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

VIII. ANEXOS

I. Memoria del trabajo realizado en el Departamento de Farmacología y Fisico-Química de las Interacciones Celulares y Moleculares, Facultad de Farmacia, Universidad Louis Pasteur, Estrasburgo, Francia.

Karina de la Torre Carbot

Memory of Brief Stay in Foreign Institution

Techniques learned during the stay:

-Determination of changes in vascular reactivity using organ chambers

-Cultures of endothelial cells

-Molecular biology techniques including Western Blot analysis

-In vivo treatments of rats and **e**xperimental design

-Confocal Microscope with fluorescence analysis

Illkirch, August 18, 2006

Approval

V.B. Schini-Kerth

Report of the work carried out

Relaxation Effects in Rat Aorta of Some Simple Virgin Olive Oil Phenols.

Several observational, epidemiological and controlled studies have associated the Mediterranean diet with a lower incidence of coronary artery disease [1-4]. Olive oil is a major constituent of the Mediterranean diet and, in addition to[5;6] oleic acid, contains a range of micronutrients, such as phenolic compounds and some effects are attributed to them [7-9].

Endothelial dysfunction, is considered the first pathological symptom of atherosclerosis and it has been demonstrated that olive oil improves the major risk factors for cardiovascular disease such as blood pressure [1;10]

The aim of the present study was to determine whether some simple olive oil phenolic compounds have a vasorelaxant capacity in the rat aorta.

The aorta was cut into rings with endothelium and suspended in organ baths containing an oxygenated Krebs bicarbonate solution for the measurement of changes in isometric tension [11].

Tyrosol, hydroxytyrosol, vanillic acid, homovanillic acid, vanillin, caffeic acid and gallic acid were used in these experiments. Penylephrine was used to cause approximately 80% of the maximal contraction before addition of a phenolic compound.

In regard with the two more abundant phenolic compounds in simple or conjugated form of olive oil, hydroxytyrosol only had a relaxing effect in the rat aortic rings of 15% at maximal concentration that was completely inhibited by the NO synthase inhibitor

nitro-L-arginine (Figure 1). In contrast, tyrosol did not induce any relaxation (see Figure 1). Vanillin induced a relaxation of about 70% with a threshold concentration of 0.6 μM; The relaxation was slightly but significantly inhibited in the presence of nitro-L-arginine (see Figure 2). All the other phenolic compounds studied (vanillic acid, homovanillic acid, caffeic and gallic acids) did not shown any relaxation.

The fact that the relaxation caused by hydroxytyrosol is totally inhibited for the nitro-L-arginine (a competitive inhibitor of NO-Synthase) suggests an endothelium-dependent relaxation due to the endothelial formation of NO. In the case of vanillin, a partial but significant inhibition was obtained with nitro-L-arginine suggesting that the relaxation involves both an endothelium-dependent component and indirect effect at the vascular smooth muscle [12]. These findings suggest that different recognition sites, with differential structural requirements, associated with different signalling mechanisms, may be involved in these different effects of vanillin.

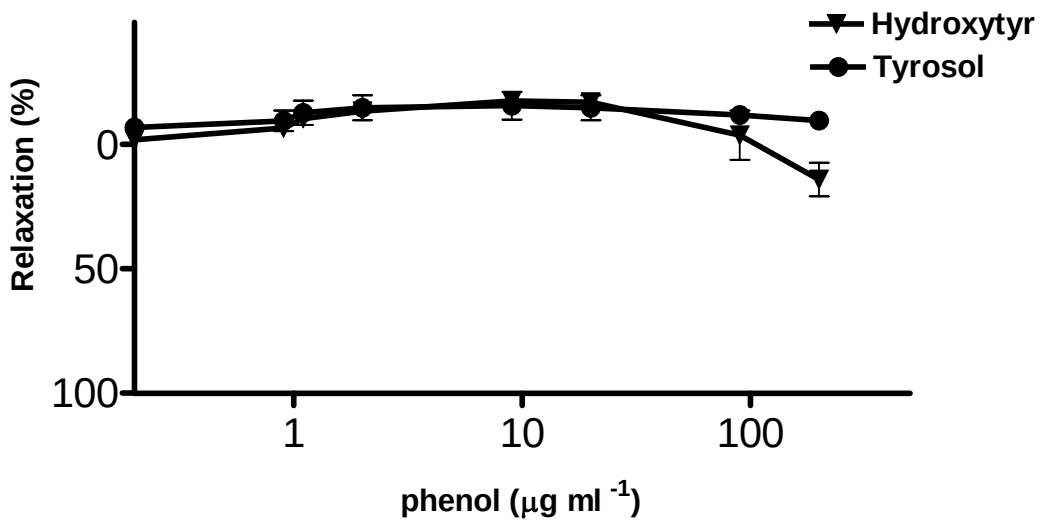
Since Olive oil extract exerts a relaxant effect in aortic rings [13], it is then expected that other phenolic compounds regularly present in olive oil are also able to relax the aorta. Additional investigations are needed to study the effects of authentic phenols present in olive oil on vascular tone.

Illkirch, August 18, 2006

Approval

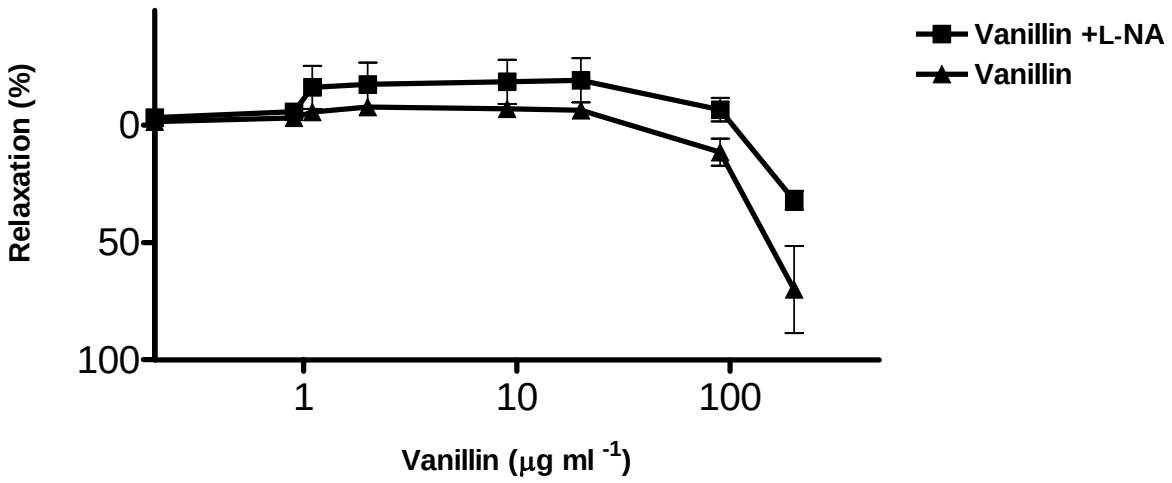
V.B. Schini-Kerth

Figure 1



Effect of hydroxytyrosol and tyrosol on rat aortic rings with endothelium contracted with Phenylephrine (10^{-7}M). Results are shown as mean ± SEM of 3 different experiments.

Figure 2



Effect of vanillin on rat aortic rings with endothelium contracted with Phenylephrine (10^{-7}M) in the absence and presence of Nitro-L-arginine (100μm) (inhibitor of NO synthase). Results are shown as mean ±SEM. of 3 different experiments

Reference List

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11. Ndiaye M, Chataigneau T, Andriantsitohaina R, Stoclet JC, and Schini-Kerth VB, Red wine polyphenols cause endothelium-dependent EDHF-mediated relaxations in porcine coronary arteries via a redox-sensitive mechanism. Biochemical and Biophysical Research Communications 310: 371-377, 2003.

12. Stoclet JC, Chataigneau T, Ndiaye M, Oak MH, El BJ, Chataigneau M, and Schini-Kerth VB, Vascular protection by dietary polyphenols. Eur.J.Pharmacol. 500: 299-313, 2004.

13. Benkhalti F, Legssyer A, Gomez P, Paz E, Lopez-Miranda J, Perez-Jimenez F, and el Boustani ES, Effects of virgin olive oil phenolic compounds on LDL oxidation and vasorelaxation activity. Therapie 58: 133-137, 2003.

II. Anexo de la publicación 1 (Supporting information)

Supporting Information

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

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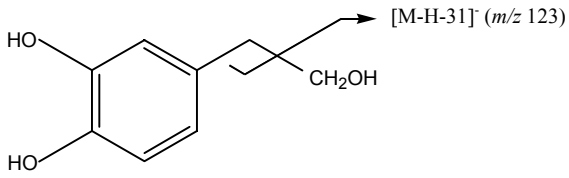
E-mail address: lamuela@.ub.edu (Rosa M. Lamuela Raventós)

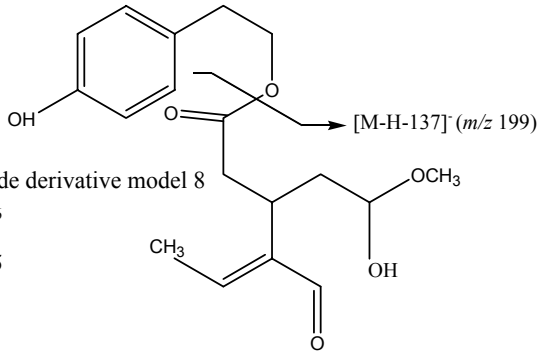
Content:

S-1. Hydroxytyrosol fragment

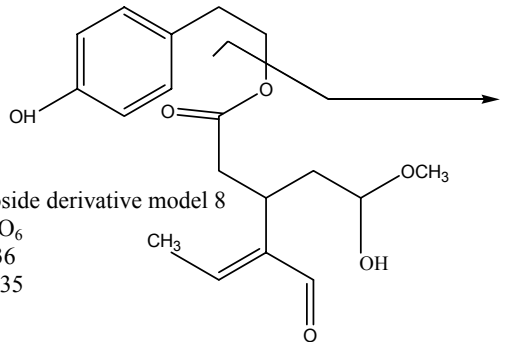


Hydroxytyrosol
$C_8 H_{10} O_3$
P.M. 154
M/Z^- 153

S-2. Ligstroside derivatives fragments



Ligstroside derivative model 8
$C_{18}H_{24}O_6$
PM: 336
M/Z: 335



Ligstroside derivative model 8
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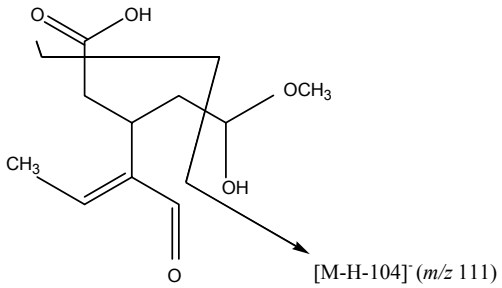

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$C_{10}H_{15}O_5$
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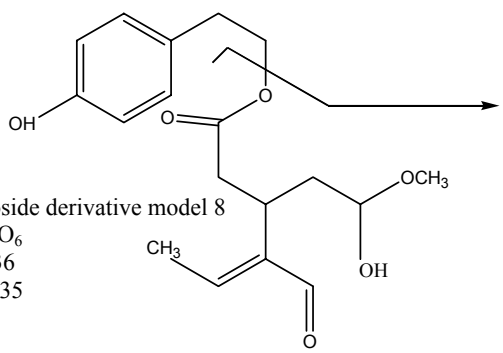
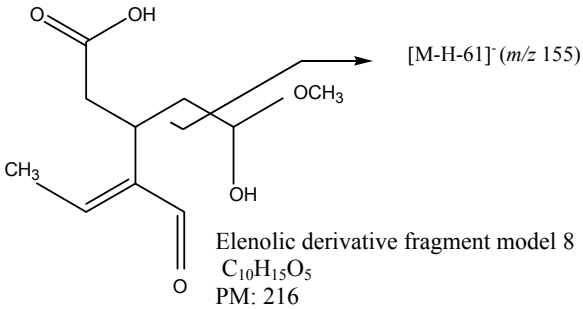
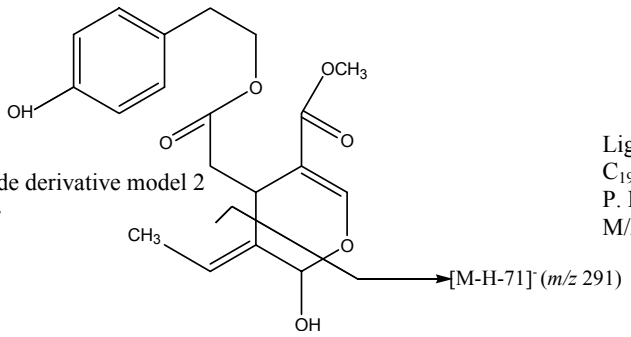
M/Z: 215



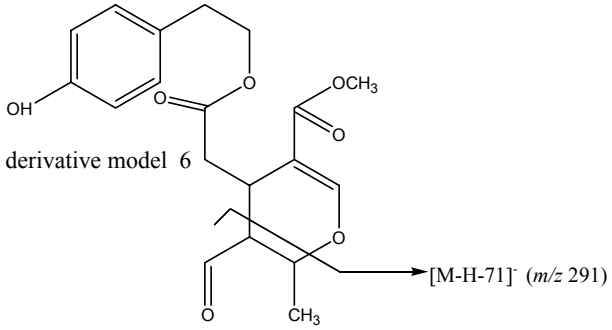
Ligstroside derivative model 8
$C_{18}H_{24}O_6$
PM: 336
M/Z: 335



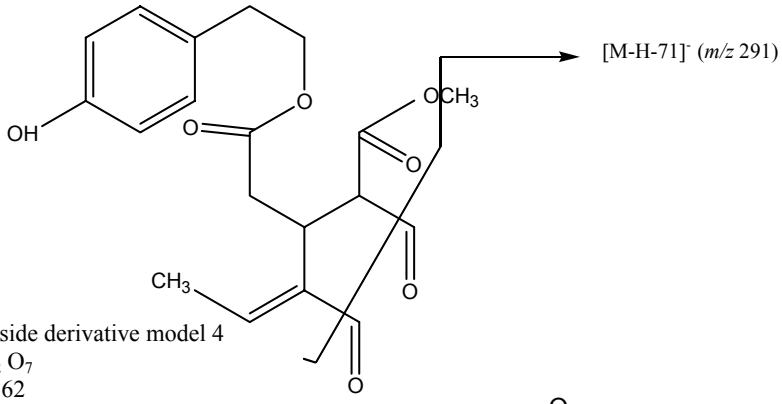
Elenolic derivative fragment model 8
$C_{10}H_{15}O_5$
PM: 216
M/Z: 215



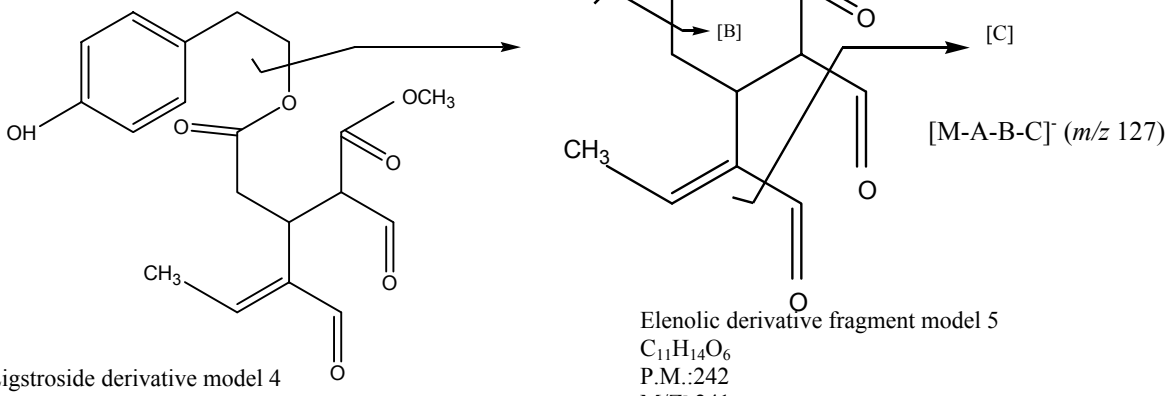
Ligstroside derivative model 2
$C_{19}H_{22}O_7$
P.M. 362
M/Z $^-$361



Ligstroside derivative model 6
C_{19} H_{22} O_7
P. M.:362
M/Z$^-$:361

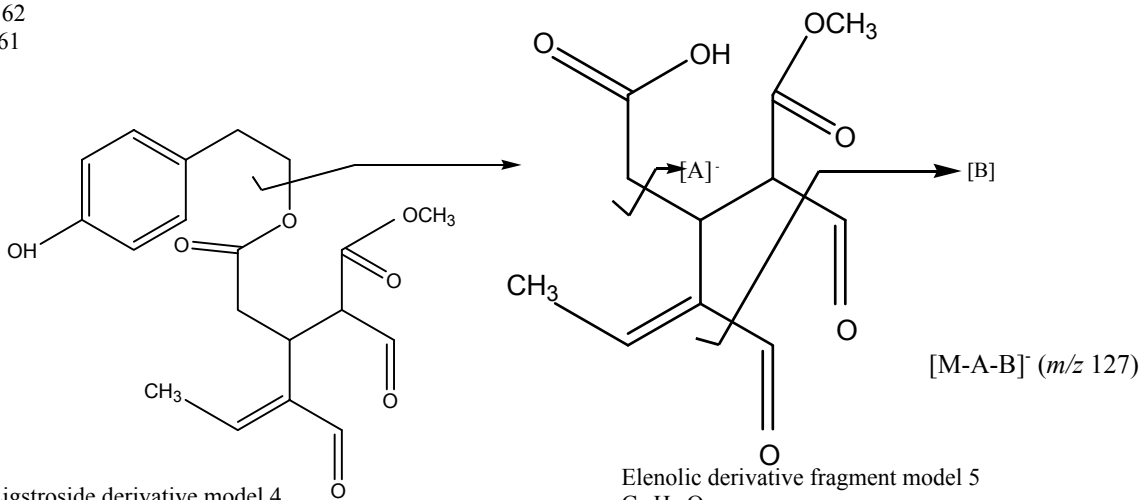


Ligstroside derivative model 4
C_{19} H_{22} O_7
P. M.:362
M/Z$^-$:361



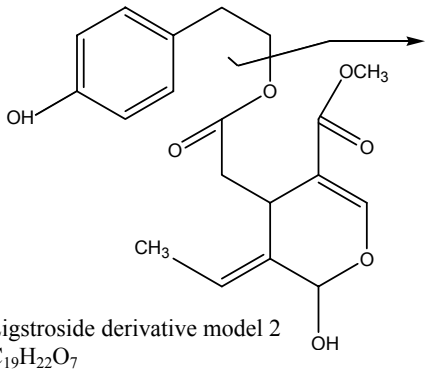
Ligstroside derivative model 4
C_{19} H_{22} O_7
P. M.:362
M/Z$^-$:361

Elenolic derivative fragment model 5
$C_{11}H_{14}O_6$
P.M.:242
M/Z$^-$:241

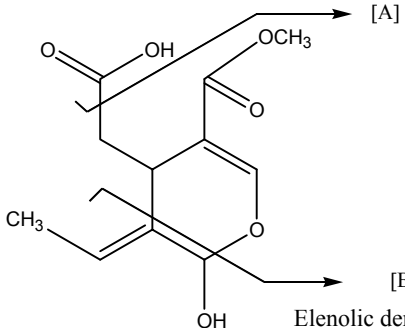


Ligstroside derivative model 4
C_{19} H_{22} O_7
P. M.:362
M/Z$^-$:361

Elenolic derivative fragment model 5
$C_{11}H_{14}O_6$
P.M.:242
M/Z$^-$:241

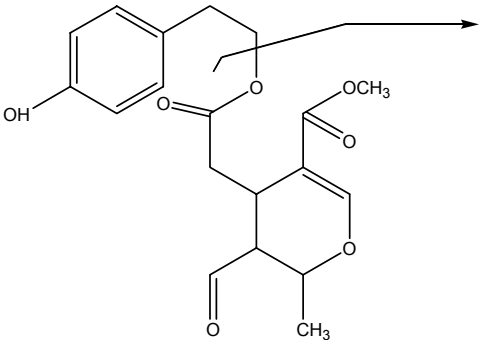


Ligstroside derivative model 2
$C_{19}H_{22}O_7$
P.M. 362
M/Z $^-$361

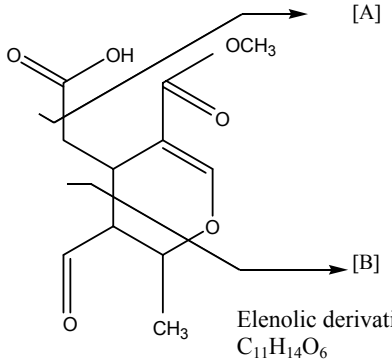


[M-A-B]$^-$ (*m/z* 127)

Elenolic derivative fragment model 2
$C_{11}H_{14}O_6$
P.M.:242
M/Z$^-$:241

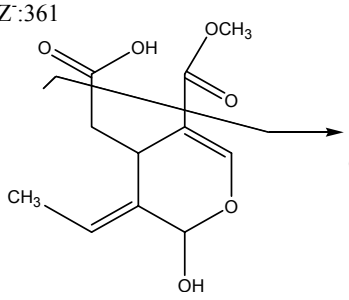


Ligstroside derivative model 6
$C_{19}H_{22}O_7$
P. M.:362
M/Z$^-$:361

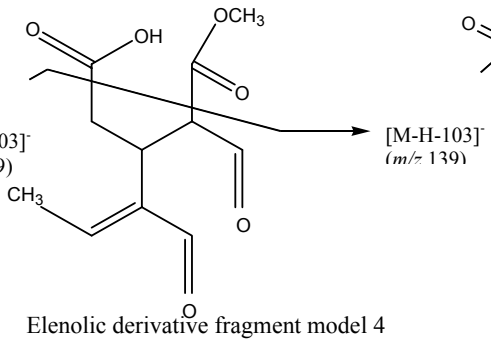


[M-A-B]$^-$ (*m/z* 127)

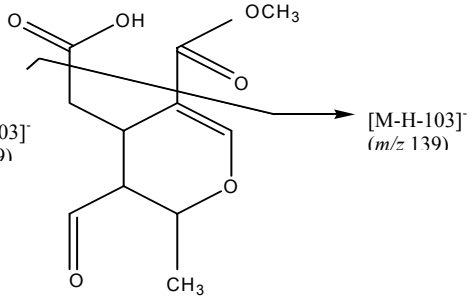
Elenolic derivative fragment model 2
$C_{11}H_{14}O_6$
P.M.:242
M/Z$^-$:241



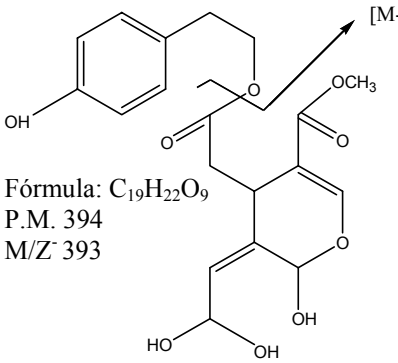
Elenolic derivative fragment model 2
$C_{11}H_{14}O_6$
P.M.:242
M/Z$^-$:241



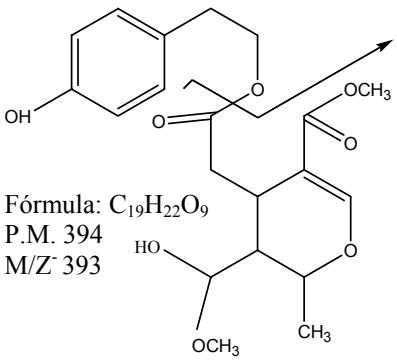
Elenolic derivative fragment model 4
$C_{11}H_{14}O_6$
P.M.:242
M/Z$^-$:241



Elenolic derivative fragment model 6
$C_{11}H_{14}O_6$
P.M.:242
M/Z$^-$:241

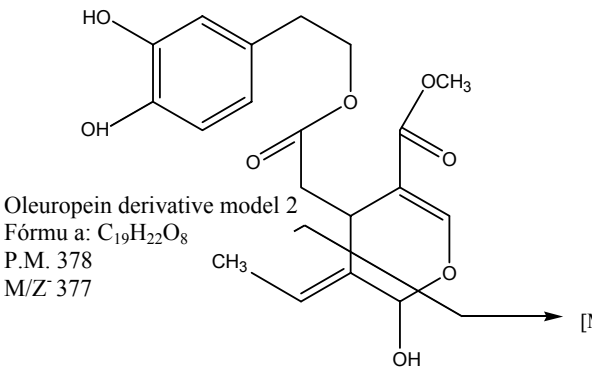


Fórmula: $C_{19}H_{22}O_9$
P.M. 394
M/Z$^-$ 393



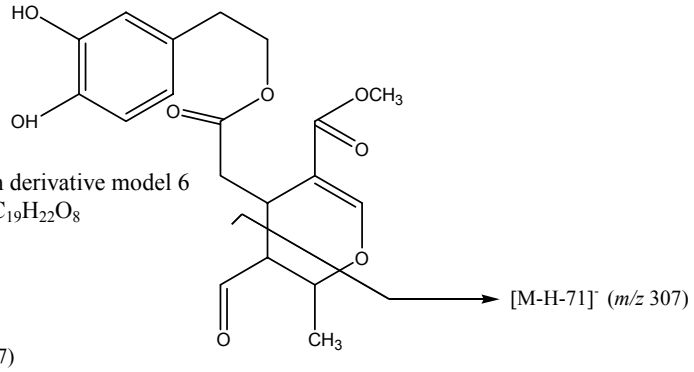
Fórmula: $C_{19}H_{22}O_9$
P.M. 394
M/Z$^-$ 393

S-3. Oleuropein derivarives fragments



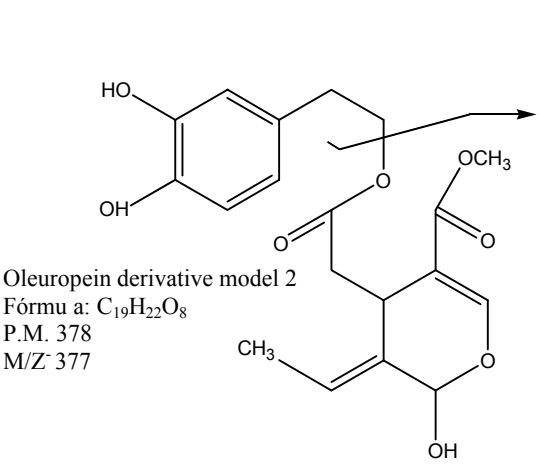
Oleuropein derivative model 2
Fórmu a: $C_{19}H_{22}O_8$
P.M. 378
M/Z^- 377

$[M-H-71]^-$ (*m/z* 307)

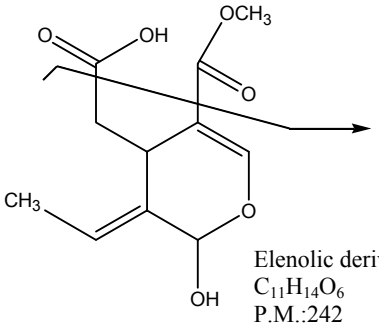


Oleuropein derivative model 6
Fórmula: $C_{19}H_{22}O_8$
P.M. 378
M/Z^- 377

$[M-H-71]^-$ (*m/z* 307)

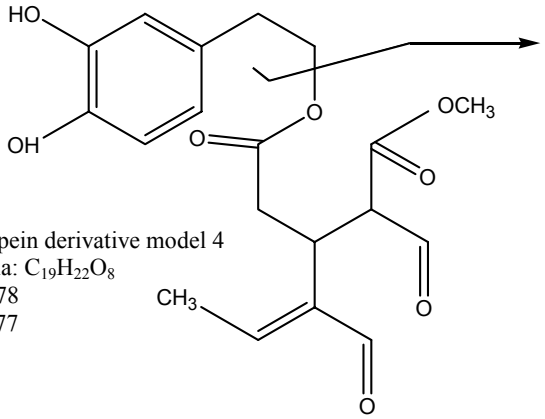


Oleuropein derivative model 2
Fórmu a: $C_{19}H_{22}O_8$
P.M. 378
M/Z^- 377

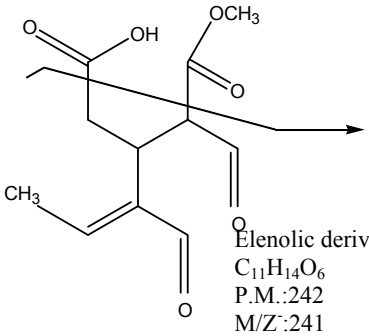


$[M-H-103]^-$ (*m/z* 139)

Elenolic derivative fragment model 2
$C_{11}H_{14}O_6$
P.M.:242
M/Z^-:241

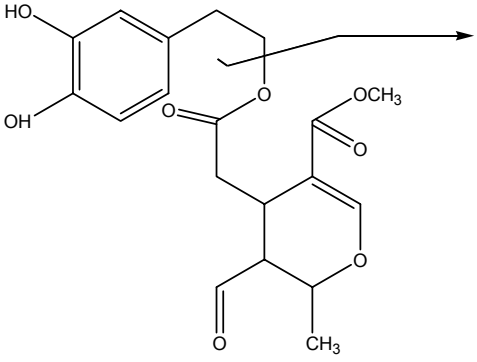


Oleuropein derivative model 4
Fórmula: $C_{19}H_{22}O_8$
P.M. 378
M/Z^- 377

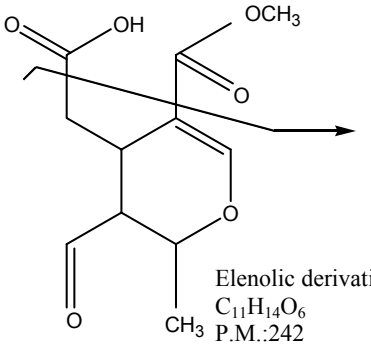


$[M-H-103]^-$ (*m/z* 139)

Elenolic derivative fragment model 4
$C_{11}H_{14}O_6$
P.M.:242
M/Z^-:241

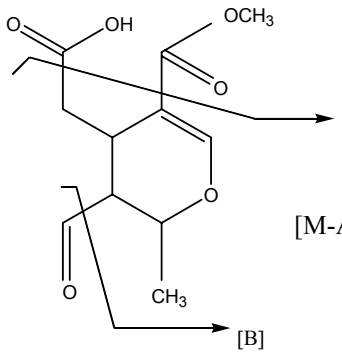


Oleuropein derivative model 6
Fórmula: $C_{19}H_{22}O_8$
P.M. 378
M/Z^- 377



$[M-H-103]^-$ (*m/z* 139)

Elenolic derivative fragment model 6
$C_{11}H_{14}O_6$
P.M.:242
M/Z^-:241



[M-A-B]$^-$ (*m/z* 111)

Elenolic derivative fragment model 6
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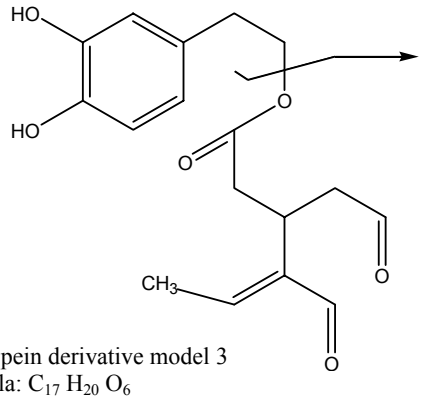
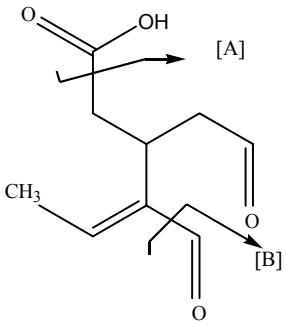
P.M.:242
M/Z$^-$:241

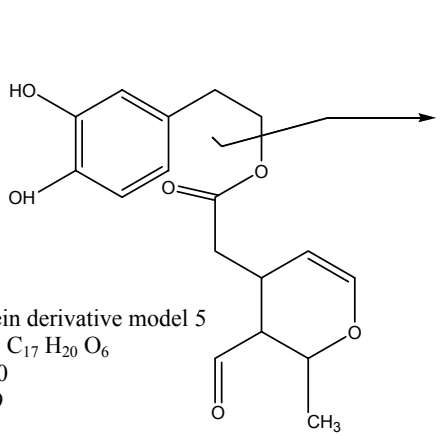


Oleuropein derivative model 3
Fórmula: $C_{17}H_{20}O_6$
P. M.:320
M/Z$^-$:319

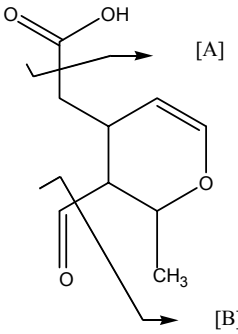


[M-A-B]$^-$ (*m/z* 111)

Elenolic derivative fragment model 3
$C_9H_{12}O_4$
P.M.:184
M/Z$^-$:183



Oleuropein derivative model 5
Fórmula: $C_{17}H_{20}O_6$
P. M.:320
M/Z$^-$:319



[M-A-B]$^-$ (*m/z* 111)

Elenolic derivative fragment model 5
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P.M.:184
M/Z$^-$:183

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Changes in the phenolic content of low density lipoprotein after olive oil consumption in men. A randomized crossover controlled trial*

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ABSTRACT

Olive oil decreases the risk of cardiovascular diseases (CVD). This effect may be due to the fatty acid profile of the oil, but it may also be due to its antioxidant content which differs depending on the type of olive oil. In this study, the concentrations of oleic acid and antioxidants (phenolic compounds and vitamin E) in plasma and low density lipoprotein (LDL) were compared after consumption of three similar olive oils, but with differences in their phenolic content. Thirty healthy volunteers participated in a placebo-controlled, double blind, crossover, randomized supplementation trial. Virgin, common, and refined olive oils were administered during three periods of 3 weeks separated by a two-week washout period. Participants were requested to ingest a daily dose of 25 mL of raw olive oil, distributed over the three meals of the day, during intervention periods. All three olive oils caused an increase in plasma and LDL oleic acid (*P*<0.05) content. Olive oils rich in phenolic compounds led to an increase in phenolic compounds in LDL (*P*<0.005). The concentration of phenolic compounds in LDL was directly correlated with the phenolic concentration in the olive oils. The increase in the phenolic content of LDL could account for the increase of the resistance of LDL to oxidation, and the decrease of the *in vivo* oxidized LDL, observed in the frame of this trial. Our results support the hypothesis that a daily intake of virgin olive oil promotes protective LDL changes in front of its oxidation.

KEY WORDS: olive oil, oleic acid, phenolic compounds, low density lipoprotein, cardiovascular risk.

Cardiovascular disease (CVD) is the main cause of death and disability in developed countries (Sans *et al.* 1997; Shaefer 2002; Kratz *et al.* 2002). The type of fat consumed can modify the plasma and low density lipoprotein (LDL) lipid profile, which is directly related to the growth of atheroma plaque (Connor 1996; Perez-Jimenez *et al.* 2002). However, the antioxidant content of the diet is also crucial, as oxidized LDL seems to be involved in atherosclerotic plaque development (Diaz *et al.* 1997).

Olive oil, rich in monounsaturated fatty acids (MUFA) and antioxidant minor components, is considered to be favourable for cardiovascular health (Mekki *et al.* 1997; Nicolaiew *et al.* 1998; Ramirez-Tortosa *et al.* 1999; Fernandez-Jarne *et al.* 2002; Covas *et al.* 2006c; Covas 2007). Linoleic acid (C18: 2) accounts for 90% of the polyunsatured fatty acids (PUFA) present in LDL and is the main substrate for oxidation. However, diets rich in oleic acid, like those in Mediterranean countries, generate LDL particles which appear to be more resistant to oxidation (Esterbauer *et al.* 1992; Reaven *et al.* 1994; Benzie 1996; Mata *et al.* 1997; Delany *et al.* 2000; Kratz *et al.* 2002; Perez-Jimenez *et al.* 2002).

Moreover, data from *in vitro* (Fitó *et al.* 2000a; Fitó *et al.* 2000b) and *in vivo* studies (Visioli & Galli 2002; Marrugat *et al.* 2004; Ruano *et al.* 2005; Covas *et al.* 2006a) show that the phenolic compounds of olive oil protect LDL from lipid peroxidation. Thus, olive oil phenolic compounds are good candidates to partially account for the prevention provided by diet on CVD Due to this, studies directed at a better understanding of the protective mechanisms of olive oil on human health must be enhanced.

To date, few studies have analyzed the effects of sustained olive oil consumption on human LDL composition. The few available data come from short-term studies (Fitó *et al.* 2002; Gimeno *et al.* 2002b; Covas *et al.* 2006a) or non-randomized trials where only virgin olive oil was used (Gimeno *et al.* 2002b). A double-blind, randomized, crossover, controlled trial

was carried out to identify the effect of similar olive oils, but with a range of phenolic content, on the levels of plasma and LDL antioxidants and oleic acid in healthy subjects. Volunteers of a religious centre, a population with regular and similar lifestyles such as physical activity and dietary habits, were involved. In the frame of this trial we have previously reported (Marrugat *et al.* 2004) a protective effect of an olive oil rich in phenolic compounds on LDL oxidation. From these results, our main hypothesis was that sustained real-life doses (25 mL /day) of raw rich phenolic olive oil could enhance the antioxidant load of the LDL, thus protecting the lipoproteins from oxidation. Here, we examined the fatty acid and antioxidant composition of the LDL after consumption of similar olive oils, but with differences in their phenolic content, in order to test our hypothesis.

Materials and methods

Study population.

An in-person screening visit was conducted to ascertain eligibility and obtain baseline data. Forty two subjects from a religious community were screened for inclusion. Nine of them were ineligible. Thus, 33 healthy volunteers, from 23 to 91 years old, with a regular lifestyle and dietary habits were included. The volunteers gave their written consent prior to participating in the study. Subjects with any of the following conditions were excluded: smoking; intake of any drug or supplements with established antioxidative properties, either in the two weeks before the onset of the study or throughout the study; obesity (body mass index >30 kg/m^2); diabetes; and any disease or condition that would impair compliance.

Diets were prepared and consumed in the religious center. Subjects maintained their regular physical activity and lifestyle throughout the study. The local institutional Review Board approved the protocol according to the Helsinki Declaration of 1975.

Olive oil composition.

Three olive oils provided by the Olive Oil Cooperative Association of Catalonia were used. They were obtained from the same harvest which means that olive fruits were of the same cultivar, recollection time, and soil. First, a virgin olive oil with a phenolic content of 825 μmol/kg caffeic acid equivalents (CAE) was selected. Then, we used a refined (phenolic content of 0 μmol/kg) and a common olive oil (phenolic content of 370 μmol/kg CAE) with similar fatty acid composition, α-tocopherol and β-carotene content, in order to match the virgin olive oil. Major and minor components of the three olive oils were examined in order to confirm their similar fatty acid and micronutrient profile, as well as the differences in their phenolic content. The characteristics of the oils are summarized in **Table 1**. The acidity value, the peroxide index, and the UV spectrophotometric index (K_{270}) were determined following the analytical methods described in the European Union Commission Regulation CE/1989/2003 (Commission Regulation 2003). Fatty acids were transformed into methyl esters and analyzed by gas chromatography (Commission Regulation 2003). α-Tocopherol was measured by high performance liquid chromatography (HPLC), as previously described (Gimeno *et al.* 2000). Phenolic compounds were measured by the Folin-Ciocalteau method (Swain & Hillis 1969; Gimeno *et al.* 2002a; Gimeno *et al.* 2002b).

Study design.

A placebo-controlled, double blind, crossover, randomized, supplementation trial was conducted. A Latin square for the three treatments was used in the crossover trial, to randomize participants into three orders of olive oil administration: virgin-common-refined (order 1), common-refined-virgin (order 2), and refined-virgin-common (order 3). The three olive oils were administered over three periods of 3 weeks, each one preceded by two-week washout periods (**Figure 1**). Participants were requested to ingest a daily raw dose of 25 mL of olive oil, distributed over the three meals of the day, during intervention periods. Refined olive oil was used as source of raw fat in washout periods. Other cooking fats were replaced by refined olive oil to mantain energy and oleic acid intake unchanged during all the study.

Daily menus were recorded, as were extra food intakes between meals, and meals eaten outside the religious center. Participants were requested to avoid a high intake of foods containing phenolic compounds such as: fruit, vegetables, tea, and red wine. Participants were managed by a trained physician who stayed at the religious center throughout the study. Participants were instructed to return the 25 mL containers every morning when they collected their next daily dose, in order to register the amount of unconsumed oil. Treatment containers, for the daily dose of olive oil to be ingested in the intervention periods, were coded, concealed from participants and investigators, and distributed to the participants. Containers were opaque in order to conceal the olive oil and to avoid its degradation.

Diets were analyzed by a nutritionist and converted into nutrients using the software Medysystems, Conaycyte S.A, Madrid Spain (Schroder *et al.* 2001). Anthropometric variables (i.e. height and weight) were recorded. Physical activity was assessed by the

Minnesota Leisure Time Physical Activity Questionnaire, which has been validated for use on Spanish men (Elosua *et al.* 2000).

Sample size and power analyses

The sample size was calculated to provide a statistical power of 80%. In order to recognize as statistically significant a difference greater than or equal to 10 minutes of change in lag time, and a change of 4 nmol/mg apo B of phenolic compounds in LDL, ten and nine subjects were necessary in each order of olive oil administration respectively. It was assumed that standard deviations were 10 min and 4 nmol/mg apo B for lag time and phenolic compounds in LDL, respectively.

Blood sampling and laboratory analyses.

Laboratory measurements were carried out on samples from fasting subjects taken: before the first washout period (baseline); before administration of the three types of oil; and after olive oil administration. To prevent oxidation and aggregation of LDL, blood samples were drawn with EDTA tubes (1g/l) and stored with saccharose (0.18mM) at -80ºC. Plasma was separated by centrifugation at 1000g at 4ºC for 15 minutes. LDL isolation was performed by sequential flotation ultra centrifugation (HAvel RJ & Eder HA an Bragdan JH 2005). All samples were stored under -80ºC until analysis.

Total cholesterol, high density lipoprotein (HDL)-cholesterol and triglyceride levels were measured by standard enzymatic methods. The fatty acid composition of plasma and LDL was measured by the method described by Rodríguez-Palmero *et al* (Rodriguez-Palmero *et al.* 1997). Fatty acid methyl esters (FAME) were prepared by alkaline hydrolysis with

sodium methylate and esterification with boron trifluoride in methanol. FAME were extracted with hexane and injected into a gas chromatograph. The coefficients of variation (CVs) obtained ranged from 3.42 to 5.25%. To determine α-tocopherol in plasma and LDL, an aliquot of the sample was deproteinized with ethanol. The analyte was then extracted with hexane and injected into an HPLC system. The coefficient of variations was 4.27% (Gimeno *et al.* 2001). Phenolic compounds in LDL were also determined by HPLC-Diode Array Detection, as previously described (Lamuela-Raventos *et al.* 1999). Briefly, acidulated LDL was applied to a Waters Oasis^{TM} HLB extraction cartridge and washed with water and 5% aqueous methanol. Phenolic compounds, measured only in LDL, were eluted with methanol, which was then evaporated under a stream of nitrogen. The residue was dissolved in acidulated water and injected into an HPLC system. The chromatogram was monitored at 280 nm and the areas of phenolic compounds were expressed as CAE. The analytical within-run precision was 5.25%, and the between-run precision was 8.8%. Oxidized LDL was measured in plasma by ELISA (ox-LDL, Mercodia AB, Uppsala, Sweden). The LDL resistance to oxidation was determined by formation of conjugated dienes after copper (5μM) oxidation of isolated LDL (Fitó *et al.* 2000b). Results of LDL parameters were expressed according to apolipoprotein B (apoB). Apo B concentrations were measured by immunoturbidimetry (Roche Diagnostics, Basel, Switzerland).

Hydroxytyrosol and tyrosol, the major olive oil phenolic compound, were measured in urine, by HPLC, as markers of compliance of the interventions. Analytical within-run precision and between-run precision were 2.9 and 3.8%; and 5.7 and 6.2% for tyrosol and hydroxytyrosol, respectively (Miró-Casas *et al.* 2001).

Statistical Methods.

The normality of variable distribution was assessed by the Kolmogorov-Smirnov test and by an analysis of skewness and kurtosis. One-factor ANOVA and the Kruskall-Wallis test were used to analyze the differences in baseline characteristics among the three groups, by order of olive oil administration. A general linear model for repeated measurements, with Tukey's correction for multiple comparisons, was used to assess washout effectiveness and the effect of each type of oil. The possible carryover effect was checked by testing a period-by-treatment interaction term in the model. Statistical significance was defined as $P<0.05$ for a two-sided test. SPSS 11.0 statistical software was used.

Results

Participants' characteristics

The subject pool at randomization consisted of 33 subjects. Two of these were withdrawn because of a post-randomization criteria of ineligibility (hypercholesterolemia), and one participant requested to be withdrawn. Thus, finally 30 subjects participated in the study. Baseline characteristics (at the beginning of the study) of the subjects are presented in **Table 2** according to the randomized order. Negligible differences in baseline characteristics were observed among the three randomly formed groups. The mean (SD) age of participants was 57.13 (19.54) years and the body mass index (BMI) was 22.92 (5.15) kg/m^2. We did not observe changes in physical activity from baseline to the end of the study.

Adverse effects

No collateral effects which could influence the participation in the study or the compliance of the participants occurred during the study period. No adverse effects were observed related to olive oil ingestion.

Dietary intake and adherence

The average of the main antioxidant (i.e. beta-carotenoid, vitamin C, alfa-tocopherol), or pro-oxidant (i.e. iron) intake, energy, and any nutrient of interest were similar in the three groups during each type of olive oil intervention (**Table 3**). Participants' alcohol intake was less than 30 g/day. Tyrosol and hydroxytyrosol in urine increased in a dose-dependent manner with the phenolic content of olive oil administered. Mean changes were 15%, 147%, and 190% for tyrosol, and 12%, 180%, and 221% for hydroxytyrosol, after refined, common, and virgin olive oil, respectively (Marrugat *et al.* 2004).

Plasma and LDL fatty acid and antioxidant content

The phenolic content of LDL was significantly higher after the virgin olive oil administration than at baseline ($P<0.005$) and pre-virgin olive oil consumption ($P<0.01$), without significant changes after refined or common olive oil interventions (**Table 4, Figure 2**). When the relative changes (as percentage) were assessed, phenolic compounds in LDL followed an increasing trend ($P<0.05$) from refined to common to virgin olive oil. The increase in phenolic compounds in LDL after virgin olive oil intervention reached significance versus that after refined olive oil intervention ($P<0.005$).

We did not observe any changes in oleic acid and α-tocopherol in plasma or in α-tocoferol in LDL among olive oil interventions. In comparison with baseline values, levels of oleic acid increased after common and virgin olive oil interventions (**Table 4**). No carryover effect was observed in any variable of interest, with the exception of oleic acid in plasma and LDL. As expected, an increase in the time-sequence for oleic acid values was observed ($P<0.05$). As has been previously described, a protective effect of olive oil phenolic compounds on LDL oxidation was observed (Marrugat *et al.* 2004). The resistance of LDL to oxidation induced by copper lag time for copper-mediated LDL oxidation increased, and the levels of *in vivo* oxidized LDL decreased in a dosis-dependent manner with the phenolic content of the olive oil administered ($P<0.05$). Mean changes were 3.2%, -5.2%, and -28.2% for *in vivo* oxidized LDL, and 2.3, 4.5%, and 5.5% for the *in vitro* lag time of LDL oxidation, after refined, common, and virgin olive oil, respectively. Changes in the lipid profile after the olive oil interventions are reflected in **Figure 3.** An increase in HDL cholesterol after virgin olive oil consumption was observed ($P=0.029$) (Marrugat *et al.* 2004).

Discussion

In Mediterranean countries, dietary fat accounts for more than the 30% of energy mostly provided by the MUFA from olive oil. The main olive oils used for dietary purposes in Mediterranean countries are virgin olive oil, obtained exclusively by physical procedures and rich in phenolic compounds, and common olive oil (Commission Regulation 2003) which is a mixture of refined (phenolic-free) and virgin olive oil. In this study, and using

three types of olive oil with high (virgin), medium (common), and null (refined) phenolic content, we observed an increase in the LDL phenolic compound content of healthy human volunteers, in a dose-dependent manner with the phenolic content of the olive oil administered. This increase in the phenolic content of the LDL was concomitant with a decrease of the *in vivo* degree of LDL oxidation, and an increase in the *ex vivo* resistence of LDL to oxidation. A dose-dependent decrease of the oxidative lipid damage with the phenolic content of the olive oil has been recently reported (Covas *et al.* 2006a). Plasma concentration of oxidized LDL has been shown to be predictive for CVD events in a general population. (Meisinger *et al.* 2005). Thus, interventions directed at controlling this variable are useful tools in the primary and secondary prevention of CVD.

As is reflected in this study and others, olive oil phenolic compounds are absorbed in humans (Visioli *et al.* 2000; Caruso *et al.* 2001; Vissers *et al.* 2002; Miró-Casas *et al.* 2003) in a dose-dependent manner with the phenolic content of the olive oil (Marrugat *et al.* 2004; Fitó *et al.* 2005; Covas *et al.* 2006a). Phenolic compounds from olive oil can bind the human LDL after virgin olive oil ingestion (De La Torre-Carbot *et al.* 2007). In a previous work, we observed that the postprandial LDL total phenolic content and LDL oxidation could be modulated by olive oil phenolic compounds in humans (Covas *et al.* 2006a). Here, we report the same phenomenum after a sustained consumption of olive oil.

At baseline, subjects did not consume only olive oil as a source of fat. Instead, they consumed preferently other vegetable oils for cooking, using olive oil for raw purposes. The increase in oleic acid in LDL observed throughout the study could be due to the consumption of all types of olive oil, given that the refined olive oil was consumed during the washout periods. From our results, olive oil consumption promoted an increase of MUFA in the LDL. MUFA are less susceptible to oxidation than PUFA (O'Byrne *et al.*

1998; Kratz *et al.* 2002). Due to this, the increase of MUFA in LDL could enhance the preservation of the phenolic compounds bound to LDL, given that they are not used to counteract the autocatalytic chain reaction of LDL fatty acid peroxidation (Gutteridge 1995). This fact could also explain the non-significant increase in LDL phenolic compounds observed after refined olive oil intervention, in front of baseline values.

Phenolic compounds can protect LDL from oxidation (Bonanome *et al.* 2000; Covas *et al.* 2000; Soler-Rivas *et al.* 2000; Espin *et al.* 2001; Marrugat *et al.* 2004; Masella *et al.* 2004; Covas *et al.* 2006a) through different mechanisms: 1) for the free radical scavenging properties (the capacity of hydrogen-donation and their ability to improve radical stability) (Stupans *et al.* 2002; Moreno *et al.* 2003; Turner *et al.* 2005); 2) by means of the strong metal-chelation capacity (Visioli *et al.* 2002); 3)Through the oxid nitric (Visioli & Galli 1998; Palmerini *et al.* 2005); 4) by stimulating antioxidant transcription and detoxification defence systems (Weinbrenner *et al.* 2004; Fitó *et al.* 2005; Masella *et al.* 2005), and 5) by modulating other enzymatic systems related with oxidation process (ciclooxygenases, lipooxigenases and NAD(P)H oxidase) (Moreno *et al.* 2003). In addition, olive oil phenolic compounds have been shown to be related with the prevention of platelet aggregation (Morton *et al.* 2000; Espin *et al.* 2001), vasodilatation (Visioli & Galli 2002), and anti-inflammation (Morton *et al.* 2000; Visioli & Galli 2002; Zern & Fernandez 2005; Miles *et al.* 2005), Thus, the protection provided by the olive oil phenolic compounds on CVD risk may be due to a combination of bioactive mechanisms.

Phenolic compounds of dietary origin have been shown to to be involved in cholesterol and lipoprotein metabolism (Zern & Fernandez 2005). In this study, we observed an increase in HDL cholesterol levels after virgin olive oil intervention. These results are in line with the recent results of the EUROLIVE study (Covas *et al.* 2006b), a large inter-

country intervention trial with three similar types of olive oils, but with differences in their phenolic content. Results of the EUROLIVE study showed a dose-dependent increase of the plasma HDL cholesterol levels with the phenolic content of the olive oil administered. Mechanisms by which phenolic compounds can enhance HDL cholesterol are at present unknown.

Phenolic compounds in olive oil may contribute to the health benefits (Visioli F. *et al.* 2004; Salvini *et al.* 2006) and Mediterrranean diet, rich in virgin olive oil, improves the major risk factors for CVD (Perez-Jimenez *et al.* 2005; Estruch *et al.* 2006)

The design and conduct of the study had strengths and limitations. One strength was that the dose administered, 25 mL per day, closely reflects real-life consumption in Southern European Mediterranean countries. Another was the crossover design, which permitted the same participants to receive all olive oils, thereby minimizing interferences with confounding variables. Our design, however, did not allow modelling the first- and second-order possible carryover effects. Another limitation was the inability to assess potential interactions between olive oil and other diet components. Measurements of dietary intake relied on self-reporting and were, therefore, subjective. Another limitation is the short duration of the intervention periods. It is unknown whether additional or different effects would have been observed over longer periods. A longer duration of the study, however, could have impaired the compliance of the participants. Also, although the trial was blinded, some participants might have identified the refined olive oil by its taste and smell.

In summary, regular consumption of olive oil increases the MUFA content of the LDL lipoprotein. Regular consumption of olive oil rich in phenolic compounds increases the LDL total phenolic content in a dose-dependent manner with the phenolic content. The

combined protective effect of the MUFA and phenolic content of the LDL could account for the decrease in LDL oxidation observed in the frame of this study.

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Table 1. Olive oil composition

	Refined	**Common**	**Virgin**
Quality parameters:			
Free acidity (% oleic acid)	0.12	0.17	0.11
Peroxide value (meq O_2/kg oil)	1.80	2.93	6.48
K_{270}	0.480	0.201	0.102
Fatty acids (%):			
C14:0	0.02	0.02	0.01
C16:0	11.25	11.78	13.18
C16:1	0.86	1.05	1.11
C17:0	0.08	0.06	0.10
C17:1	0.15	0.13	0.22
C18:0	2.52	2.59	1.76
C18:1	73.46	75.65	73.37
C18:2	9.97	7.17	9.02
C20:0	0.45	0.39	0.33
C18:3	0.73	0.70	0.48
C20:1	0.34	0.29	0.28
C22:0	0.12	0.10	0.10
C24:0	0.05	0.04	0.04
MUFA(%)	74.83	77.14	74.98
PUFA(%)	10.68	7.86	9.50
SFA(%)	14.49	15.00	15.52
Phenolic compounds (μmol/kg CAE*)	0	370	825
α-Tocopherol (mol/kg)	65.88	48.22	47.98

*CAE=Caffeic Acid Equivalents

Table 2. Baseline characteristics (Mean (SD)) by sub-groups of subjects depending on the order of olive oil administration.

Clinical Parameter	**Order 1** †	**Order 2**†	**Order 3**†	***P***
Age (year)	54.8 (21.4)	61.0 (19.2)	56.6 (19.3)	0.80
*BMI (kg/m^2)	24.2 (3.5)	23.2 (3.3)	23.6 (2.9)	0.22
Waist-hip ratio	0.92 (0.05)	0.89 (0.04)	0.89 (0.05)	0.40
Triglycerides (mmol/L)	1.1 (0.5)	1.2 (0.4)	1.0 (0.5)	0.68
Total cholesterol (mmol/L)	5.4 (1.1)	5.7 (1.0)	5.9 (1.2)	0.25
‡HDL cholesterol (mmol/L)	1.4 (0.3)	1.4 (0.3)	1.5 (0.3)	0.66
Glucose (mmol/l)	4.4 (0.7)	4.2 (0.5)	4.6 (0.9)	0.59
Plasma α-Tocopherol (μmol/ml)	4.2×10^{-2} (1.38×10^{-2})	4.7×10^{-2} (1.2×10^{-2})	5.1×10^{-2} (1.1×10^{-2})	0.22
Plasma oleic acid (mmol/L)	2.1 (0.5)	2.0 (0.9)	2.0 (0.6)	0.77
LDL α-Tocopherol (μmol/mg apo B)	2.1×10^{-2} (0.31×10^{-2})	1.9×10^{-2} (0.38×10^{-2})	1.7×10^{-2} (0.45×10^{-2})	0.59
LDL Phenolic compounds (nmol CAE/mg apoB)	8.7 (3.2)	7.9 (2.3)	7.8 (1.5)	0.29
LDL oleic acid (μmol/mg apo B)	0.31 (0.1)	0.25 (0.05)	0.31 (0.1)	0.34
Physical activity (kJ/day)	1410 (966)	1648 (879)	1886 (1518)	0.62

†Orders of olive oil administration (n=number of participants): Order 1, Virgin-common-refined (n=11);

Order 2, common-refined-virgin (n=9); Order 3, refined-virgin-common (n=10)

*BMI, body mass index; †LDL, low density lipoprotein; ‡HDL, high density lipoprotein

Table 3. Mean (standard deviation) daily intake of nutrients in each dietary period

	OLIVE OIL ADMINISTERED			
n= 30	**Refined** (0 mg/μmol/ kg CAE)	**Common** (370 μmol/ kg CAE)	**Virgin** (825μmol/ kg CAE)	***P***
Energy (kJ)	9567 (937)	9639 (1079)	9668 (966)	0.84
Protein (%)	20.4 (1.8)	20.2 (1.8)	20.3 (1.6)	0.70
Fat (%)	37.5 (4.1)	37.7 (4.5)	40.0 (4.8)	0.60
Carbohydrate (%)	41.9 (5.1)	41.8 (4.9)	41.6 (5.5)	0.89
MUFA (%)	20.2 (2.8)	20.1 (2.9)	20.2 (2.8)	0.96
PUFA (%)	4.5 (0.5)	4.4 (0.5)	4.4 (0.4)	0.55
SFA (%)	13.9 (2.0)	13.9 (2.2)	13.8 (1.9)	0.82
α-tocopherol (mg)*	8.4 (1.9)	8.3 (2.4)	8.6 (2.3)	0.66
Vitamin C (mg)	227 (99)	228 (102)	229 (97)	0.75
Phenolic compounds (mg)*	14.9 (4.8)	14.4 (5.3)	14.7 (5.8)	0.66
β-Carotene (μg)	2385 (354)	2337 (361)	2420 (332)	0.74

CAE, phenolic content in caffeic acid equivalents; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

* This amount excludes the phenolic compounds and α-tocopherol taken with the oils studied

Table 4. Content in α-tocopherol, phenolic compounds and oleic acid at baseline and after each dietary period (mean and standard deviation)

(n=30)	**Baseline**	**Post-consumption of refined olive oil**	**Post-consumption of common olive oil**	**Post-consumption of virgin olive oil**
Plasma				
α-Tocopherol (μmol/ml)	4.8×10^{-2} (1.1×10^{-2})	4.6×10^{-2} (1.2×10^{-2})	4.4×10^{-2} (1.5×10^{-2})	4.4×10^{-2} (1.1×10^{-2})
Oleic acid (mmol/L)	2.02 (0.10)	2.05 (0.07)	2.12 (0.07)	2.05 (0.10)
LDL				
α-Tocopherol (μmol/mg apo B)	1.90×10^{-2} (0.49×10^{-2})	1.95×10^{-2} (0.58×10^{-2})	1.94×10^{-2} (0.53×10^{-2})	1.92×10^{-2} (0.51)
Phenolic compounds (nmol CAE /mg apo B)	7.88 (2.55)	9.16 (3.7)	9.55 (4.27)	10.44 (4.0)*#
Oleic acid (μmol/mg apoB)	0.31(0.14)	0.32 (0.12)	0.39 (0.14)*	0.39 (0.14)

# $P < 0.05$ for linear trend from refined to common to virgin olive oil.

* Denotes significant differences with baseline values ($P<0.005$)

Legends for figures

Fig 1. Time-line for the study design.

WO, wash-out; B, Baseline

Fig. 2 Phenolic content in LDL at the beginning of the study (baseline) and before and after each olive oil intervention.

The superscripts denote significant differences [a] ($P<0.005$) [b]($P<0.01$)

Fig 3. Mean (SD) levels of total cholesterol, HDL cholesterol, and triglycerides.

*Significant differences ($P=0.029$)

		Type of intervention (Week)					
Order	Baseline	1st washout (1-2 weeks)	1st olive oil intervention (3-5 weeks)	2nd washout (6-7 weeks)	2nd olive oil intervention (8-10 weeks)	3rd washout (11-12 weeks)	3rd olive oil intervention (13-15 weeks)
1	**B**	**WO**	Virgin	**WO**	Common	**WO**	Refined
2			Common		Refined		Virgin
3			Refined		Virgin		Common

Figure 1

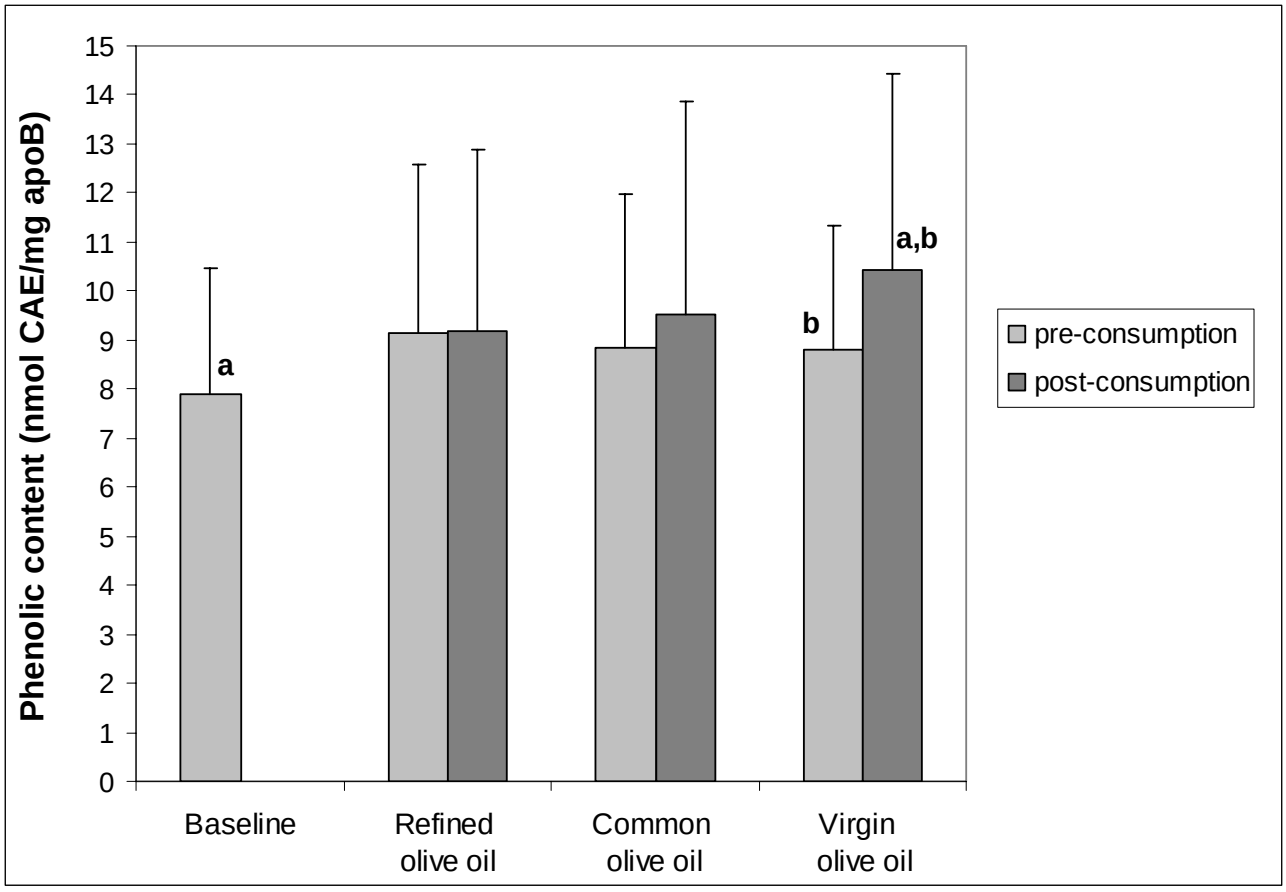
Phenolic content (nmol CAE/mg apoB)
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
a
b
a,b
Baseline
Refined olive oil
Common olive oil
Virgin olive oil
pre-consumption
post-consumption

Figure 2

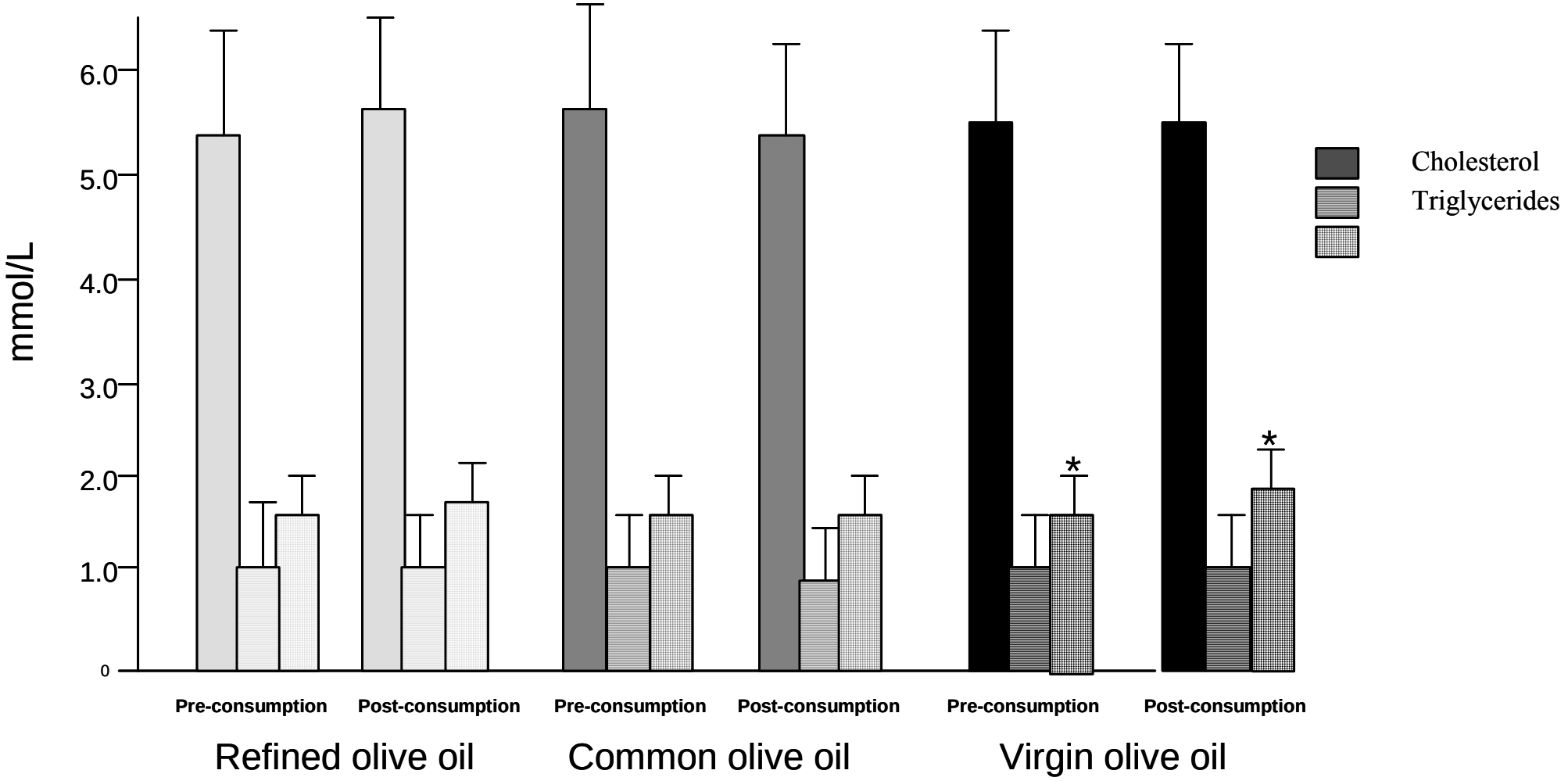
mmol/L
6.0
5.0
4.0
3.0
2.0
1.0
0
Cholesterol
Triglycerides
*
*
Pre-consumption
Post-consumption
Pre-consumption
Post-consumption
Pre-consumption
Post-consumption
Refined olive oil
Common olive oil
Virgin olive oil

Figure 3

Abbreviations used

apoB, apolipoprotein B; CAE, caffeic acid equivalents; CVD, cardiovascular disease; FAME, fatty acid methyl esters; HDL, high density lipoprotein; HPLC, high performance liquid chromatography; LDL, low density lipoprotein ; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

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IV. Resultados de la evaluación del índice de peróxidos de aceites utilizados en el estudio de EUROLIVE

ESTUDIO INTERNO DE ESTABILIDAD EUROLIVE

INDICE DE PERÓXIDOS

	ACEITE					
	A		B		C	
MESOS	NEVERA	CONGELADOR	NEVERA	CONGELADOR	NEVERA	CONGELADOR
T=0 (30/1/03)	5,70		12,8		3.2	
T=3 (30/4/03)	6,18	5,90	11,2	11,70	3,49	2,98
T=6 (30/7/03)	7,90	10,60	14,6	13,30	7,60	8,30
T=9 (30/10/03)	7,30	6,60	13,0	9,40	5,20	2,70
T=12 (30/1/04)	9,87	8,07	14,6	12,56	9,30	6,05

CÓDIGOS: *A-430 (COMERCIAL)*
B-580 (VIRGEN)
C-750 (REFINADO)

ESTUDIO EXTERNO DE ESTABILIDAD EUROLIVE

		ACEITES					
		INDICES DE PERÓXIDOS					
Centro de origen	**Fecha llegada**	**430**		**580**		**750**	
		media	sd	media	sd	media	sd
Berlín, Alemania (Charité)	19/12/2002	**7,2**	0,37	**12,5**	0,48	**4,2**	0,22
Charité, Alemania (Berlín)	14/04/2003	**6,5**	0,74	**11,1**	1	**3,6**	1,04
Itàlia	24/01/2003	**11,2**	0,08	**24,4**	0,98	**7,9**	0,74
Finlàndia	18/02/2003	**8,5**	0,49	**14,3**	1,24	**3,6**	0,09
Finlandia	25/03/2003	**8,29**	1,63	**9,1**	1,09	**3,02**	0,87
Finlandia	24/04/2003	**6,2**	0,51	**10**	0,83	**3,6**	1
Bergholz, Alemania	18/02/2003	**9,5**	0,16	**15,0**	0,93	**4,1**	0,01
IMIM	08/05/2003	**6,4**	0,49	**9,4**	0,18	**2,4**	0,15
IMIM	16/05/2003	**9,5**	0,22	**14,2**	0,23	**3,3**	0,84

CÓDIGOS *430 (COMERCIAL)*
580 (VIRGEN)
750 (REFINADO)

V. Presentaciones realizadas durante el doctorado

Poster:

"Characterisation and quantification of phenolic compouns in olive oils by Solid Phase Extraction, HPLC-Diode Array Detection and HPLC-MS Detecion". Durante el "1st International Conference on Polyphenols and Health". Del 18-21 de Noviembre de 2003. Vichy, Francia. Autores: K. de la Torre-Carbot, E. Gimeno, A. I. Castellote, R. M. Lamuela-Raventós, M. C. López-Sabater.

Characterisation and Quantification of Phenolic Compounds in Olive Oils by Solid Phase Extraction, HPLC-Diode Array Detection and HPLC-Mass Spectrometry Detection.

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Introduction

Phenolic compounds are an important group of natural compounds in plants (1). The main phenolic compounds in olive fruit are secoiridoid derivatives, phenyl acids, phenyl alcohols and flavonoids (2). Phenolic content of virgin olive oil is affected by the variety, location and degree of ripeness (2-4). Phenolic compounds present in olive oil are basic to its quality and nutritional properties (5-8). However, phenolic compounds are removed when the oil is refined (9).

Traditionally, the phenolic fraction of olive oil has been isolated by liquid-liquid extraction (10). Nevertheless, this method is laborious. Some attempts to isolate these compounds by solid-phase extraction (SPE) have been made (11,12), but this fraction has not fully obtained when cartridges are used (13).

Total phenolic compounds in oil are determined quantitatively mainly by the Folin-Ciocalteau method. However, this method is nonspecific (14).

Phenolic compounds usually founded in olive oil.	
Simple phenols	Hydroxytyrosol
	Tyrosol
	Vainillic acid
	Cafeic acid
	Vainillin
	Sirigic acid
	p-Coumaric acid
	o-Coumaric acid
	Ferulic acid
	Hyroxytyrosol acetate
	Tyrosol acetate
	Elenolic acid (4 forms)
Oleuropein family	Oleuropein aglicon (4 forms)
Ligstroside family	Ligstroside aglicon (4 forms)
Lignans	1-hydroxypinoresinol
	Pinoresinol
	1-acetoxypinoresinol
Flavonoids	Luteolin
	Apigenin
	Metoxiluteolin

Objective

The aim of this study was 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.

Experimental

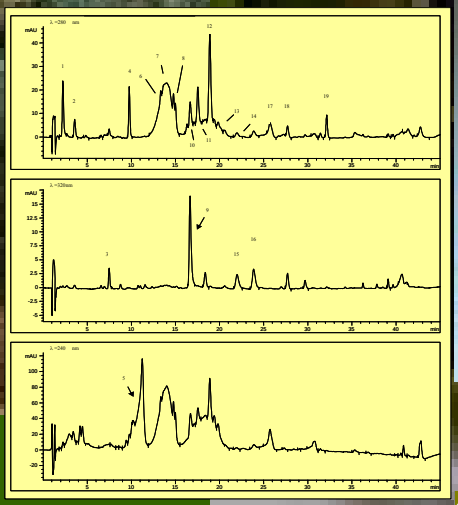
Extraction: the polar fraction was obtained from small amounts of oil sample, using a SPE diol cartridge, washing with *n*-hexane to eliminate apolar compounds, and eluting with a combination of methanol, water and acetonitrile,

Chromatographic conditions HPLC-DAD
Aparatus: Hewlett Packard-1050 Series liquid chromatograph with an automatic injector and DAD 1050 M coupled with a Chemstation HP.
- Column: 5-µm particle size C_{18} Luna column, 15 cm x 2.00 mm I.D.
- λ=280, 240 and 320 nm.
- Mobile phase: acidulated water and acetonitrile.
- Total time: 35 minutes.

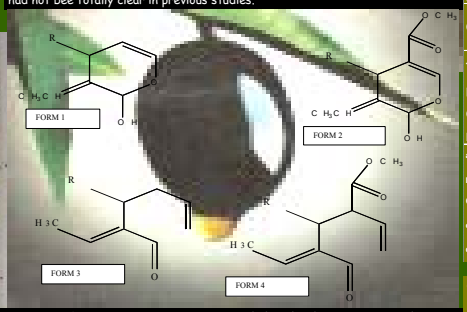
Chromatographic conditions HPLC-MS.
Aparatus: Waters-2690 HPLC (Waters Chromatography, Mildford, MA. USA) with a quadrupole Platform II MS (Micromas, Manchester, UK) equipped with APCI in the negative-ion mode.
- Nebulising gas: nitrogen
- Temperature: 400ºC.
- Corona voltage: 2.5 kV
- Extraction voltage: -30 V.
- Mass range of *m/z*: 100-700.

Results

Eighteen compounds were identified and quantified



Four basic forms of ligstroside and oleuropein aglycons were elucidated, which had not bee totally clear in previous studies.



FORM	R=H (elenolic acid)	R = hydroxytirosol (oleuropein family)	R = tyrosol (ligstroside family)
1.- Decarboxylated form, closed ring	EDA	3,4-DHPEA-EDA	p-HPEA-EDA
2.- Complete form, closed ring	EA	3,4-DHPEA-EA	p-HPEA-EA
3.- Dialdehiyic-decarboxylate form, open ring	EDiDA	3,4-DHPEA-EDiDA	p-HPEA-EDiDA
4.- Dialdehydic form, open ring	EDiA	3,4-DHPEA-EDiA	p-HPEA-EDiA

Quantification: Phenolic compounds were divided and expressed each group with a representative compound.

Method validation

Table 1
Repeateability (n=6) and reproducibility (n=12)

Compound	Repeatability Mean (ppm)	Repeatability RSD (%)	Reproducibility Mean (ppm)	Reproducibility RSD (%)
1 Hydroxytyrosol	7.14	6.99	7.84	7.48
2 Tyrosol	2.99	4.11	2.90	9.49
3 Elenolic acid derivative	0.11	2.15	0.10	2.69
4 p-Coumaric acid	18.28	4.28	18.86	4.59
5 Elenolic acid	405.19	4.05	403.56	4.87
6 Dihaldehydic form of elen(	48.07	4.83	50.74	5.61
7 3,4-DHPEA-EDA	115.25	5.62	119.24	6.81
8 p-HPEA-EDA	17.44	6.56	19.38	8.70
9 Luteolin	3.30	4.56	3.42	5.34
10 3,4-DHPEA-EDiDA	11.66	6.31	11.70	7.56
11 p-HPEA-EDiDA	18.03	6.59	18.15	6,80
12 Pinoresinol	6,70	4.53	6.59	5.42
13 10-hydroxyoleuropein	2.25	5.02	2.27	5.37
14 10-hydroxyligstrosid	3.36	4.88	3.32	5.64
15 Apigenin	1.49	5.32	1.50	5.92
16 Methoxiluteolin	1.40	4.65	1.48	8.52
17 3,4-DHPEA-EDiA	12.92	3.63	14.40	8.59
18 p-HPEA-EDiA	6.16	4.87	6.45	5.01
19 Unknown	9.81	2.89	9.90	2.90

Table 2
Linearity, sensitivity and recovery studies.

Compound	Linear Regresion[b]	r[a]	LOD[c] (ppm)	LOQ[d] (ppm)	Recovery mean (%)
Tyrosol	y = 22.01 x - 5.08	0.999	0.28	0.39	115
p-Coumaric acid	y = 270.52x - 0.83	0.999	$7x10^{-3}$	0.02	105
Oleuropein	y = 8.36 x - 3.62	0.999	2.06	2.36	76
Luteolin	y=70.123x - 90.37	0.999	0.34	0.40	103

[a] correlation coefficients of the regression equation

[b] y = ax + b where x is the phenolic compound concentration (ppm) and y is the peak area

[c] LOD = Limit of Detection

[d] LOQ = Limit of Quantification

Conclusions.

- We have developed a simple and reproducible SPE, HPLC-DAD and HPLC-MS method to characterise and quantify the phenolic compounds present in virgin olive oil.
- The method proposed is faster and recovers the phenolic compounds better than most of the previous methods described in the literature.
- The method is suitable for routine analysis of different types of olive oil.

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Poster:

"Levels of Total Phenolic Compounds in LDL After Olive Oil Consumption" Durante el "3rd Euro Fed Lipid Congress". Del 5-8 de septiembre de 2004, Edimburgh University, Scotland. Autores: K. de la Torre-Carbot, S. Morera, J. L. Chávez, A. I. Castellote, R. M. Lamuela-Raventós, M.I. Covas, M. Fitó, M. Farré-Albaladejo, M. C. López- Sabater.

Levels of Total Phenolic Compounds in LDL After Olive Oil Consumption

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INTRODUCTION

There is increasing evidence that oxidative modification of low density lipoprotein (LDL) plays a key role in the development of atherosclerosis (1). On the other hand the effect of dietary fatty acids and antioxidants on the resistance of lipoprotein to oxidation is well-known (2-4).

Virgin olive oil, obtained exclusively by physical procedures, contains high amounts of antioxidants, mainly phenolic compounds and vitamin E and its principal fatty acid present is oleic acid, which is less susceptible to oxidation.

In animal and in vitro studies, olive oil phenolic compounds have been shown to be strong antioxidants (5-7).

In this study, the bioavailability and capacity of olive oil phenolic compounds to bind LDL were evaluated. Other objective was to evaluate in what moment the highest incorporation of phenolic compound to the LDL is produced

EXPERIMENTAL

SUBJECTS: 12 healthy male volunteers of 20 a 22 years and a 22,9 media of body mass index. Three different types of olive oil were used: with a scarce (refined), medium (common) and high (virgin) phenolic compounds content. All of them with similar quantities of oleic acid, vitamin E and other minority components.

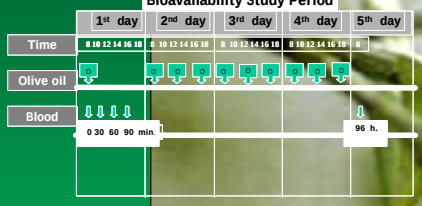
Two Latin squares of 3 x 3 for the three different treatments were used in the crossover clinical trial to randomise participants into six orders of olive oil administration. Prior to each intervention volunteers followed a 7-day washout period. Refined olive oil was the only source of fat for raw and cooking purposes during the washout period and for cooking purposes during intervention periods.

At 8 a.m. on day 1, volunteers were provided with 25 ml of one of the three olive oils. Olive oil was the sole source of phenols during the following 24 h. On day 2, 3, and 4, subjects received the same olive oil dose per day, but distributed among meals. Venous blood was collected at baseline, at 30, 60, 90 minutes, and at day 5 at fasting state.

The voluntiers were interned in the Lipid and Cardiovascular Epidemiology Unit of the Municipal Institute of Medical Investigation (IMIM),Barcelona, Spain, where the meals were supplied and venous blood was collected.

During these days a nutritionist controlled and instructed them on excluding several foods, rich in phenolic compounds from their diets

Bioavailability Study Period



LABORATORY MEASUREMENTS

- **Fatty acids in LDL** *(Bondía et al, 1994)*
- **Alfa-tocopherol in LDL** *(Gimeno et al, 2001)*
- **Phenolic compounds in LDL** *(Lamuela et al, 1999)*

RESULTS

Content in fatty acids and α-tocopherol in LDL at baseline and after the 5th day olive oils consumption.

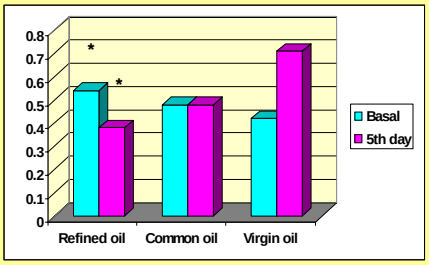
	Baseline	Post-refined	Post-common	Post-virgin
Oleic acid (μg/mg apo B)	235.64 (101.27)	246.19 (106.75)	268.22 (133.32)	222.6 (69.77)
Palmitic acid (μg/mg apo B)	210.19 (86.43)	210.83 (83.24)	219.36 (99.78)	184.44 (52.59)
Stearic acid (μg/mg apo B)	67.55 (24.05)	69.12 (30.18)	85.67 (45.02)	63.42 (20.45)
Linoleic acid (μg/mg apo B)	425.44 (132.62)	479.20 (201.41)	492.46 (193.74)	416.17 (121.72)
Arachidonic acid (μg/mg apo B)	74.38 (28.62)	75.02 (29.78)	78.86 (33.56)	66.16 (27.22)
α-tocopherol (μg/mg apo B)	6.94 (0.37)	7.44 (1.76)	7.63 (1.48)	6.98 (1.16)

Content in phenolic compounds (ng/g total fatty acids) in LDL after a consumption of 25ml of different types of olive oil.

	0 min.	30 min.	60 min.	90 min.	P for linear trend
Refined olive oil	0.54* (0.25)	0.44 (0.21)	0.46 (0.23)	0.37* (0.14)	0.025
Common olive oil	0.48 (0.28)	0.38 (0.18)	0.49 (0.23)	0.38 (0.16)	0.280
Virgin olive oil	0.42 (0.23)	0.43 (0.17)	0.58 (0.48)	0.56 (0.31)	0.094

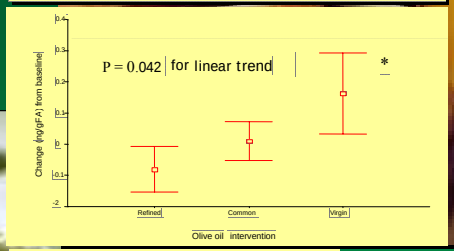
* Denotes significant differences (p<0.05)

Content in phenolic compounds (ng/g total fatty acids) in LDL before and after 5th day olive oils intervention.



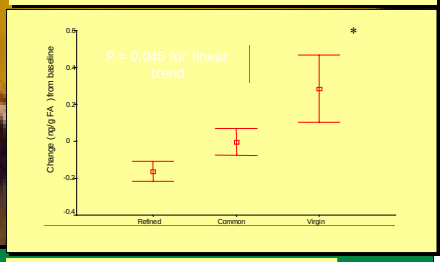
*Denotes significante differences (p<0.05)

Change in levels of the phenolic compounds in LDL at 60 minutes after olive oils intervention



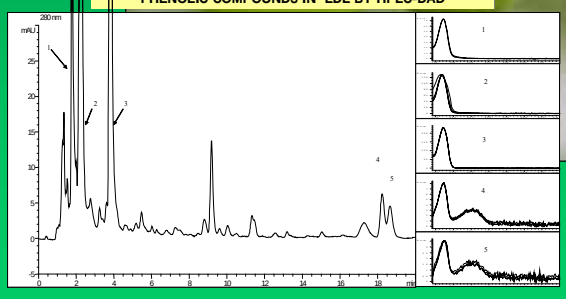
* Denotes significant differences (p<0.05) as compared to baseline

Change in levels of the phenolic compounds in LDL at the 5th after olive oils intervention



* Denotes significant differences (p<0.05) as compared to baseline

PHENOLIC COMPOUNDS IN LDL BY HPLC-DAD



CONCLUSIONS

- The total phenolic content of LDL increased in a dose dependent manner with the phenolic content of the olive oil administered.
- The significant decrease in the phenolic content of LDL after a consumption of refined olive oil (90 minutes and 5th dat) may be caused for a strict phenolic compounds-low diet.
- Phenolic compounds in LDL had a linear tendency to increase after 60 minutes of consumption of virgin olive oil compared to baseline values.
- Phenolic compounds in LDL had a linear tendency to increase after the 5th day of virgin olive oil consumption compared to baseline values. Consecutively, an accumulative effect was observed.
- Thus, the beneficial effect of olive oil may be due not only to their monounsaturated matrix, but also to the combined effects of these antioxidants compounds.

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Presentación oral del trabajo:

"Caracterización y Cuantificación de los Compuestos Fenólicos en Aceites de Oliva Vírgen por Cromatografía Líquida de Alta Eficacia y Espectrometría de Masas". Durante el Congreso Internacional sobre Aceite de Oliva y Salud, CIAS 2004. Del 21 al 23 de octubre de 2004, Jaén, Andalucía, España. Autores: K. de la Torre-Carbot, O. Jaúregui, E. Gimeno, A. I. Castellote, R. M. Lamuela-Raventós, M. C. López-Sabater.

CIAS

2004

Congreso Internacional sobre **Aceite de Oliva y Salud**

Karina de la Torre Carbot

Los compuestos fenólicos contenidos en el aceite de oliva, han recibido considerable atención en los últimos años, ya que son esenciales para la calidad y propiedades nutritivas de este alimento. Estos compuestos están relacionados con la estabilidad del aceite y con propiedades sensoriales como el color y la astringencia. Además, varios estudios han sugerido que dichos compuestos pueden ofrecer un papel protector contra afecciones como las enfermedades cardiovasculares y el cáncer. La composición en compuestos fenólicos del aceite de oliva, se ve afectada por las condiciones ambientales del cultivo, la variedad y grado de maduración del fruto y el tipo de extracción del aceite. En este estudio se presentan los perfiles fenólicos de aceites de oliva virgen. Para este fin, se realizó la extracción de dichos compuestos en fase sólida, utilizando hexano para eliminar los compuestos apolares y usando metanol:agua y acetonitrilo como eluyentes. El extracto fue analizado por medio de cromatografía líquida de alta eficacia con detector de fotodiodos (HPLC-DAD), y espectrometría de masas con presión atmosférica de ionización en modo negativo (LM-MS-APCI).

Los secoiridoides conformaron el mayor porcentaje de los compuestos fenólicos totales. Dichos compuestos son derivados de la oleuropeina, demetiloleuropeina y glucósidos del ligstrósido que se encuentran en el fruto. Durante el proceso de extracción del aceite de oliva, los enlaces glucosídicos se rompen y los aglicones pasan al aceite, también ocurren otras modificaciones parciales, lo cual genera isoformas en la estructura elenólica, pero conservando el anillo fenólico. El resto de los compuestos fenólicos minoritarios, están formados por flavonoides, ácidos fenólicos y otros compuestos no carboxílicos.

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Poster:

“Determination and Quantification of Virgin Olive Oil Phenolic Metabolites in Human Low Density Lipoproteins by HPLC-MS/MS” Durante las “JAI”, 2005, del 15 al de noviembre 2005, Barcelona. Autores: K. de la Torre-Carbot, J. L. Chávez-Servín, O. Jauregui, A. I. Castellote, R. M. Lamuela-Raventós, M. I. Covas, M.C. López-Sabater.

Determination and Quantification of Virgin Olive Oil Phenolic Metabolites in Human Low Density Lipoproteins by HPLC-MS/MS

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Introduction

LDL peroxidation is an essential step in the development of atherosclerosis, and numerous studies have shown that olive oil phenols are potent inhibitors of LDL oxidation. It has been detected glucoronide, sulphate and methyl conjugates of hydroxytyrosol and tyrosol in plasma and urine in human experiments.

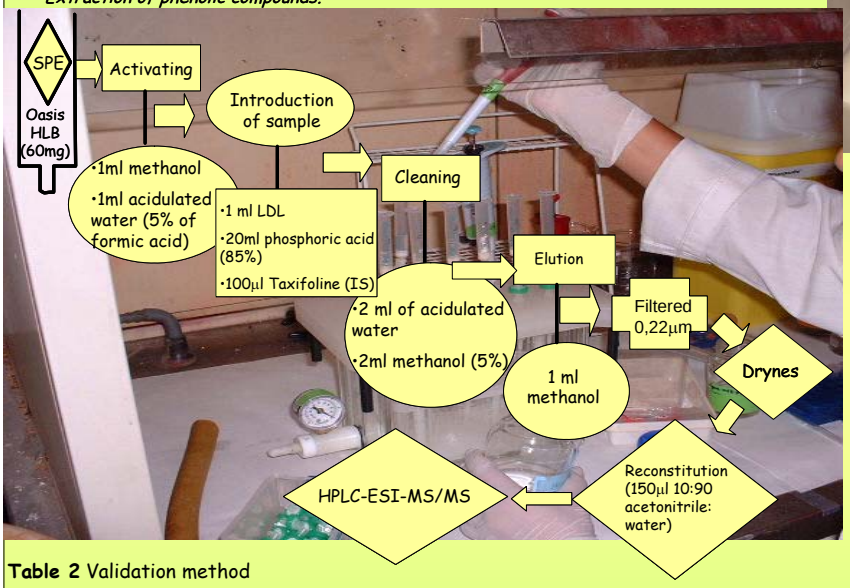
Objective

In this study we report the development and validation of an analytical method for quantitative determination of some olive oil phenolic compounds and posible metabolites in LDL

Experimental

Sample conditions. Comercial LDL with known concentrations of hydroxytyrosol and homovainillic acid standards were used to carry out the validation assays.

Extraction of phenolic compounds.



HPLC-ESI-MS/MS conditions and quantification.

Equipment: Agilent 1100HPLC (Waldbronn, Germany) API3000 triple-quadrupole MS

Column:C18 Luna, 5 cm x 2 mm I.D. 3 µm particle size (Phenomenex, UK).

Temperature column: 40°C

Mobil phase: binary solvent acidified water (acid formin 0.1%) as fase A and acetonitrile as fase B

Injection volume: 20µl

Sourse type: Turbo Spray

Source temperature: 300°C

Gradient

Time (min)	Fase A	Fase B
1	95	5
3	85	15
5	85	15
6	100	0
7	100	0

Table 2 Validation method

Compound	LOD[a] (ng/ml)	LOQ[b] (ng/ml)	% Recovery	Linearity	Repetibility CV	Reproducibility CV
Hydroxytyrosol	0,48	1,61	75	>0.99	<4	<7
Homovainillic acid	4,44	14,81	90	>0.99	<6	<8

[a]LOD=Limit of Detection
[b]LOQ=Limit of Quantification

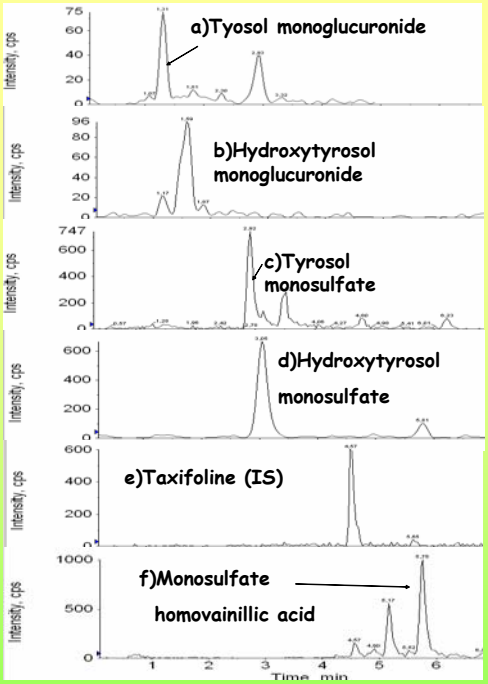
APLICATION

Table 1 Metabolites found in LDL 60 minutes post-consumption of 50 ml extra virgin olive oil.

Metabolite	Q1/Q3	RT	LDL concentration (ng/mL)
Hydroxytyrosol monoglucuronide	329/153	1.32	24.27
Hydroxytyrosol monosulfate	233/153	1.59	NQ
Tyrosol monoglucuronide	313/137	2.91	14.87
Tyrosol monosulfate	217/137	3.03	14.87
Monosulfate, homovainillic acid	261/181	5.77	46.96

[a]No quantificable (NQ)

APLICATION



Conclusions

An analytical method for determine olive oil phenolic compounds and possible metabolites in LDL has been developed. The simultaneous quantification method using HPLC-ESI-MS/MS is specific, sensitive and accurate for the determination of these components and to investigate their bioavailability and metabolism, in view of their consideration as potential antioxidant agents in LDL. This method is suitable and can be used in further studies and investigations.

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