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Liquid chromatography with electrospray ionisation mass spectrometric detection of phenolic compounds from *Olea europaea*

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Abstract

The results demonstrate the potential of electrospray ionisation mass spectrometry for the specific detection of phenolic species in olives. Phenolic compounds were detected with greater sensitivity in the negative ion mode, but results from positive and negative ion modes were complementary with the positive ion mode showing structurally significant fragments. This is demonstrated by the identification of oleuropein and isomers of verbascoside. The structure of the latter were confirmed by retention, mass spectral and nuclear magnetic resonance data. These isomers have not previously been reported in olive.

Keywords: Olives; Phenolic compounds; Oleuropein; Verbascoside

1. Introduction

Olives constitute an important dietary source of phenolic compounds [1] and their recovery from the fruit typically involves an aqueous alcohol extraction from a powdered sample obtained by freeze–drying or treatment of the drupe with liquid nitrogen [2–5]. Preliminary clean-up of the extract by liquid–liquid extraction with a non-polar solvent is used to remove lipoidal material and pigments following which the phenolic compounds are partitioned into ethyl acetate. In an early paper, Vàzquez Roncero et al. [6] reported the main phenolic compounds in olive pulp using spectrophotometric and chromatographic methods, primarily thin-layer chromatography (TLC), with an extensive range of solvents and colorimetric reagents to distinguish between different classes of phenolics. More recently, the identification of phenolic substances in olive fruit and oil has usually been based on correspondence of retention data obtained using high-resolution techniques [7–11] and, less commonly, on a combination of retention and mass spectrometric data using newer soft-ionisation techniques. Data on the latter are limited [12,13] and the present study reports the application of liquid chromatography–mass spectrometry (LC–MS) to the identification of selected phenolics in olive fruit. This has allowed the identification of isomers of

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verbascoside in olive which have not previously been reported. Despite the successes of LC–MS caution is necessary in interpretation of data as shown by the in-source formation of dimer ions of oleuropein.

2. Experimental

2.1. Sample preparation and LC–MS

Olive extracts were prepared and analysed by LC–MS as previously described [14] except that aqueous acetic acid (2%, v/v) and methanol–acetonitrile (50:50, v/v), served as solvents A and B, respectively for the gradient elution program which involved an initial 15 min isocratic elution with 5% solvent A, followed by a linear ramp to 30% solvent A at 40 min with further linear ramps to 40% solvent A (at 50 min), 52% solvent A (at 55 min) and 70% solvent A (at 65 min).

2.2. Isolation of phenolic compounds by semi-preparative high-performance liquid chromatography (HPLC)

Semi-preparative HPLC analyses were performed with two Waters 510 pumps, a Rheodyne 7125 injector equipped with a 1000-μl sample loop, and a YMCPak ODS-AQ column (10 mm×250 mm; 5.0 μm) using methanol–water–acetic acid (10:90:1, v/v/v) as solvent A and methanol–acetic acid (100:1, v/v) as solvent B. A Waters 481 variable-wavelength detector with a 2.3 mm path flow cell served to monitor the column eluent. Waters Maxima software was used to control both pumps and process output from the detector. Analyses were conducted using a stepwise linear gradient from 22.2% solvent B to 44.4% solvent B at 30 min, isocratic for 5 min, and ramped to 100% solvent B at 55 min. A constant flow-rate of 4.0 ml min^{-1} was used for all analyses, and the UV–Vis detector was set at 280 nm. The mobile phases were continuously sparged with helium during the analyses to prevent resaturation by air.

Undiluted sample extract was injected into the semi-preparative HPLC system approximately 10 times and the peaks eluting at 21.08 min and 26.32 min, referred to as fraction A and fraction B, respectively, were collected in scintillation vials. The A and B fractions were collected in two separate flasks by transfer with methanol–water–acetic acid (50:50:1, v/v/v). The two extracts were then rotary evaporated to dryness using a dry ice–acetone cold finger with the water bath at 35°C when thin films of both substances were visible. Fraction A was further dried under high vacuum and stored under nitrogen at −16°C.

Fraction B which contained a late-eluting shoulder was dissolved in methanol–water–acetic acid (50:50:1, v/v/v; 1 ml) and injected (500 μl per injection) into the same semi-preparative HPLC system using isocratic elution with methanol–water–acetic acid (40:60:1, v/v/v) at 4.0 ml min^{-1}. The main peak was collected (fraction B1) before commencement of elution of the shoulder and treated as for fraction A.

2.3. Identification of fractions A and B1

Electrospray mass spectrometric analyses in the positive and negative ion modes were conducted on fractions A and B1 using a Fisons' Instruments VG Quattro II triple quadrupole with dedicated electrospray source. Samples were introduced by a 25 μl injection through a Rheodyne 7125 injector, into a solvent stream of methanol–water (50:50, v/v) at 50 μl per minute. Both fractions yielded sodium adduct peaks at m/z 647 in the positive ion mode, and $[M-H]^-$ peaks at m/z 623 in the negative ion mode.

TLC was also performed on fractions A and B1 and was based on the method used by Nakamura et al. [15]. Merck, C_{18} bonded to silica gel 60, 10 cm×5 cm, gel thickness 0.25 mm TLC plates were first eluted with methanol, marking the direction of the elution, and allowed to dry in air for 20 min. Fractions A and B1 in conjunction with the phenolic standard oleuropein were applied to the plate as a small spot under a stream of nitrogen to evaporate the solvent. The plate was eluted with methanol–water–acetic acid (50:50:1, v/v/v) in the same direction as the initial methanol elution. The plate was dried under a stream of nitrogen and then sprayed with 20% aqueous sulphuric acid and heated with a hot air gun until charring of the spots occurred. Fractions A, B1 and oleuropein eluted at R_F 0.57, 0.43 and 0.38, respectively.

2.4. Tandem mass spectrometry

Fractions corresponding to the oleuropein (retention time 27.5 min) and verbascoside (retention time 22.6 min) peaks were used for flow injection tandem mass spectrometric analysis (elution conditions as described for LC–MS in Section 2.1). Samples were analysed on the Quattro II mass spectrometer as described above (see LC–MS). The samples were injected as collected into a solvent flow of 50% aqueous acetonitrile using a Rheodyne 7123 injection valve at a flow-rate of 10 μl min^{-1}. The mass resolution for MS1 and MS2 for the triple quadrupole mass spectrometer was set to 0.7 u. Argon was used as the collision gas.

2.5. Nuclear magnetic resonance spectroscopy

Samples (1 mg) collected from semi-preparative HPLC were prepared in d_4 methanol (0.4 ml) with and without 1% trifluoroacetic acid (TFA) for NMR spectroscopy. NMR spectra were collected on a Varian INOVA 500 MHz instrument using 4 mm tubes with an acquisition time of 2.084 s, and a pulse delay of 3.000 s. A 7.1 μs (90°) pulse was used for all spectra.

3. Results and discussion

Electrospray ionisation allows the simultaneous detection of both phenolic and non-phenolic species such as elenolic acid (retention time 19.4 min) and elenolic acid glucoside (retention time 11.5 min), degradation products of oleuropein (retention time 27.5 min), which accumulate during olive maturation [3]. Extracted ion mass chromatograms (Fig. 1) further demonstrate the potential of MS for the specific detection of phenolic species as previously reported [20]. Although positive (not shown) and negative ion (Fig. 1) analysis are complementary, the latter showed better sensitivity and selectivity for the acidic and phenolic compounds. For example, Fig. 1A shows the selective detection of elenolic acid whereas additional peaks occurred in the positive ion mode. Nevertheless, some structurally significant fragmentation, particularly of the phenolic glycosides was produced in the positive ion mode which may assist in structural assignments. Fig. 1 also illustrates the selective detection of elenolic acid glucoside (B), ligstroside (C), oleuropein (D) and verbascoside (E). Of particular interest is the observation of two peaks (vide infra) at retention times of 22.6 min and 24.9 min in Fig. 1E. These peaks were also observed using positive ion mode but with more background interference.

The negative ion spectrum of oleuropein (the major phenolic in olive) showed a molecular ion at m/z 539 but with no structural information and tandem MS was performed on the m/z 539 and 541 ions (negative and positive mode, respectively). These spectra (not reported here) proved useful in confirming the proposed fragmentation scheme (Fig. 2) for oleuropein. In contrast the positive ion spectrum derived from oleuropein (Fig. 3) yielded a wealth of information, including a strong molecular ion peak at m/z 541. Strong fragments arising from cleavage of the glycosyl bond at either side of the oxygen atom were also observed at m/z 379 and 361 (base peak). The mechanism of glycosyl bond cleavage in oligosaccharides has been thoroughly investigated [16] and is applicable to the present situation. A fragment at m/z 225 was also observed and may arise from a McLafferty-type rearrangement of the ester function. A strong peak at m/z 137 has been attributed to the phenol fragment, $C_8H_9O_2$ of the glycoside molecule. The relative abundance of this peak can be explained by the highly stabilised cation structure resulting from rearrangement of the original ion. This fragment allows characterisation of the phenol moiety of the aglycone.

Also observed in LC–MS, due to the large intensity of the oleuropein peak was the non-covalent dimer $[2M+H]^+$. This was observed in both the positive and negative ion modes (Fig. 3), but, the dimer is more obvious in the negative ion mode (m/z 1079). The peak at m/z 637 is attributed to $(M+HSO_4)^-$ resulting from some residual sulphuric acid in the HPLC system from a previous buffer. In the positive ion mode, there are peaks present at m/z 901 and 721 corresponding to $[2M+H\text{-glucose}]^+$ and $[2M+H\text{-2glucose}]^+$. Mass chromatograms of m/z 541, 721, 901 and 1081 indicated that these ions co-elute. There are no negative ions corresponding to the m/z 721 and 901, this supports the contention that these ions are generated in the electrospray source, either

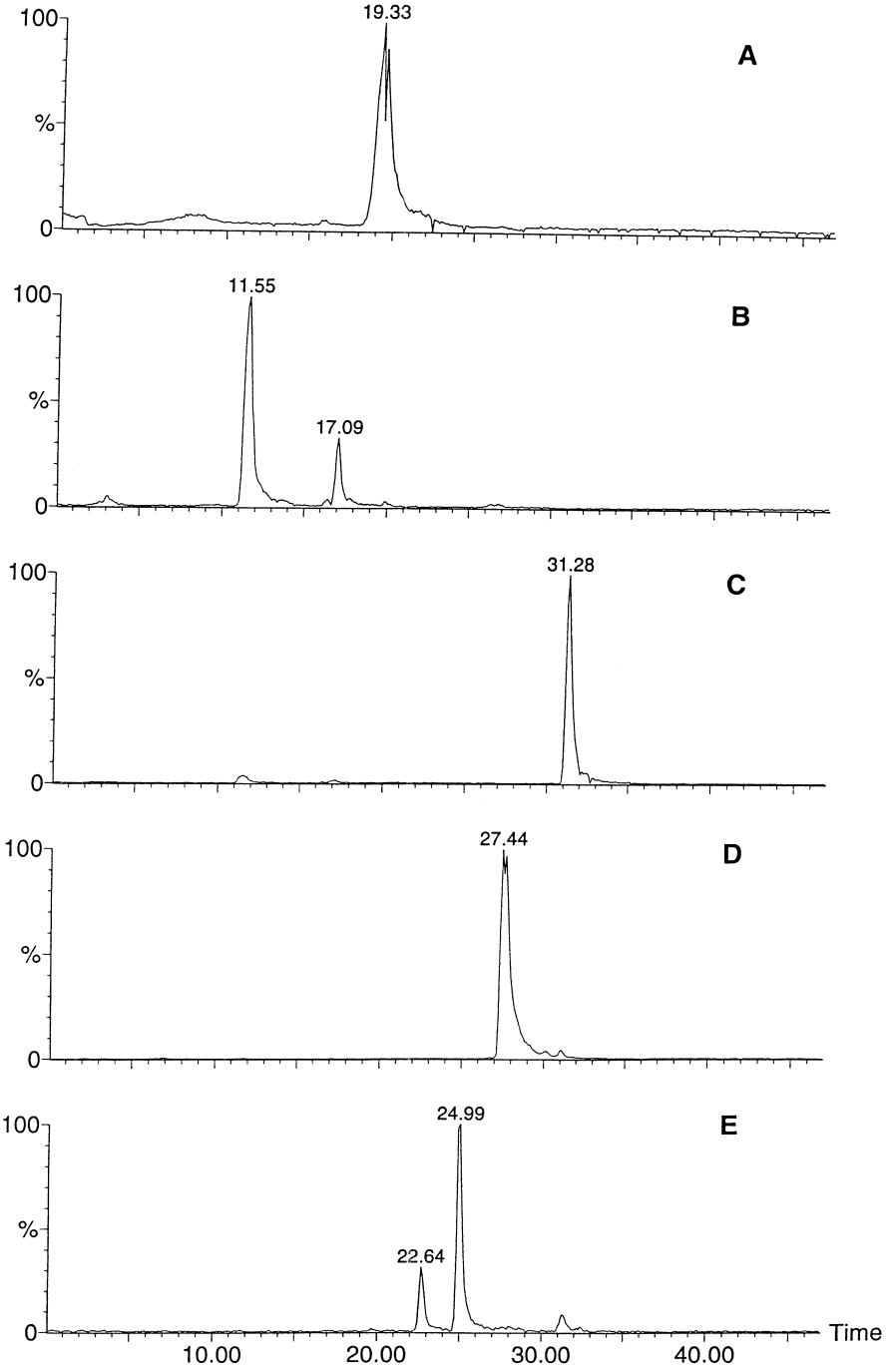


Fig. 1. Extracted ion mass chromatograms in negative ion mode. Values of m/z used were (A) 241 $[M-H]^-$ (elenolic acid); (B) 403 (elenolic acid glucoside); (C) 523 (ligstroside); (D) 539 (oleuropein); and (E) 623 (verbascoside).

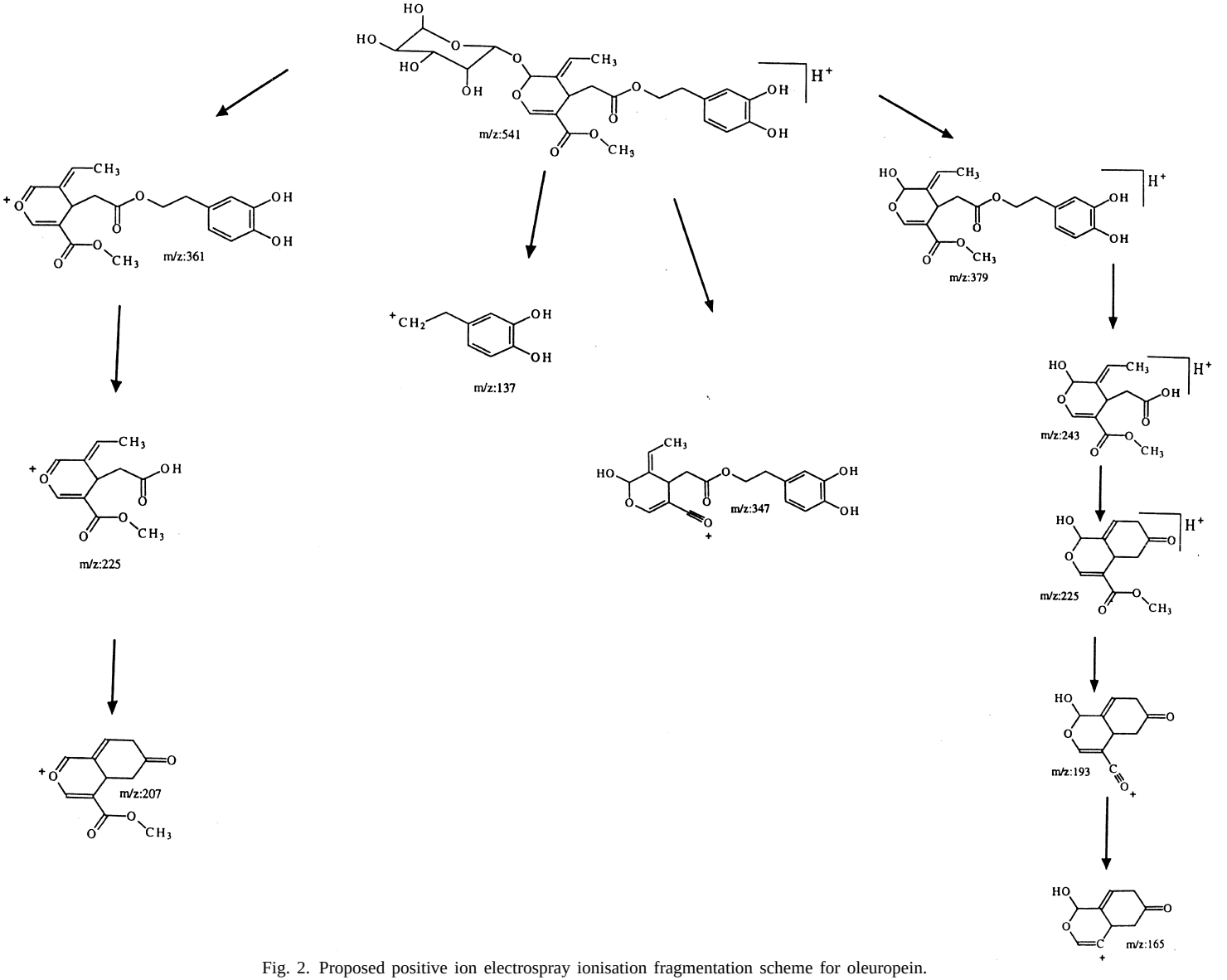
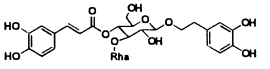
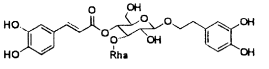
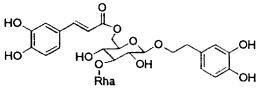
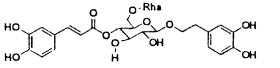


Fig. 2. Proposed positive ion electrospray ionisation fragmentation scheme for oleuropein.

Table 1
Literature and experimental ^{1}H-NMR data[a] for verbascoside and its isomers

Proton	Fraction A[b]	Fraction B1	Verbacoside standard[c]	Verbacoside (Acteoside) [18]	Acteoside isomer [18]	Forsythiaside [21]
						
Rhamnose-CH_3	1.18[d], d,	1.24, d,	1.08, d,	1.10, d,	1.26, d,	1.23, d,
	J=7 Hz	J=7 Hz	J=6 Hz	J=6 Hz	J=6 Hz	J=6 Hz
Ar–Ch_2	2.79, t[e],	2.77, t[e],	2.79, t[e],	2.78, t,	2.77, t,	2.80, t,
	J=12 Hz	J=13 hz	J=11 Hz	J=7 Hz	J=7 Hz	J=7 Hz
H1 of glucose	4.37, d,	4.33, d,	4.37, d,	4.36, d,	4.33, d,	4.35, d,
	J=8 Hz	J=8 Hz	J=8 Hz	J=8 Hz	J=8 Hz	J=8 Hz
Rhamnose-anomeric H	5.18, s	5.17, s	5.18, s	5.17, s	5.18, s	4.63, s[f]
Ar–C=CH	6.27, d,	6.28, d,	6.27, d,	6.28, d,	6.28, d,	6.30, d,
	J=16 Hz	J=16 Hz	J=16 Hz	J=15 Hz	J=16 Hz	J=15 Hz
Aromatic H	6.5–7.1	6.5–7.1	6.5–7.1	6.4–7.1	6.4–7.1	6.53–7.10
Ar–CH=C	7.58, d,	7.55, d,	7.58, d,	7.57, d,	7.54, d,	7.56, d,
	J=16 Hz	J=16 Hz	J=16 Hz	J=15 Hz	J=16 Hz	J=15 Hz

[a] All spectra recorded in d_4 MeOH.
[b] +1% TFA.
[c] Rha=rhamnose.
[d] 1.10 ppm in absence of TFA.
[e] At 500 MHz some fine structure observed.
[f] Assignment as recorded in Nishibe et al. [21]. A further peak was observed at 5.26 ppm.

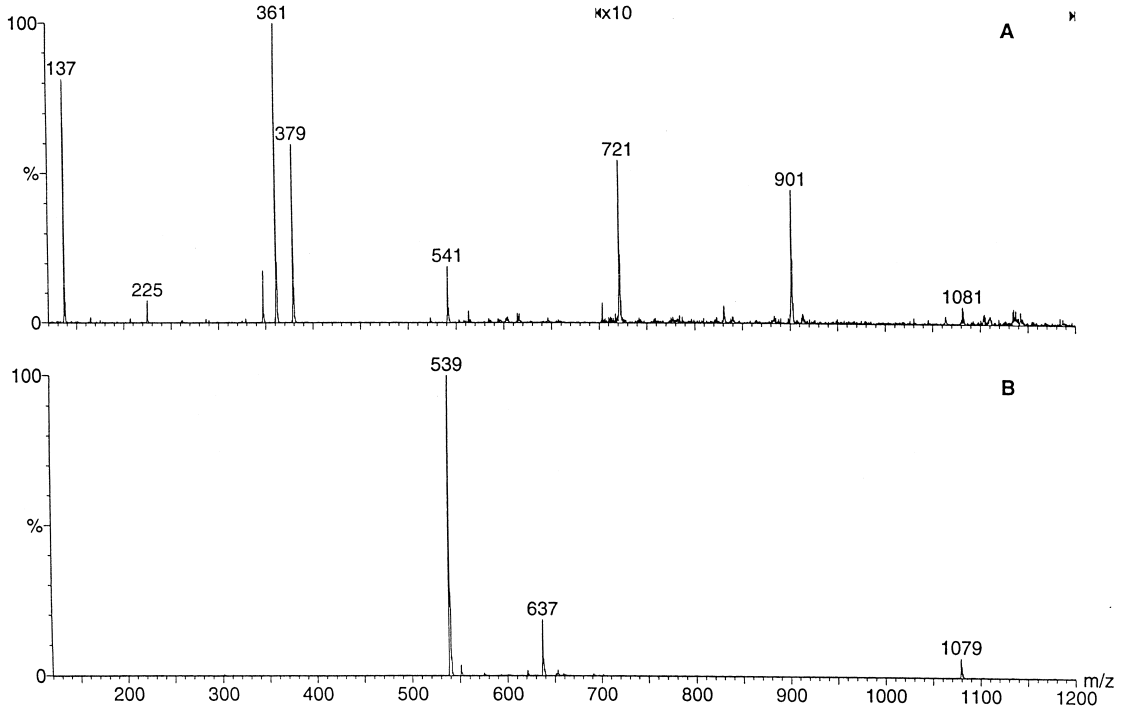


Fig. 3. Electrospray ionisation of oleuropein. (A) Positive ion mass spectrum. The region above m/z 700 is magnified 10 times. (B) Negative ion mass spectrum.

from the loss of glucose from the dimer or the aggregation of oleuropein molecular ions and fragments within the electrospray source. De Nino et al. [13] have observed a peak in olive leaf extracts that was attributed to a diglucose homologue of oleuropein which eluted at a different time to the oleuropein, there was no equivalent diglucose peak observed in the current study.

For all extracts of the cv. *Manzanillo*, two peaks were observed in extracted ion chromatograms at m/z 623 (Fig. 2E) and showed identical mass spectra, consistent with the structure of verbascoside (Table 1). The base peak in the negative ion mode was the pseudomolecular ion with few other fragment ions, whereas the positive ion spectrum showed some structural information with major peaks at m/z 479, 471 and 325. These correspond to the loss of the rhamnose sugar, the hydroxytyrosol and the loss of both the rhamnose and the hydroxytyrosol, respectively. As the negative ion mass spectrum showed no structural information, tandem mass spectrometric analysis was performed on the m/z 623 ion, and the tandem mass spectra of both compounds were very similar with the only ions of significant intensity at m/z 461 and 161. The similarity of the tandem mass spectra for the two compounds is strong evidence that the two peaks are due to isomers of verbascoside. Based on fragmentation in structurally similar compounds [17], the equivalent fragmentation in verbascoside (Fig. 4) by the loss of caffeic acid would produce the ion at m/z 461, and a ketene as the neutral fragment. The peak at m/z 161 results from a proton transfer and the production of an anionic ketene. The spectra are consistent with the compounds being isomers of verbascoside, a heterosidic ester of caffeic acid and hydroxytyrosol.

3.1. Verbascoside – structural assignment

Verbascoside may exist as a pair of geometric isomers arising from the configuration about the C=C double bond of the caffeic acid moiety. Alternatively, linkage isomers are possible due to the different attachments of the sugar to the aglycone

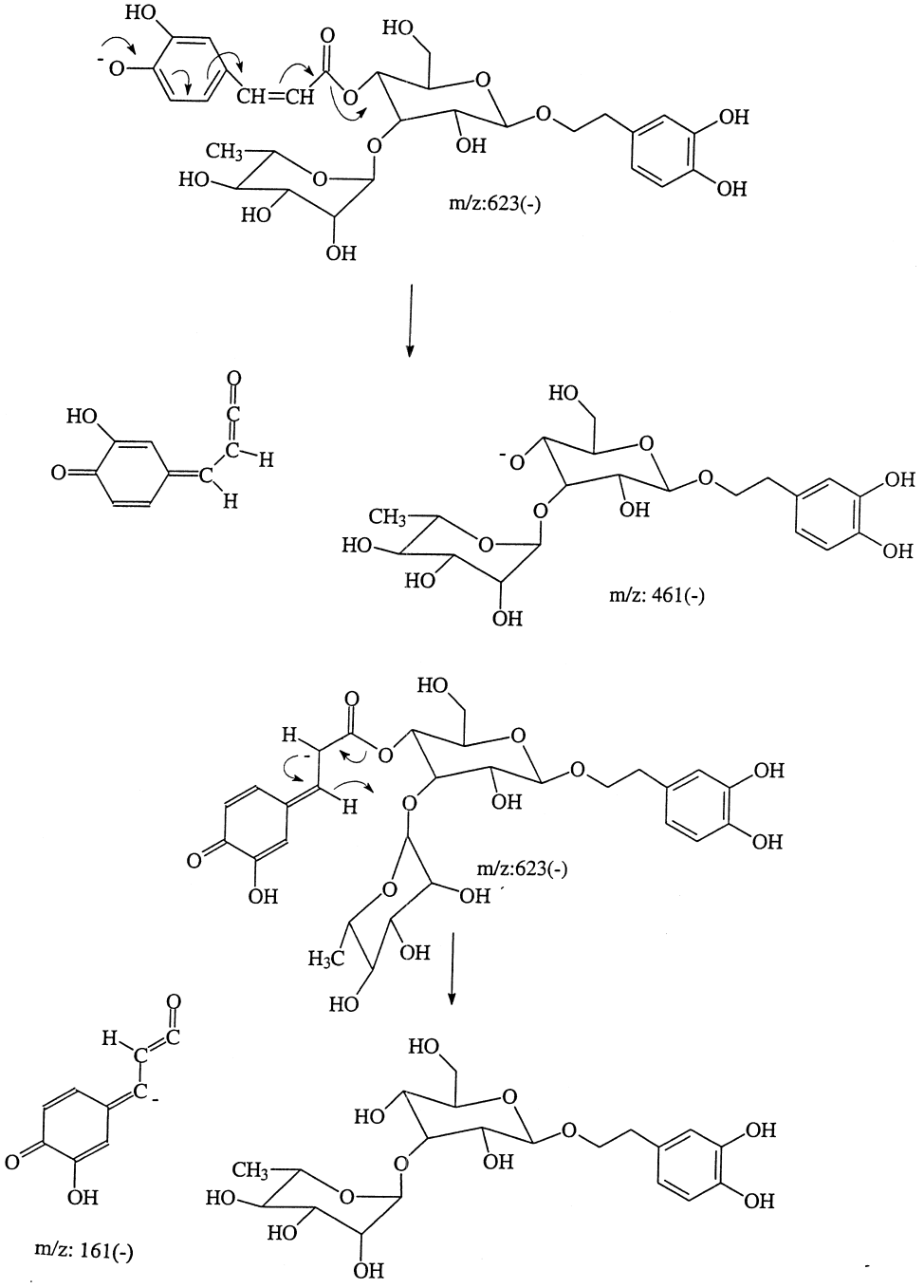


Fig. 4. Proposed negative ion collision-induced dissociation (CID) fragmentation of verbascoside.

that could account for the observed mass spectral and chromatographic behaviour. There has been no previous comment regarding the existence of such isomers of verbascoside in olive fruit although a structurally identical compound named acteoside has been isolated from other species [15,18–20]. There has been considerable confusion regarding the structure of this compound which will be referred to here

as verbascoside. On the other hand, forsythiaside (3,4-dihydroxy-β-phenethyl-*O*-α-L-rhamnopyranosyl-(1→6)-4-*O*-caffeoyl-β-D-glucopyranoside) is a genuine isomer of verbascoside which has been isolated from *Forsythia* [21]. A third isomer (Table 1) has been identified [18] in which the rhamnosyl and caffeoyl groups are attached to C-3 and C-6 of the glucose moiety, respectively. The authors referred to this compound as an ''acteoside isomer''.

In the present study the peak eluting at 22.6 min (fraction A) was confirmed as verbascoside by comparison of retention, mass spectral and ^{1}H-NMR (Table 1) data with that of a pure verbascoside standard[1]. Based on previous NMR analyses [18,21] of verbascoside and its isomers, fraction B1 (eluting at 24.9 min) has been assigned as the ''acteoside isomer'' identified by Miyase et al. [18]. This deduction has been based upon comparison of the chemical shift of the methyl group for fraction B1 at 1.24 ppm, with the literature value of 1.26 ppm for the acteoside isomer. The possibility of this fraction being forsythiaside was eliminated since its spectrum did not show the broad singlet peak at 5.26 ppm identified by Nishibe et al. [21] nor the singlet at 4.63 ppm attributed to the rhamnose anomeric proton. An alternative isomer where the alkene protons are in a *cis* configuration may be excluded on the basis of the proton–proton coupling constant (16 Hz) which indicates *trans* (*Z*) geometry.

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[1]Verbascoside standard provided by Dr. Emi Okuyama of Chiba University, Chiba, Japan.

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