ELSEVIER

Food Chemistry 74 (2001) 377–383

Food Chemistry

www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods Section

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

Maria Tasioula-Margari*, Otu Okogeri

Department of Chemistry, Section of Industrial and Food Chemistry, University of Ioannina, Ioannina 45110, Greece

Received 17 October 2000; received in revised form 3 March 2001; accepted 3 March 2001

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 C_{18} 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 α-tocopherol can be determined in one run. Phenolic compounds were totally extracted whereas an average recovery of 80% was achieved for α-tocopherol. Validation of method is reported for olive oil samples with varying concentrations of α-tocopherol and phenolic compounds.

Keywords: Virgin olive oil; Extraction; HPLC; Phenolic compounds; α-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, α-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). α-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, & Miniati, 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 β- and γ-tocopherols which cannot be resolved by RP-HPLC. Recently however, separation of α-, β-, γ-, and δ-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 α-tocopherol which fluoresce to a much lesser extent than free α-tocopherol. A saponification step is therefore needed to convert α-tocopherol esters to their free alcohol form with the risk of partial loss of tocopherols.

* Corresponding author. Fax: +30-651-98795.
E-mail address: mtasioul@cc.uoi.gr (M. Tasioula-Margari).

0308-8146/01/$ - see front matter
PII: S0308-8146(01)00176-5

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 α-tocopherol concentrations of extracts and to facilitate their detection using UV detector. A reversed phase C_{18} column was chosen since β- and γ-tocopherols, which cannot be resolved on RP C_{18} 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 N_2 below −20°C prior to analysis.

2.2. Reagents and standards

Acetonitrile, methanol, hexane, isopropanol (2-propanol) filtered through a 1.5-μm 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-α-tocopherol and β-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°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°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-μl injection loop was used. The column used was an Apex octadecyl 104 C_{18} (25×0.4 cm ID) with 5-μm packing (Jones Chromatography limited, Colorado, USA).

2.6. HPLC conditions

Detection was performed at 280 nm for both phenols and α-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°C. The sample injection volume was 20 μl. 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 α-tocopherol and phenols

The initial α-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 α-tocopherol contents were then calculated from integrated peak areas of the

samples and the calibration curve of α-tocopherol standard. Good linearity was achieved in the range 50–400 mg kg^{-1} ($r^2 = 0.999$).

Recovery of α-tocopherol was determined by subjecting olive oil samples to extraction and by comparing extracted amounts of α-tocopherol to the initial amounts determined above (without extraction). Six olive oil samples of different initial α-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 α-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: 14.8min) and peak 6 (RT: 16.3min). 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. γ- And β-tocopherols overlap on a RP C_{18}

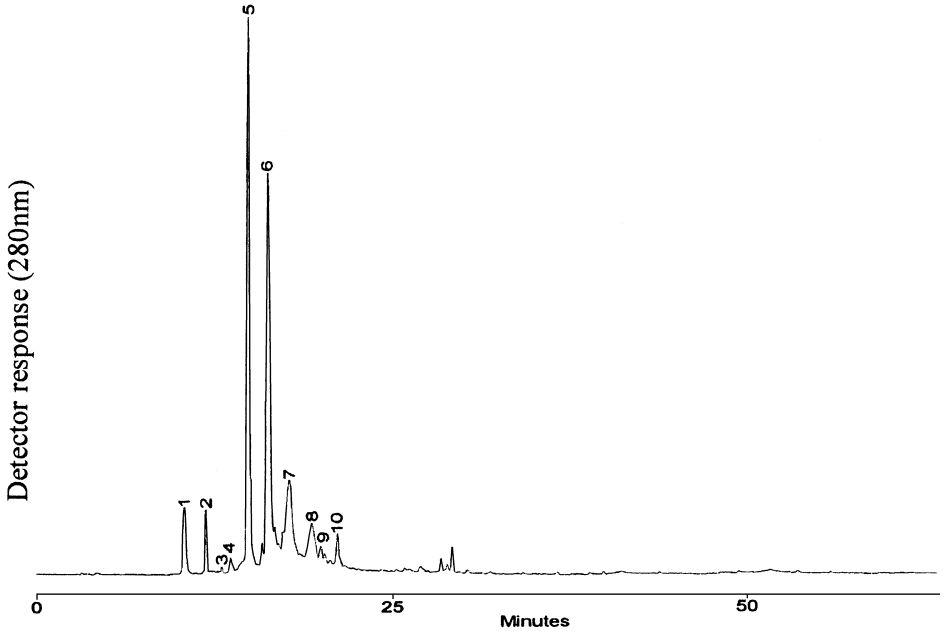

Fig. 1. Reversed phase HPLC separation of virgin olive oil phenolic compounds extracted from oil/hexane solution using 60% aqueous methanol. $\lambda = 280$ nm. Peak numbers: (1) hydroxytyrosol; (2) tyrosol; (3) vanilic acid; (4) syringic acid; (5) hydroxytyrosol derivative; (6) tyrosol derivative; (7–10) complex phenolic compounds. Sensitivity 0.01.

column but their content in olive oil is very low. Interest was therefore focused on α-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 α-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 α-tocopherol extracted were not remarkably affected by variations in methanol/isopropanol ratio. Using IPA/MeOH

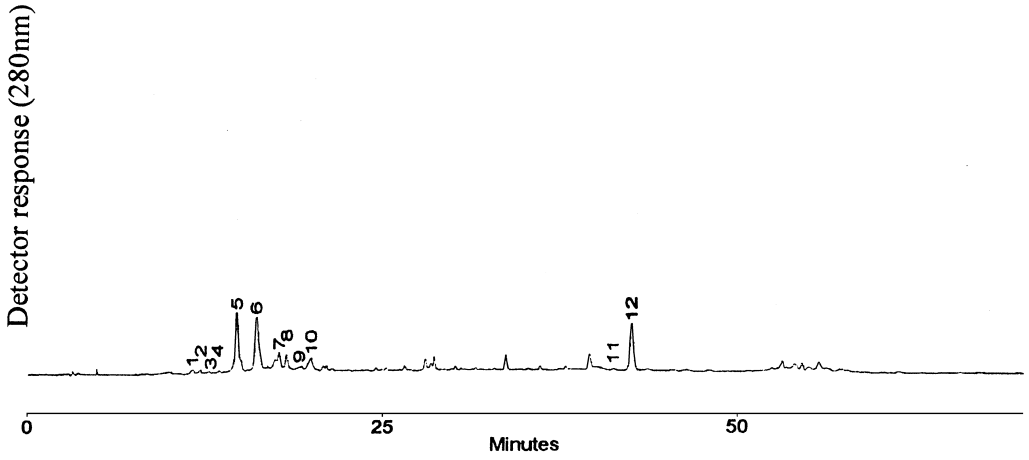

Fig. 2. Reversed phase HPLC separation of virgin olive oil sample prior to extraction. $\lambda = 280$ nm. Peak numbers: (1) hydroxytyrosol; (2) tyrosol; (3) vanilic acid; (4) syringic acid; (5) hydroxytyrosol derivative; (6) tyrosol derivative; (7–10) complex phenolic compounds; (11) β-tocopherol; α-tocopherol. Sensitivity 0.01.

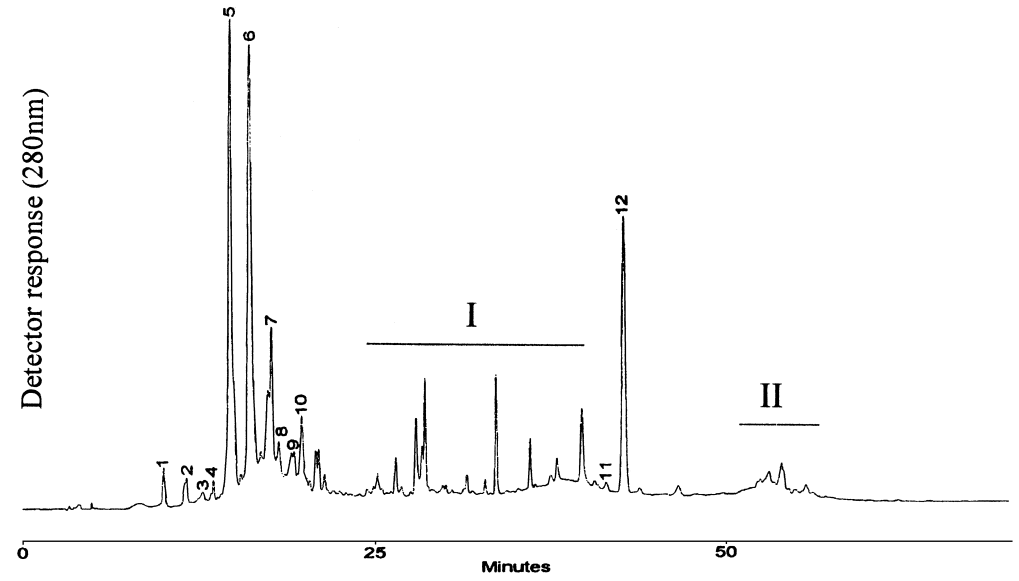

Fig. 3. Reversed phase HPLC separation of virgin olive oil fraction extracted using 2×25 ml MeOH and 2×25 ml MeOH/IPA (80:20). $\lambda = 280$ nm. Peak numbers: (1) hydroxytyrosol; (2) tyrosol; (3) vanilic acid; (4) syringic acid; (5) hydroxytyrosol derivative; (6) tyrosol derivative; (7–10) complex phenolic compounds; (I) unknown; (11) β-tocopherol; (12) α-tocopherol; (II) triglycerides. Sensitivity 0.01.

ratios of 10:90 (v/v) the lowest amount (56 mg kg^{-1}) of α-tocopherol was extracted, whereas using 20:80 and 30:70 (v/v), the highest amount was extracted (59 mg/kg; Table 1). However the amount of oil transferred to the extracts increased as the volume of isopropanol in methanol increased. Hence, extraction with an isoprapanol/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 α-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 α-tocopherol from virgin olive oil using absolute methanol and isopropanol-methanol mixtures of varying ratios

First extraction[a]		Second extraction[b]			Total α-Toc extracted (mg/kg)	Recovery[c] (%)
Transferred oil (%)	α-Toc (mg/kg)	IPA/MeOH ratio	Transferred oil (%)	α-Toc (mg/kg)		
2.1	65.5	10:90	2.7	56	122	84
2.1	66.0	20:80	3.8	59	125	86
2.2	66.7	30:70	4.4	59	126	87
2.4	67.6	40:60	6.4	58	126	87
2.3	66.8	50:50	9.6	58	125	86

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

Table 2
Recovery of α-tocopherol from virgin olive oil samples of varying initial α-tocopherol contents, using four extraction steps[a]

Sample	Initial α-tocopherol (mg/kg±S.D.)[b]	Amount extracted (mg/kg±S.D.)[b]	Recovery (%)
Oil 1	116.82±5.63	94.44±5.58	81
Oil 2	125.52±6.57	101.07±2.76	80
Oil 3	134.80±4.60	104.40±3.60	77
Oil 4	155.75±2.93	123.07±3.77	79
Oil 5	184.80±4.20	141.60±3.40	77
Oil 6	195.05±5.03	166.20±6.60	85

[a] 2×25 ml methanol and 2×25 ml methanol/isopropanol (20:80, v/v).
[b] $n=3$.

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

Sample	Peak 5[b]			Peak 6[b]		
	Initial (mg/kg±S.D.)[c]	Extracted (mg/kg±S.D.)[c]	Recovery (%)	Initial (mg/kg±S.D.)[c]	Extracted (mg/kg±S.D.)[c]	Recovery (%)
Oil 1	42.83±2.10	44.48±2.31	104	48.32±1.50	48.50±1.15	100
Oil 2	81.42±4.63	80.66±2.05	99	84.27±4.88	85.29±2.10	101
Oil 3	94.81±4.79	96.12±2.53	101	108.22±7.10	111.41±1.88	103

[a] 2×25 ml methanol and 2×25 ml methanol/isopropanol (20:80, v/v).
[b] Expressed as tyrosol.
[c] $n=3$.

Table 4
Validation of extraction method for virgin olive oil samples with varying phenolic contents

Sample No.	MeOH/H_20 (60/40)[a]				100% MeOH and IPA/MeOH (20/80)[b]			
	Peaks 1–4[c]	Peak 5[c]	Peak 6[c]	Peak 7–10[c]	Peaks 1–4[c]	Peak 5[c]	Peak 6[c]	Peaks 7–10[c]
1	31.14	586.67	431.38	104.58	20.23	606.68	426.13	116.81
2	14.42	10.30	40.32	11.59	3.36	26.63	38.47	17.50
3	36.17	133.76	166.77	68.91	22.52	142.04	165.72	85.64
4	24.29	218.41	299.74	88.03	18.23	216.02	269.29	103.24
5	9.87	20.77	87.47	15.64	3.54	20.71	97.65	23.62
6	6.27	17.94	152.00	19.66	4.67	20.71	204.19	26.06
7	7.96	5.56	207.03	8.35	3.73	2.96	147.96	24.63
8	9.99	143.17	162.40	57.90	2.37	106.53	165.72	83.48
9	18.89	201.82	201.79	107.62	12.71	207.15	218.98	117.63
10	10.12	79.84	154.48	45.25	6.07	103.57	153.88	53.32
Average (%)	4.21	35.29	47.37	13.13	2.38	35.52	46.16	15.94

[a] Extraction was performed on oil/hexane solution using 3×20 ml MeOH/H_20 (60:40, v/v; Section 2.3).
[b] Extraction was performed directly on oil samples using 2×25 ml MeOH and 2×25 ml MeOH/IPA (80:20, v/v; Section 2.4).
[c] Peak area×10^4.

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_3OH/H_2O (80:20, v/v) as the most efficient extraction solvent for simple and hydrolyzable phenolic compounds. However, Angerosa et al. (1995) proposed the use of pure methanol due to low recovery of some simple phenols when CH_3OH/H_2O (80:20, v/v) 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 isopropanol/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/acetonitle 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, α-tocopherol and phenolic compounds can be sufficiently concentrated and therefore analysed simultaneously by HPLC/UV. An average recovery of 80% was achieved for α-tocopherol whereas recovery of phenols exceeded 98% in most cases.

References

Abidi, S. L., & Mounts, T. L. (1997). Reversed-phase high-performance liquid chromatographic separation of tocopherols. *Journal of Chromatography A*, *782*, 25–32.

Akasbi, M., Shoeman, D. W., & Csallany, A. S. (1993). High-performance liquid chromatography of selected phenolic compounds in olive oils. *Journal of the American Oil Chemist's Society*, *70*, 367–370.

Andrikopoulos, N. K., Brueschweller, H., Felber, H., & Taeschler, Ch. (1991). HPLC analysis of phenolic antioxidants, tocopherols and triglycerides. *Journal of the American Oil Chemist's Society*, *68*, 359–364.

Angerosa, F., d'Alessandro, N., Konstantinou, P., & Di Giacinto, L. (1995). GC-MS evaluation of phenolic compounds in virgin olive oil. *Journal of Agriculture and Food Chemistry*, *43*, 1802–1807.

Boskou, D. (1996). *Olive oil: chemistry and technology*. Champaign, IL: AOCS Press (pp. 59–60).

Brenes, M., Garcia, A., Garsia, P., Rios, J. J., & Carrido, A. (1999). Phenolic compounds in Spanish olive oils. *Journal of Agriculture and Food Chemistry*, *47*, 3535–3540.

Carpenter, A. P. Jr. (1979). Determination of tocopherols in vegetable oils. *Journal of the American Oil Chemist's Society*, *5*, 668–671.

Dionisi, F., Prodolliet, J., & Tagliaferri, E. (1995). Assessment of olive oil adulteration by reversed-phase high-performance liquid chromatography/amperometric determination of tocopherols and tocotrienols. *Journal of the American Oil Chemist's Society*, *72*, 1505–1511.

Gutfinger, T. (1981). Phenols in olive oils. *Journal of the American Oil Chemist's Society*, *58*, 966–968.

Hoehler, D., Frohlich, A. A., Marquardt, R. R., & Stelsovsky, H. (1998). Extraction of tocopherol from serum prior to reversed-phase liquid chromatography. *Journal of Agriculture and Food Chemistry*, *46*, 973–978.

Indyk, H., & Woollard, D. C. (1986). Antioxidant analysis in edible oils and fats by normal-phase high-performance liquid chromatography. *Journal of Chromatography*, *356*, 401–408.

IUPAC. (1987). *Standard methods for the analysis of oils, fats and derivatives*. *Method 2.411* (7th ed.). International Union of Pure and Applied Chemistry, London: Blackwell Scientific Publication.

Montedoro, G., Servili, M., Baldioli, M., & Miniati, E. (1992a). Simple and hydrolyzable phenolic compounds in virgin olive oil. 1. Their extraction, separation, and quantitative and semiquantitative evaluation by HPLC. *Journal of Agriculture and Food Chemistry*, *40*, 1571–1576.

Montedoro, G., Servili, M., Baldioli, M., & Miniati, E. (1992b). Simple and hydrolyzable phenolic compounds in virgin olive oil. 2. Initial characterization of the hydrolyzable fraction. *Journal of Agriculture and Food Chemistry*, *40*, 1577–1580.

Okogeri, O., & Tasioula-Margari, M. (2000). Evaluation of Greek virgin olive oil phenolic compounds by HPLC/UV and GC/MS. In *2000 IFT annual meeting book of abstracts* (p. 113). Dallas, Texas, USA. Illinois: Institute of Food Technologists.

Papadopoulos, G., & Boskou, D. (1991). Antioxidant effect of natural phenols on olive oil. *Journal of the American Oil Chemist's Society*, *68*, 669–671.

Pirisi, F. M., Angioni, A., Cabras, P., Garau, V. L., di Teulada, M. T. S., dos Santos, M. K., & Bandino, G. (1997). Phenolic compounds in virgin olive oils. 1. Low-wavelength quantitative determination of complex phenols by high-performance liquid chromatography under isocratic elution. *Journal of Chromatography A*, *768*, 207–213.

Satue, M. T., Huang, S-W., & Frankel, E. N. (1995). Effects of natural antioxidants in virgin olive oil on oxidative stability of refined, bleached, and deodorized olive oil. *Journal of the American Oil Chemist's Society*, *72*, 1131–1137.

Tsimidou, M., Papadopoulos, G., & Boskou, D. (1992). Determination of phenolic compounds in virgin olive oil by reversed-phase HPLC with emphasis on UV detection. *Food Chemistry*, *44*, 53–60.

Visioli, F., & Galli, C. (1994). Oleuropein protects low density lipoprotein from oxidation. *Life Science*, *55*, 1965–1971.

Visioli, F., Bellomo, G., Montedoro, G., & Galli, C. (1995). Low density lipoprotein oxidation is inhibited in vitro by olive oil constituents. *Atherosclerosis*, *117*, 25–32.

Wahyuni, W. T., & Jinno, K. (1988). Separation of tocopherols on various chemically bonded phase in microcolumn liquid chromatography. *Journal of Chromatography*, *448*, 398–403.