Characterisation of phenolic extracts from olive pulp and olive pomace by electrospray mass spectrometry

Susana M Cardoso,[1,2] Sylvain Guyot,[3] Nathalie Marnet,[3] José A Lopes-da-Silva,[1] Catherine MGC Renard[3] and Manuel A Coimbra[1]*

[1]*Departamento de Química, Universidade de Aveiro, P-3810-193 Aveiro, Portugal*
[2]*Escola Superior Agrária de Bragança, Instituto Superior Politécnico de Bragança, P-5301-855 Bragança, Portugal*
[3]*Unité de Recherches Cidricoles — Biotransformations des Fruits et Légumes, INRA, BP 35327, F-35653 Le Rheu Cédex, France*

Abstract: Methanol extracts of olive pomace (two-phase olive oil extraction) and olive pulp were analysed by reverse phase HPLC and the eluted fractions were characterised by electrospray ionisation mass spectrometry. This technique allowed the identification of some common phenolic compounds, namely, verbascoside, rutin, caffeoyl-quinic acid, luteolin-4-glucoside and 11-methyl-oleoside. Hydroxytyrosol-1′-β-glucoside, luteolin-7-rutinoside and oleoside were also detected. Moreover, this technique enabled the identification, for the first time in *Olea europaea* tissues, of two oleoside derivatives, 6′-β-glucopyranosyl-oleoside and 6′-β-rhamnopyranosyl-oleoside, and of 10-hydroxy-oleuropein. Also, an oleuropein glucoside that had previously been identified in olive leaves was now detected in olive fruit, both in olive pulp and olive pomace. With the exception of oleoside and oleuropein, the majority of phenolic compounds were found to occur in equivalent amounts in olive pulp and olive pomace. Oleoside was the main phenolic compound in olive pulp (31.6 mg g^{-1}) but was reduced to 3.6 mg g^{-1} in olive pomace, and oleuropein (2.7 mg g^{-1} in the pulp) almost disappeared (<0.1 mg g^{-1} in the pomace). Both these phenolic compounds were degraded during the olive oil extraction process.
© 2004 Society of Chemical Industry

INTRODUCTION

Olive pomace is an industrial by-product originating from the olive oil production process, which is obtained by squeezing the olive fruit without any chemical treatment. In the triphasic process the resultant pomace is quite dry and is usually used for the extraction of olive pomace oil with hexane, which has a high added value. However, the use of this extraction process also produces vegetative waters, which contain powerful pollutants.[1] Owing to the environmental problems, many of the industries involved in olive oil production are now using the biphasic extraction system.[2,3] In this environmentally friendly process, only the oil and residue phases are produced. However, owing to the retention of the fruit's water in the residue, the olive pomace obtained (78–83% w/w of olive weight, containing 2.3–3.4% fat) is very wet (54–62% moisture) and, because of the energy required in the drying process, does not

have a significant commercial value.[2] The possibility of recovering biological products from this residue is an attractive way of valorising it.

Data reported on olive fruit indicate significant amounts of phenolic compounds, which are predominantly secoiridoids, such as oleuropein, demethyloleuropein, ligstroside, and their hydrolytic derivatives, oleuropein aglycones, oleoside-11-methyl ester, elenoic acid, hydroxytyrosol and tyrosol.[4–8] In addition, olive fruit contains flavonol glucosides, in particular rutin and luteolin-7-glucoside, anthocyanins such as cyanidin and delphinidin glycosides, and derivatives of hydroxycinnamic acids, with predominance of verbascoside.[4–8] These phenolic compounds have also been detected in olive pomace (three-phase olive oil extraction), although the quantity of secoiridoids can be diminished by the malaxation process of the olives during olive oil production.[9,10] Oleuropein and hydroxytyrosol have been shown to have important

biological properties such as antimicrobial, hypogly-caemic, hypolipidemic, hypocholesterolic, antioxidant and free radical-scavenging actions.[11-15] The association of these properties with the prevention of several diseases such as atherosclerosis and heart diseases has raised interest in these phenolic compounds.

The analysis of phenolic extracts from olive and related products has been mostly performed by reverse phase HPLC coupled with diode array detection (DAD). More recently, the association of this methodology with ionisation mass spectrometry has proved to be useful in the identification of the major compounds in olive phenolic extracts.[16-20] Moreover, this methodology has been of great help in the identification of new compounds even when present in trace amounts.[21] In order to contribute to the knowledge of the phenolic compounds present in olive pomace, the reversed phase HPLC/DAD separation of a methanol extract was performed, the collected fractions were characterised by electrospray ionisation mass spectrometry (ESI-MS^n) and the structures of new compounds were identified by MS^2 and MS^3 experiments.

MATERIALS AND METHODS
Phenolic standards
Caffeoyl-quinic acid, vanillic acid, protocatechuic acid, syringic acid, oleuropein, luteolin, luteolin-7-glucoside, cinnamic acid and tyrosol were purchased from Sigma Chemical Co (St Louis, MO, USA).

Solvents and reagents
n-Hexane, methanol, acetone and acetonitrile, all of chromatographic grade, were purchased from Bio-solve BV (Valkenswaard, The Netherlands). Glacial acetic acid was also purchased from Biosolve Ltd. Folin–Ciocalteu reagent was purchased from Merck (Darmstadt, Germany). Deionised water was obtained with a Milli-Q water system (Millipore, Bedford, MA, USA).

Sample origin
The analyses were performed on two samples of the same batch: olive pomace and olive pulp (*Olea europaea* L var Verdial). The samples were collected at Prolagar, an olive oil factory in Mirandela, Portugal. A representative sample (2 kg, 912 olives) was collected before processing. These olives were stoned, immersed in 0.5% NaF solution and homogenised in a mixer. In the factory, olives from the same batch were crushed with a hammer mill, malaxed (slowly mixed) in a sequential extractor at a temperature close to 40 °C for 45 min and separated from the olive oil in a continuous two-phase centrifugation system. At this stage the olive pomace was collected. After collection, this residue was immediately immersed in 0.5% NaF. After arriving at the laboratory, the samples were immediately frozen, freeze-dried and kept at −20 °C until used.

Extraction of phenolic compounds
The extraction procedure used for the olive pulp and olive pomace was adapted from that of Guyot *et al*.[22] Just before extraction the freeze-dried materials were sieved through a 700 μm filter to remove the non-pulverised peel and the stones that were present in the olive pomace. Each sample (30 g of powder) was defatted with *n*-hexane, which was subsequently discarded, and the residue was extracted with 300 ml of methanol. The solution was filtered, concentrated, frozen at −20 °C and freeze-dried to give the non-purified methanol extract. The resulting residue was extracted with acetone/water (6:4 v/v). Acetone was eliminated as described for methanol and the aqueous solution was frozen and freeze-dried to obtain the non-purified aqueous acetone extract. The insoluble residue was abundantly washed with water, frozen and freeze-dried.

Estimation of amount of smashed seed hull in olive pomace
To relate the amount of material extracted from olive pomace with olive pulp, a procedure to allow the estimation of the amount of olive hull in olive pomace material was designed. As this lignified material contains low amounts of the typical phenolic compounds found in the pulp,[5,23] it should be omitted from the basis of calculation of starting material rich in phenolic compounds. An olive pomace representative sample was freeze-dried and defatted and the resulting residue was sieved through a 300 μm filter in order to separate the smashed seed and stone particles still present from the pulp material. The seeds and stones that are resistant to the grinding and malaxing, remain as coarser particles which are retained on the sieve, while the purified pulp passes through. The sieving through the 300 μm filter was only possible after defatting, since otherwise the fat present in the material would immediately have collapsed the fine pores of the filter. This procedure resulted in a residue without visible smashed particles, which accounted for 90.1% of the defatted residue.

Purification of methanol and acetone extracts
The purification step was performed on Sep Pack C18 cartridges (5 g, Waters, Milford, MA, USA). The cartridges were preconditioned by sequential treatment with methanol, H$_2$O and 2% acetic acid. Two fractions of the phenolic compounds were recovered by elution of the cartridges with methanol/water/acetic acid (50:48:2 v/v/v) followed by methanol/acetic acid (98:2 v/v). The fractions corresponding to 50 and 100% methanol extractions (MeOH 50 and MeOH 100 respectively) were concentrated to an aqueous suspension, frozen and freeze-dried.

Colorimetric quantification of total phenolic compounds by Folin–Ciocalteu method
The total concentration of phenolic compounds in the non-purified and purified extracts was determined by

an adaptation of the Folin–Ciocalteu method[24] by dispersing the non-purified and purified extracts by sonication in aqueous acetic acid (2.5% v/v) and using a calibration curve of oleuropein standard (0–70 µg).

Reverse phase HPLC conditions

HPLC analysis was performed using a Waters 2690 separation module equipped with an autosampler and a cooling system, set to 4 °C, and a Waters 996 photodiode array detector. Data acquisition and remote control of the HPLC system were done by Millennium 32 version 3.20 software (Waters, Milford, MA, USA). The column was a 250 mm × 4 mm id, 5 µm bead diameter, end-capped Purospher RP 18 column (Merck) maintained at 30 °C. The mobile phase comprised (A) 2.5% acetic acid and (B) acetonitrile, which were previously degassed and then continuously sparged with high-purity helium during analysis to prevent air resaturation. The solvent gradient started with 97% A and 3% B, reaching 91% A at 4 min, 85% A at 15 min, 79% A at 75 min, 70% A at 80 min and 10% A at 85 min, followed by an isocratic plateau for 5 min and a return to initial conditions.

For the HPLC analysis the purified methanol extracts (5 mg) were dissolved in 1 ml of methanol/acetic acid (99:1 v/v). All samples were filtered through a 0.45 µm Teflon membrane (Millipore) and 10 µl of each solution was injected.

HPLC characterisation of phenolic compounds

Compounds for which standards were available were first identified by comparison of the retention times and UV/vis spectra of the corresponding peaks. As on-line LC/MS does not give enough time to examine in detail the MS^n patterns of the various fragments, the 27 peak-forming fractions were collected prior to their characterisation by electrospray ionisation mass spectrometry ($ESI-MS^n$).

HPLC quantification of phenolic compounds

Quantification of the identified compounds was performed by correlating the measured peak area with the calibration curves obtained with reference compounds. Oleuropein and hydroxytyrosol glucoside were quantified according to their absorbances at 280 nm. In accordance with Mulinacci et al,[8] hydroxytyrosol glucoside was quantified using tyrosol as reference compound. Oleoside and its derivatives were evaluated at 240 nm using oleuropein as reference. Caffeoyl-quinic acid was evaluated at 320 nm using caffeoyl-quinic acid as reference. The flavones luteolin-7-glucoside, luteolin-4-glucoside, luteolin-7-rutinoside and rutin were evaluated at 340 nm and expressed with the extinction coefficient of luteolin-7-glucoside.

ESI-MS

The mass spectrometry system was an LCQ DECA ion trap mass spectrometer (Thermofinnigan, San Jose, CA, USA) equipped with an ESI source and run by Xcalibur® (Thermofinnigan, San Jose, CA, USA) version 1.2 software. Infusion analyses were performed in negative mode with an ion spray voltage of approximately 4500 kV, a −60 V orifice voltage, a 225 °C capillary temperature, a 50 au (arbitrary units) sheath nitrogen gas flow rate and a nominal mass range up to m/z 1800. Although phenolic compounds give lower-intensity peaks in negative than in positive mode, negative ion electrospray was used because cleaner spectra were obtained. Samples corresponding to collected HPLC peaks were directly introduced into the ESI source by a built-in syringe pump at a flow rate of 10 µl min^{−1}.

RESULTS AND DISCUSSION

Isolation and purification of phenolic compounds

The yields of mass and phenolic compounds extracted from olive pulp and olive pomace using methanol and aqueous acetone are presented in Table 1. Ponderal yields were calculated on a dried, defatted and dehulled basis to facilitate comparisons between olive pulp and olive pomace. Indeed, the two samples had different oil contents and, in contrast with the olive pulp, the olive pomace still contained smashed seed hulls, the weight of which was estimated as 9.9% of defatted olive pomace.

For both samples the methanol extract was the most significant one, representing 68 and 66% of the dried, defatted and dehulled olive pulp and olive pomace respectively. The aqueous acetone extract of both samples consisted of about 10% of the methanol extract. The remaining residues represented 23 and 28% of the olive pulp and olive pomace respectively. Mass recovery of the extracts and residues was about 98% for olive pulp and total for olive pomace.

The total phenolics of each extract were expressed as oleuropein equivalents and the values are shown in Table 1. Depending on the considered material (pulp or pomace), the non-purified methanol and aqueous acetone extracts showed total polyphenol proportions in the range of 20–36%. After C18 cartridge purification the phenolic content was raised, with a phenolic recovery of 97–99% in the methanol extracts (MeOH 50 and MeOH 100) and total recovery in the acetone extracts (Acetone 50 and Acetone 100). The sum of the amounts of phenolic compounds obtained in methanol and acetone extracts, calculated on a dried, defatted and dehulled starting material basis, was similar (154 mg g^{−1} for olive pulp and 146 mg g^{−1} for olive pomace). For both samples, most of the phenolic material was present in the MeOH 50 fraction, which represented 73% of the extractable phenolics and was therefore chosen for further investigation.

Separation of phenolic compounds by reverse phase HPLC

The MeOH 50 fractions of olive pomace and olive pulp were fractionated and analysed by HPLC/DAD

Table 1. Yields of mass and phenolic compounds in extracts and purified fractions (C18 cartridges)

Fraction	Mass (% of dry weight)	Total phenolics[a] (mg g^{-1} fraction)	Total phenolics recovered (%)	Total phenolics[b] (mg g^{-1} pulp or pomace)
Olive pulp				
Non-purified methanol extract	68[c]	204	—	—
MeOH 50	22[d]	752	82	111
MeOH 100	5[d]	716	17	23
Non-purified acetone extract	7[c]	295	—	—
Acetone 50	33[d]	726	81	16
Acetone 100	13[d]	459	20	4
Olive pomace				
Non-purified methanol extract	66[c]	198	—	—
MeOH 50	18[d]	904	83	106
MeOH 100	5[d]	601	14	18
Non-purified acetone extract	6[c]	356	—	—
Acetone 50	35[d]	830	82	18
Acetone 100	14[d]	480	19	4

[a] Values expressed as oleuropein equivalents, as result of Folin–Ciocalteu assay.
[b] Values expressed as mg phenolic compounds (oleuropein equivalents, as determined by Folin-Ciocalteu assay) g^{-1} dried, defatted and dehulled material.
[c] Yield expressed as percentage of dried, defatted and dehulled starting material (olive pulp or olive pomace).
[d] Yield expressed as percentage of non-purified extract (methanol or acetone).
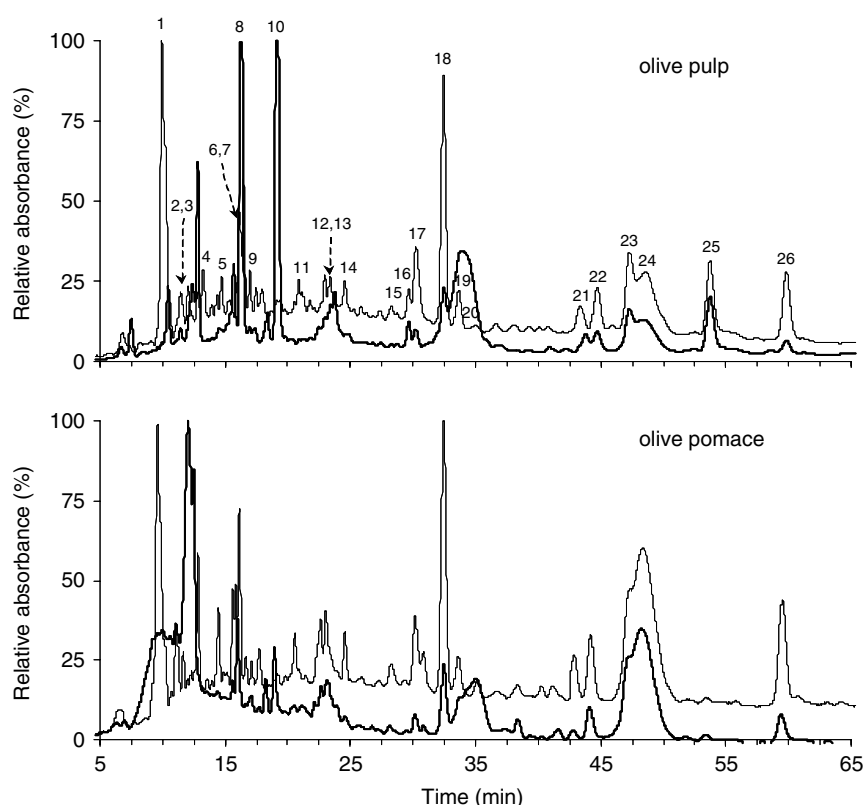

Figure 1. Chromatographic profiles of MeOH 50 fractions of olive pomace and olive pulp at 240 nm (bold curves) and 280 nm (light curves). The numbers on the figure correspond to the fractions that were collected and analysed by ESI-MS.

and the respective chromatograms obtained at 240 and 280 nm are presented in Fig 1. To improve the resolution of the chromatogram, fraction 27 was not included. Both samples had a high number of resolved fractions, suggesting a large variety of compounds. Although reasonable chromatographic separation was achieved, a significant rise of the baseline was observed in the first part of the chromatogram, suggesting co-elution of some compounds. The chromatographic profiles of olive pomace and olive pulp at 240 and 280 nm (Fig 1) differed mostly in the relative abundance of the various peak-forming compounds. This was more evident at 240 nm, related to the presence of secoiridoid derivatives, namely fractions

Table 2. Identification of HPLC-eluting fractions from MeOH 50 (pulp or pomace) extract and correspondence with results obtained by mass spectrometry analysis

Fraction number	Retention time (min)	Ident[a]	Predominant negative ion [M − H]⁻	Main fragments by ESI-MS[b]	Compound
1	10.0	B	315	153, 135, 179, 161	Hydroxytyrosol-1′-β-Glucoside
2a	11.2	—	421	389, 241, 239, 165, 195	Unknown
2b	11.2	—	407	389, 375, 357, 313, 161	Unknown
3	11.7	—	NI		—
4	12.8	—	NI		—
5	14.5	—	763	565, 341	Unknown
6	15.5	C	353	191, 179, 161	Caffeoyl-quinic acid
7	15.8	—	NI		—
8	16.2	B, C	389	345, 209	Oleoside
9	17.2	—	NI		—
10	17.8	B	595		Unknown
11	20.7	—	377	197, 179, 153	Oleuropein aglycone derivative
12	22.9	—	383; 257		Unknown
13a	23.3	B	403		11-Methyl-oleoside
13b	23.3	B	151	123, 108	4-Hydroxyphenylacetic acid
14	24.7	—	NI		—
15a	28.5	C	555	537, 403, 393	Unknown[c]
15b	28.5	—	579	337, 547, 561, 529	Unknown
16	30.6	B	609	301, 179	Rutin
17	31.0	B	593	447, 285	Luteolin-7-rutinoside
18a	32.6	A	447	285	Luteolin-7-glucoside
18b	32.6	B	623	461, 315, 135, 161, 297	Verbascoside
19	33.3	B	593	447, 285	Luteolin-7-rutinoside (isomer)
20	35.3	B, C	701	539, 377, 307	Oleuropein glucoside
21	43.2	B	447	285	Luteolin-4-glucoside
22	44.6	C	551	507, 341, 532, 389, 281	Unknown[c]
23	47.2	—	335	199, 181, 153	Unknown
24	48.9	—	663	479, 295	Unknown
25	53.9	A	539	377, 197, 153	Oleuropein
26	59.7	C	535	491, 389, 265, 325	Unknown[c]
27	75.2	A	285		Luteolin

[a] Identification was based on the following: A, retention time and DAD spectrum consistent with those of authentic standard; B, MS data consistent with literature; C, MS with fragmentation.

[b] Ordered by decreasing intensity.

[c] Compounds to be elucidated in the present study.

NI, no main [M − H]⁻ identified.

The notation a,b for a peak number in the fraction number column indicates co-eluted compounds.

8 and 10 (240 nm) and fraction 25 (240 and 280 nm), which were detected as intense peaks only in olive pulp.

Only three of the 27 fractions matched with the nine standard compounds used (see Table 2), namely fractions 18, 25 and 27, attributed to luteolin-7-glucoside, oleuropein and luteolin respectively, which correspond to compounds usually found in olives.[23,25,26] However, tyrosol, vanillic acid and syringic acid, which were among the standards and are also frequently found in phenolic extracts from olive,[17,27] although not always,[4,6] were not detected. The HPLC-eluting fractions were analysed by ESI-MS to complete the identification of the phenolic compounds in olive pulp and olive pomace.

Analysis of HPLC fractions by ESI-MS

The identification of the corresponding compounds was based on the search of the main [M − H]⁻ ion together with the interpretation of its collision-induced dissociation (CID) fragments. Table 2 summarises the data obtained for each of the analysed fractions. In some fractions the ionic species [M − H]⁻ was not observed (marked with 'NI' in Table 2), probably because the solvent and/or MS conditions were not favourable to its ionisation. Also, 11 of the 26 detected molecular ions were compounds not yet known in *O europaea*.

The compounds previously identified by HPLC/DAD were confirmed by ESI-MS. Luteolin (peak 27) showed an intense molecular ion at m/z 285 and for its derivative luteolin-7-glucoside (peak 18a) a molecular ion at m/z 447 and also a strong fragment at m/z 285 were obtained. The identification of peak 25 as oleuropein was corroborated by detection of the molecular ion at m/z 539 and its aglycone fragment at m/z 377. Fraction 11 shows ions characteristic of oleuropein aglycone or one of its isoforms.[28]

The comparison of the ESI-MS data with literature data also allowed the identification of hydroxytyrosol-1'-β-glucoside (peak 1), 11-methyloleoside (peak 13a), hydroxylphenylacetic acid (peak 13b), three derivatives of luteolin (peaks 17, 19 and 21), verbascoside (peak 18b), oleoside (fraction 8) and oleuropein glucoside (fraction 20).

The main compound in fraction 1 had a molecular ion at m/z 315 and fragment ions at m/z 153, 135, 180 and 161, which suggested the presence of a hydroxytyrosol hexoside. To our knowledge, three isomers of hydroxytyrosol glucoside have been characterised by NMR in olive fruit and olive oil.[29] These isomers have also been detected in different table olive varieties,[30] in which hydroxytyrosol-4-β-glucoside was the most abundant compound. This isomer was also the only one detected by Romero *et al*[9] in olive pulp, olive pomace and waste waters. However, the similarity between the fragmentation profile of the molecular ion at m/z 315 in fraction 1 to that published by De Nino *et al*[21] allowed us to infer that this compound should be hydroxytyrosol-1'-β-glucoside.

Fractions 13a and 13b were attributed to 11-methyloleoside and 4-hydroxylphenylacetic acid respectively based on their specific and characteristic molecular ions described in the literature for *O europaea*.[23] The high absorbance at 240 nm of fraction 10 can possibly be attributed to a secoiridoid derivative.

The presence of a fragment at m/z 285 is diagnostic of luteolin derivatives. According to the molecular ion at m/z 593 and its main fragments observed for both fractions 17 and 19, luteolin-7-rutinoside could be proposed for both fractions. To our knowledge, the flavone luteolin-7-rutinoside was previously detected only in olive leaves[23] and its ESI-MS data were similar to those of peaks 17 and 19. Since the luteolin-7-rutinoside detected by Ryan *et al*[23] eluted before luteolin-7-glucoside in HPLC reverse phase conditions, the same compound was tentatively attributed to fraction 17. Thus fraction 19 may correspond to a non-described

isomer of that compound, probably with a different linkage position to the sugar. The MS analysis of fraction 21 demonstrated a molecular ion at m/z 447, suggesting the presence of a luteolin hexoside. Four luteolin glucosides have already been detected in olives: luteolin-7-glucoside and its three isomers[23] luteolin-4-glucoside, luteolin-6-glucoside and luteolin-8-glucoside. According to those authors, luteolin-4-glucoside was the only one eluting after luteolin-7-glucoside in HPLC reverse phase conditions and that was the reason why fraction 21 was attributed to that isomer.

ESI-MS of fraction 18b indicated a molecular ion at m/z 623 and various fragments that are in accordance with the fragmentation of verbascoside. These results were also corroborated with the fragmentation profile of verbascoside described by Ryan *et al*.[18]

The comparison of the MS data with literature data was also possible for the compounds detected in fraction 8 (oleoside) and fraction 20 (oleuropein glucoside). However, as they had not been detected previously in olive pulp, the interpretation of their structures will be discussed in more detail. Also, the structures of some new oleoside derivatives corresponding to fractions 15a, 22 and 26 will be elucidated.

Structure determination of fraction 8

The mass spectrum of fraction 8, eluted at 16.2 min, displayed an intense peak at m/z 389 which formed two major fragments by CID, one at m/z 345 and the other at m/z 209 (Fig 2). The former corresponded to the loss of 44 Da, which can be justified by the elimination of a CO_2 molecule of a carboxylic group, and the latter can be attributed to the Z fragment of a hexose (loss of 180 Da). This hexose residue was attributed to glucose in accordance with the literature.[16,23] The presence of a hexose moiety was also supported by the detection of minor ionic species at m/z 161 and 179 in the ESI-MS^2 spectrum shown in Fig 2 (inset) and major ones in the ESI-MS^3 spectrum of the ion at m/z 345 (results not shown). These results are in agreement with the
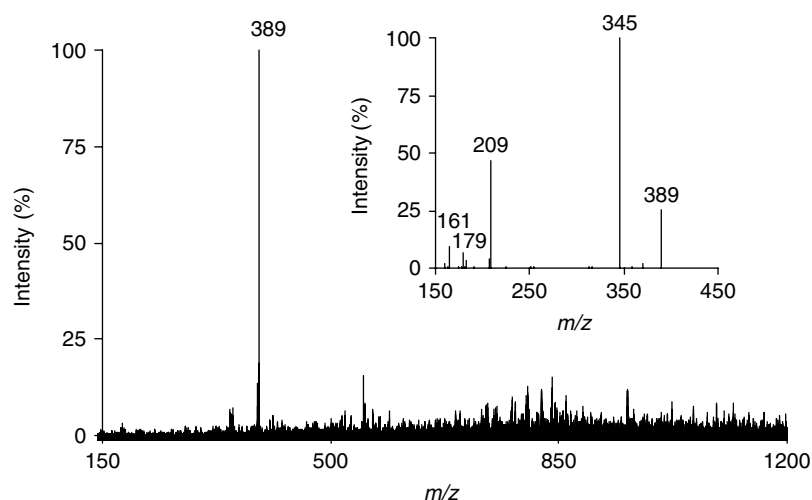

Figure 2. ESI-MS spectrum of fraction 8 and (inset) ESI-MS^2 spectrum of molecular ion at m/z 389.

presence of oleoside, which has a molecular mass of 390 Da (represented by the fragment at *m/z* 389 in Fig 4). This compound has previously been detected in olive leaves by the use of ESI-MS in positive mode.[21] However, to our knowledge, its presence in olive fruit is now demonstrated for the first time.

Structure determination of fraction 22

A derivative of oleoside, also not yet reported to occur in *O europaea*, was found in fraction 22. The mass spectrum of that fraction showed a strong peak at *m/z* 551 (Fig 3a) whose MS[2] fragmentation spectrum indicated various ionic species (Fig 3a, inset). As discussed for oleoside, the principal fragment was originated by the loss of 44 Da, giving rise to the intense signal at *m/z* 507. The ionic species at *m/z* 389, representing the oleoside structure, was also observed in the ESI-MS spectrum of the molecular ion. Its formation was accomplished by the loss of a hexose moiety (162 Da), suggesting that the compound was a hexoside derivative of oleoside. Moreover, the presence of a fragment at *m/z* 341, which corresponds to a disaccharide, indicated that this hexose molecule should be linked to the sugar moiety of oleoside (tentative structure of the molecular ion in Fig 4).

The presence of the fragment at *m/z* 251 is characteristic of a $(1 \rightarrow 6)$ disaccharide,[31–33] and a low-intensity signal at *m/z* 221 can be diagnostic of a β isomer.[33] According to Fig 3b, it is probable that the oleoside derivative detected in fraction 22 was a 6'-β-hexopyranosyl-oleoside, possibly 6'-β-glucopyranosyl-oleoside, with a scheme of fragmentation in negative mode as represented in Fig 4.

Structure determination of fraction 26

Fraction 26 of the chromatogram was a distinct and relatively intense peak. Its mass spectrum showed a high-intensity ion at *m/z* 535 that has not been detected so far in *O europaea* (Fig 5). The ESI-MS[2] spectrum of that ion (Fig 5, inset) demonstrated some similarities to the spectral profile of the two compounds already described (Figs 2 and 3a, insets). Namely, the main signal was obtained by the loss of 44 Da (ion at *m/z* 491), and an ionic species corresponding to the oleoside ion (*m/z* 389) was also detected. In this case the oleoside fragment was originated by the elimination of 146 Da, which can be justified by the Y fragmentation of a deoxyhexose molecule (fragment Y in the tentative structure represented in Fig 5). In agreement with this hypothesis, in Fig 5 (inset) the signal observed
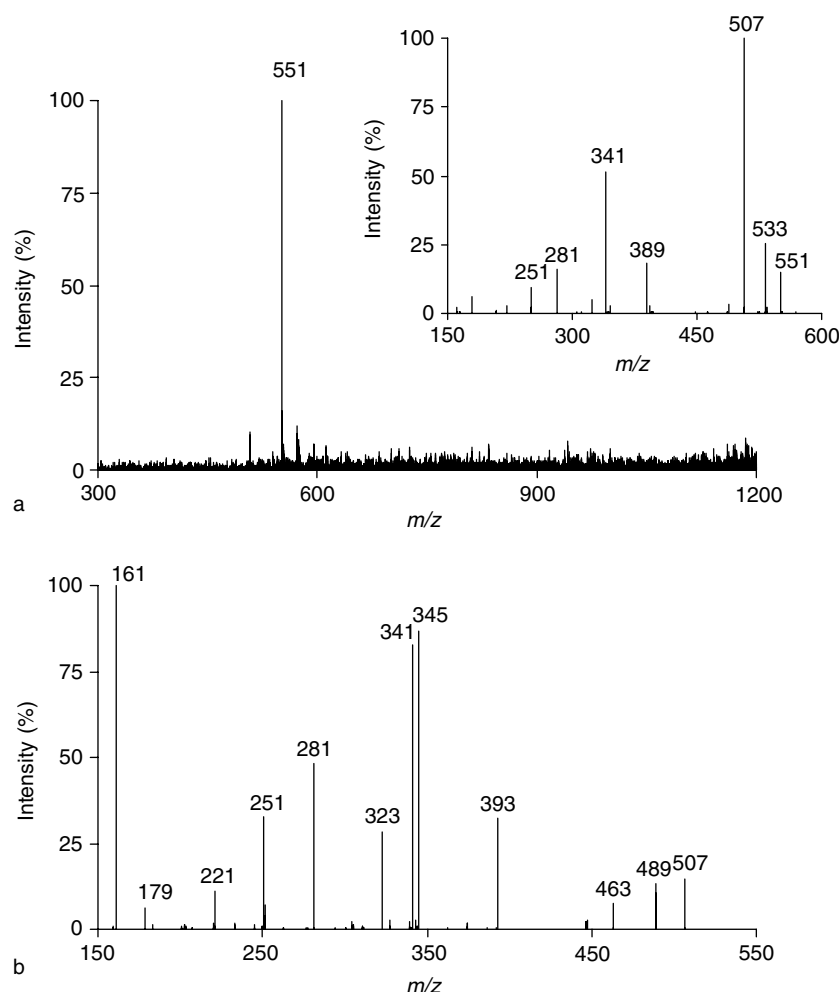

Figure 3. Mass spectra of fraction 22: (a) ESI-MS spectrum and (inset) [551] ESI-MS[2] molecular ion spectrum; (b) [551→507] ESI-MS[3] spectrum.
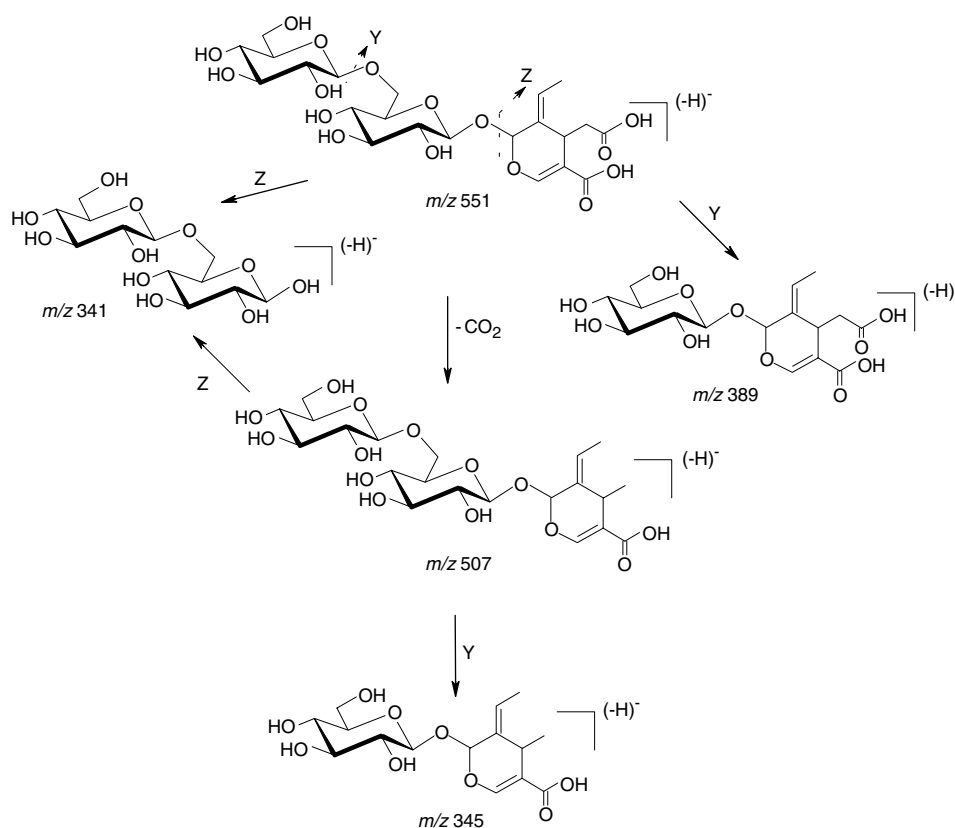
Figure 4. Proposed scheme for fragmentation of molecular ion at *m/z* 551 of fraction 22.
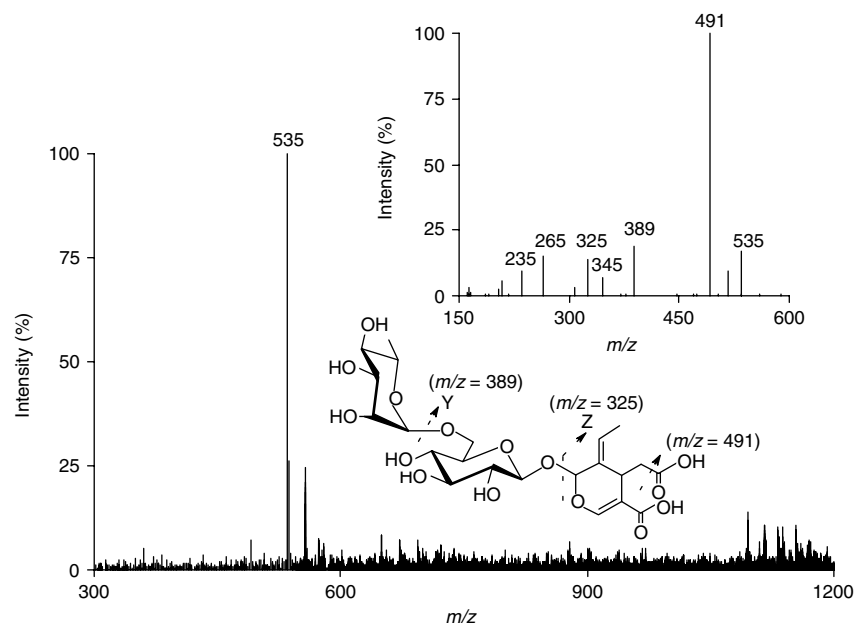

Figure 5. ESI-MS spectrum of fraction 26 and (inset) ESI-MS^2 spectrum of molecular ion at *m/z* 491. The tentative structure of the compound is also shown.

at *m/z* 325 (fragment Z) can correspond to a deoxyhexose—hexose disaccharide residue, which should correspond to a rhamnose-glucose residue, one of the most common disaccharides found in phenolic compounds.[34] The 535 MS^2 spectrum also showed the fragments of the disaccharide moiety that correspond to a pattern similar to that for glucose-(1→6)-glucose disaccharide residue, suggesting the presence of a rhamnose-(1→6)-glucose. In this manner, it can be concluded that this new compound is a 6′-deoxyhexopyranosyl-oleoside, possibly 6′-*β*-rhamnopyranosyl-oleoside.

Structure determination of fraction 20

The ESI-MS analysis of fraction 20 showed an [M − H]$^-$ ion at *m/z* 701 (results not shown). Its MS^2
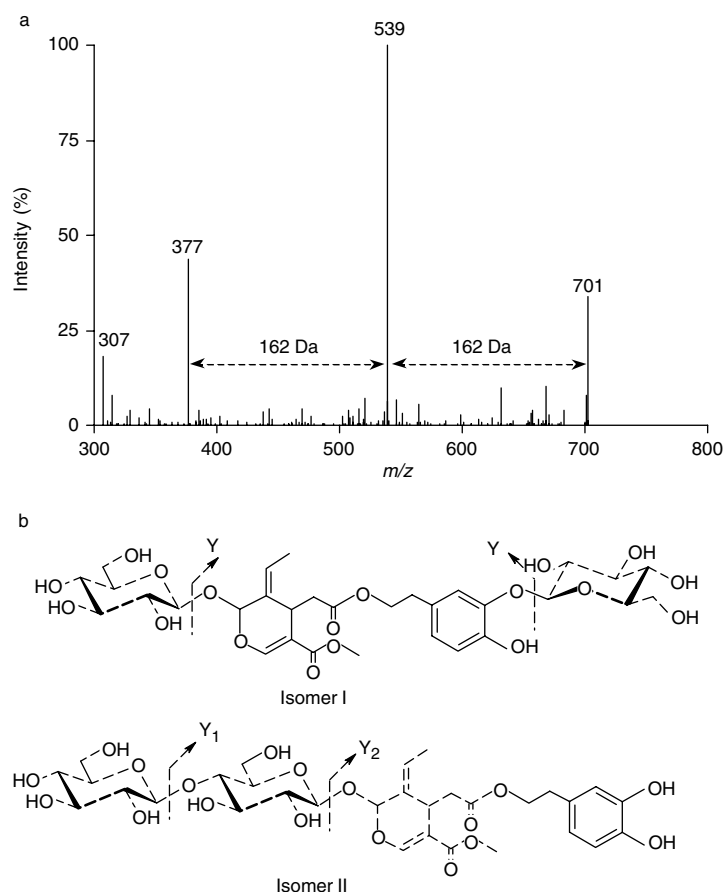
Figure 6. (a) ESI-MS^2 spectrum of molecular ion at *m/z* 701. (b) Structure of two diglucoside homologues of oleuropein.

fragmentation profile showed a main ionic species at *m/z* 539 that was formed by the loss of 162 Da, and another intense peak at *m/z* 377 indicative of the elimination of another hexose unit (Fig 6a). These two main fragments correspond to oleuropein and its aglycone respectively and together they support the hypothesis of a hexose derivative of the oleuropein structure. To our knowledge, hexose derivatives of oleuropein have never been described in olive fruit. However, angustifolioside A (isomer I represented in Fig 6b) was already described to occur in the family Oleaceae.[35] Also, De Nino *et al*[16] have proposed the presence of the same isomer in olive leaves, although their results did not allow the exact structural determination, and the exclusion of isomer II. In the present study the loss of 162 Da of the molecular ion at *m/z* 701 can fit for both isomer structures: the consecutive or simultaneous elimination of a Y-type hexose fragment would be possible for the two compounds, explaining the fragments at *m/z* 539 and 377. However, from the oleoside derivatives discussed in Figs 3 and 5, it can be expected that, if isomer II was present, the *O*-dihexosyl ion (at *m/z* 341) together with its fragments would have appeared in the ESI-MS^2 spectrum of the molecular ion. Yet, the total absence of those species was confirmed, indicating that the isomer present in fraction 20 is angustifolioside A (isomer I in Fig 6b). These results together with those of De Nino *et al*[16] suggest that *O europaea* has the same glucoside derivative of oleuropein that is present in *Fraxinus angustifolia*.[35]

Structure determination of fraction 15a

The MS analysis of fraction 15a showed a predominant [M − H]$^-$ signal at *m/z* 555. As for the oleoside derivatives, it was not possible to find any MS data in the literature about this compound. Alternatively, its structure elucidation was only based on its ESI-MS^n analysis. Fig 7 shows the ESI-MS^2 spectrum of the ion at *m/z* 555. The main fragment ion represented in the spectrum was obtained by the loss of 18 Da (ion at *m/z* 537), suggesting that the compound has an OH group that is easily removed. Also, another two intense peaks at *m/z* 393 and 403 could be observed. The first corresponded to the aglycone (loss of 162 Da) and the latter was equivalent to the mass of an 11-methyl-oleoside moiety. The aglycone at *m/z* 393 was already detected as 10-hydroxy-oleuropein aglycone in olive oil using mass spectrometry,[36] which indicates that this compound should be 10-hydroxy-oleuropein. In this manner the fragments at *m/z* 537 and 393 arose from the loss of water from the 10-OH group and from the Y-type cleavage of the molecule respectively. The fragment ion at *m/z* 403 could be originated by a cleavage X together with the loss of water. To our knowledge, 10-hydroxy-oleuropein has not previously been detected in any tissue of *O europaea*.
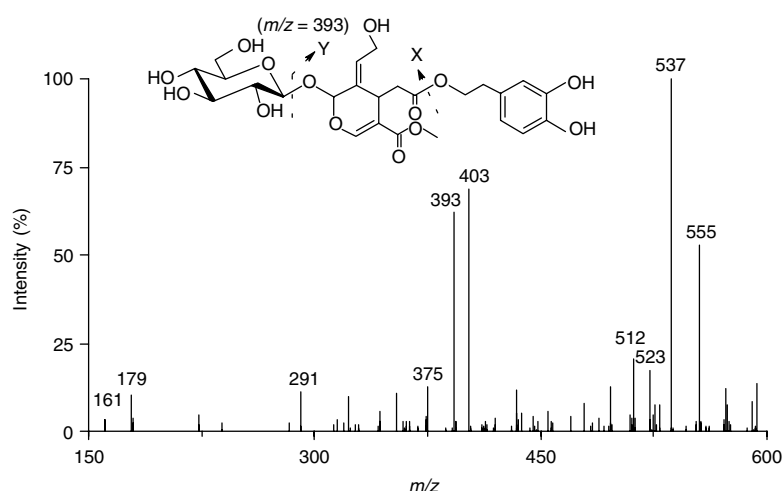
Figure 7. ESI-MS^2 spectrum of molecular ion at *m/z* 555. The structure of the molecular compound is also represented.

Table 3. Quantification of main identified compounds (mg g^{-1}) in MeOH 50 extracts of olive pulp and olive pomace

Fraction number	Compound	Olive pulp	Olive pomace
1	Hydroxytyrosol-1′-β-glucoside	6.4 (0.4)	6.5 (0.3)
6	Caffeoyl-quinic acid	0.10 (0.01)	0.09 (0.01)
8	Oleoside	31.6 (7.9)	3.6 (0.1)
16	Rutin	0.73 (0.03)	0.66 (0.03)
17	Luteolin-7-rutinoside	0.10 (0.01)	0.32 (0.01)
18[a]	Luteolin-7-glucoside + verbascoside	2.0 (0.1)	2.1 (0.0)
19	Luteolin-7-rutinoside (isomer)	0.41 (0.03)	0.44 (0.01)
21	Luteolin-4-glucoside	0.37 (0.03)	0.47 (0.01)
22	6′-β-Glucopyranosyl-oleoside	6.6 (0.2)	5.0 (0.0)
25	Oleuropein	2.7 (0.14)	V
26	6′-β-Rhamnopyranosyl-oleoside	3.8 (0.2)	6.5 (0.1)
Total		54.8	25.7

Phenolic compounds were determined as the mean value of two independent assays measured in duplicate. Values in parentheses represent the standard deviation. Values are expressed as mg phenolic g^{-1} dried, defatted and dehulled starting material. V, vestigial quantity.
[a] Quantified as luteolin-7-glucoside.

By the same reasoning (loss of respectively 32, ie a methoxyl, and 18, ie a hydroxyl, to give the ion at 389), fractions 2a and 2b were respectively hydroxy and methoxy derivatives of oleoside, which were not investigated further.

Quantification of main identified compounds separated by HPLC

The amounts of the major identified compounds are shown in Table 3. Demethyloleuropein, tyrosol, hydroxytyrosol and vanillic acid, which are frequently detected in olive pulp, were not found in this sample. As the profile of olive pulp phenolics and derivatives can be influenced by various factors such as olive cultivar, climatic conditions, degree of maturation and agronomic practices,[9] the absence of these compounds can be accepted as possible. According to Table 1, the compounds identified in Table 3 represent 49% of the total phenolics present in the MeOH 50 extract of olive pulp, but only 24% of those present in the MeOH 50 extract of olive pomace. These results can be related to the possible modification of olive phenolics during olive oil extraction. The amounts of hydroxytyrosol-1′-β-glucoside, caffeoyl-quinic acid and flavones, with the exception of rutin, were not greatly affected by the olive oil extraction process (Table 3). The secoiridoids were more affected by the extraction process: oleuropein was one of the main compounds in olive pulp (2.7 mg g^{-1} sample) but only a vestigial compound in olive pomace. This result suggests that oleuropein could be extracted to the oil phase, which is supported by its detection in the oil,[37] although this compound is mostly soluble in water. Alternatively, oleuropein could be degraded during the crushing and malaxation of the olives, as its glycosydic linkage is easily hydrolysed by β-glucosidases, producing oleuropein aglycone which is more hydrophobic and consequently more soluble in the oil. This is corroborated by the frequent detection of oleuropein aglycone and its isomer 3,4-(dihydroxyphenyl)ethanol elenoic acid ester (3,4-DHPEA-EA) in olive oil.[9–11,26,38,39] Moreover, it is well known that oleuropein aglycone can be modified due to the keto–enol tautomeric equilibrium that involves the ring opening of secoiridoids,

usually originating secoiridoid derivatives, such as the dialdehydic form of elenoic acid linked to hydroxytyrosol (3,4-DHPEA-EDA) or to tyrosol (*p*-DHPEA-EDA).[9–11,26,38,39] In this way the lower quantity of oleuropein in olive pomace, when compared with olive pulp, is probably correlated with the formation of these compounds, which are major phenolics in olive oil.

Oleoside and its derivatives were very significant compounds in the phenolic extract of both samples. In olive pulp, oleoside was evaluated at $31.6\,\mathrm{mg\,g^{-1}}$ sample. However, its concentration was drastically diminished to $3.6\,\mathrm{mg\,g^{-1}}$ in the olive pomace, indicating a loss of approximately 89% during oil extraction. As for oleuropein, this can probably be explained by its degradation during the malaxation of the pastes, with a concomitant loss to the oil or accumulation of newly formed oleoside derivatives in the olive pomace, which were not quantified or detected at 280 nm. The accumulation of oleoside derivatives in the olive pomace is in accordance with the different profiles demonstrated for the two samples at 240 nm (Fig 1). Compounds such as $6'$-β-rhamnopyranosyl-oleoside and the majority of luteolin derivatives, namely luteolin-7-rutinoside, showed higher concentrations in olive pomace than in olive pulp. As the contribution of the reserve endosperm was not taken into account in the calculations of olive pomace phenolics, the higher amounts of these compounds suggest their presence in this tissue.

CONCLUSION

The analysis of the methanol extract by ESI-MS^n allowed the detection of the common phenolic compounds but also detected unusual ones. Moreover, these techniques were very useful in the structure elucidation of new compounds, which were mainly hexoside derivatives of oleoside and oleuropein. The described data surely contribute to a better understanding of phenolic extracts from olive and its residue obtained after olive oil extraction. Also, most of the phenolic compounds, including hydroxytyrosol glucoside, which can have biological activities, are not degraded during olive oil extraction, suggesting that the olive pomace from the two-phase system can be a good source of those compounds, as is olive pulp.

ACKNOWLEDGEMENTS

REFERENCES

1 Saviozzi A, Riffaldi R, Levi-Minzi R, Scagnozzi A and Vanni G, Decomposition of vegetation-water sludge in soil. *Bioresource Technol* **44**:223–228 (1993).

2 Civantos L, Comparación entre sistemas de extracción, in *Obtención del Aceite de Oliva Virgen*, Ed by Civantos L. Editorial Agrícola Española, Madrid, pp 189–201 (1999).

3 Di Giovacchino L, Resultados obtenidos de la extracción del aceite de las aceitunas com un nuevo decantador de dos fases. *Olivae* **50**:42–44 (1994).

4 Amiot M-J, Fleuriet A and Macheix J-J, Importance and evolution of phenolic compounds in olive during growth and maturation. *J Agric Food Chem* **34**:823–826 (1986).

5 Servili M, Baldioli M, Selvaggini R, Miniati E, Macchioni A and Montedoro G, Phenolic compounds of olive fruit: one- and two-dimensional nuclear magnetic resonance characterization of nuzhenide and its distribution in the constitutive parts of fruit. *J Agric Food Chem* **47**:12–18 (1999).

6 Esti M, Cinquanta L and La Notte E, Phenolic compounds in different olive varieties. *J Agric Food Chem* **46**:32–35 (1998).

7 Romani A, Mulinacci N, Pinelli P, Vincieri FF and Cimato A, Polyphenolic content in five Tuscany cultivars of *Olea europaea* L. *J Agric Food Chem* **47**:946–967 (1999).

8 Mulinacci N, Romani A, Galardi C, Pinelli P, Giaccherini C and Vincieri FF, Polyphenolic content in olive oil waste waters and related olive samples. *J Agric Food Chem* **49**:3509–3514 (2001).

9 Romero C, Brenes M, Garcia P and Garrido A, Hydroxytyrosol 4-β-D-glucoside, an important phenolic compound in olive fruits and derived products. *J Agric Food Chem* **50**:3835–3839 (2002).

10 Vierhuis E, Servili M, Baldioli M, Schols HA, Voragen AGJ and Montedoro GF, Effect of enzyme treatment during mechanical extraction of olive oil on phenolic compounds and polysaccharides. *J Agric Food Chem* **49**:1218–1223 (2001).

11 Visioli F and Galli C, Olive oil phenols and their potential effects on human health. *J Agric Food Chem* **46**:4292–4296 (1998).

12 Visioli F and Galli C, The effect of minor constituents of olive oil on cardiovascular disease: new findings. *Nutr Rev* **56**:142–147 (1998).

13 Arouma OI, Deiana M, Jenner A, Halliwell B, Kaur H, Banni S, Corongiu F, Dessí MA and Aeschbach R, Effect of hydroxytyrosol found in extra virgin olive oil on oxidative DNA damage and on low-density lipoprotein oxidation. *J Agric Food Chem* **46**:5181–5187 (1998).

14 Le Tutor B and Guedon D, Antioxidative activities of *Olea europaea* L. leaves and related phenolic compounds. *Phytochemistry* **31**:1173–1178 (1992).

15 Bisignano G, Tomaino A, Cascio RL, Crisafi G, Uccella N and Saija A, On the in-vitro antimicrobial activity of oleuropein and hydroxytyrosol. *J Pharmaceut Pharmacol* **51**:971–974 (1999).

16 De Nino A, Lombardo N, Perri E, Procopio A, Raffaelli A and Sindona G, Direct identification of phenolic glucosides from olive leaf extracts by atmospheric pressure ionization tandem mass spectrometry. *J Mass Spectrom* **32**:533–541 (1997).

17 Ryan D, Robards K and Lavee S, Changes in phenolic content of olive during maturation. *Int J Food Sci Technol* **34**:265–274 (1999).

18 Ryan D, Robards K, Prenzler P, Jardine D, Herlt T and Antolovich M, Liquid chromatography with electrospray ionisation mass spectrometric detection of phenolic compounds from *Olea europaea*. *J Chromatogr A* **855**:529–537 (1999).

19 Ryan D, Robards K and Lavee S, Determination of phenolic compounds in olives by reversed-phase chromatography and mass spectrometry. *J Chromatogr A* **832**:87–96 (1999).

20 De Nino A, Mazzotti F, Perri E, Procopio A, Raffaelli A and Sindona G, Virtual freezing of the hemiacetal–aldehyde equilibrium of the aglycones of oleuropein and ligstroside present in olive oils from *Carolea* and *Coratina* cultivars by ionspray ionization tandem mass spectrometry. *J Mass Spectrom* **35**:461–467 (2000).

21 De Nino A, Mazzotti F, Morrone SP, Perri E, Raffaelli A and Sindona G, Characterization of Cassanese olive cultivar through the identification of new trace components by ionspray tandem mass spectrometry. *J Mass Spectrom* **34**:10–16 (1999).

22 Guyot S, Marnet N, Larada D, Sannoner P and Drilleau J-F, Reversed-phase HPLC following thiolysis for quantitative estimation and characterization of the four main classes of compounds in different tissues zones of a French cider apple variety (*Malus domestica* Var. Kermerrien). *J Agric Food Chem* **46**:1698–1705 (1998).

23 Ryan D, Antolovich M, Herlt T, Prenzler PD, Lavee S and Robards K, Identification of phenolic compounds in tissues of the novel olive cultivar Hardy's Mammoth. *J Agric Food Chem* **50**:6716–6724 (2002).

24 Singleton V and Rossi JA, Colorimetry of total phenolics with phosphomolybdic–phosphotungstic acid reagents. *Am J Enol Vitic* **16**:144–158 (1965).

25 Limiori R, Consonni R, Ranalli A, Bianchi G and Zetta L, ^{1}H NMR study of phenolics in the vegetation water of three cultivars of *Olea europaea*: similarities and differences. *J Agric Food Chem* **44**:2040–2048 (1996).

26 Servili M, Baldioli M, Selvaggini R, Miniati E, Macchioni A and Montedoro G, High-performance liquid chromatography evaluation of phenols in olive fruit, virgin olive oil, vegetation waters, and pomace and [1]D- and [2]D-nuclear magnetic resonance characterization. *J Am Oil Chem Soc* **76**:873–882 (1999).

27 Marsilio V, Campestre C and Lanza B, Phenolic compounds change during California-style ripe olive processing. *Food Chem* **74**:55–60 (2001).

28 Gariboldi P, Jommi G and Verotta L, Secoiridoids from *Olea europaea*. *Phytochemistry* **25**:865–869 (1986).

29 Bianco A, Mazzei RA, Melchioni C, Romeo G, Scarpati ML, Soriero A and Uccella N, Microcomponents of olive oil—III. Glucosides of 2(3,4-dihydroxyphenyl)ethanol. *Food Chem* **63**:461–464 (1998).

30 Bianco A and Uccella N, Biophenolic components of olives. *Food Res Int* **33**:475–485 (2000).

31 Garozzo D, Giuffrida M and Impallomeni G, Determination of linkage position and identification of the reducing end in linear oligosaccharides by negative ion fast atom bombardment mass spectrometry. *Anal Chem* **62**:279–286 (1990).

32 Mulroney B, Peel JB and Traeger JC, Theoretical study of deprotonated glucopyranosyl disaccharide fragmentation. *J Mass Spectrom* **34**:856–871 (1999).

33 Mulroney B and Traeger JC, Determination of both linkage position and anomeric configuration in underivatized glucopyranosyl disaccharides by electrospray mass spectrometry. *J Mass Spectrom* **30**:1277–1283 (1995).

34 Ribéreau-Gayon P, *Les Composés Phénoliques des Végétaux*. Dunod, Paris (1968).

35 Çalis I, Hosny M, Khalifa T and Nishibe S, Secoiridoids from *Fraxinus angustifolia*. *Phytochemistry* **33**:1453–1456 (1993).

36 Caruso D, Colombo R, Patelli R, Giavarini F and Galli F, Rapid evaluation of phenolic component profile and analysis of oleuropein aglycon in olive oil by atmospheric pressure chemical ionization mass spectrometry (APCI-MS). *J Agric Food Chem* **48**:1182–1185 (2000).

37 Montedoro G, Servili M, Baldioli M and Miniati E, Simple and hydrolyzable phenolic compounds in virgin olive oil. 1. Their extraction, separation, and quantitative and semiquantitative evaluation by HPLC. *J Agric Food Chem* **40**:1571–1576 (1992).

38 Pirisi FM, Cabras P, Cao CF, Migliorini M and Muggelli M, Phenolic compounds in virgin olive oil. 2. Reappraisal of the extraction, HPLC separation, and quantification procedures. *J Agric Food Chem* **48**:1191–1196 (2000).

39 Angerosa F, D'Alessandro N, Corana F and Mellerio G, Characterization of phenolic and secoiridoid aglycons present in virgin olive oil by gas chromatography–chemical ionization mass spectrometry. *J Chromatogr A* **736**:195–203 (1996).