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Analytical Methods Section

Determination of phenolic compounds in virgin olive oil by reversed-phase HPLC with emphasis on UV detection

M. Tsimidou, G. Papadopoulos & D. Boskou

Laboratory of Food Chemistry and Technology, Faculty of Chemistry, Aristotle University of Thessaloniki, Thessaloniki 54006, Greece

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A method is described for the chromatographic separation and quantitative determination of phenolic compounds in virgin olive oil by high-performance liquid chromatography (HPLC) using gradient elution. For the determination of individual compounds molar absorptivities at 280 nm are calculated. A technique based on the use of four external standards (tyrosol, vanillic acid, syringic acid, *o*-coumaric acid) is applied in order to minimize errors due to different λ_{max} and ϵ_{280}. The method was applied successfully to olive oil samples.

INTRODUCTION

Phenolic compounds which occur in plants and foods of plant origin are important for their effect on the flavour of foods (Kozlowska *et al.*, 1983; Seo & Morr, 1984) and beverages (Lea, 1982; Salagoity-Auguste & Bertrand, 1984) and also for their antioxidant activity in fatty foods (Hammerschmidt & Pratt, 1978; Ramarathnam *et al.*, 1988). The presence of phenolic compounds in seeds and processed foods may also cause a deterioration of colour (Sosulski, 1979). Binding of oxidized phenols with essential amino acids has been reported to form complexes difficult to assimilate by animals and man (Davies *et al.*, 1978; Subba Rau *et al.*, 1972; Herrmann, 1990).

Virgin olive oil is a stable oil. Its stability has been related to the triglyceride composition, the presence of α-tocopherol (Bauernfeind, 1980) and recently to the presence of phenolic compounds such as hydroxytyrosol, tyrosol, caffeic acid and others (Chimi *et al.*, 1988). These compounds conventionally characterized as 'polyphenols' are part of the polar fraction which is usually obtained from the oil by extraction with methanol–water mixtures (Cortesi & Fedeli, 1983). There is evidence that the stability of the oil to autoxidation is partly due to the total polyphenol content (Gutfinger, 1981). The latter, however, is determined by conventional colorimetric methods using the Folin-Ciocalteau reagent which is not specific (Smit *et al.*, 1955; Solinas & Cichelli, 1981) and it cannot be used successfully as a quality criterion. Previous work in this laboratory (Papadopoulos & Boskou, 1991) showed that phenols present in small quantities are more effective in retarding oxidation while tyrosol, which is the major phenol, is only slightly active. Therefore, a specific method for the determination of individual phenolic compounds in the polar fraction is needed.

Packed and capillary gas chromatography has been used for the separation of plant phenolic compounds (Schulz & Herrmann, 1980; Forcadell *et al.*, 1987) but this technique has some drawbacks (high oven temperature, derivatization) and it cannot be widely applied. In the last decade, high performance liquid chromatography (HPLC) procedures have also been applied for the analysis of plant phenols (Villeneuve *et al.*, 1982; Andersen & Pedersen, 1983). In these works reversed-phase-HPLC (RP-HPLC) coupled with UV detection is mainly used and well-established techniques are presented. However, the literature concerning the HPLC analysis of the phenolic compounds in virgin olive oil is limited and the quantitative results reported are often ambiguous since they are based on the use of one internal standard or only a few external standards (Cortesi *et al.*, 1981; Solinas & Cichelli, 1982).

This work is part of a thorough study on the phenolic compounds in virgin olive oil. It was undertaken to improve the chromatographic conditions usually em-

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ployed and proposes a quantification procedure with a limited number of characteristic phenols as external standards to minimize errors arising from UV detection.

MATERIALS AND METHODS

Samples

Greek virgin olive oil samples were provided by a plant located in the area of Athens or collected by the authors from various regions in Greece.

Reagents and standards

Methanol (Chromasolv) was obtained from Riedel de Häen (Seelze, Germany). Ethanol and acetic acid (pro-analysis) and acetonitrile were purchased from Merck (Darmstadt, Germany). The water used for HPLC analysis was purified by successive application of reversed osmosis and filtration through active carbon and ion exchange resin.

Protocatechuic, vanillic, *p*-coumaric, *o*-coumaric and 3,4-dihydroxyphenylacetic acids were purchased from Sigma Co. (St Louis, USA), caffeic acid (97%) and *p*-hydroxybenzoic acid (99%) from Fluka (Buchs, Switzerland), tyrosol (98%) from Aldrich Co. (Milwaukee, Wisconsin, USA). Hydroxytyrosol (3,4-dihydroxyphenylethanol) was prepared by acidic hydrolysis of oleuropein according to a method described by Constante and Roncero (1980). Oleuropein was kindly offered by Dr R. M. Duran (Instituto de la Grasa y Derivados, Seville, Spain).

HPLC apparatus

All 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).

Sample preparation

The polar fraction of virgin olive oil was obtained in the following manner (Cortesi *et al.*, 1981): oils (50 g) dissolved in hexane (50 ml) were extracted with methanol–water (60:40, v/v) (3 × 30 ml). Each extract was treated once with hexane (50 ml). The three extracts were combined and the solvent evaporated to dryness using a Rotavapor (40°C) and transferred quantitatively with methanol into a volumetric flask (5 ml). The repeatability of the extraction technique was checked by determining the total phenol content with the Folin-Ciocalteau reagent (Swain & Hillis, 1969).

Table 1. Mobile phase for separation of phenolic compounds

Segments	Time (min)	Mobile phase (MeOH in H_2O + 3% acetic acid, v/v)
1	0	6
2	60	37
3	70	100
4	90	100
5	105	6
6	130	6

For five replicate extractions the coefficient of variation (CV%) was found to be 4·6%.

HPLC determination of phenolic compounds

The chromatographic separation was achieved on a Spherisorb ODS-2, 5 μm column (250 × 4·6 mm i.d.), obtained from Anachem (Luton, Bedfordshire, UK) at room temperature. Gradient elution at a flow rate of 1 ml min^{-1} was used (Table 1).

Segments 3 and 4 were omitted in the chromatography of standards. The back pressure was below 2400 psi. Ultraviolet detector and integrator settings were: 280 nm, 0·002 AUFS and attenuation × 32, chart speed 0·25 cm min^{-1}, peak threshold × 235, respectively. Identification of peaks was based on relative retention time and spiking.

Construction of standard curves

Calibration plots were established using three series of standards over the range 5–80 ng per 10 μl injected and for tyrosol and hydroxytyrosol 5–800 ng per 10 μl injected.

Series 1: 3,4-dihydroxyphenylacetic acid, syringic acid, tyrosol, *p*-hydroxyphenylacetic and cinnamic acids
Series 2: protocatechuic acid, *p*-hydroxybenzoic acid
Series 3: hydroxytyrosol

The solutions of these standards were made in MeOH/3% v/v aqueous acetic acid (6:94, v/v) (pH=2·64).

UV spectra of phenols

The UV spectra for phenols were scanned and molar absorptivities were measured using a Pye Unicam SP 8000UV recording spectrophotometer against a blank of MeOH/3% v/v aqueous acetic acid (6:94, v/v).

RESULTS AND DISCUSSION

Column and elution system

Separation on 5 μm particles seemed to be advanta-

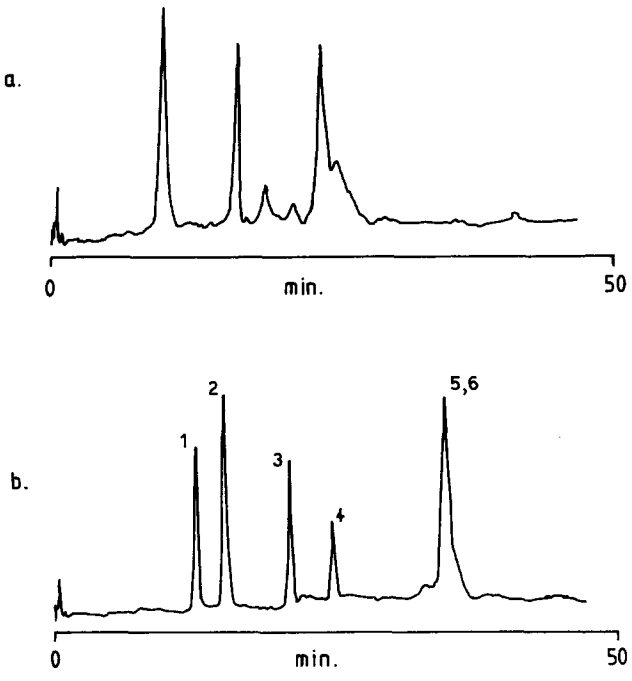

Fig. 1. Effect of acetic acid on the elution of phenols. Chromatographic conditions: column ODS-2, 5 μm (250 × 0·4 mm); MeCN–H_2O (a) and MeCN–H_2O plus 0·2% CH_3COOH (b), 3% MeCN (0 min), 10% MeCN (10 min), 15% MeCN (40 min); 1 ml min^{-1}; 0·05 AUFS; att × 16; rise time × 1·0; chart speed × 0·25 cm min^{-1}; peaks: resorcinol, 1; protocatechuic acid, 2; tyrosol, 3; *p*-hydroxyphenylacetic acid, 4; caffeic acid, 5; syringic acid, 6.

geous over that carried out on 10 μm packings. Therefore, a column packed with ODS-2, 5 μm material was used throughout the experimental work. Gradient elution proved to be necessary in order to separate the large number of compounds with similar polarity within a reasonable length of time. Preliminary work with mixtures of acetonitrile–water and methanol–water with the addition of small amounts of acetic acid 0·2% (pH = 3·13), showed that serious disturbances of the column performance and lengthy equilibration times often occurred with an acetonitrile–water system. This phenomenon was mainly observed when methanol was used between sample injections and at the end of the working day. This is possibly due to hydrophobic phenomena arising from the incompatibility of stationary and mobile phase. It appears that methanol is absolutely necessary to eliminate high molecular weight substances absorbing at 280 nm.

Therefore in routine column conditioning, methanol is recommended instead of acetonitrile. The addition of acetic acid was also essential to suppress dissociation of the phenolic compounds and enhance the strength and selectivity of the elution system. This is clearly shown in Fig. 1.

Separation of phenol standards

The main aim of the elution system developed in this study was to separate all the phenols which, according to the literature, have significant antioxidant activity and occur in considerable amounts in olive oil. These compounds are presented in Table 2. The protection factors reported in the table were determined in the

Table 2. Antioxidant activity of the phenolic compounds in virgin olive oil as indicated by protection factors, PF_n[a] at 63°C

Phenols[b]	PF_{70}	PF_{20}
3,4-Dihydroxyphenylacetic acid[c]	>10·6	>18·4
Hydroxytyrosol	9·5	15·2
Caffeic acid	5·2	5·7
Protocatechuic acid	2·3	2·7
Syringic acid	1·4	1·5
p-Hydroxyphenylacetic acid	1·2	1·3
p-Hydroxybenzoic acid	1·1	1·3
Tyrosol	1·1	1·2
Vanillic acid	1·0	1·0
o-Coumaric acid	1·1	1·0
p-Coumaric acid	1·1	1·2

[a] PF_n are calculated from the time needed for peroxide value of refined olive oil to attain n, T_{70}=456h, T_{20}=264h.
[b] Phenolic compounds added in oil (200 ppm).
[c] This compound was used as standard (Constante *et al.*, 1980) although its presence in olive oil has not been confirmed.

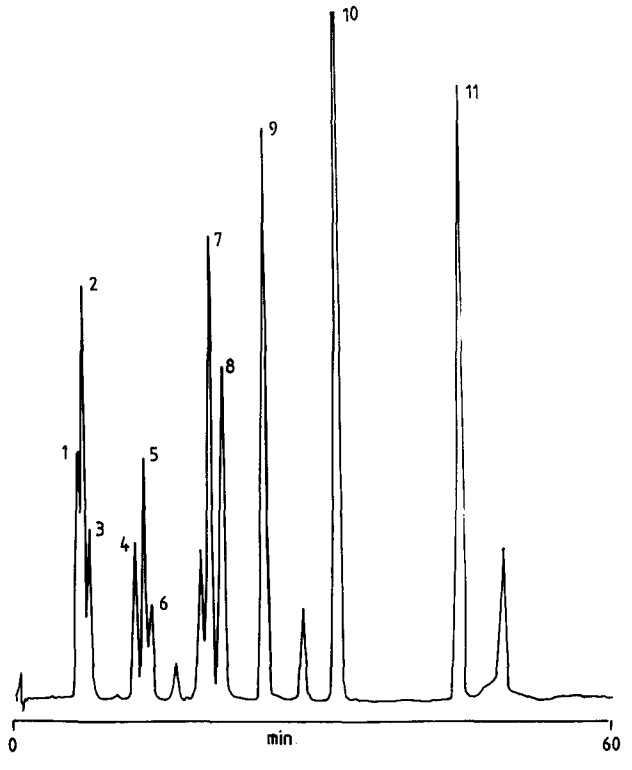

Fig. 2. HPLC analysis of a standard mixture of phenols. Chromatographic conditions as in 'Materials and Methods' section except for: 0·005 AUFS; att × 16, peak threshold × 18; peaks: hydroxytyrosol, 1; protocatechuic acid, 2; 3,4-dihydroxyphenylacetic acid, 3; tyrosol, 4; *p*-hydroxybenzoic acid, 5; *p*-hydroxyphenylacetic acid, 6; vanillic acid, 7; caffeic acid, 8; syringic acid, 9; *p*-coumaric acid, 10; *o*-coumaric acid, 11.

Table 3. Ultraviolet spectra of standards

Phenol	λ_{max} (nm)	ϵ_{280}[a]
p-Hydroxyphenylacetic acid	275	1515·2
Tyrosol	276	1517·2
Hydroxytyrosol	281	2307·6
3,4-Dihydroxyphenylacetic acid	281	3109·2
p-Hydroxybenzoic acid	256	4143·6
Protocatechuic acid	260,295	4160·2
Vanillic acid	261,293	5210·0
Caffeic acid	323,300	10791·4
Syringic acid	275	10891·1
p-Coumaric acid	306	11475·4
o-Coumaric acid	277,325	17704·9

[a] Measurements were taken in solutions of MeOH–H_2O plus 3% CH_3COOH (6:94, v/v) in 1 cm standard cuvettes.

authors' laboratory (Papadopoulos & Boskou, 1991). As protection factor the ratio $PF_n = T_n/T_n°$ is described (Sonntag, 1979). In this equation T_n is the time for peroxide value of fats to attain n and $T_n°$ is the T_n in the control test. Figure 2 illustrates the chromatographic analysis of a mixture of 11 phenols. It is clear that although some of the investigated compounds have similar polarities their resolution is feasible. The retention times of 3,4-dihydroxyphenylacetic acid and protocatechuic acid are very close to that of hydroxytyrosol and the separation is not satisfactory. This is, however, of minor importance from a practical point of view because both these acids are present in trace amounts in olive oil (Fig. 4).

UV detection and quantification of phenols

Maximum wavelength and molar absorptivities of standards

Ultraviolet detection of virgin olive oil phenols is usually carried out at 280 nm (Cortesi *et al.*, 1981; Solinas & Cichelli, 1982). This wavelength is characteristic for aromatic acids (Schwarzenbach, 1982). However, scanning UV spectra showed that 280 nm was not the maximum wavelength for all of the phenols studied. Table 3 presents λ_{max} and molar absorptivities for these compounds.

According to these results different detection limits are expected for each compound quantified with UV detection at 280 nm. Furthermore, the composition expressed as peak area percent gives a distorted picture of the real situation in the polar fraction. On the other hand, quantitative studies based on internal standards with λ_{max} other than 280 nm, such as protocatechuic, vanillic, *o*-coumaric and caffeic acids give lower values for the phenolic compounds under investigation.

The use of simultaneous multiple UV detection could be a solution. However, this instrumentation is not always available. Successive detections at different wavelengths is not practical because the chromatography of phenols is time consuming. Hence, the detection at 280 nm could be a compromise because most of the phenols examined absorb considerably at this wavelength. It is obvious that the use of an internal standard is inadequate for quantitative studies, bearing in mind that not only maximum wavelengths but also molar absorptivities differ (Table 3).

Since a great number of external standards is required for a more precise quantification, the investigated phenols were grouped on the basis of the slopes of the regression lines of standard curves. In this way the number of standards required which are not always available, e.g. hydroxytyrosol, was reduced significantly.

Standard curves

The standard curves (Fig. 3) demonstrate that the eleven phenols fall into four groups:

Group A: hydroxytyrosol, tyrosol, 3,4-dihydroxyphenylacetic acid, *p*-hydroxyphenylacetic acid
Group B: protocatechuic, *p*-hydroxybenzoic, vanillic acids
Group C: caffeic, syringic, *p*-coumaric acids
Group D: *o*-coumaric acid

Linearity of standard curves was very good as indicated by correlation coefficients. Regression data are presented in Table 4. With the above grouping a

Table 4. Regression data of detector response against amount of phenols

Phenol	Number of injections	Correlation coefficient	Slope areas × 10^4 per ng injected	Intercept area × 10^4
Hydroxytyrosol	4	0·988	5275·0	−21·2
Protocatechuic acid	5	0·990	11995·0	1·2
3,4-Dihydroxyphenylacetic acid	5	0·999	6305·1	0·2
Tyrosol	5	0·999	5142·5	−3·8
p-Hydroxybenzoic acid	5	0·990	12343·3	3·9
p-Hydroxyphenylacetic acid	5	0·999	3369·3	0·8
Vanillic acid	5	0·999	12952·6	−0·5
Caffeic acid	5	0·999	21190·4	1·7
Syringic acid	5	0·999	21057·7	1·4
p-Coumaric acid	5	0·987	24525·4	4·3
o-Coumaric acid	5	0·999	38307·1	2·2

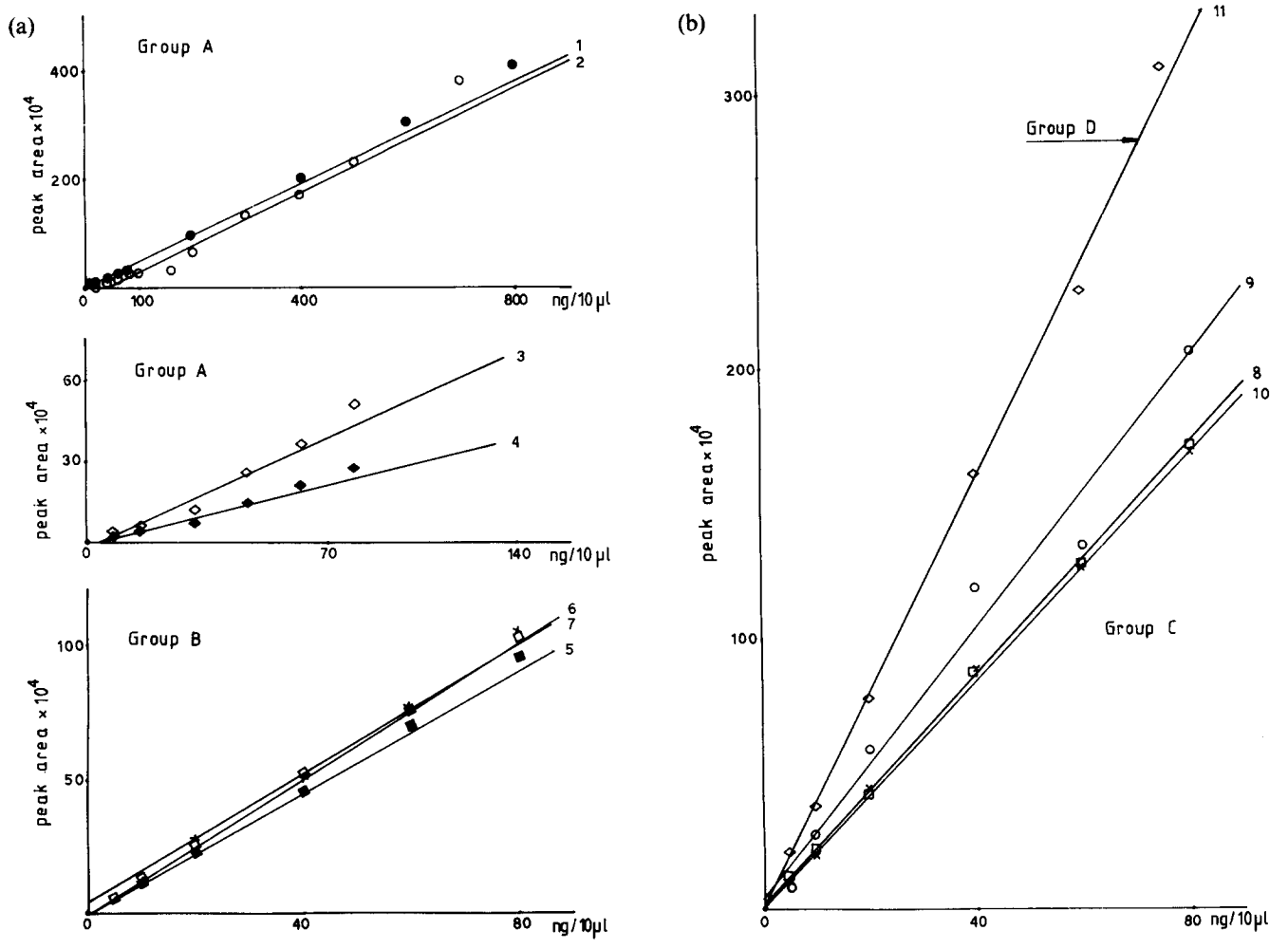

Fig. 3. Standard curves of phenols (peak area recorded versus amount injected). Group A: hydroxytyrosol, 1 (●); tyrosol, 2 (○); 3,4-dihydroxyphenylacetic acid, 3 (◇); *p*-hydroxyphenylacetic acid 4, (◆). Group B: protocatechuic acid, 5 (■); *p*-hydroxybenzoic acid, 6 (□); vanillic acid, 7 (×). Group C: caffeic acid, 8 (□); *p*-coumaric acid, 9 (○); syringic acid, 10 (×). Group D: *o*-coumaric acid, 11 (◇).

significantly smaller number of external standards is needed, namely: tyrosol for group A, vanillic acid for group B, syringic acid for group C and *o*-coumaric acid for group D. These four phenols have been reported to be present in Italian olive oils (Cortesi *et al.*, 1981; Solinas & Cichelli, 1982) and they were also tentatively identified in most of the Greek olive oil samples examined in the authors' laboratory.

Detection limits

Detection limits are shown in Table 5. The detection limit is defined as the amount of standard required to give a peak height of twice the height of base line under a peak threshold set up at 235.

Repeatability

The repeatability of peak area data is presented in Table 6. The CV values were acceptable and below 3% for most phenols. Tyrosol and *p*-hydroxyphenylacetic acid produced a comparatively higher CV (4·1%) probably due to low ϵ_{280} values. The CV% of *o*-coumaric acid (3·9%) can be explained by the sharp change of gradient composition.

Applications

The proposed technique was applied to the determination of individual phenols present in the polar fraction

Table 5. Ultraviolet detection limits for the standard phenols

Phenol	Detection limit (ng injected)
Hydroxytyrosol	5
Protocatechuic acid	3
3,4-Dihydroxyphenylacetic acid	4
Tyrosol	5
p-Hydroxybenzoic acid	4
p-Hydroxyphenylacetic acid	6
Vanillic acid	3
Caffeic acid	2
Syringic acid	2
p-Coumaric acid	2
o-Coumaric acid	1

Table 6. Peak area repeatability data for standard phenols

Phenol	Number of injections	CV%
Hydroxytyrosol	4	2·2
Protocatechuic acid	5	2·7
3,4-Dihydroxyphenylacetic acid	5	2·5
Tyrosol	5	4·1
p-Hydroxybenzoic acid	5	2·4
p-Hydroxyphenylacetic acid	5	4·1
Vanillic acid	5	2·5
Caffeic acid	5	3·0
Syringic acid	5	1·4
p-Coumaric acid	5	0·9
o-Coumaric acid	5	3·9

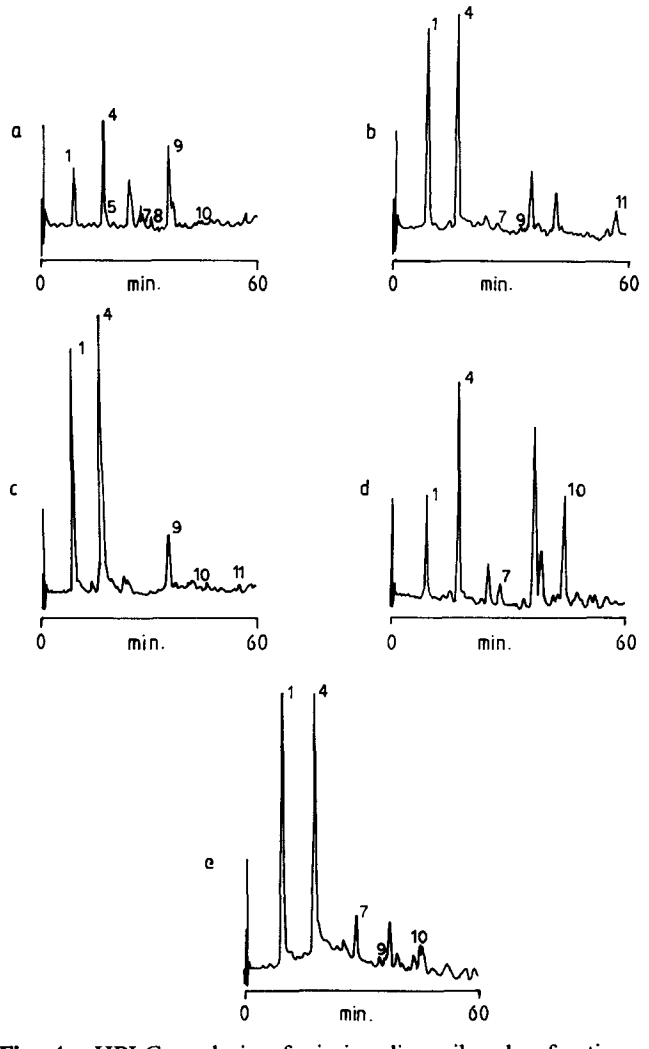

Fig. 4. HPLC analysis of virgin olive oil polar fraction. Chromatographic conditions as in 'Materials and Methods' section. Peaks as in Fig. 2. Sample 1, a; Sample 2, b; Sample 3, c; Sample 4, d; Sample 5, e.

of virgin olive soils. Characteristic chromatograms and their quantitative data are presented in Fig. 4 and Table 7.

As shown in the table the use of one external standard, e.g. syringic acid, may be a serious source of error due to the different ϵ_{280} of the various phenols. The different molar absorptivities also cause a misleading chromatogram when the concentration is expressed as percent peak area.

On the contrary, when four external standards are used, quantitative results appear to be more accurate. These data differ considerably from quantitative results reported for Italian olive oils. The difference has to be attributed not only to varietal and origin characteristics of the samples but also to the quantitative techniques applied.

CONCLUSIONS

Ultraviolet detection is widely used in the analysis of plant phenols by HPLC. This mode of detection presents inherent limitations due to the different λ_{max} and ϵ_{280} of individual compounds. These restrictions are seriously considered in this work which, it is hoped, will contribute to a more accurate estimation of phenols present in virgin olive oil. The linear gradient system satisfactorily resolves the compounds under investigation within a reasonable length of time; the reproducibility is very good and the use of acetonitrile is avoided. The quantitative procedure minimizes errors arising from absorption differences, where more specific detectors are not available. It should be also stressed that hydroxytyrosol which is readily oxidized is not commercially available and it has to be synthesized or obtained from hydrolysis of oleuropein. Thus, grouping of the standards and the use of tyrosol instead of hydroxytyrosol appear to be very convenient.

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Table 7. Comparison of quantitative results for olive oil phenols obtained with different external standards (ppm)

	Samples																			
	1				2				3				4				5			
	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d
Hydroxytyrosol	1·3	1·0	0·2	12·6	4·4	2·9	0·8	27·2	5·6	3·0	0·8	23·2	2·4	1·5	0·4	3·9	12·5	7·6	1·8	33·3
Tyrosol	1·6	1·6	0·4	22·0	3·7	3·7	1·0	36·4	6·2	6·2	1·7	48·2	3·3	3·3	0·8	22·6	7·1	7·1	1·7	37·6
p-Hydroxybenzoic acid	tr	tr	tr	0·5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Vanillic acid	0·1	0·1	tr	3·0	tr	tr	tr	1·3	—	—	—	—	0·2	0·2	tr	2·6	0·3	0·3	0·2	4·6
Caffeic acid	tr	tr	tr	1·7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Syringic acid	0·3	0·3	0·3	18·5	tr	tr	tr	0·6	0·3	0·3	0·3	9·0	—	—	—	—	tr	tr	tr	0·9
p-Coumaric acid	tr	tr	tr	0·7	—	—	—	—	tr	tr	tr	1·0	1	0·9	0·6	16·2	0·2	0·2	0·2	3·0
o-Coumaric acid	—	—	—	—	tr	tr	tr	2·5	tr	tr	tr	1·2	—	—	—	—	—	—	—	—

a = Eleven standards.
b = Tyrosol, vanillic acid, syringic acid, *o*-coumaric acid for groups A, B, C, D, respectively.
c = Syringic acid.
d = % of peak area recorded.
tr = trace: <0·1 ppm.

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