparisons described above remained significant. The significance of the association was maintained when stratifying by center.

Oxidized LDL was negatively associated with the difference in the oleic/linoleic ratio ($r= 0.327$, $p<0.001$): for every increase of 1 mg/g in the oleic/linoleic ratio, oxidized LDL decreased by 1.9 U/L. Both covariates made independent contributions to explain the levels of oxidized LDL after olive oil consumption, the overall model explaining the 10.7% of the variation (Table 5).

Isoprostanes were inversely correlated with the difference in the oleic/linoleic ratio: for every increase of 1 mg/g in the oleic/linoleic ration, $F_{2\alpha}$-isoprostanes decreased by 4.2 μg/L. There was no relationship between adjusted isoprostanes and difference in arachidonic acid between interventions. $F_{2\alpha}$-isoprostanes, adjusted by baseline levels, were, however, directly related with levels of arachidonic acid after olive oil consumption ($B=0.024$, 95% CI 0.002-0.046; $p= 0.030$). The overall model, with difference in oleic/linoleic ratio age and arachidonic acid in LDL simultaneously as independent variables explains a 7.6% of the isoprostanes variation ($r= 0.276$, $p=0.004$); difference in oleic/linoleic ratio is the variable with the strongest relationship to isoprostanes (Table 5). Further adjustments by other covariates (as. energy intake,

change in fat intake from baseline, oil administration sequence) did not modify the trend. No relationship was observed with the other variables, included uninduced dienes.

Discussion

In this study, sustained consumption of olive oil, in replacement of other fats, at a real life dose of 25 mL per day, increased the oleic acid content of the LDL as well as the MUFA/PUFA and oleic/linoleic acid ratios in LDL. Changes in the LDL fatty acid composition were inversely related with the oxidative lipid and LDL damage. These results were independent of the type of the olive oil consumed.

Higher MUFA intake has been associated with a more favorable cardiovascular risk profile [25]. In our study, carbohydrate consumption decreased with the increase in olive oil consumption. Low carbohydrate/high fat diets typically increase HDL cholesterol levels versus high carbohydrate/low fat diets [26-28]. In agreement with this, and with our previous results [21, 29, 30], we observed an increase in serum HDL-cholesterol after olive oil ingestion. Benefits of olive oil consumption on the lipid profile were also reflected in the reduction of total/HDL cholesterol and LDL/HDL cholesterol ratios. The total/HDL cholesterol ratio is an established efficient indicator of lipid atherogenesis, reflecting the balance of cholesterol transport in and out of the arterial intima [31]. In a recent report with data from the 20-year follow up Framingham Offspring Study, total/HDL cholesterol and LDL/HDL cholesterol ratios have been reported to be the most efficient lipid parameters for predicting coronary artery disease (CHD) [32]. We observed a decrease in F_2 –isoprostanes from the beginning to the end of the study. In a previous work (21) we did not observe differences in this oxidative biomarker when comparing values before and after each one of the three olive oils

intervention period. Thus, perhaps a long-term consumption of olive oil is required to observe changes in F2-isoprostanes plasma concentrations.

Dietary fat can modulate the susceptibility of LDL to oxidative modification. Most of the studies comparing the effect of a MUFA-rich diet with that of a PUFA-rich one concluded that MUFA-rich diets reduced the susceptibility of LDL to oxidation [33]. Thus, oleate rich LDL appeared to be more resistant to oxidation than linoleate-enriched LDL. In the above mentioned studies [33], the susceptibility of LDL oxidation was measured through an *in vitro* test: the formation of conjugated dienes promoted by copper oxidation of the LDL. Here, we report a reduction of the *in vivo* LDL oxidative damage associated with an enrichment of oleic acid in LDL, as a consequence of a sustained olive oil consumption in a large sample size population. The oxidative modification of LDL plays a key role in atherosclerosis and CHD development. Oxidation of the lipids and lipoproteins present in LDL leads to a change in the lipoprotein conformation by which LDL is better able to enter the monocyte/macrophage system of the arterial wall, and promote the atherosclerotic process [35]. The change in the conformation of the LDL when oxidized is measured by the levels of *in vivo* circulating oxidized LDL. It is currently thought that oxidized LDL is more damaging to the arterial wall than native LDL [36]. In several studies, but not in all [37], elevated concentrations of circulating oxidized LDL show a positive relationship with the severity of acute coronary events [38, 39]; are independently associated with carotid intima-media thickness [40]**;** and are predictors for CHD both in CHD patients [41] and in the general population [42]. In our study we also observed an inverse relationship between the oleic/linoleic acid ratio in LDL and the plasma concentration of isoprostans. Plasma F2-isoprostanes are considered to be a systemic marker of oxidative stress [43] and high levels of circulating F2-isoprostanes have been shown to be predictors of cardiac events

in CHD patients [44]. **T**he increase in the oleic/linoleic acid ratio in LDL has been shown to promote~~s~~ favorable changes in inflammatory markers. Tsimikas et al [45] showed that an increase in the oleic/linoleic acid ratio in LDL induced less monocyte chemotaxis and adhesion when exposed to oxidative stress.

In summary, real-life daily doses of olive oil (25 mL/day) increased oleic acid and the oleic/linoleic acid ratio in LDLs and improved the cardiovascular risk lipid profile**.** This increase in the oleic/linoleic acid ratio was inversely related with the degree of lipid and LDL oxidation. Our study adds further evidence to recommend the use of olive oil as a source of fat in order to achieve benefits against classical and novel risk factors or cardiovascular disease.

Acknowledgements

The authors thank the EUROLIVE investigators whose complete list is reported in [21]. All authors have been involved in study design. Dr. Nascetti, Dr. Cicero, Prof. Gaddi and Dr. Covas analyzed data and written the paper. Prof. Gaddi coordinated the Bologna research unit, while Dr. Covas the whole study. Dr. Elosua analyzed physical activity questionnaires and provided a critical revision of the manuscript. Dr. Lopez-Sabater and Dr. de la Torre analyzed olive oil. Prof. Salonen, Dr. Nyyssonen, Dr. Kaikkonen and Dr. Mursu performed oxidative and antioxidative biomarkers. Prof. Zunft, Prof. Bäumler, Prof. Poulsen and Dr. Koenbich were involved in patients enrollment and management.

No one author has conflict of interest in the publication of this paper.

References

1. Perez-Jimenez F, Alvarez de Cienfuegos G, Badimon L, Barja G, Battino M, Blanco A, Bonanome A, Colomer R, Corella-Piquer D, Covas I, Chamorro-Quiros J, Escrich E, Gaforio JJ, Garcia Luna PP, Hidalgo L, Kafatos A, Kris-Etherton PM, Lairon D, Lamuela-Raventos R, Lopez-Miranda J, Lopez-Segura F, Martinez-Gonzalez MA, Mata P, Mataix J, Ordovas J, Osada J, Pacheco-Reyes R, Perucho M, Pineda-Priego M, Quiles JL, Ramirez-Tortosa MC, Ruiz-Gutierrez V, Sanchez-Rovira P, Solfrizzi V, Soriguer-Escofet F, de la Torre-Fornell R, Trichopoulos A, Villalba-Montoro JM, Villar-Ortiz JR, Visioli F. International conference on the healthy effect of virgin olive oil. Eur J Clin Invest. 2005;35(7):421-4.
2. Trichopoulou A, Vasilopoulou E. Mediterranean diet and longevity. Br J Nutr 2000;84 Suppl 2:S205-209.
3. Hooper L, Griffiths E, Abrahams B, et al on behalf of the UK Heart Health and Thoracic Dietitians Specialist Group of the British Dietetic Association. Dietetic guidelines: diet in secondary prevention of cardiovascular disease. J Hum Nutr Dietet 2004;17:337–349.
4. Kay CD, Kris-Etherton PM, West SG. Effects of antioxidant-rich foods on vascular reactivity: review of the clinical evidence. Curr Atheroscler Rep. 2006,8(6):510-22.
5. Perez-Jimenez F, Lopez-Miranda J, Mata P. Protective effect of dietary monounsaturated fat on arteriosclerosis: beyond cholesterol. Atherosclerosis 2002;163(2):385-398.
6. Moreno JJ, Mitjavila MT. The degree of unsaturation of dietary fatty acids and the development of atherosclerosis. J Nutr Biochem 2003;14(4):182-95.

7. Visioli F, Galli C. Antiatherogenic components of olive oil. Curr Atheroscler Rep 2001;3(1):64-67.
8. Visioli F, Galli C. Antiatherogenic components of olive oil. Curr Atheroscler Rep. 2001;3(1):64-7
9. Perona JS, Cabello-Moruno R, Ruiz-Gutierrez V. The role of virgin olive oil components in the modulation of endothelial function. J Nutr Biochem. 2006;17(7):429-45.
10. Visioli F, Bogani P, Grande S, Detopoulou V, Manios Y, Galli C. Local food and cardioprotection: the role of phytochemicals. Forum Nutr. 2006;59:116-29.
11. Mensink RP, Zock PL, Kester AD, Katan MB. Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: a meta-analysis of 60 controlled trials. Am J Clin Nutr 2003;77(5):1146-1155.
12. Stark AH, Madar Z. Olive oil as a functional food: epidemiology and nutritional approaches. Nutr Rev 2002;60(6):170-176.
13. Kratz M, Cullen P, Kannenberg F et al. Effects of dietary fatty acids on the composition and oxidizability of low-density lipoprotein. Eur J Clin Nutr 2002;56(1):72-81.
14. Gimeno E, Fito M, Lamuela-Raventos RM et al. Effect of ingestion of virgin olive oil on human low-density lipoprotein composition. Eur J Clin Nutr 2002;56(2):114-120.
15. Ochoa JJ, Quiles JL, Ramirez-Tortosa MC, Mataix J, Huertas JR. Dietary oils high in oleic acid but with different unsaponifiable fraction contents have different effects in fatty acid composition and peroxidation in rabbit LDL. Nutrition 2002;18(1):60-65.

16. Plantinga EA, Beynen AC. The influence of dietary fish oil vs. sunflower oil on the fatty acid composition of plasma cholesteryl-esters in healthy, adult cats. J Anim Physiol Anim Nutr 2003;87(11-12):373-379.
17. Ochoa JJ, Quiles JL, Ramirez-Tortosa MC, Mataix J, Huertas JR. Dietary oils high in oleic acid but with different unsaponifiable fraction contents have different effects in fatty acid composition and peroxidation in rabbit LDL. Nutrition 2002;18(1):60-65.
18. Kratz M, Gulbahce E, von Eckardstein A, et al. Dietary mono- and polyunsaturated fatty acids similarly affect LDL size in healthy men and women. J Nutr 2002;132(4):715-718.
19. Perona JS, Canizares J, Montero E, Sanchez-Dominguez JM, Ruiz-Gutierrez V. Plasma lipid modifications in elderly people after administration of two virgin olive oils of the same variety (Olea europaea var. hojiblanca) with different triacylglycerol composition. Br J Nutr 2003;89(6):819-826.
20. Carmena R, Ascaso JF, Camejo G, et al. Effect of olive and sunflower oils on low density lipoprotein level, composition, size, oxidation and interaction with arterial proteoglycans. Atherosclerosis 1996;125(2):243-55.
21. Covas MI, Nyyssönen K, Poulsen HE, et al. for the EUROLIVE Study Group. The effect of polyphenols in olive oil on heart disease risk factors: a randomized trial. Ann Intern Med 2006;145(5):333-341.
22. Gimeno E, Castellote AI, Lamuela-Raventós RM, de la Torre MC, López-Sabater MC. The effects of harverst and extraction methods on the antioxidant content (phenolics, α-tocopherol and β-carotene) in virgin olive oil. Food Chemistry 2002;78:207-211.

23. Fito M, Gimeno E, Covas MI, Miro E et al. Postprandial and short-term effects of dietary virgin olive oil on oxidant/antioxidant status. Lipids 2002;37(3):245-251.

24. Bondia EM, Castellote AI, Lopez MC, Rivero M. Determination of plasma fatty acid composition in neonates by gas chromatography. J Chromatogr B Biomed Appl 1994;658(2):369-74.

25. Kris-Etherton PM, Pearson TA, Wan Y, Hargrove RL, Moriarty K, Fishell V, et al. High-monounsaturated fatty acid diets lower both plasma cholesterol and triacylglycerol concentrations. Am J Clin Nutr 1999;70: 1009-15.

26. Connor WE, Connor SL. Should a low-fat, high carbohydrate diet be recommended for everyone? the case for a low-fat, high-carbohydrate diet. New Engl J Med 1997;337:562-567

27. Brinton EA, Eisenberg S, Breslow JL. A low-fat diet decreases high density lipoprotein (HDL) cholesterol level by decreasing HDL apolipoprotein transport rates. J Clin Invest 1990;85:144-151.

28. Katan MB, Grundy SM, Willet WC. Should a low-fat, high-carbohydrate diet be recommended for everyone? Beyond low-fat diets. New Engl J Med 1997; 337: 563-566.

29. Bondia-Pons I, Schroder H, Covas MI, et al. Moderate consumption of olive oil by healthy European men reduces systolic blood pressure in non-Mediterranean participants. J Nutr 2007;137(1):84-7.

30. Machowetz A, Poulsen HE, Gruendel S, et al. Effect of olive oils on biomarkers of oxidative DNA stress in Northern and Southern Europeans. FASEB J. 2007;21(1):45-52.

31. Kannel WB, Wilson PWF. Efficacy of lipid profiles in prediction of coronary disease. Am Heart J 1992;124:768-774.
32. Byung-Ho N, Kannel W, D´Agostino RB. Search for an optimal atherogenic lipid risk profile: from the Framingham study. Am J Cardiol 2006; 97: 372-375.
33. López-Miranda J, Badimon L, Bonanome A, Lairon D, Kris-Etherton PM, Mata P, Pérez-Jiménez F. Monounsaturated fat and cardiovascular risk. Nutr Rev 2006; 64 (S):S2-S12.
34. Esterbauer H, Striegl G, Puhl H, Rotheneder M. Continuous monitoring of *in vitro* oxidation of human low density lipoprotein. Free Rad Res Commun 1989;6:67-75.
35. Witztum JL. The oxidation hypothesis of atherosclerosis. Lancet. 1994;344:793-795.
36. Navab M, Berliner JA, Watson AD, Hama SY, Territo MC, Lusis AJ, et al. The Ying and Yang of oxidation in the development of the fatty streak. Arterioscler Thromb Vasc Biol. 1996;16:831-42.
37. Wu T, Willet WC, Rifai N, Shai I, Manson JAE, Rimm EB. Is plasma oxidized low-density lipoprotein, measured with the widely used antibody 4E6, an independent predictor of coronary heart disease, among U.S. men and women? J Am Coll Cardiol 2006; 48:973-9.
38. Holvoet P, Mertens A, Verhamme P, Bogaerts K, Beyens G, Verhaeghe R, et al. Circulating oxidized LDL is a useful marker for identifying patients with coronary artery disease. Arterioscler Thromb Vasc Biol 2001;21:844-8.
39. Weinbrenner T, Cladellas M, Covas MI, Fitó M, Tomás M, Sentí M, et al. High oxidative stress in patients with stable coronary heart disease. Atherosclerosis 2003; 168: 99-106.

40. Liu ML, Ylitalo K, Salonen R, Salonen JT, Taskinen MR. Circulating oxidized low-density lipoprotein and its association with carotid intima-media thickness in asymptomatic members of familial combined hyperlipidemia families. Arterioscler Thromb Vasc Biol 2004;24:1492-7.
41. Toshima S, Hasegawa A, Kurabayashi M, Itabe H, Takano T, Sugano J, et al. Circulating oxidized low density lipoprotein levels. A biochemical risk marker for coronary heart disease. Arterioscler Thromb Vasc Biol 2000; 20: 2243-7.
42. Meisinger C, Baumert J, Khuseyinova N, Loewel H, Koenig W. Plasma oxidized low-density lipoprotein, a strong predictor for acute coronary heart disease events un apparently healthy, middle-aged men from the general population. Circulation 2005; 112:651-7.
43. Halliwell B, Whiteman M. Measuring reactive species and oxidative damage *in vivo* and in cell culture: how should you do it and what do the results mean? Br J Pharmacol. 2004; 142:231-255.
44. Tsimikas S, Philis-Tsimikas A, Alexopoulos S, Sigari F, Lee C, Reaven PD. LDL isolated from Greek subjects on a typical diet or from American subjects on an oleate-supplemented diet induces less monocyte chemotaxis and adhesion when exposed to oxidative stress. Arterioscler Thromb Vasc Biol 1999;19(1):122-130.

Table 1. Characteristics of the olive oils administered.

	Type of olive oil:		
	LPC	MPC	HPC
Quality parameters			
Free acidity (% oleic acid)	0.03	0.08	0.18
Peroxide value (mEq O_2/kg)	4.12	5.89	11.28
Fatty acids (%)			
C14:0	0.01	0.01	0.01
C16:0	10.63	10.50	10.63
C16:1	0.88	0.86	0.88
C17:0	0.05	0.05	0.04
C17:1	0.09	0.09	0.09
C18:0	3.27	3.13	2.84
C18:1	79.08	79.80	80.60
C18:2	4.64	4.21	3.35
C20:0	0.39	0.39	0.35
C18:3	0.58	0.58	0.58
C20:1	0.26	0.25	0.25
C22:0	0.11	0.10	0.10
C24:0	0.01	0.02	0.02
α-Tocopherol (ppm)	229	228	228
Phenolic compounds (ppm)	2.7	164	366
Squalene (mg/g)	3.0	3.2	3.4
β-sitosterol (mg/g)	1.4	1.5	1.5

Table 2 – Dietary intake characteristics, as mean nutrient intake and differences, at the beginning and at the end of the study.

Component (g)		Mean	SD	Absolute change	P value
Carbohydrates	baseline	275,8	91,1		
	end	256,9	84,4	-18,8	<0,001
Protein	baseline	88,7	27,5		
	end	85,5	26,8	-3,1	0,137
Total fat	baseline	86,4	30,6		
	end	95,1	34,6	+8,6	0,002
SFA	baseline	33,7	15,4		
	end	32,6	14,6	-1,1	0,360
MUFA	baseline	30,9	12,6		
	end	39,8	16,1	+8,9	<0,001
PUFA	baseline	12,1	6,2		
	end	12,3	6,0	+0,2	0,630
Cholesterol (mg)	baseline	329,7	149,5		
	end	310,9	151,6	-13,5	0,243
Oleic acid	baseline	27,5	11,3		
	end	34,8	15,1	+7,3	<0,001
Alcohol	baseline	81,0	191,0		
	end	74,3	191,0	-6,7	0,292
% Kcal					
Energy (Kcal)	baseline	2275,3	654,3		
	end	2366,3	1258,8	+91,0	0,302
Carbohydrates	baseline	48,5	8,2		
	end	45,2	9,4	-3,3	<0,001
Protein	baseline	16,0	3,9		
	end	15,2	3,6	-0,8	0,020
Total fat	baseline	34,1	6,9		
	end	37,5	10,3	+3,4	<0,001
SFA	baseline	13,0	3,7		
	end	12,6	3,5	-0,4	0,207
MUFA	baseline	12,3	4,0		
	end	15,9	5,9	+3,6	<0,001
PUFA	baseline	4,8	2,1		
	end	4,8	1,9	0,0	0,902

Saturated fat (SFA); Monounsaturated fat (MUFA), Polyunsaturated fat (PUFA)

Table 3 Lipid values and oxidative stress markers before and after intervention

	Base line		End		p
	Mean	DS	Mean	DS	
Cholesterol (mg/dL)	182,7	40,2	184,4	42,1	ns
Triacylglycerols (mg/dL)	95,6	49,0	91,4	44,5	ns
HDL cholesterol (mg/dL)	47,3	11,1	50,5	12,6	<0,001
LDL cholesterol (mg/dL)	116,5	36,7	115,7	38,2	ns
Glucose (mg/dL)	85,7	9,7	87,3	10,8	0,035
Oxidized LDL (U/L)	49,4	22,8	47,2	22,4	ns
Serum-LDL uninduced conjugated dienes (umol/mmol cholesterol)	11,8	3,5	11,7	3,8	ns
Plasma- $F_{2\alpha}$-isoprostanes (ng/L)	29,2	6,7	28,0	6,9	<0,01

Table 4 – Baseline and final fatty acids content of LDL in the studied subjects. Absolute values are given as mg/g of LDL-apolipoprotein B100.

		Baseline			End		
Fatty acids		Mean	SD	5% trimmed mean	Mean	SD	5% trimmed mean
Oleic	mg/gApo B 100	147,30	74,82	140,61	166,66**	80,12	160,48
	%	21,12	2,66	21,09	22,99**	2,81	22,96
Linoleic	mg/gApo B 100	303,29	150,82	293,01	312,62	156,19	302,50
	%	43,24	4,90	43,33	42,22*	4,41	42,36
Palmitic	mg/gApo B 100	144,72	75,24	137,86	150,80	74,39	144,70
	%	20,78	2,70	20,83	20,56	2,18	20,47
Stearic	mg/gApo B 100	48,07	23,27	46,11	49,71	24,80	47,73
	%	7,00	1,46	6,85	6,83	1,63	6,64
Arachidonic	mg/gApo B 100	54,65	31,14	51,68	53,85	27,42	51,85
	%	7,86	1,18	7,80	7,39**	1,52	7,33
Oleic/linoleic ratio		0,50	0,14		0,55**	0,12	

* $p < 0.01$; ** $p < 0.001$

Table 5 Linear regression coefficients (standard error) of the relations between oxidative stress parameters (Oxidized LDL and $F_{2\alpha}$-isoprostanes) with difference in oleic/linoleic ratio. Adjusted for age and arachidonic Acid in LDL (only Model B).

Model A	**Ox-LDL (U/L)**
Change in oleic/linoleic (Units)	-15.942 (8.072)*
Age-(year)	0.411 (0.106)**
Constant	33.955 (3.766)**
	R = 0.327
	R^2 = 0.107
Model B	**F2α-isoprostanes (µg/L)**
Change inoleic/linoleic (units)	-4.208 (1.897)*
Age(year)	-0.0520 (0.026)*
Arachidonic Acid in LDL	0.0241 (0.011)*
(mg/g Apo B)	
Constant	28.563 (0.908)**
	R = 0.276
	R^2 = 0.076

Change in oleic/linoleic ratio = difference in the oleic/linoleic ratio in LDL between baseline and after olive oil consumption; Ox-LDL= oxidized LDL; $F_{2\alpha}$-isoprostanes= Plasma $F_{2\alpha}$-isoprostanes after olive oil ingestion

*p < 0.05; **p <0.001