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On the determination of minor phenolic acids of virgin olive oil by RP-HPLC

By **M. Tsimidou*** (1), **M. Lytridou** (1), **D. Boskou** (1), **A. Pappa-Louisi** (2), **F. Kotsifaki** (3) and **C. Petrakis** (3)

(1) Laboratory of Food Chemistry and Technology, Faculty of Chemistry, Aristotle Univ. of Thessaloniki, 54006 Thessaloniki, Greece.
(2) Laboratory of Physical Chemistry, Faculty of Chemistry, Aristotle Univ. of Thessaloniki, 54006 Thessaloniki, Greece.
(3) Mediterranean Agronomic Institute of Chania (MAICH) Chania, Greece.

RESUMEN

Sobre la determinación de ácidos fenólicos minoritarios de aceite de oliva virgen mediante RP-HPLC

El objetivo de este estudio fue la detección y determinación de cuatro ácidos fenólicos minoritarios presentes en la fracción polar del aceite de oliva. Los ácidos gálico, homovanílico, caféico y ferúlico fueron separados mediante RP-HPLC y detectados usando tres sistemas diferentes. Se determinaron los límites de detección para los cuatro componentes. Se encontró que los detectores UV a longitud de onda variable son más apropiados que los detectores de batería de diodos para la información cuantitativa. Sin embargo, el detector de batería de diodo fue necesario para la información cualitativa de componentes desconocidos en el aceite de oliva. Para la determinación de compuestos fenólicos sensibles, presentes en cantidades minoritarias, fue necesario el uso de un sistema más sensible, como es el detector electroquímico.

PALABRAS-CLAVE: Aceite de oliva virgen – Ácido fenólico – Cromatografía líquida de alta eficacia – Detector de batería de diodos – Detector electroquímico – Detector UV.

SUMMARY

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The objective of this study was the detection and determination of four minor phenolic acids present in the polar fraction of olive oil. Gallic, homovanillic, caffeic and ferulic acids, were separated on RP-HPLC and detected by using three different systems. Detection limits were determined for the four compounds. Variable wavelength UV detectors were found to be more suitable than diode array for quantitative information. However, diode array was required for qualitative information for unknown components present in olive oil. For the determination of sensitive phenolic compouds present in minor amounts the use of a more sensitive system, such as the electrochemical detector, was necessary.

KEY-WORDS: Diode array detector – Electrochemical detector – High performance liquid chromatography – Phenolic acid – UV detector – Virgin olive oil.

1. INTRODUCTION

The polar fraction of virgin olive oil, obtained by extraction with methanol-water mixtures, contains phenolic compounds which are conventionally characterised as «polyphenols» (Cortesi & Fedeli, 1983; Harborne, 1989). Phenolic constituents seem to be of great importance for the quality assessment of olive oil because their presence is related to the shelf life of the oil and its flavour (Vázquez Roncero, 1978; Gutfinger, 1981; Constante & Roncero, 1981; Gutierrez *et al.,* 1989; Tsimidou *et al.,* 1992a).

Previous work in this laboratory (Blekas *et al.,* 1995) showed that even small quantities of some phenolic compounds may retard considerably the oxidation rate of olive oil triacylglycerols. Therefore, there is a need for a sensitive, reproducible analytical procedure by wich both separation and quantification of individual phenols could be feasible. The most promising method, currently applied for the determination of olive oil phenols, is RP-HPLC coupled with UV, diode array or electrochemical detection (Tsimidou *et al.,* 1992b; Montedoro *et al.,* 1992; Akasbi *et al,* 1993).

There are discrepancies among the workers on the level of most phenolic constituents of olive oil (Cortesi *et al,* 1981; Solinas & Cichelli, 1982; Solinas, 1987; Nergiz & Unal, 1991; Montedoro *et al.,* 1992; Tsimidou *et al,* 1992b). Characteristic are the cases of the following minor phenolic compounds. Gallic acid, not usually reported as an olive oil constituent, is considered by Mannino *et al.,* (1993) as a major component of the polar fraction. Similarly, great differences can be observed in the results reported for homovanillic (Solinas & Cichelli, 1982; Akasbi *et al.,* 1993), caffeic and ferulic acid (Solinas & Cichelli, 1982; Nergiz & Unal, 1991; Montedoro *et al,* 1992).

Apart from the expected natural variability among samples, analytical parameters may influence significantly phenol quantification (Tsimidou *et al.,* 1992a). Comparative studies among various chromatographic methods using different detection systems are lacking. Such studies are necessary to explore the extent to which qualitative and quantitative results depend on parameters applied when an olive oil sample is analyzed.

This work is a part of a thorough study on the phenolic compounds of olive oil. It aims at comparing the detection limits of selected minor phenolic acids of olive oil by three different HPLC detection systems, UV, diode array and electrochemical. The phenols tested were homovanillic, ferulic, gallic and caffeic acid. The last three compounds are known as natural antioxidants.

2. EXPERIMENTAL

2.1. Reagents and standards

1. HPLC - diode array detection: The phenolic compounds used as standards were: tyrosol (4-hidroxyphenylethanol) and cinnamic acid from Aldrich Chem. Co (Milwaukee, WI, USA); caffeic, gallic, p-hydroxyphenylacetic, vanillic, o-coumaric, p-coumaric, syringic, protocatechuic, homovanillic, ferulic and p-hydroxybenzoic acids from Sigma Chemical Co. (St Louis USA). The extraction solvents used were: hexane from Mallinckrodt and methanol (Spectranal) from Ridel de Häen (Seelze, Germany). The elution solvents used for HPLC analysis were: methanol (Gradient grade) from Riedel de Häen (Seelze, Germany), water (HPLC grade) and acetic acid (glacial) from Merck (Darmstadt, Germany).

2. HPLC - UV or electrochemical detection: The phenolic compounds used as standards were as previously described. The solvents used for the extraction of the phenolic compounds were methanol (pro-analysis) and hexane (pro-analysis), both from Riedel de Häen (Seelze, Germany). The elution solvents used for HPLC were methanol (HPLC Chromasolv) from Lab. Scan Analytical Sciences (Dublin, Ireland). The water used was purified by successive application of reversed osmosis and filtration through active carbon and ion exchange resin. The acetic acid (glacial) was purchased from Riedel de Häen (Seelze, Germany).

2.2. Apparatus

1. HPLC - diode array detection: The analysis of polyphenols was carried out on a Hewlett Packard Model 1090 series II liquid chromatograph with a quaternary solvent delivery system and auto-injector, coupled with a UV-Vis diode-array detector. The Hewlett Packard Chemist Station 3D software package was used for data aquisition and analysis.

2. HPLC system with UV-detection: All HPLC analytical separations were performed with a Spectra Physics liquid chromatograph (Model 8800) equipped with a variable wavelength UV detector (Spectra Chrom 100) and an electronic integrator (Spectra Physics, Model 4290); injection was by means of a Rheodyne injection valve (Model 7125) with 10 μl fixed loop (Rheodyne, California, USA).

3. HPLC system with electrochemical detection: The liquid chromatograph consisted of a Shimadzu LC-9A solvent delivery unit with a double-plunger reciprocating pump, a Model 7125 syringe loading sample injector fitted with a 20 μl loop (Rheodyne, Cotati, CA) and a Gilson Electrochemical Detector (Model 141).

The working electrode was a small disc of glassy carbon (3 mm diameter) and the reference electrode was Ag/AgCl (3.0 mol/l NaCl). The electrochemical detector was interfaced to an AT-compatible computer (AS1 386) via a 14-bit AD-DA card.

2.3. Methods

Sample preparation

The method used was described by Vazquez Roncero *et al.* (1976). 50 g of oil dissolved in hexane (50 ml) was extracted with methanol: water (60:40, v/v, 3x30 ml). The three extracts were combined and treated once with hexane (50 ml). The solvent was evaporated to dryness in a flash evaporator (40ºC); the residue which contained the phenolic compounds of olive oil is conventionally called «polar fraction of olive oil». This residue in methanol was transferred into a volumetric flask (5 ml) and aliquots of this solution were used for reversed-phase HPLC analysis.

Reversed-phase HPLC determination of phenolic compounds

1. HPLC - diode array detection: The chromatographic separation of the complex virgin olive oil polar fraction, was achieved on a Lichrospher 100 RP-18, 5 mm column (250 x 4 mm i.d.) (Hewlett Packard) connected with a 100 RP-18, 5 μm guard column, (Hewlett Packard) at 37ºC. The gradient elution as described by Tsimidou *et al* (1992), with some modifications, consisted of a binary solvent system with water: acetic acid, 98:2, v/v (solvent A) and methanol (solvent B) at a flow rate of 1 ml/min. The gradient composition was: 5%B (0-10 min); 5-70%B (10-45 min); 70%B (45-65 min); 70-5% B (65-90 min); 5%B (90-115 min) and the injection volume was 10 μl. Absorptions were recorded at five different wavelengths: 256, 278, 295 and 325 nm with bandwith 6 nm and at 280 nm with bandwith 20 nm while the reference wavelength was set to 400 nm with bandwith 80 nm. The initial threshold setting was -2 (signal in DAD 1 mAu), the attenuation 8 and the peakwidth 0.2 min. These five wavelengths were chosen by scanning the olive oil samples in the region 190-820 nm.

2. HPLC system with UV-detection: The chromatographic separation was achieved on a Macherey-Nagel RP-18, 5 μm (250 x 4.6 mm i.d.)

column at room temperature by gradient elution at a flow rate of 1 ml/min. Solvent A was aqueous acetic acid, 3%, v/v and solvent B was methanol. The gradient programme was: 6-37% B (0-60 min); 37-100%B (60-70 min); 100%B (70-90 min); 100-6%B (90-105 min) and 6% B (105-130 min). The wavelengths used for the UV-detection were 280 and 235 nm. Ultraviolet detector and integrator settings were 0.005 AUFS and attenuation x 32, peak threshold x 235 and chart speed 0.25 cm/min. Identification of peaks was based on relative retention times and spiking.

3. HPLC system with electrochemical detection: The separation was achieved on the same Macherey-Nagel column at room temperature. The composition of the isocratic elution system was arranged so that all the phenolic compounds were eluted at approximately the same time as with the previous system. The initial composition of the isocratic system was aqueous acetic acid 3%: methanol, 94:6, v/v. The flow rate was 1ml/min. The electrode potential was maintained at + 0.8 V *versus* the reference electrode (Akasbi *et al,.* 1993).

Calibration curves

Calibration plots for the HPLC-UV detection system were established using two standards, caffeic acid and tyrosol over the range 5-80 ng per 10 μl injected (for caffeic acid) and 5-800 ng per 10 μl injected (for tyrosol) (Tsimidou *et al.,* 1992b). The standard solutions were prepared in the mobile phase.

UV-spectra of phenols

The UV spectra for phenols were scanned using an U-2000 HITACHI spectrophotometer (Hitachi Ltd, Tokyo, Japan) against a blank of aqueous acetic acid, 3%: methanol, 94:6, v/v. All standard solutions were made in the above solvent (pH=2.64).

3. RESULTS AND DISCUSSION

Maximum wavelength of standard phenols

Detection of phenolic compounds in virgin olive oil is usually carried out at 280 nm by UV detector (Cortesi *et al,* 1981; Solinas & Cichelli, 1982). This is a wavelength of general use and it is not the maximum for all the phenolic compounds. The λmax and ε values at various wavelengths of the phenolic compounds studied in this work are presented in Table I. The above λmax were also confirmed by diode array scanning.

Detection limits

Detection limits were found for gallic acid (derivative of benzoic acid), homovanillic acid (derivative of phenylacetic acid), caffeic and ferulic acid (derivatives of cinnamic acid). As detection limit was considered a signal to noise ratio of 2. The detection limits were studied at the general wavelength 280 nm and also at other characteristic wavelengths. Detection limits are shown in Table II.

Gallic acid, absorbs only at 280 nm and has a satisfactory detection limit and good repeatability with both UV detection and diode array detection. The detection limit with the electrochemical detector was found to be a hundred times lower. It is obvious that the determination of this compound depends greately on the sensitivity of the detection system so that it may hardly be detected with a UV or a diode array system. Homovanillic acid also absorbs at 280 nm but presents comparatively high detection limit in both UV detection and diode array systems because of its low ϵ_{280} value. The detection limit for homovanillic acid is significantly improved using electrochemical

Table I
Absorbance data for standard phenolic compounds

Phenolic compound	λmax (nm)	$^{\epsilon}$λmax	$^{\epsilon}$280
Gallic	270.6	9112.6	9112.6
Protocatechuic	**259.0**/293.0	**10670.1**/5412.4	4329.9
Tyrosol	275.2	1519.3	1519.3
p-hydroxyphenylacetic	274.4	1700.5	1700.5
Vanillic	**260.2**/291.4	**16685.4**/8595.5	7471.9
Homovanillic	278.4	2785.4	2785.4
Caffeic	319.4	28558.6	5225.2
Syringic	273.4	10646.8	10646.8
p-coumaric	308.6	16776.6	10989.0
Ferulic	321.4	20686.3	9803.9
o-coumaric	**276.2**/321.8	**17352.9**/8676.5	17352.9

detection and this may explain why this compound has been mainly reported in studies where such a sensitive detector is used (Akasbi *et al.,* 1993).

The derivatives of cinnamic acid have their maxima at wavelengths other than 280 nm. Caffeic acid has a low detection limit both at its λmax (325 nm) and at 280 nm (Table III). Therefore, the absence of this acid in reported results based on UV detection means that it does not occur at all or at least it is present in trace amounts. The detection limits obtained with diode array detection system was many folds higher. In this case the use of a UV or electrochemical detector seems to be essential.

Table II
Detection limits of minor phenolic acids

Phenolic acid	Detection limits ± CV% (ng/10 μl)		
	Detection system		
	Diode array (at λmax) *n=3*	UV (at λmax) *n=5*	electro-chemical *n=3*
Gallic	8±4.0	1±1.3	0.01±7.5
Homovanillic	44±2.9	4±7.6	0.1±4.7
Caffeic	10±1.2	1±2.1	0.05±13.2
Ferulic	10±6.5	1±5.9	0.5±6.7

n= replication number

Similar observations can be made for ferulic acid for both spectrometric detectors. Electrochemical detector still produces better detection limits (twice lower) albeit not as good as with the other phenolic compounds. This may be partially due to long retention times needed (36 min) and poor resolution. According to Mannino *et al.* (1993) the choice of the oxidation potential is critical for the detection limit and phenolic compounds containing one –OH group are detected at higher values of oxidation potentials. Still for ferulic acid the use of a value of 0.8 V was not the appropriate (Concerton & Chapital, 1983).

The results presented so far, show that differences in the sensitivity between various detection systems should be seriously taken into consideration when quantitation of minor phenolic compounds is necessary. UV is a detector of general use and seems to be more appropriate than diode array for quantitative information. UV detection provides a relatively simple and easy to carry out method in routine analysis. A diode array detection system can be additionally used when qualitative information for unknown samples is needed.

Electrochemical detection provides high sensitivity and selectivity. It is extremely useful for the quantification of specific compounds the level of which cannot be easily determined by other means (Macrae, 1988; Hart, 1990; Bernwieser & Sontag, 1990; Mannino *et al.*, 1993; Akasbi *et al.,* 1993). Besides, some additional information about the antioxidant activity of the compounds can be obtained (Mannino *et al.,* 1993). There are, however, certain disadvantages of electrochemical detector: a) A gradual absorption of phenols and their oxidation products takes place on the electrode and reduces the repeatability and sensitivity of the detector. Therefore, a regular cleaning and polishing of the electrode is necessary (especially after application of high concentrations).

b) Since the oxidation potential is characteristic for each compound the choice of its value for a natural product sample can be critical. The aplication of a high potential, as in the case of ferulic acid, causes unwanted oxidations, increased baseline noise and reduced sensitivity. Therefore, specific conditions for electrochemical measurement must be found for each particular case; this requires a certain knowledge of electrochemistry and some experience.

Electrochemical detection should be rather applied only to specific phenolic compounds which are readily oxidised and occur in minute quantities.

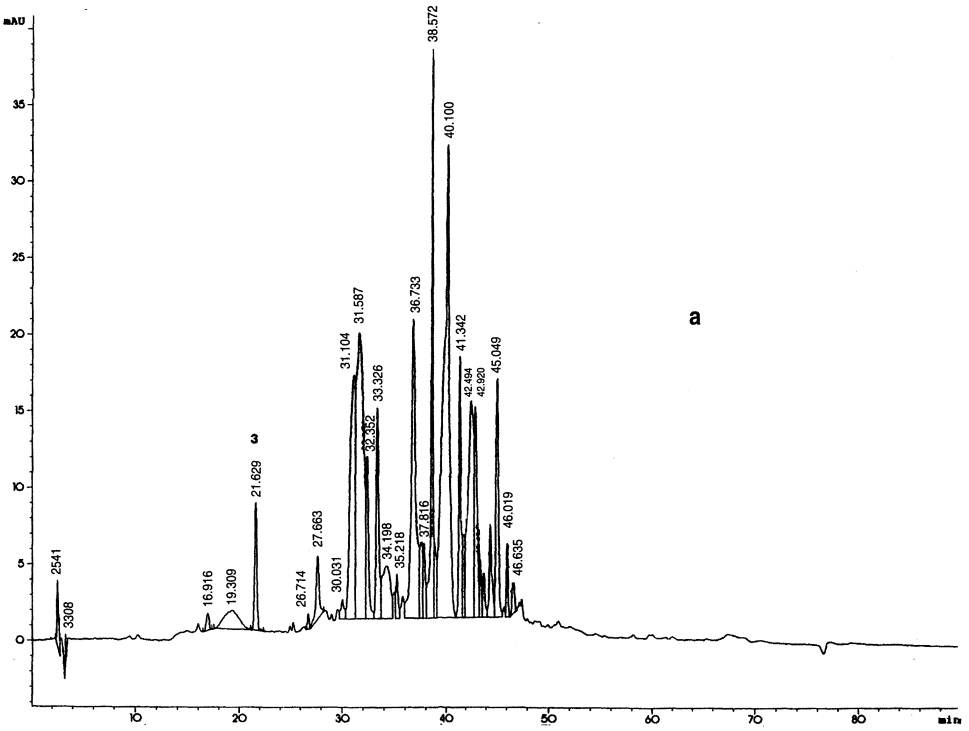
Application to virgin olive oil samples

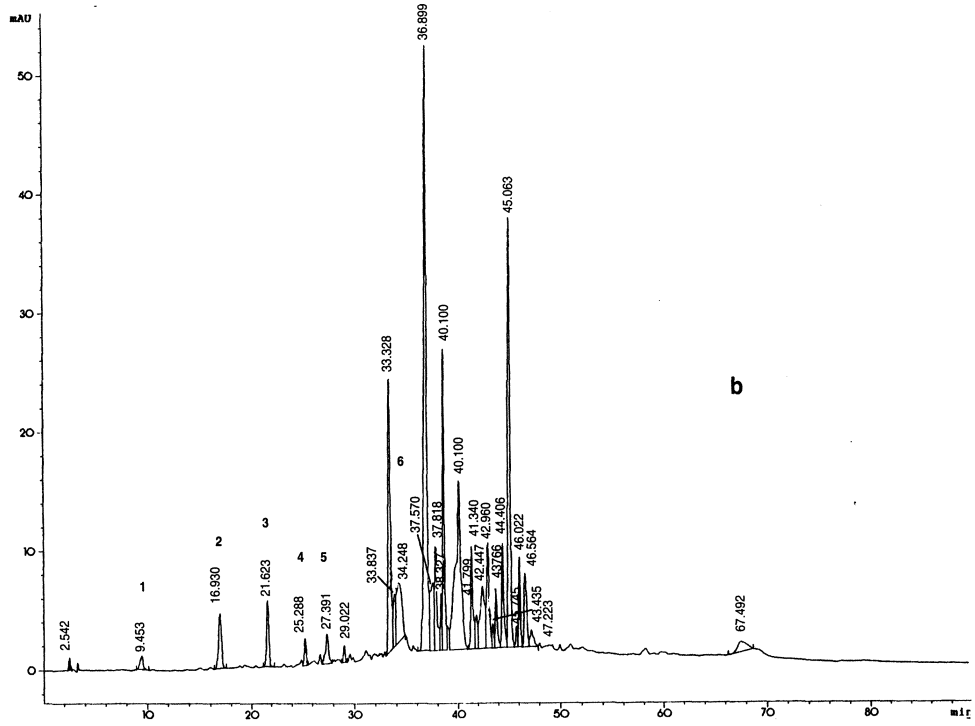
The chromatographic systems coupled with UV and diode array detectors were used for the determination of the phenolic compounds present in the polar fraction of virgin olive oil samples. Electrochemical detector was not included because of practical problems related to gradient elution. Two samples were chosen to be analysed, one extracted by a classical press system (sample 1) and another by centrifugation (sample 2). Detection was carried out at 280, 256, 295 and 325 nm (with diode array system) and 280 and 325 nm (with UV detector). Each analysis was carried out in duplicate. Hydroxytyrosol, tyrosol, vanillic, syringic, p-coumaric and o-coumaric acid were detected with both systems. Detection and determination of gallic, homovanillic and ferulic acid was feasible only by UV detector. Caffeic acid was not detected by both detection systems. Figure 1a, b, c and d show characteristic differences between chromatograms recorded at various wavelengths which may be used for the identification of the phenolic components of olive oil. The amounts recorded for the minor phenolic acids of olive oil samples (Table IV) were few times higher than the detection limits given in Tables II and III.

Table III
Detection limits of cinnamic acid derivatives

Phenolic acid	Detection limits ± CV% (ng/10 μl) at 280 nm	
	Detection system	
	Diode array *n=3*	UV *n=5*
Caffeic	20±6.1	1±1.9
Ferulic	20±3.3	1±9.1

n= replication number





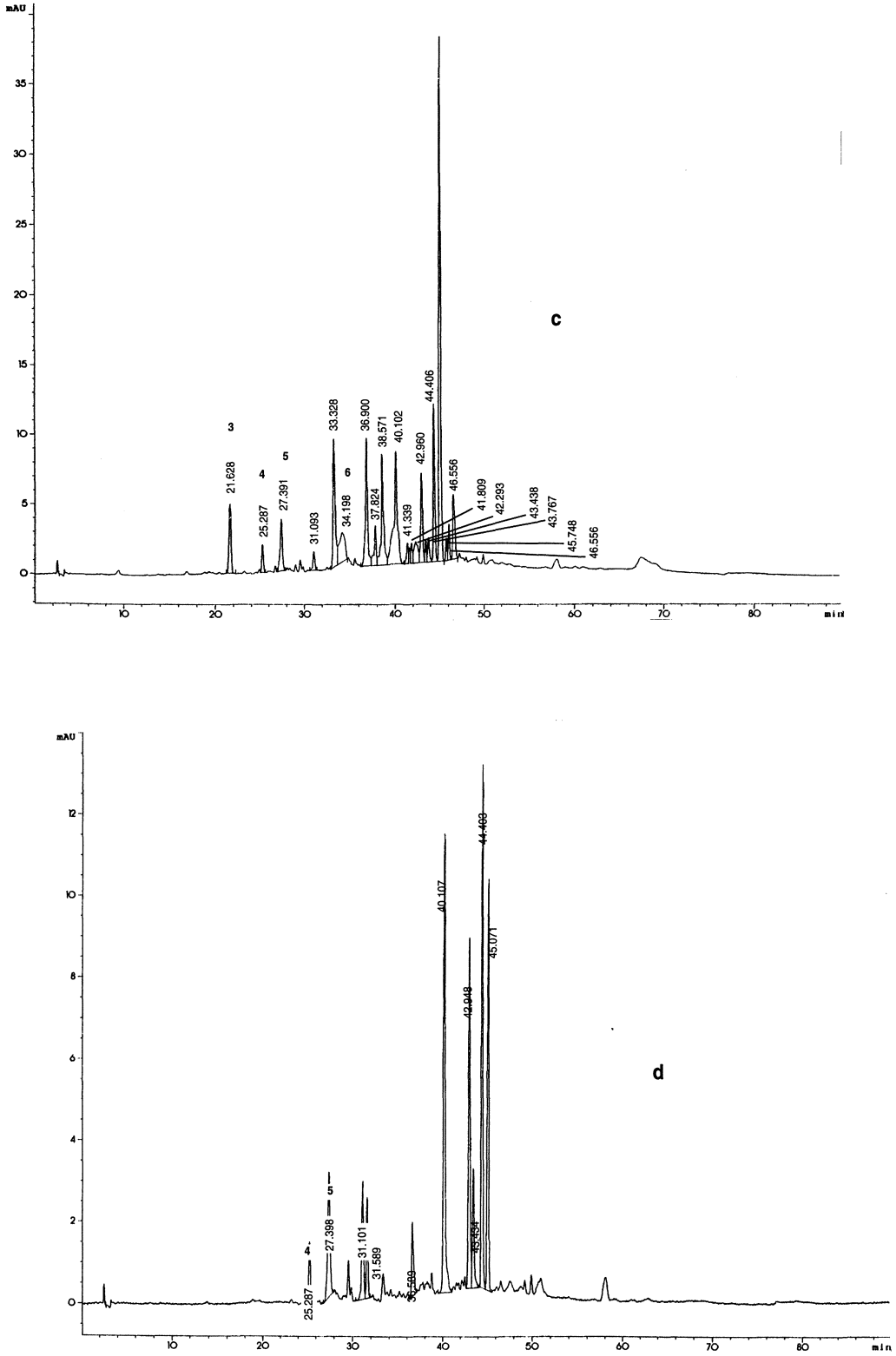


Figure 1
RP-HPLC of the polar fraction of an olive oil (sample 1); Detection at 256 nm (a); 280 nm (b); 295 nm (c) and 325 nm (d). Peak identification: Hydroxytyrosol, 1; tyrosol, 2; vanillic acid, 3; syringic acid, 4; p-coumaric acid, 5; o-coumaric acid, 6 (Experimental conditions as described in the experimental part for diode array detection)

Table IV
Quantitation of gallic, homovanillic and ferulic acids in virgin olive oil samples using UV detection

	Sample 1	Sample 2
	ng/10 μl injected	
Phenolic acid	**λ= 280 nm (or 325 nm)**	
Gallic	8.8[a]	5.5[a]
Homovanillic	58.7[b]	16.7[b]
Ferulic	21.1 (10.9)[a]	14.1 (5.0)[a]

a= quantitation based on the use of caffeic acid as standard
b= quantitation based on the use of tyrosol as standard

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