Simultaneous determination of phenolic compounds and tocopherols in virgin olive oil using HPLC and UV detection

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Abstract

A simple procedure is described for the simultaneous extraction and HPLC determination of phenols and tocopherols in virgin olive oil. The extraction was carried out using methanol and an isopropanol-methanol mixture. Separation was achieved on a reversed phase C18 column with acetic acid/water-methanol-acetonitrile-isopropanol mixture under gradient elution. Detection was accomplished with UV detection at $\lambda = 280$ nm. Using this method, simple and complex phenols as well as $\alpha$-tocopherol can be determined in one run. Phenolic compounds were totally extracted whereas an average recovery of 80% was achieved for $\alpha$-tocopherol. Validation of method is reported for olive oil samples with varying concentrations of $\alpha$-tocopherol and phenolic compounds.

Keywords: Virgin olive oil; Extraction; HPLC; Phenolic compounds; $\alpha$-Tocopherol

1. Introduction

Virgin olive oil produced from olives of good quality is consumed unrefined. Thus virgin olive oil contains phenolic compounds which are usually removed from other vegetable oils during various stages of refining. In virgin olive oil, $\alpha$-tocopherol dominates with its composition reaching 95% of the total tocopherol (Boskou, 1996). Both phenols and tocopherols contribute to the remarkable stability of the oil (Papadopoulos & Boskou, 1991; Satue, Huang, & Frankel, 1995). $\alpha$-Tocopherol and phenolic compounds such as oleuropein have been reported as having beneficial biological activity (Visioli & Galli, 1994; Visioli, Bellomo, Montedoro, & Galli, 1995). The isolation and quantitation of the above compounds are therefore of high importance.

Quantitative determination of phenolic compounds in oil is usually performed according to Folin-Ciocalteau colorimetric method (Gutfinger, 1981). However, this method is not specific, as it gives no indication of the nature of the phenolic compounds present. Separation and identification of individual phenolic compounds has been performed by gas-liquid chromatography (GLC) and mass selective detector (Angerosa, d’Alessandro, Konstantinou, & Giacinto, 1995). One problem associated with GLC is that non-volatile phenolic compounds require derivatization prior to the quantitation step. Reversed-phase HPLC currently represents the most popular and reliable technique for the analysis of phenolic compounds. The technique has been mainly used with UV detection (Montedoro, Servili, Baldioli, & Mimiti, 1992a; Pirisi et al., 1997). Amperometric detection has also been used (Akasbi, Shoeman, & Csallany, 1993).

Quantitative determination of tocopherols is commonly performed by normal or reversed phase HPLC (Wahyuni & Jinno, 1988). Normal phase chromatography is advantageous for resolving $\beta$- and $\gamma$-tocopherols which cannot be resolved by RP-HPLC. Recently however, separation of $\alpha$-, $\beta$-, $\gamma$-, and $\delta$-tocopherols has been achieved with an octadecyl polyvinyl alcohol column. (Abidi & Mounts, 1997). HPLC detectors used to measure tocopherols include ultraviolet, fluorescence and amperometric (Carpenter, 1979; Dionisi, Prodolliet, & Tagliaferri, 1995; IUPAC, 1987). Fluorescence detection is more sensitive and selective than UV detection (Hoehler, Frohlich, Marquardt, & Stelsovsky, 1998). However, it is not suitable for detecting the ester form of $\alpha$-tocopherol which fluoresce to a much lesser extent than free $\alpha$-tocopherol. A saponification step is therefore needed to convert $\alpha$-tocopherol esters to their free alcohol form with the risk of partial loss of tocopherols.

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Reports on simultaneous analysis of phenolic substances and tocopherols by HPLC (Andrikopoulos, Brueschweller, Felber, & Taeschler, 1991; Indyk & Woollard, 1986) dealt with synthetic phenolic antioxidants and tocopherols either in standard mixtures or directly in oil samples with no prior extraction or clean up. With direct injection, most compounds of lower concentrations and extinction coefficients cannot be detected. No reference was found in the literature on the simultaneous determination of natural phenolic compounds and tocopherols after their co-extraction from vegetable oils.

The aim of this study was to develop a simple method for the simultaneous determination of phenols and tocopherols in virgin olive oil by HPLC with UV detection. Extraction with various solvent mixtures was investigated during attempts to increase both phenolic and \( \alpha \)-tocopherol concentrations of extracts and to facilitate their detection using UV detector. A reversed phase C18 column was chosen since \( \beta \)- and \( \gamma \)-tocopherols, which cannot be resolved on RP C18 column, are negligible in quantity in virgin olive oil.

2. Materials and methods

2.1. Materials

Extra virgin olive oil samples from Lianolia variety olives grown in the region of Preveza, Greece were used. The samples were filtered and stored under \( \text{N}_2 \) below \( \text{C}0\text{C} \) prior to analysis.

2.2. Reagents and standards

Acetonitrile, methanol, hexane, isopropanol (2-propanol) filtered through a 1.5-\( \mu \)m filter, acetic acid, and water were all of HPLC grade and were purchased from Merck (Darmstadt, Germany). Methanol and hexane for oil extraction were pro-analysis grade and were purchased from Merck (Darmstadt, Germany). The standards dl-\( \alpha \)-tocopherol and \( \beta \)-tocopherol were purchased from Merck (Darmstadt, Germany); caffeic acid was purchased from Merck-Schuchardt (Hohenbrunn, Germany); tyrosol, syringic acid, and ferulic acid were purchased from Sigma-Aldrich (Steinheim, Germany); and triolein was purchased from Sigma Chemical Company (St.Louis, Mo. USA).

2.3. Extraction of phenolic compounds

Phenolic compounds were extracted from virgin olive oil according to the method described by Gutfinger (1981). Oil (10 g), were dissolved in 50 ml hexane and the solution was extracted successively with three 20-ml portions of 60% aqueous methanol. The combined extracts were brought to dryness in a rotary evaporator at \( 40\text{C} \) and the residue was dissolved in 5 ml methanol, and submitted to chromatographic analysis.

2.4. Co-extraction of phenolic compounds and tocopherols

Samples of 10 g extra virgin olive oil were extracted at room temperature with two 25-ml portions of absolute methanol. The residue was extracted again under the same conditions with two 25-ml portions of methanol/isopropanol (80:20, v/v). The extracts were combined and brought to dryness in a vacuum rotary evaporator at \( 40\text{C} \). The residue was dissolved in 5 ml of a methanol/isopropanol/hexane mixture (1:3:1, v/v/v) and analysed for phenolic and tocopherol content by HPLC.

2.5. HPLC apparatus

A Shimadzu model HPLC system (Shimadzu Corporation, Kyoto, Japan) consisting of a solvent delivery module (LC-10AD) with a double plunger reciprocating pump, UV-VIS detector (SPA-10A), column oven (CTO-10A) and 20-\( \mu \)l injection loop was used. The column used was an Apex octadecyl 104 C18 (25\( \times \)0.4 cm ID) with 5-\( \mu \)m packing (Jones Chromatography limited, Colorado, USA).

2.6. HPLC conditions

Detection was performed at 280 nm for both phenols and \( \alpha \)-tocopherol. The elution solvents used were A (2% acetic acid in water), B (methanol), C (acetonitrile) and D (isopropanol). The samples were eluted according to the following gradient: 95% A/5% B in 2 min; 60% A/10% B/30% C in 8 min; 25% B/75% C in 22 min, and this percentage was maintained for 10 min; 40% C/60% D in 10 min; and this percentage was maintained for 15 min; 25% B/75% C in 2 min, and finally, 95% A/5% B in 3 min. Flow rate was 1 ml/min and run time, 70 min. The run was performed at 32\( \text{C} \). The sample injection volume was 20 \( \mu \)l. Identification of compounds was achieved by comparing their retention time values with those of standards. Data was collected and processed using Class-VP Chromatography Laboratory Automated software (Shimadzu Corporation).

2.7. Quantitation and recovery of \( \alpha \)-tocopherol and phenols

The initial \( \alpha \)-tocopherol content of samples was determined by diluting approximately 200 mg of olive oil in 1 ml methanol/isopropanol/hexane mixture (1:3:1, v/v/v) and analyzing the sample solution by HPLC. Concentrations of initial \( \alpha \)-tocopherol contents were then calculated from integrated peak areas of the
samples and the calibration curve of $\alpha$-tocopherol standard. Good linearity was achieved in the range 50–400 mg kg$^{-1}$ ($r^2 = 0.999$).

Recovery of $\alpha$-tocopherol was determined by subjecting olive oil samples to extraction and by comparing extracted amounts of $\alpha$-tocopherol to the initial amounts determined above (without extraction). Six olive oil samples of different initial $\alpha$-tocopherol content were used. Extraction was performed as described in Section 2.4, and analysis was performed by HPLC.

Recovery study was also carried out for hydroxytyrosol derivative and tyrosol derivative. Both compounds were the most abundant phenols present in all samples, and could be detected (and therefore quantified) directly in oil samples without any extraction. Recovery of the above compounds was determined by comparing the amounts of phenols extracted from oil samples, to the amounts found prior to extraction. Three replicates were carried out for each sample. Means with standard deviations are reported.

3. Results and discussion

3.1. HPLC analysis

Eluting mixture of acetic acid/water-methanol-acetonitrile-isopropanol was tested at various compositions in an attempt to resolve the extracted phenols and $\alpha$-tocopherol by HPLC. The composition of the eluent was repeatedly adjusted until satisfactory resolution was achieved. Fig. 1 shows the separation of phenolic compounds extracted from oil/hexane solution using 60% aqueous methanol, while Fig. 2 depicts representative chromatograms of original olive oil sample. Simple (peaks 1–4) and complex (peaks 5–10) phenolic compounds were eluted first with a mobile phase mixture of acetic acid/water-methanol-acetonitrile. Retention times for both simple and complex phenols varied between 10.3 min and 21.1 min. Secondly, less polar tocopherols (peaks 11 and 12, RT: 42.2 min and 43.6 min, respectively) were eluted with the use of less polar methanol/acetonitrile mixture and finally, triglycerides (RT: 52.8–55.6 min) were eluted using acetonitrile/isopropanol as the mobile phase. In all samples analysed, the most abundant compounds were those of peak 5 (RT: 14.8 min) and peak 6 (RT: 16.3 min). These compounds have a complex phenolic nature and correspond to a hydroxytyrosol derivative and a tyrosol derivative (Okogeri & Tasioula-Margari, 2000).

A solvent mixture consisting of methanol-isopropanol-hexane 1:3:1 (v/v/v) was used for preparing samples prior to injection. The mixture was developed on the basis of solvent efficiency. UV detection fixed at $\lambda = 280$ nm was used for both phenols and tocopherols. Detection of virgin olive oil phenols is usually achieved at $\lambda = 280$ nm even though this is not the maximum wavelength for all the phenols (Tsimidou, Papadopoulos, & Boskou, 1992). Tocopherols detection has also been carried out at 295 nm (Carpenter, 1979). However, detection at 280 nm may be a compromise because most phenols and tocopherols absorb considerably at this wavelength. $\gamma$- And $\beta$-tocopherols overlap on a RP C$_{18}$

Fig. 1. Reversed phase HPLC separation of virgin olive oil phenolic compounds extracted from oil/hexane solution using 60% aqueous methanol. $\lambda = 280$ nm. Peak numbers: (1) hydroxytyrosol; (2) tyrosol; (3) vanillic acid; (4) syringic acid; (5) hydroxytyrosol derivative; (6) tyrosol derivative; (7–10) complex phenolic compounds. Sensitivity 0.01.
column but their content in olive oil is very low. Interest was therefore focused on \( \alpha \)-tocopherol, which is the main tocopherol species in virgin olive oil.

3.2. Simultaneous extraction and recovery studies

Olive oil phenols are usually extracted with water/methanol mixtures from hexane solutions. The advantage of aqueous methanol extraction of antioxidants from hexane solutions is that the oil is mostly excluded. On the other hand, extraction of tocopherol cannot be achieved from oil/hexane solution as tocopherol is retained in the hexane solution. Extraction was consequently applied directly to oil samples without dissolution in hexane. Isopropanol/methanol mixtures of varying composition were tested. Representative chromatograms of olive oil extract is shown in Fig. 3. In comparison to Fig. 2, it can be observed that phenolic compounds (peaks 1–10) and \( \alpha \)-tocopherol (peak 12) were remarkably concentrated and therefore can be quantitated with higher precision.

In attempts to achieve higher extraction yield, samples were subjected to a total of six extractions, using three portions of methanol and three portions of methanol/isopropanol mixtures of increasing polarity (Table 1). It was observed that the amounts of \( \alpha \)-tocopherol extracted were not remarkably affected by variations in methanol/isopropanol ratio. Using IPA/MeOH...
ratios of 10:90 (v/v) the lowest amount (56 mg kg\(^{-1}\)) of \(\alpha\)-tocopherol was extracted, whereas using 20:80 and 30:70 (v/v), the highest amount was extracted (59 mg kg\(^{-1}\); Table 1). However, the amount of oil transferred to the extracts increased as the volume of isopropanol in methanol increased. Hence, extraction with an isopropanol/methanol ratio of 20:80 (v/v) was used for further analyses due to the relatively high recovery yield (86%) and lower amount (3.8%) of oil transferred to extracts.

Further recovery studies carried out to ensure the effectiveness of the extraction procedure showed that four extraction steps, two with absolute methanol and two with isopropanol/methanol ratio of 20:80 (v/v) gave a recovery yield as high as 85% for \(\alpha\)-tocopherol (Table 2) and >98% for the main phenolic compounds corresponding to peaks 5 and 6 (Table 3). As can be seen from Tables 2 and 3, the reproducibility of the method is satisfactory with relative standard deviations being less than 6% in all cases. Four extraction steps were therefore considered satisfactory and were carried out in further analyses. It should also be noted that with the exclusion of water from the extraction solvents, the formation of an emulsion between oil and water-methanol layer, which can adversely effect recovery yield, was prevented.

Table 1
Extraction of \(\alpha\)-tocopherol from virgin olive oil using absolute methanol and isopropanol-methanol mixtures of varying ratios

<table>
<thead>
<tr>
<th>Transferred oil (%)</th>
<th>(\alpha)-Toc extracted (mg/kg)</th>
<th>IPA/MeOH ratio</th>
<th>Transferred oil (%)</th>
<th>(\alpha)-Toc (mg/kg)</th>
<th>Total (\alpha)-Toc extracted (mg/kg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>65.5</td>
<td>10:90</td>
<td>2.7</td>
<td>56</td>
<td>122</td>
<td>84</td>
</tr>
<tr>
<td>2.1</td>
<td>66.0</td>
<td>20:80</td>
<td>3.8</td>
<td>59</td>
<td>125</td>
<td>86</td>
</tr>
<tr>
<td>2.2</td>
<td>66.7</td>
<td>30:70</td>
<td>4.4</td>
<td>59</td>
<td>126</td>
<td>87</td>
</tr>
<tr>
<td>2.4</td>
<td>67.6</td>
<td>40:60</td>
<td>6.4</td>
<td>58</td>
<td>126</td>
<td>87</td>
</tr>
<tr>
<td>2.3</td>
<td>66.8</td>
<td>50:50</td>
<td>9.6</td>
<td>58</td>
<td>125</td>
<td>86</td>
</tr>
</tbody>
</table>

\(a\) Performed with 3\(\times\)25 ml absolute methanol.
\(b\) Performed with 3\(\times\)25 ml isopropanol/methanol (IPA/MeOH) mixtures of varying ratios.
\(c\) Oil sample initially contained 145 mg/kg \(\alpha\)-tocopherol.

Table 2
Recovery of \(\alpha\)-tocopherol from virgin olive oil samples of varying initial \(\alpha\)-tocopherol contents, using four extraction steps

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial (\alpha)-tocopherol (mg/kg±S.D.)(^a)</th>
<th>Amount extracted (mg/kg±S.D.)(^b)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil 1</td>
<td>116.82±5.63</td>
<td>94.44±5.58</td>
<td>81</td>
</tr>
<tr>
<td>Oil 2</td>
<td>125.52±6.57</td>
<td>101.07±2.76</td>
<td>80</td>
</tr>
<tr>
<td>Oil 3</td>
<td>134.80±4.60</td>
<td>104.40±3.60</td>
<td>77</td>
</tr>
<tr>
<td>Oil 4</td>
<td>155.75±2.93</td>
<td>123.07±3.77</td>
<td>79</td>
</tr>
<tr>
<td>Oil 5</td>
<td>184.80±4.20</td>
<td>141.60±3.40</td>
<td>77</td>
</tr>
<tr>
<td>Oil 6</td>
<td>195.05±5.03</td>
<td>166.20±6.60</td>
<td>85</td>
</tr>
</tbody>
</table>

\(a\) 2\(\times\)25 ml methanol and 2\(\times\)25 ml methanol/isopropanol (20:80, v/v).
\(b\) \(n=3\).

Table 3
Recovery of hydroxytyrosol derivative and tyrosol derivative using four extraction steps

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peak 5(^b)</th>
<th>Peak 6(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial (mg/kg±S.D.)(^c)</td>
<td>Extracted (mg/kg±S.D.)(^c)</td>
</tr>
<tr>
<td></td>
<td>Initial (mg/kg±S.D.)(^c)</td>
<td>Extracted (mg/kg±S.D.)(^c)</td>
</tr>
<tr>
<td>Oil 1</td>
<td>42.83±2.10</td>
<td>44.48±2.31</td>
</tr>
<tr>
<td>Oil 2</td>
<td>81.42±4.63</td>
<td>80.66±2.05</td>
</tr>
<tr>
<td>Oil 3</td>
<td>94.81±4.79</td>
<td>96.12±2.53</td>
</tr>
</tbody>
</table>

\(a\) 2\(\times\)25 ml methanol and 2\(\times\)25 ml methanol/isopropanol (20:80, v/v).
\(b\) Expressed as tyrosol.
\(c\) \(n=3\).
In order to evaluate the effectiveness of the extraction procedure for phenolic compounds, 10 virgin olive oil samples, with polyphenol contents (expressed as tyrosol) ranging from 45–501 mg kg$^{-1}$, were subjected to extraction using both aqueous methanol after dissolution in hexane, and the solvent system of the proposed method (Table 4). In comparison to the aqueous methanol extraction method, the recommended method extracted lower amounts (43% lower) of compounds corresponding to peaks 1–4, approximately the same amount of compounds corresponding to peaks 5 and 6, and higher amounts (18% higher) of compounds corresponding to peaks 7–10. It has been demonstrated that the concentration of phenols in extracts were dependent on extraction solvents and the class of phenolic compounds present in olive oil (Montedoro et al., 1992a, 1992b; Angerosa et al., 1995). Montedoro et al. (1992a) reported a mixture of CH$_3$OH/H$_2$O (80:20, v/v) as the most efficient extraction solvent for simple and hydrolysable phenolic compounds. However, Angerosa et al. (1995) proposed the use of pure methanol due to low recovery of some simple phenols when CH$_3$OH/H$_2$O was used. Results from Table 4 suggest that simple phenols (peaks 1–4) were better extracted with 60% aqueous methanol while complex phenols (peaks 7–10) were better extracted with methanol and iso-propanol/methanol (20:80, v/v) mixture. Peaks 5 and 6 were not substantially affected by extraction solvents.

In addition, the recommended method extracted a relatively large number of unknown peaks (Fig. 3, fraction I) which were eluted with the use of methanol/acetonitrile mixture. Compounds present in this fraction may have complex phenolic structure. The phenolic fraction of olive oil is complex and numerous unidentified compounds may be determined with improvements in analytical techniques (Brenes, Garcia, Rios, & Carrido, 1999). The characterisation of fraction I is in progress.

In conclusion, using the above proposed method, $\alpha$-tocopherol and phenolic compounds can be sufficiently concentrated and therefore analysed simultaneously by HPLC/UV. An average recovery of 80% was achieved for $\alpha$-tocopherol whereas recovery of phenols exceeded 98% in most cases.

### References


