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Determination of phenolic compounds in olives by reversed-phase chromatography and mass spectrometry

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Abstract

A method based on extraction from freeze-dried material and clean up by solid-phase extraction was optimized for recovery of phenolic compounds from olive fruit. The extracted compounds were characterised by reversed-phase liquid chromatography using both ultraviolet, fluorescence and electrospray ionization mass spectral detection. Using this approach, oleuropein was confirmed as the major phenolic in olive fruit. Other compounds whose presence was confirmed include tyrosol, syringic, ferulic and homovanillic acids, quercetin-3-rhamnoside, ligstroside and isomers of verbascoside. Elenolic acid and its glucoside, which are not phenolic but are closely related, were also identified in sample extracts.

Keywords: Olives; Phenolic compounds; Oleuropein

1. Introduction

The beneficial effects of olive consumption have been attributed partly to the phenolic content of the fruit and its associated antioxidant activity [1,2]. The phenolics constitute a complex mixture in both olive fruit and derived products (notably oil) and a considerable amount of data has been amassed on olive oil using high resolution techniques [3–6]. There have been few reports of the phenolic content of olive fruit, although the work of Amiot et al. [7,8] is notable. Despite the use of high resolution techniques, preliminary extraction and fractionation remains as an essential step in the analysis of both oil and drupe for phenolic content.

Recovery of the phenols from the fruit is more exacting than that from oil, as the fruit represents a less homogeneous sample and has a higher enzyme activity. A typical procedure involves an aqueous alcohol extraction from a powdered sample obtained by freeze-drying or treatment of the drupe with liquid nitrogen [2,7,9]. Preliminary clean-up of the extract by liquid–liquid extraction with a non-polar solvent is used to remove lipoidal materials and pigments, following which the phenolic compounds are partitioned into ethyl acetate.

The extracted phenols have been fractionated by classical low-pressure column chromatography, for example, on Sephadex LH2O [10]. However, the poor efficiency of such separations has favoured the development of reversed-phase high-performance liquid chromatography (RPLC) which currently represents the most popular and reliable technique for

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phenolic analysis. Compound elution is typical of RPLC, that is, polar compounds (e.g., phenolic acids) elute first, followed by those of decreasing polarity. In one of the early reports [11] on the RPLC of phenolic compounds, different mixtures of acetic acid, water and methanol were used to separate members of several classes of phenols, and the effects of organic modifier on selectivity were deduced. Since then, numerous mobile phases have been employed with different modifiers (usually methanol, acetonitrile or tetrahydrofuran), acids (acetic or formic acid) and/or salts (ammonium phosphate). Detection has been largely restricted to the measurement of ultraviolet absorption, typically at 280 nm, although other wavelengths including 340 nm have been used. Further information is contained in a comprehensive review of the analytical chemistry of olive phenolics [2].

Despite the obvious successes of RPLC in the analysis of olives, the complete chemical nature of the phenolic component has not been elucidated. This paper describes the RPLC of olive phenolics following a simpler extraction procedure in which olive fruit was freeze-dried and phenolics were extracted with carbonate solution. Clean-up was achieved by a combination of liquid–liquid- and solid-phase extraction. After separation by RPLC, the eluted phenolic compounds were detected by photodiode array and fluorescence detection. On-line mass spectrometry served as a valuable technique for structural elucidation of the eluted species.

2. Experimental

2.1. Reagents

Reagents from the following sources were used without further purification: methanol (EM Science), acetic acid (Ajax Chemicals), Folin and Ciocalteu's phenol reagent 2.0 *N* (Sigma), hexane (Biolab Scientific) and hydrochloric acid (32%; Ajax Chemicals). Phenolic standards were obtained as follows: oleuropein was from Extrasynthese (Genay, France); caffeic, chlorogenic, *p*-coumaric and vanillic acids were from Sigma; tyrosol was from Aldrich and phenol (analytical reagent grade) was from Mallinckrodt. All water used for RPLC analyses, and for sample and standard preparation was purified using reverse osmosis. Acetone supplied by Ajax Chemicals was used for cleaning purposes.

2.2. Instrumentation

2.2.1. Reversed-phase liquid chromatography

RPLC analyses were performed using a Perkin Elmer binary LC pump 250 equipped with a 20 μl loop injector. A Perkin Elmer LC-235 diode array detector and a Perkin Elmer LC-240 fluorescence detector connected in series served to monitor the column eluent. The RPLC system was interfaced to a model number DCM-1488E Lasernet Computer Systems computer with a 20 MByte hard disk. Chromatograms were generated using an LX-300 printer.

A number of mobile-phase gradients were examined. Gradient profile I (Table 1) was used with a Varian C_{18} column (15 cm×4.0 mm; 4.5 μm) for routine analyses where solvent A is methanol–acetic acid (100:1, v/v) and solvent B is water–acetic acid (100:1, v/v). Gradient II comprised solvent A, sulfuric acid (1×10^{-3} *M*) and solvent B, acetoni-

Table 1
Gradient elution programs used for reversed-phase high performance liquid chromatography

Time (min)	% Solvent A	% Solvent B
Gradient I		
Initial	20	80
30.0	80	20
35.0	80	20
50.0	100	0
55.0	100	0
Gradient II		
0.00	85	15
12.00	85	15
35.00	66	34
40.00	85	15
Gradient III		
0.00	80	20
5.00	80	20
35.00	20	80
37.00	0	100
40.00	0	100
42.00	80	20

For details of solvents A and B, refer to Section 2.

trile. A constant flow-rate of 1.0 ml min^{-1} was used for all analyses. The eluent was monitored by UV at 280 nm (sensitivity, 0.1), whilst an excitation wavelength of 280 nm and an emission wavelength of 320 nm were used for fluorescence detection. The mobile phases were degassed under vacuum using Alltech Nylon 66 membranes, 47 mm×0.45 μm prior to RPLC analysis. Once degassed, solvents A and B were continuously sparged with high purity helium (60 kPa) to prevent resaturation by air. Solvents were pumped through the RPLC system for at least 30 min each day prior to sample injection to ensure that a stable baseline had been attained and that the system had equilibrated.

2.3. Mass spectrometry

Mass spectrometric analyses were performed using a Fisons Instrument VG Quatro II triple quadrupole mass spectrometer in the flow injection analysis (FIA) mode using electrospray ionization (ESI). The solvent flow-rate was 10 μl min^{-1} and the loop injection volume was 20 μl. Phenolic standards at a concentration of 100 ppm in methanol–water (50:50, v/v) were analysed in both the positive and negative ion mode. The effect of cone voltage on the ionization was examined at 30 and 50 V.

2.4. Liquid chromatography–mass spectrometry

Sample extracts were analysed using a Hewlett Packard model 1090 liquid chromatograph and a Quattro II quadrupole mass spectrometer (MS) (Micromass, Altrincham, Cheshire, UK) by ESI. A Waters C_{18} column (15 cm×2 mm), thermostatted at an oven temperature of 35°C, was used. Milli-Q water with 0.1% formic acid and RPLC-grade methanol (Mallinckrodt) with 0.1% formic acid served as solvents A and B, respectively, for the gradient elution program (Table 1, gradient III). An injection volume of 10 μl and a constant flow-rate of 0.200 ml min^{-1} were used for each analysis, with a split ratio of approximately 10:1 (UV detector–MS). The UV was monitored at 280 nm and the UV trace was acquired by the Masslynx Data System (Micromass) along with the mass spectral data.

2.5. Standards

Standards for RPLC and mass spectrometry were prepared in methanol–water (50:50, v/v) as individual solutions (100 μg ml^{-1}) and as part of a combined mixture at the following concentrations: caffeic, *p*−coumaric and vanillic acids, 25 μg ml^{-1}; oleuropein, 350 μg ml^{-1} and tyrosol, 100 μg ml^{-1}. All standards were filtered (0.45 μm) prior to RPLC injection.

2.6. Sample preparation and storage

Olives were picked randomly from trees of different cultivars (Manzanillo and Cucco) from an approximately 60-year-old olive grove in Wagga Wagga at various stages of physiological development, as reflected by skin coloration. Olive samples of at least 50 g were selected for each fruit colour. The olives were refrigerated at 4°C prior to processing. The fruit was pitted by hand and freeze-dried over a two–three day interval according to the moisture content of the fruit samples. The freeze-dried olives were blended into a fine powder using a general purpose electric blender. The powdered samples were stored in screw top plastic jars that were kept in desiccators prior to analysis, which was performed as soon as practicable, although there were no notable changes observed in freeze-dried samples that were stored for up to four months.

To determine the effects of a heat drying treatment, fruit from a single tree was pooled, randomly selected and used in one of three treatments. The first sample was processed as above by freeze-drying while the other two samples were dried in an air-circulated oven at either 40 or 60°C but otherwise treated as for the freeze-dried samples.

For RPLC analysis, a powdered olive sample (1 g) was reconstituted with sodium carbonate solution (5 ml; 1 *M*). The solution was swirled and left to sit for 15 min at ambient temperature (optimized in the ranges 10–60 min and from ambient temperature to 60°C). This mixture was then filtered using a buchner funnel apparatus fitted with Advantec 55 mm filter paper and a hand pump. The olive mass was recovered and transferred to the same conical flask to which a further 5 ml portion of carbonate solution

was added. The flask was again swirled, left to stand for 15 min and filtered using fresh filter paper. Both filtrates were combined and transferred to a 25-ml separating funnel. The combined filtrate was washed once with hexane (5 ml) to remove lipoidal material [as verified by thin-layer chromatography (TLC) of the washed filtrate on silica gel 60] and the aqueous phase was recovered and adjusted to a pH of approximately 4 (optimized over the range of pH values from 1.5 to 7.3) with hydrochloric acid (4–5 ml; 3 *M*). The acidified extract was subjected to solid-phase extraction (SPE) using a Waters reversed-phase (C_{18}) Sep-Pak cartridge that had been conditioned with methanol (6 ml) followed by nanopure water (6 ml). Phenolic compounds were eluted using methanol–water (5 ml; 50:50, v/v). Sample extracts were collected in screw top scintillation vials and stored at 4°C, where necessary, prior to RPLC analysis. The eluate was diluted (1:10) with the same solvent and filtered using 0.45 μm Cameo 25AS acetate filters and Terumo plastic syringes before being injected into the RPLC system. Peak identification was based on retention time and spiking of sample extracts with authentic materials in conjunction with spectral data.

The stability of oleuropein was established by spiking sample extracts. Recovery of phenolic compounds was checked at each stage of the procedure by measurement of the ultraviolet absorbance of extracts at 280 nm and following reaction with Folin–Ciocalteu reagent as follows. Eluent from SPE (0.5 ml, after appropriate dilution, but usually 1:10) or a phenolic standard was mixed with Folin–Ciocalteu reagent (5 ml, diluted 1:10, v/v, with nanopure water) and sodium carbonate solution (4 ml, 1 *M*). The mixture was heated for 15 min at 45°C in a water bath. The absorbance of the solutions was measured at 765 nm against a reagent blank. Based on these measurements, recovery of phenolic substances using the recommended procedure was estimated to exceed 95%.

3. Results and discussion

Recovery of phenols from extracted oil presented practical problems due to emulsion formation and resulting low recoveries of the analytes. On the other hand, preliminary drying was a necessary prerequisite for direct extraction from the fruit. There were no significant differences in recovery of the three major phenols between the different drying procedures. Furthermore, based on comparisons of peak areas, the RPLC profiles were not affected by any of the heat treatments. The majority of purple and black fruit samples yielded red/brown extracts upon acidification, in contrast to the golden-brown extracts obtained from green olive fruit. This can be attributed to acid-catalysed condensation of leucoanthocyanidins, which exist in higher concentrations in purple and black fruits, to yield red phlobaphenes [12].

Clean-up of the extract by SPE using C_{18} Sep-Pak cartridges was preferable to liquid–liquid extraction with ethyl acetate, a solvent with good selectivity for phenolic compounds, but which proved to be a problem due to emulsification and associated low recoveries. Using the preferred procedure, the reproducibility of the method based on measurement of peak areas was ±5%.

3.1. Chromatographic analysis

RPLC using gradient I provided baseline separation of the six standards oleuropein, tyrosol, caffeic, chlorogenic, *p*-coumaric and vanillic acids, with the exception of chlorogenic and caffeic acids (Table 2). These standards were selected on account of their structural diversity and polarity differences and their documented presence in *Olea europaea* [2]. The presence of acetic acid in the mobile phases suppressed the dissociation of the phenolic compounds and enhanced the selectivity of the elution system, as noted by Tsimidou et al. [13]. All six standards were detected at 280 nm following RPLC whereas only caffeic, chlorogenic and *p*-coumaric acids were detected at 340 nm. The observed fluorescence of the phenolic standards accorded with their absorption behaviour. Thus, of the six standards, only tyrosol, vanillic acid and oleuropein fluoresced at 320 nm following excitation at 280 nm, whereas chlorogenic, caffeic and *p*-coumaric acids fluoresced at 380 nm (excitation wavelength, 340 nm).

The diversity of phenolic compounds in olives is demonstrated by the chromatograms shown in Fig. 1. The advantage of gradient II is the ability to use low

Table 2
Retention data, spectral characteristics from photodiode array detection and electrospray ionization mass spectral data for phenolic compounds analysed in the positive and negative ion modes at 5O V

	Caffeic acid	Chlorogenic acid	*p*-Coumaric acid	Oleuropein	Tyrosol	Vanillic acid
Molecular mass	180	354	164	540	138	168
Retention time (min), gradient I	6.8	7.0	9.9	14.8	3.6	6.1
Wavelength (nm) of maximum absorption	325	329	312	279	274	260
Positive ion						
$[M+H]^+$	n.o.	355	165	541	n.o.	169
$[M+Na]^+$	n.o.	377	187	563	n.o.	n.o.
Dimer	361	n.o.	n.o.	n.o.	n.o.	n.o.
Other	**163**, loss of OH	**163**, $C_9H_6O_3$	**147**, loss of OH	901,?	297,?	**214**,?
	135, loss of COOH		119, loss of COOH	721,?	257,?	186, water adduct
	117, loss of OH and COOH			361, loss of sugar moiety	239,?	151, loss of OH
				137, $C_8H_9O_2$	**121**, loss of OH	
Negative ion						
$[M-H]^-$	**179**	**353**	**163**	**539**	**137**	**167**
Dimer	359	n.o.	327	n.o.	n.o.	n.o.
Other	215, adduct	97, solvent cluster		195, $C_{10}H_{11}O_4$	271,?	201,?
				97, solvent cluster	235,?	113,?
					183,?	
					173, adduct	
					97, solvent cluster	

The base peak is shown in boldface.
n.o., not observed.

wavelength detection with enhanced sensitivity for some phenolic compounds (see Fig. 1). However, gradient I is more convenient for general application and was used in routine analyses. Diode array detection was particularly valuable in confirming the phenolic nature of eluted species from their characteristic spectra (Table 2). For example, the peak eluting at 14.8 min (Fig. 1, gradient 1) was confirmed as oleuropein from comparison of retention and spectral data with those of the corresponding standard. The detection wavelength used in RPLC is dependent not only upon the class of phenolic compound but also on the particular member of such classes [2]. Comparisons of chromatograms generated at different detection wavelengths support the use of 280 nm as a compromise for the detection of phenolics [13,14]. Dectection at 280 nm (Fig. 1) shows considerably more detail than detection at other wavelengths in the range 250 to 360 nm. Other wavelengths can be useful for particular purposes. For instance, oleuropein is not detected at 340 nm and detection at this wavelength (not illustrated) provided enhanced resolution of the smaller peaks that eluted at between 12 and 19 min in Fig. 1, gradient I.

UV detection also provided much more information than fluorescence detection, since most phenolics absorb at 280 nm whereas significantly fewer phenolics fluoresce following absorption at this wavelength. Fluorescence detection at 320 nm following excitation at 280 nm appeared the best compromise for general profiling. Chromatograms obtained under these conditions were dominated by a series of early eluting peaks (retention time, 1–5 min) including tyrosol, with a further later-eluting peak due to oleuropein whereas chromatograms collected, for example, at a fluorescence wavelength of 380 nm (absorption 340 nm) showed very few, if any, peaks. These observations may explain the fact that no literature methods have been reported for the profiling of phenolics in olives or olive oil using fluorescence detection. Alternatively, the use of fluorescence detection can be exploited for the more selective and sensitive detection of certain phenolics, for example, tyrosol and oleuropein.

Chromatograms such as those shown in Fig. 1

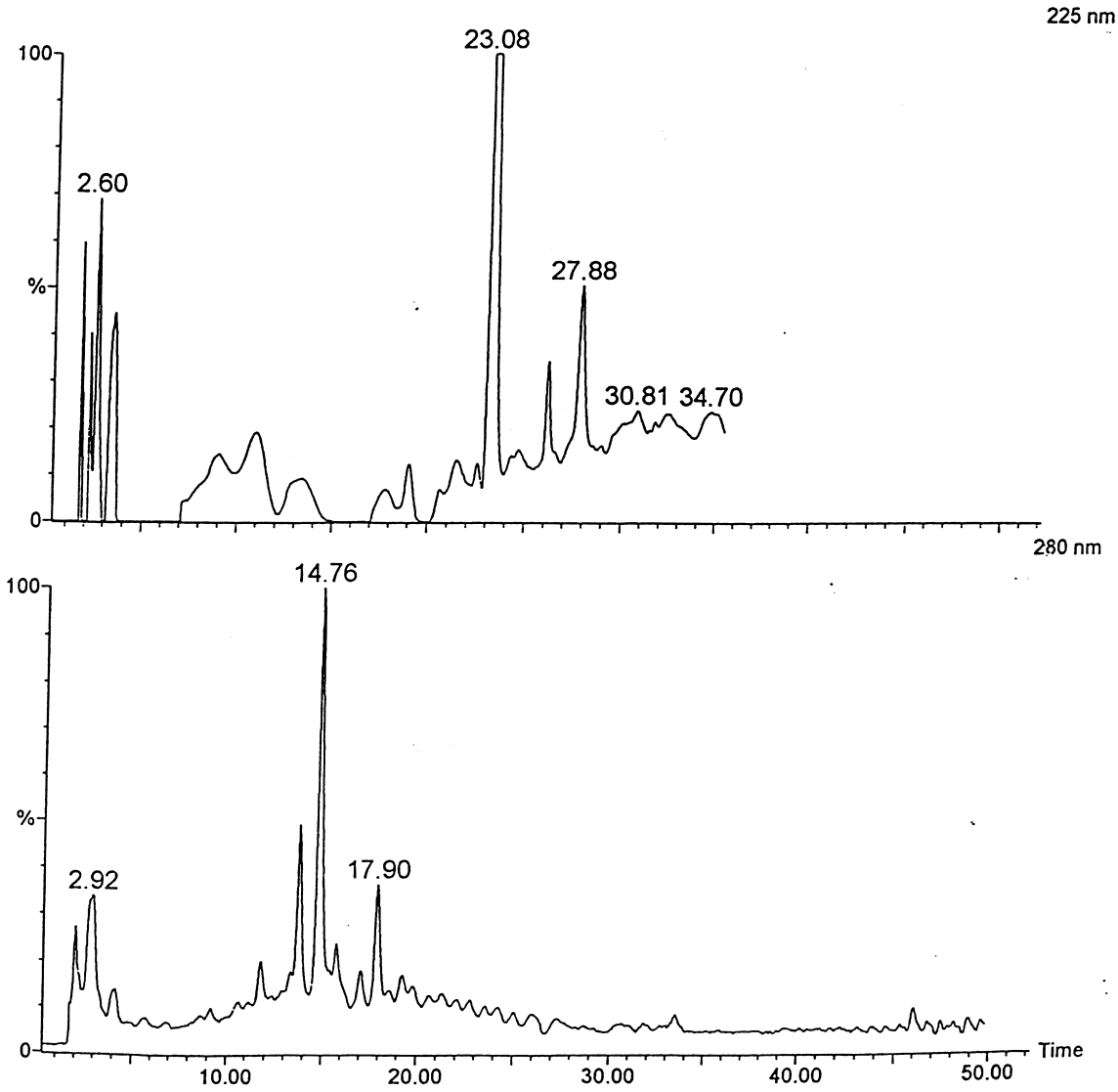

Fig. 1. Chromatograms comparing mobile phase gradients I (bottom) and II (top) for the RPLC of phenolic compounds in olive. Chromatograms were obtained with a Waters C_{18} column (15 cm×2 mm) thermostatted at 35°C and with UV detection at 280 nm (gradient I) or 225 nm (gradient II). Sensitivity, 0.01 for both chromatograms.

provide a quantitative fingerprint of the phenolic profile of a sample, which may be used for comparison with other samples at different stages of maturation. Nevertheless, the comparison of large numbers of chromatograms is a difficult process due to the numerous minor changes that arise in individual chromatographic profiles. For example, profiles of Manzanillo olives harvested at black maturation one week apart were markedly different in the later stages of the chromatograms, with more peaks present in samples harvested at the later date. Although not used in this study, such comparisons are facilitated by the use of multivariate-based pattern recognition programs [15].

Chromatographic profiles using UV detection at 280 nm were qualitatively similar for the two olive

varieties at all stages of maturation and showed little variation from the chromatograms shown in Fig. 1. There were quantitative differences, however, between the two varieties in the distribution of phenolics observed between 12 and 19 min. These compounds included oleuropein (retention time, 14.8 min) and ligstroside (17.9 min), which were observed in all Manzanillo and Cucco fruit. The oleuropein content of Manzanillo fruit was higher than that of Cucco fruit at all stages of maturation, in accordance with the observations of Amiot et al. [7] who noted an inverse relationship between fruit size and oleuropein content. A verbascoside isomer (12.0 min) was present in all Manzanillo samples at all stages of maturity, but its concentration varied significantly between fruits harvested from different regions. This same compound was rarely detected in Cucco samples and then only at certain stages of development. Tyrosol was present in Cucco fruit at a considerably reduced level to that in Manzanillo, unlike ligstroside, which was relatively more abundant in the Cucco fruit.

3.2. Mass spectrometry

The application of ESI was evaluated for the six standard phenolic compounds in both positive and negative ion modes at a cone voltage of 50 V using methanol–water (50:50, v/v) as the solvent. This represents the first report of the ESI spectra for several of these compounds. ESI overcomes the lack of analyte volatility by the direct formation or emission of ions from the surface of a condensed phase. Hence, it eliminates the need for neutral molecule volatilization prior to ionization and generally minimises thermal degradation of the molecular species. Spectra are summarized in Table 2 for both the positive and negative ion modes.

Deprotonated molecular ions represented the base peak in the negative ion spectra of all six phenolic species, as shown in Table 2. In contrast, protonated molecular ion peaks were observed in the positive ion spectra of only four of the compounds, namely, oleuropein, chlorogenic, *p*-coumaric and vanillic acids and in no case represented the base peak. Sodium adduct peaks were present in the positive ion spectra of oleuropein, chlorogenic acid and *p*-coumaric acid, as well as a dimer peak for caffeic acid. Such protonated dimers may be formed through various reaction pathways including protonation of a monomer followed by association with a neutral species or formation of a neutral dimer followed by protonation. In an analogous fashion, deprotonated dimer ions may form in the negative ion mode and were observed for caffeic and *p*-coumaric acids.

3.3. LC–MS

Extracts from several olive samples were examined by LC–MS (Gradient III conditions) using ESI in the positive and negative ion modes to generate total ion current (TIC) chromatograms (Fig. 2). The presence or otherwise of particular phenols was determined by extraction of mass spectra and use of reconstructed mass chromatograms. Thus, the elution of oleuropein at 16.5 min using gradient III was confirmed by the presence of very clean and distinct peaks in both the positive and negative ion mass chromatograms at m/z 541 and 539, respectively, with a sodium adduct at m/z 563 in the positive ion mass chromatogram.

Demethyloleuropein has previously been reported [8] in the olive pulp of some cultivars and a scan of the TIC chromatogram (negative ion mode) at m/z 525, corresponding to the deprotonated molecular ion of demethyloleuropein, produced a strong peak at 18.9 min. However, based on structural considerations, demethyloleuropein is predicted to elute prior to its methylated analogue, oleuropein, under reversed-phase conditions and this has been confirmed by Amiot et al. [8]. Hence, it is unlikely that the peak at 18.9 min is demethyloleuropein. On the other hand, scanning at m/z 525 and 547 (positive ion mode) and m/z 523 (negative ion mode) produced a single peak in each of the mass chromatograms at 18.9 min. These m/z values correspond to the protonated molecular ion, sodium adduct and deprotonated molecular ion of ligstroside, respectively. Furthermore, the mass spectrum of the peak at 18.9 min was consistent with the assignment of this peak as ligstroside.

The two glycoside compounds luteolin-7-glucoside and quercetin-3-rhamnoside reported by Vlahov [16] to be present in olive pulp share the same molecular mass. Nevertheless, a peak at 14.2 min in reconstructed mass chromatograms using m/z 449 or

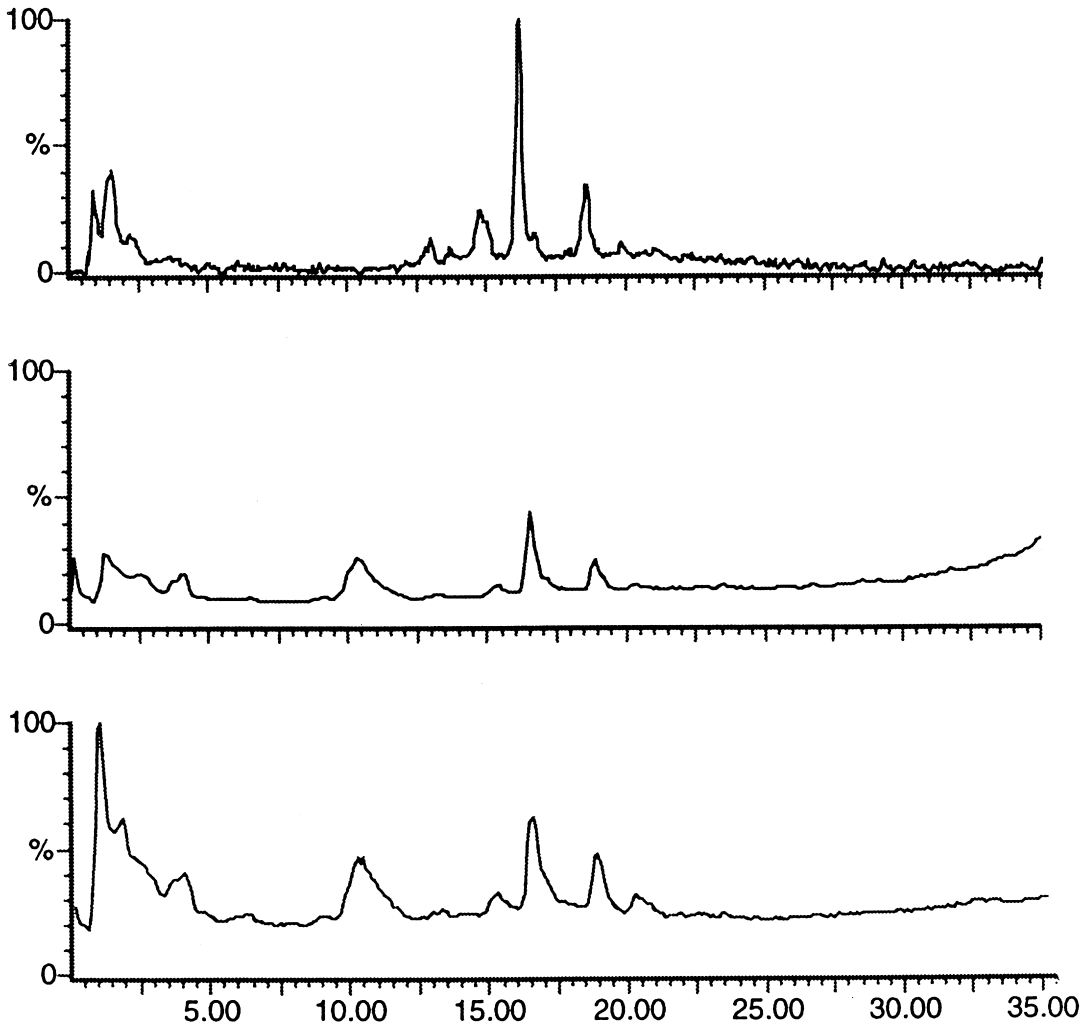

Fig. 2. Chromatograms of an extract of green Cucco fruit obtained with gradient III and comparing UV detection at 280 nm with total ion current detection in negative and positive ion mode (top to bottom).

447 (positive and negative ion mode, respectively) was concluded to arise from quercetin-3-rhamnoside, based on an examination of the respective mass spectra, which showed peaks due to the aglycone, quercitin (302 amu) in both positive and negative ion modes but no peak corresponding to luteolin (286 amu).

Reconstructed mass chromatograms using either m/z 625 (positive ion mode) or m/z 623 (negative ion mode) showed peaks at 13.7 and 15.3 min for all Manzanillo extracts. Mass spectra of both substances were identical and exhibited the molecular ions plus the sodium adduct peak at m/z 647 in the positive ion mode. These spectra are consistent with the compounds being isomers of verbascoside. There are a number of possible structures and these will be the subject of a separate communication.

A peak was observed in the TIC chromatograms (positive and negative ion mode), at about 10.4 min, of a similar size to the oleuropein peak in some samples. However, the peak was not detected by absorption of radiation at 280 nm, which suggests that it was not phenolic. Mass spectral data support the identification of this peak as elenolic acid, which

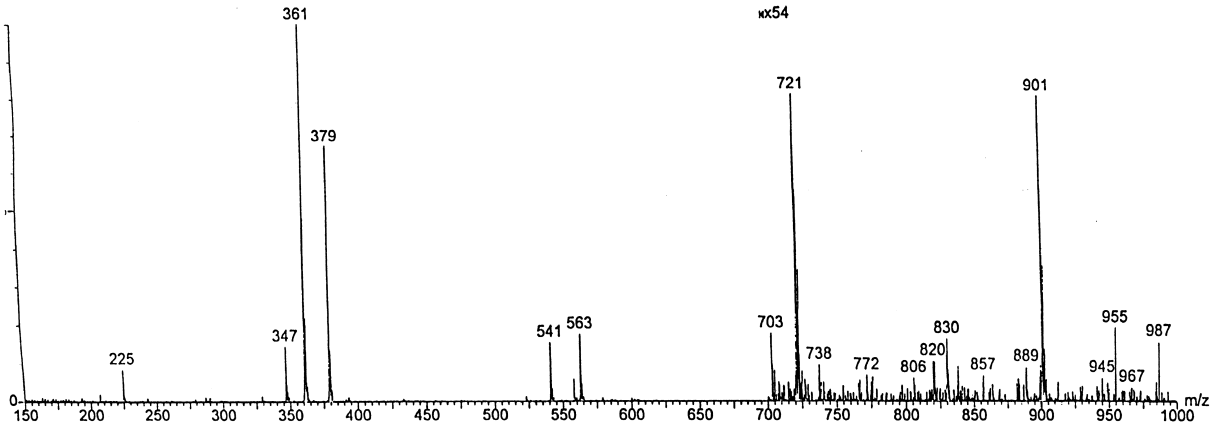

Fig. 3. Mass spectrum of peak eluting at 16.5 min (gradient III). This peak is oleuropein with a molecular mass of 540.

Table 3
Phenolic compounds identified in olives using RPLC and LC–MS

Compound	Molecular mass	Retention time (min) Gradient I	Retention time (min) Gradient III	Major ESI− peaks	Major ESI+ peaks	Sodium adduct
p-Hydroxybenzoic acid	138		I			
Tyrosol	138	3.6	I			
4-Hydroxyphenylacetic acid	152		I			
Protocatechuic acid	154		I			
p-Coumaric acid	164	9.9	I			
Hydroxytyrosol	168		I			
Vanillic acid	168	6.1	I			
Caffeic acid	180	6.8	I			
Homovanillic acid	182		1.3	181	183	205
Ferulic acid	194		1.1	193	n.d.	217
Syringic acid	198		1.1	197		
Sinapic acid	224		I			
Elenolic acid	242		10.4	241	243	265
Cyanidin	278		I			
Luteolin	286		I			
Chlorogenic acid	354	7.0	n.d.			
Apigenin-7-glucoside	432		I			
Cyanidin-3-glucoside	440		I			
Luteolin-7-glucoside	448		I			
Quercetin-3-rhamnoside	448		14.2	447	449	
Ligstroside	524	18.0	18.9	523	525, 363, 345	547
Demethyloleuropein	526		I			
Oleuropein	540	14.8	16.5	539	541	n.d.
Rutin	610		I			
Verbascoside, isomer 1	624	12.0	13.7	623, 241	625	647
Verbascoside, isomer 2	624	13.4	15.3	623, 241	625	647
Cyanidin-3-rutinoside	697		I			

I=mass spectral data were inconclusive; n.d. not detected.

arises from the presence of elenolic acid glucoside (retention time, 6.4 min), a major degradation product of oleuropein [8], which accumulates during olive maturation. The identification is based on peaks arising at m/z 241 and 265 in the negative and positive ion reconstructed mass chromatograms, which correspond to the deprotonated molecular ion and sodium adduct, respectively.

A collection of small peaks occurring between the retention times of 47 and 50 min (using gradient I conditions) was confirmed by LC–MS as phthalate esters. Such artefacts arise due to the use of plastic equipment for sample storage and extraction including SPE. Examination of the LC–MS data showed peaks in the mass spectrum of oleuropein at m/z 721 and 901 (see Fig. 3) that arise as an artefact in the electrospray, and our studies in this area are continuing. Compounds whose presence in olive fruit (Table 3) was confirmed include tyrosol, syringic, ferulic and homovanillic acids, quercetin-3-rhamnoside, oleuropein, ligstroside and isomers of verbascoside. Elenolic acid and its glucoside were also identified in sample extracts.

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