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

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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 C18 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. © 2006 Elsevier B.V. All rights reserved.

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 [3–9,13,19,35,36]. However, characterizations of phenolic compounds metabolites in LDL remain scarce [37]. Moreover, existing studies were carried out after enzymatic hydrolysis and fail to provide any information about metabolic profiles [9,17].
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, syringaldehyde, 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 Extrasynthese (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 (Waldborn, 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 C18 Luna column, 5 cm × 2.0 mm I.D. with a C18, 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 1.98 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 5 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 (7 <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-
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 was adjusted 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 multiplex 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 ion-spray 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 (N2) 10 (arbitrary units), curtain gas (N2) 12 (arbitrary units), collision gas (N2) 4 (arbitrary units), DP and CE for MRM and double MRM experiments are shown in Table 1. Focusing potential was calibrated so that mass accuracy specifications and sensitivity were achieved over the entire mass range. Prior to its use, the instrument was checked to meet the specifications and tolerances. Moreover, 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 C18 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. Shown 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 homovanillic acid. The entire process was performed under conditions of darkness using brown glass material.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Transitions</th>
<th>DP (V)</th>
<th>CE (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxytyrosol</td>
<td>153 → 123</td>
<td>−30</td>
<td>−20</td>
</tr>
<tr>
<td>Homovanillic acid</td>
<td>181 → 137</td>
<td>−25</td>
<td>−15</td>
</tr>
<tr>
<td>Taxifolin</td>
<td>305 → 281</td>
<td>−30</td>
<td>−30</td>
</tr>
<tr>
<td>Hydroxytyrosol monoglucuronide</td>
<td>329 → 153, 153 → 123</td>
<td>−25, −30</td>
<td>−25, −15</td>
</tr>
<tr>
<td>Hydroxytyrosol monosulphate</td>
<td>233 → 153, 153 → 123</td>
<td>−25, −30</td>
<td>−25, −15</td>
</tr>
<tr>
<td>Tyrosol glucuronide</td>
<td>313 → 137, 137 → 93</td>
<td>−10, −10</td>
<td>−25, −25</td>
</tr>
<tr>
<td>Tyrosol sulphate</td>
<td>217 → 137, 137 → 93</td>
<td>−10, −10</td>
<td>−25, −25</td>
</tr>
<tr>
<td>Homovanillic acid sulphate</td>
<td>261 → 181, 181 → 137</td>
<td>−25, −30</td>
<td>−20, −15</td>
</tr>
</tbody>
</table>

* Declustering potential.

† Collision energy.
fic 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 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 analyzing 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 satisfactorily covered by the polar “range” in which eluted process is carried out.

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

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 CH2OH 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 CH2OH 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 glucuronides 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

![Image](380x643 to 578x773)

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

<p>| Table 2 | Metabolites found in LDL 60 min post-consumption of 50 mL extra virgin olive oil |
|---------|---------------------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Q1/Q3 transition (m/z)</th>
<th>LDL concentration (mg/g ApoB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxytyrosol monoglucuronide</td>
<td>329/153</td>
<td>1.59</td>
</tr>
<tr>
<td>Hydroxytyrosol monosulfate</td>
<td>253/153</td>
<td>3.03</td>
</tr>
<tr>
<td>Tyrosol glucuronide</td>
<td>313/157</td>
<td>1.32</td>
</tr>
<tr>
<td>Tyrosol sulfate</td>
<td>237/157</td>
<td>2.91</td>
</tr>
<tr>
<td>Homovanillic acid sulfate</td>
<td>261/157</td>
<td>5.77</td>
</tr>
</tbody>
</table>

* Monitored transition for each metabolite analysis (Q1/Q3) in quantification process.

Retention time.
Table 3

<table>
<thead>
<tr>
<th>Compound</th>
<th>Intra-day precision RSD (%) (n = 10)</th>
<th>Inter-day precision RSD (%) (n = 30)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 ng/mL</td>
<td>100 ng/mL</td>
<td>500 ng/mL</td>
</tr>
<tr>
<td>Hydroxytyrosol</td>
<td>3.72</td>
<td>1.49</td>
<td>1.78</td>
</tr>
<tr>
<td>Homovanillic acid</td>
<td>7.51</td>
<td>4.31</td>
<td>2.46</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>LOD a (ng/mL)</th>
<th>LOQ b (ng/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxytyrosol</td>
<td>0.24</td>
<td>0.81</td>
<td>81.9</td>
</tr>
<tr>
<td>Homovanillic acid</td>
<td>3.05</td>
<td>10.18</td>
<td>101.3</td>
</tr>
</tbody>
</table>

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

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 to 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

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