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Direct Identification of Phenolic Glucosides from Olive Leaf Extracts by Atmospheric Pressure Ionization Tandem Mass Spectrometry

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Pneumatically assisted electrospray (or ionspray) coupled with liquid chromatography was applied to the identification of the phenolic glucoside content of olive leaf directly from the crude extracts. The mass spectra of the positive ions provide insights into the composition of the phenolic constituents. Oleuropein, ligstroside and a disaccharide containing the hydroxytyrosol moiety were found in olive leaf of *Olea europea* L. cv. Cassanese and their structures were thoroughly determined by tandem mass spectrometry. © **1977 by John Wiley & Sons, Ltd.**

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INTRODUCTION

Olea europaea L. is a typical Mediterranean tree widely cultivated for the production of oil and table olives. Extensive studies have been performed to identify (i) the chemical components of the secondary metabolism of this plant[1] to improve the organoleptic quality of the edible oil[2] and table olives,[3] (ii) the substances that influence host acceptance to *Bactrocera oleae*[4] and (iii) the pharmacological properties of some metabolites.[5]

Phenolic glycosides are very important minor constituents in olive trees because they contribute to certain plant properties, such as the colour of olive fruit,[6] the nutritional value and stability of the oil[7] and their resistance to microorganisms.[8] The leaves of *Olea europaea* have been studied, among others, for their therapeutic effects.[9] This peculiar physiological action has also prompted the identification of the putative active principle, a secoiridoid glucoside[9] called oleuropein, of typical bitter taste (**1**, Scheme 1), which was the major constituent of the extracts.[10] Oleuropein and its derivatives may act as, among others, antioxidant, hypoglycemic and anticholesteric agents, and therefore several other phenolic compounds, present in smaller amounts in leaves, have been isolated and identified, such as demethyloleuorpein,[11] ligstroside,[12] verbascoside,[13] oleoside dimethyl ester,[14] non-glycosidic secoiridoids[14] and oleuroside.[15] The complexity of the chromatographic profiles of the polar fraction of the olive leaf extracts clearly indicates that many other phenolic derivatives could be present in the natural matrix; on the other hand, it cannot be excluded that both extraction procedures and sample treatments do not alter the structure of some of the secondary metabolites originally present in the plant tissues. There is a need, therefore, for a reliable method for the direct analysis of the leaf extracts before any purification step. The goal is the direct characterization of olive leaf metabolites by mass spectrometry (MS) either to allow 'chemical labelling' of the particular cultivar or to provide a fast and virtually non-destructive method for the identification of those biologically and pharmacologically important molecules worth isolating.

EXPERIMENTAL

Oleuropein glycoside was obtained from Extrasynthèse (Z. I. Lyon Nord, Genay, France).

Plant material

Leaves of *Olea europaea* L. cv. Cassanese were collected at the beginning of July from a 15-year-old olive tree growing in the Olive Culture Field of the Istituto Sperimentale per l'Olivicoltura in Rende (Italy).

Fresh foliage (2.5 g) of *Olea europaea* L. cv. Cassanese was extracted with methanol (2 × 25 ml). The methanolic extract was concentrated in vacuum under a stream of nitrogen, keeping the temperature below 35 °C until it reached a syrupy consistency and partitioned in acetonitrile–hexane (4:6, v/v). Evaporation to dryness afforded a yellowish foam which was dissolved in 25 ml of methanol.

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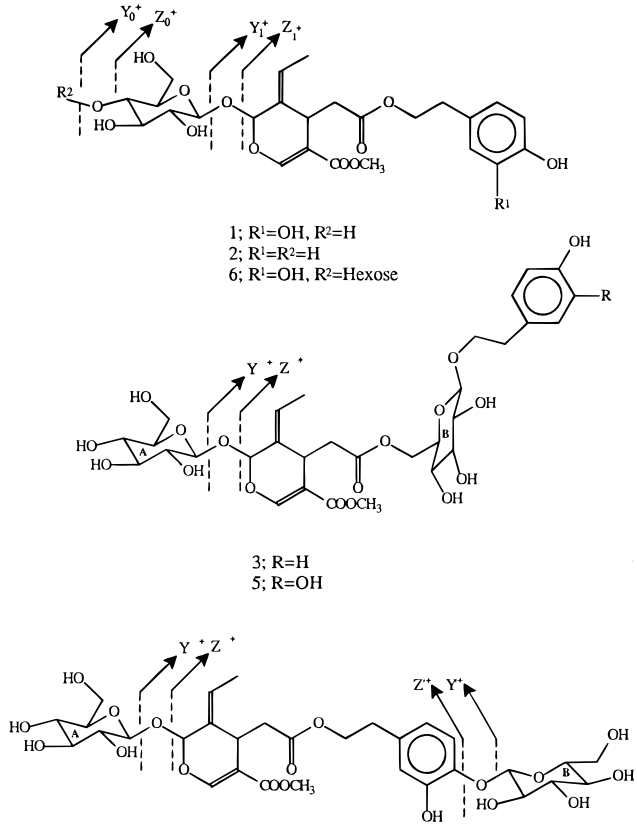


1; R^1=OH, R^2=H
2; R^1=R^2=H
6; R^1=OH, R^2=Hexose

3; R=H
5; R=OH

Scheme 1

Mass spectrometry

The ionspray (pneumatically assisted electrospray, ISI) mass spectra were acquired on a Perkin-Elmer Sciex API III Plus mass spectrometer (Sciex, Thornhill, ONT, Canada) equipped with a Perkin-Elmer Series 200 dual solvent delivery system (Perkin-Elmer, Norwalk, CT, USA) and a Perkin-Elmer LC-295 UV detector using a Perkin-Elmer C_{18} 30 mm × 4.6 mm i.d. column (3 μm particle size). The separation was performed using a gradient of acetonitrile and water containing 0.1% of formic acid. The ionspray spectra in the positive-ion mode were obtained under the following conditions: ionspray voltage, 5.5 kV; orifice voltage, 55 V; scan range, *m/z* 100–900; scan rate, 5.3 ms u^{-1}; no interscan delay; and resolution >1 u. Product ions from selected precursors were formed by collision-induced dissociation (CID) with argon in the collision cell of the PE Sciex API III Plus instrument, and mass analysed using the second analyser of the instrument under the same experimental condition as described above for the ionspray spectra. Other experimental conditions for CID included: collision energy, 20 eV; collision gas thickness[16] (CGT), 240×10^{13} molecules cm^{-2}; and scan range, *m/z* 20–900.

The fast atom bombardment (FAB) mass spectra were acquired on a VG ZAB-2F mass spectrometer equipped with a standard gun operated with a neutral xenon beam of 8 keV and a neutral current of 10 μA. Samples were prepared from 1 μl of a methanolic solution of the analyte mixed with 1 μl of ammonium chloride–glycerol (1:6, w/w) solution. Mass-analysed ion kinetic energy (MIKE) and MIKE-CID spectra were acquired by upwards scanning of the electrostatic analyser at a constant accelerating potential of ~8 kV, fixing the magnetic field at the appropriate value for the transmission of the selected reactant ions into the second field-free region of the instrument. The CID experiments were carried out by admitting helium into the collision cell and by reducing the intensity of the beam of the sampled ion (see the table in Fig. 3).

RESULTS AND DISCUSSION

The soft ionization methods introduced in the last decade[17] have extended the applicability of MS to any type of molecules regardless of their volatility and molecular mass. Accordingly, highly polar biologically[18,19] and pharmacologically[20] active compounds and components from natural extracts[21,22] have been thoroughly investigated by MS. Moreover, the combined use of multistage ion analysers coupled with various MS ionization methods allows the structure determination of the components of complex mixtures without any previous separation.[23,24]

Ionspray[25,26] was used to nebulize and ionize the high-performance liquid chromatographic (HPLC) eluate in the atmospheric pressure source of the instrument, where the ionization of the analytes is driven by the field at the needle tip that disperses the emerging charged droplets.[27]

A methanolic solution of young olive leaf from *Olea europaea* L. cv. Cassanese gave the reconstructed chromatogram shown in Fig. 1(A). As expected the chro-

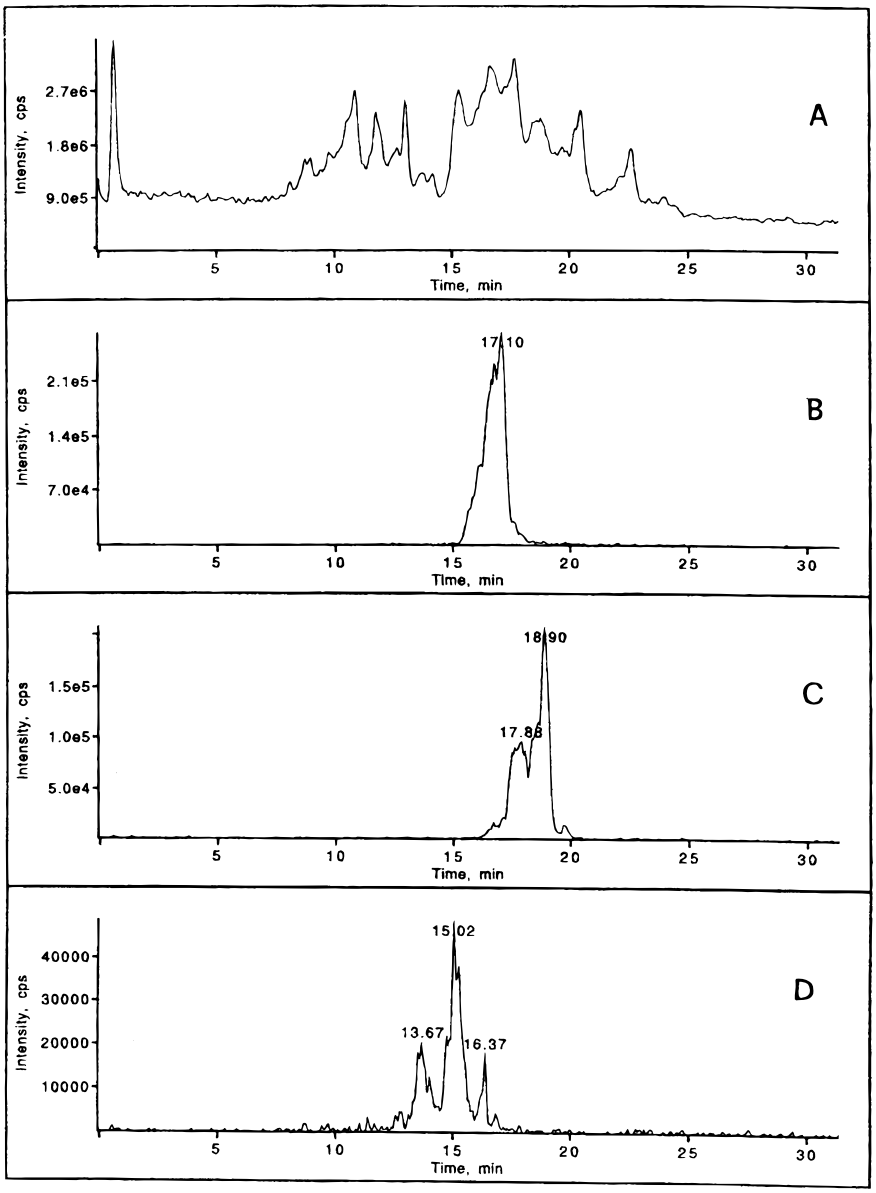


Figure 1. (A) Reconstructed ion chromatograms obtained from 20 µl of a methanolic extract of young olive leaf from *Olea europea* L. cv. Cassanese. Total ion current in the range 100–900 units plotted *vs*. elution time. Extracted ion chromatograms from (B) 558.2 u, (C) 541.2 u and (D) 720.2 u are also shown.

matographic performance was not improved (see Experimental) since the goal of the measurements was the rapid evaluation of the structure of the sampled analytes mainly based on the high specificity of an MS analysis, which is even more enhanced when HPLC is combined with tandem mass spectrometry (MS/MS).

Spectra selected on the basis of the reconstructed ion chromatograms [Fig. 1(B)–(D)] indicated the presence of the compound which might correspond to oleuropein (**1**, Scheme 1). Since **1** is readily available as a pure standard, its mass spectrometric behaviour was evaluated under the same experimental conditions as for the olive leaf extracts. ISI generally produces, in the positive-ionization mode, mass spectra of analytes of relative molecular mass M_r where the protonated $[M + H]^+$ species are accompanied by $[M + C]^+$ ions due to the attachment of cations (C), usually ammonium and/or alkali metals, to the neutral, even in the absence of added salts.[16,28] Moreover, intense peaks corresponding to $[M + NH_4]^+$ ions have been found in FAB mass spectra of glycosides,[29] whose presence, in the absence of added salt,[30] has been attributed to impurities present in the matrix.[31] It is also well known that carbohydrates tend to form, by desorption ionization methods, stable ionized adducts with amines[32] and that the determination of their molecular mass by FABMS can be conveniently carried out with diethanolamine as the matrix.[33]

In the case of **1**, the mass spectrum displayed peaks at *m/z* 541 (7%), 558 (100%) and 563 (42%) due to $[M + H]^+$, $[M + NH_4]^+$ and $[M + Na]^+$ species, respectively. The protonated and ammoniated species were chosen as parents for low-energy CIDs and the tandem mass spectra thus obtained are shown in the Fig. 2(A) and (B), respectively. Both precursors produce the same spectrum with minor differences in the relative

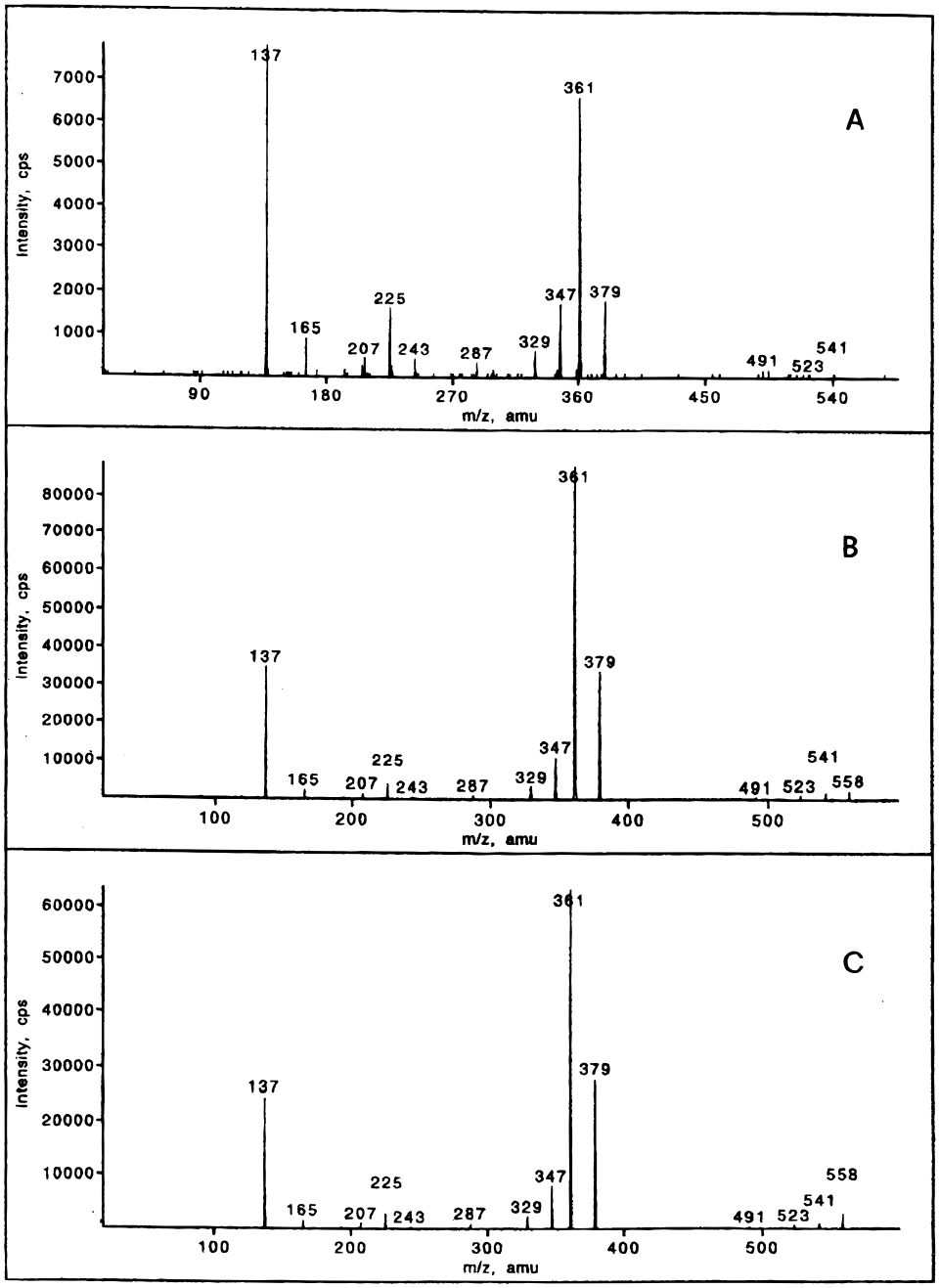
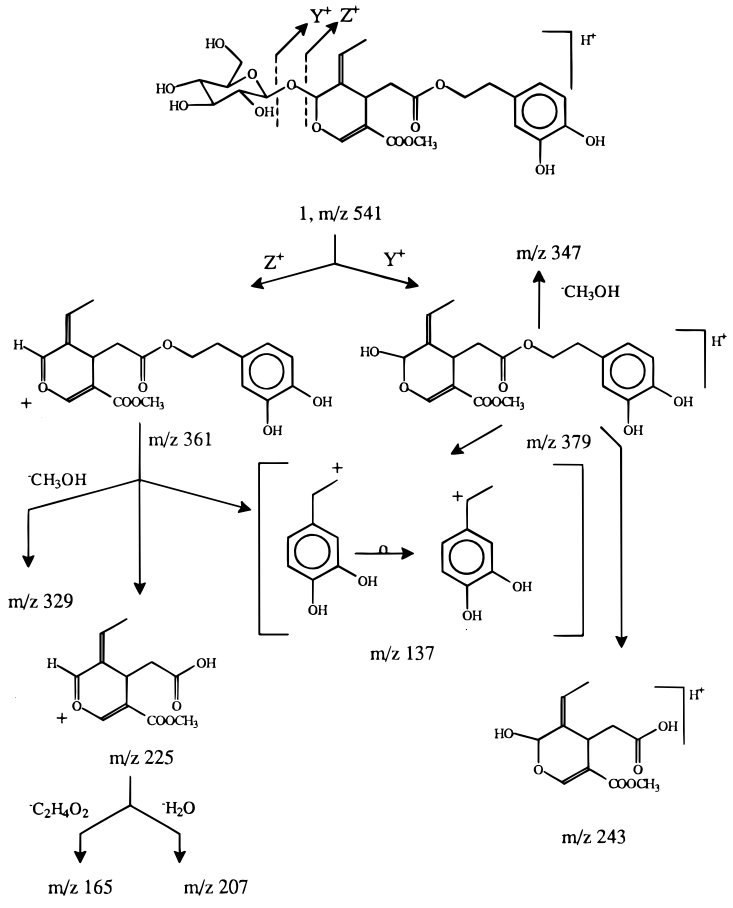


Figure 2. ISI tandem mass spectra of oleuropein (**1**). Product ion spectra (A) from $[M+H]^+$ and (B) from $[M+NH_4]^+$ precursors obtained from the commercial standard. Product ion spectrum (C) from $[M+NH_4]^+$ precursors obtained from the extracted ion chromatogram shown in Fig. 1(B).

abundances of product ions. From an analytical point of view, therefore, it is worth sampling the $[M+NH_4]^+$ ions since they provide higher sensitivity to the measurements.

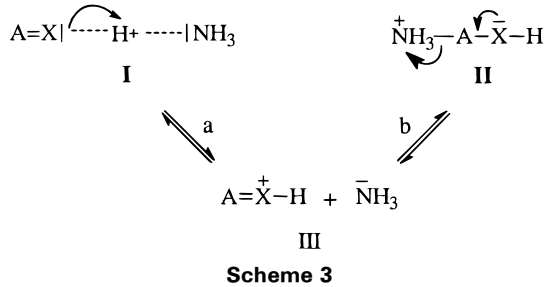
The main difference in the spectra in Fig. 2(A) and (B) is represented by the relative height of the ions at *m/z* 137 deriving from the hydroxytyrosol moiety, whereas the relative abundances of the *m/z* 379 and 361 ions are nearly the same. The latter species correspond to the Z^+ and Y^+ fragments involving cleavage of a glycosyl bond in protonated carbohydrates[34] (Scheme 2) and are of great importance in structure determination. The mechanism of glycosyl bond cleavage in oligosaccharides has been thoroughly evaluated,[35] in addition to the possible gas-phase structure of amine adducts with organic molecules formed by chemical ionization MS.[36,37] The formation of Z^+ and Y^+ ions is not necessarily driven by a localized charge site,[38] whereas the $[M+NH_4]^+$ species could be described either as proton-bound heterodimers (**I**) or a 'covalent adduct' (**II**), according to the structural features of the analyte (AX, Scheme 3).

Type I adducts of should dissociate into $[M+H]^+$ species (**III**) when the proton affinity (*PA*) of the analyte (AX) is higher than that of ammonia.[39–41] In this case the elimination of neutral ammonia, in ISI measure-



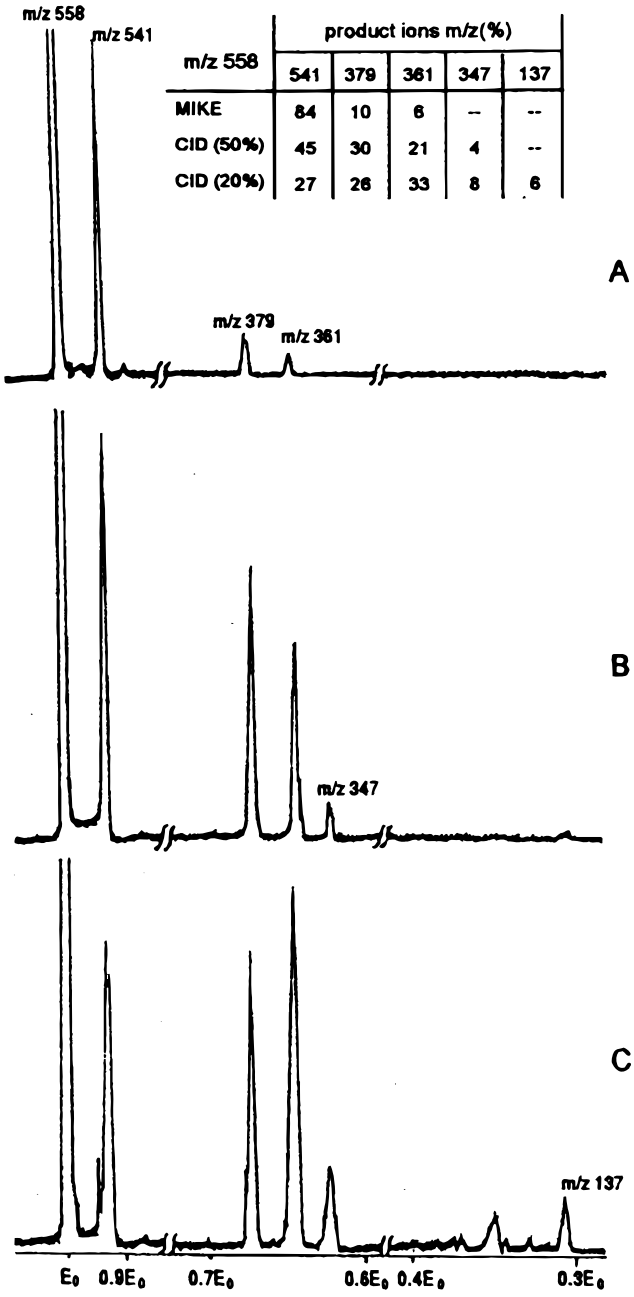
Scheme 2

ments, can also take place in the ionization region leading to $[M + H]^+$ species only, as verified for modified nucleosides.[42] If the reacting species were described by the hetero proton-bound dimer **I**, with a single hydrogen bond, the elimination of a neutral molecule of ammonia (Scheme 2, path a) would be hampered by the *PA* difference between ammonia and that estimated for an aliphatic alcohol.[43] Structure **II** might be formed by a nucleophilic attack of ammonia on one of the two ester functions of oleuropein. Assuming that the reacting $[M + H]^+$ intermediate is formed according to path b (Scheme 3), the tetrahedral intermediate thus obtained could explain the observed reactivity. It has to be considered, however, that the $[M + NH_4]^+$ ions obtained by FAB from glycerol solutions of methyl α-D-glucopyranoside and ammonium chloride preferentially release, under high-energy collisions, ammonia followed by consecutive losses of water neutrals.[43] In this case the formation of covalent adducts can hardly be suggested, whereas the higher *PA* of the monosaccharide with respect to ammonia in reasonably due to the formation



Scheme 3

of multiple hydrogen-bonded structures. Furthermore, if the observed gas-phase cleavage of the glycosidic bond of oleuropein has occurred, as suggested,[35] remote from the localization site of the ammonium moiety, ammoniated fragments, different from the observed Y and Z ions, should also be formed. It can be assumed, therefore, that the $[M + NH_4]^+$ species of oleuropein might correspond to a mixture of different structures and react, in the gas phase, through the elimination of neutral ammonia to give a transient $[M + H]^+$ which possesses enough internal energy to undergo further fragmentation. The main differences observed in the CID spectra of $[M + H]^+$ and $[M + NH_4]^+$ [Fig. 2(A) and (B)] are due to the more extensive fragmentation displayed by the former. This can be easily interpreted if the product ions are formed by consecutive reaction paths (Scheme 2) taken by the Z^+ and Y^+ intermediates possessing different internal energies. The fragments at *m/z* 243 and 255, among others, are probably due to a McLafferty-type rearrangement of the ester function involving the hydroxytyrosol moiety of Y^+ and Z^+ precursors, respectively. The species at *m/z* 137 is a very significant fragment since it allows the characterization of an important part of the aglycone, i.e. the phenol moiety. It may be formed as suggested in Scheme 2 or directly from ionized **1** or from other ionic intermediates such as *m/z* 379. The relative abundance of *m/z* 137 reflects its highly stabilized benzyl cation structure into which the originally formed ion may rearrange. In conclusion, ISI MS/MS of oleuropein provides evidence for (i) the monosaccharide unit (Y^+ and Z^+ fragments),



m/z 558	product ions m/z(%)				
	541	379	361	347	137
MIKE	84	10	6	--	--
CID (50%)	45	30	21	4	--
CID (20%)	27	26	33	8	6

Figure 3. FAB tandem mass spectra of $[M + NH_4]^+$ from oleuropein (**1**). (A) MIKE and (B) and (C) CID spectra taken at different internal energies of the precursors.

(ii) the aglycone moiety (*m/z* 243, 225 and 207, 165) and (iii) the phenolic constituent (*m/z* 137). These ions can be considered as 'markers' of the phenolic components of olive leaves which could allow their identification in complex mixtures by MS.

Oleuropein (**1**) is the major phenolic constituent of olive leaves. The extracted ion profile in Fig. 1(B) includes the species at *m/z* 558 present in the crude mixture. The mass spectrum taken at 17.10 min displayed an abundant *m/z* 558 ion and a minor peak at *m/z* 414. These two species are essentially those contributing to the ion current in Fig. 1(B); the lower *m/z* species is more abundant at the onset whereas the other dominates around the top of the distribution. The tandem mass spectrum of the *m/z* 558 ion from the olive extracts [Fig. 2(C)] is entirely superimposable with that of standard oleuropein [**1**, Fig. 2(B)], whose presence in the olive leaves was expected.

The interpretation of the ISI tandem mass spectra in Fig. 2 is based, as previously mentioned, on the formation of transient $[M + H]^+$ intermediates which possess enough internal energy to undergo complete conversion into product ions. A very small peak at *m/z* 541 due to the release of NH_3 is present in the spectrum of **1**. A relatively more stable protonated species could be formed by varying the experimental conditions. The $[M + NH_4]^+$ of **1**, produced in the sector instrument by FAB of standard oleuropein in ammonium chloride–glycerol solution, dissociates spontaneously [MIKE, Fig. 3(A)] into ions of *m/z* 541, 379 and 361. The elimination of neutral ammonia (*m/z* 541) represents, under these conditions, the base peak of the MIKE spectrum.

When the internal energy of the same precursor is enhanced by high-energy collisions, the relative ratio of *m/z* 541 and Y^+ (*m/z* 379) and Z^+ (*m/z* 361) fragments changes drastically. Interestingly, the FAB tandem mass spectrum obtained under the maximum conditions [Fig. 3(C)] is very similar to that obtained by ISI MS/MS [Fig. 2(B) and (C)], thus supporting the fragmentation mechanism suggested above.

A deoxy analogue of **1**, the glucoside **2** (Scheme 1), named ligstroside,[12] was isolated from plants of the family of Oleaceae and thoroughly characterized by spectroscopic data;[44,45] its ammonium adduct, if **2** was a component of the olive leaf extract, would appear at *m/z* 542. The latter is the only ionic species present in the peak centred at 18.84 min in Fig. 1(C), whereas it is accompanied by the ions at *m/z* 428 in the shoulder co-eluting at 17.88 min. The tandem mass spectrum of the *m/z* 542 ion (Fig. 4) is very similar to that of the $[M + NH_4]^+$ of **1** [Fig. 2(B)] with the difference that all the most intense peaks are downshifted by 16 u. The spectrum contains all the useful markers of a secoiridoide glycoside such as **1** and **2** (Scheme 1). The Z and Y fragments at *m/z* 345 and 363 indicate the presence of a hexose unit, which is probably the same as that of oleuropein. The aglycone moiety is identified by the fragments at *m/z* 225, 207 and 165 and, finally, the abundant species at *m/z* 121 can be assigned to the phenolic moiety of ligstroside (**2**). It is possible to conclude that **2** can be easily identified in the crude extract of olive leaf by the LC/MS/MS method employed here.

Diglucoside metabolites have been found in the family of Oleaceae. The nüzhenide **3** (Scheme 1) is present in the seeds of *Olea europea*[44,46] and in other genera of Oleaceae,[47,48] whereas the homologue angustifolioside A (**4**) is present in the leaf of *Fraxinus angustifolia*.[45] Diglucoside homologues of oleuropein (**1**) have never been isolated from olive leaves. From the data discussed above, it appears that the ionspray MS/MS technique could allow the identification of trace components even though not completely separated in the chromatographic stage of the combined analysis. The extracted ion chromatogram in Fig. 1(D) is related to ionic species of *m/z* 720, which might correspond to the $[M + NH_4]^+$ of a diglucoside homologue of **1**. The different scans taken within the ion current distribution showed that the species at *m/z* 720 predominates in the first two peaks where it is accompanied by other lower *m/z* ionic species, whereas it is negligible around the peak centred at 16.37 min. The diglucoside of interest could therefore be present in the leaf extract together with other co-eluted phenolic components. The tandem mass spectrum of the *m/z* 720 ion (Fig. 5) remarkably provides, in a straightforward manner, structural information. The presence of two hexose units, a hydroxytyrosol moiety and the aglycone is unambiguously proved by the pairs of peaks at *m/z* 541/523 and at *m/z* 379/361, by the species at *m/z* 137 and by the fragments at *m/z* 225 and 243, respectively. Since **3** and **4** (Scheme 1) have already been found in Oleaceae, the species under investigation could correspond to the diglucoside **4** or **5**, the latter being the hydroxylated homologue of the nüzhenide **3**. Conversely, the species at *m/z* 720 might represent the $[M + NH_4]^+$ ions of a disaccharide derivative of oleuropein of type structure **6**.

The presence of **5** can be excluded since the pair of ions at *m/z* 541/523 can reasonably correspond to Y

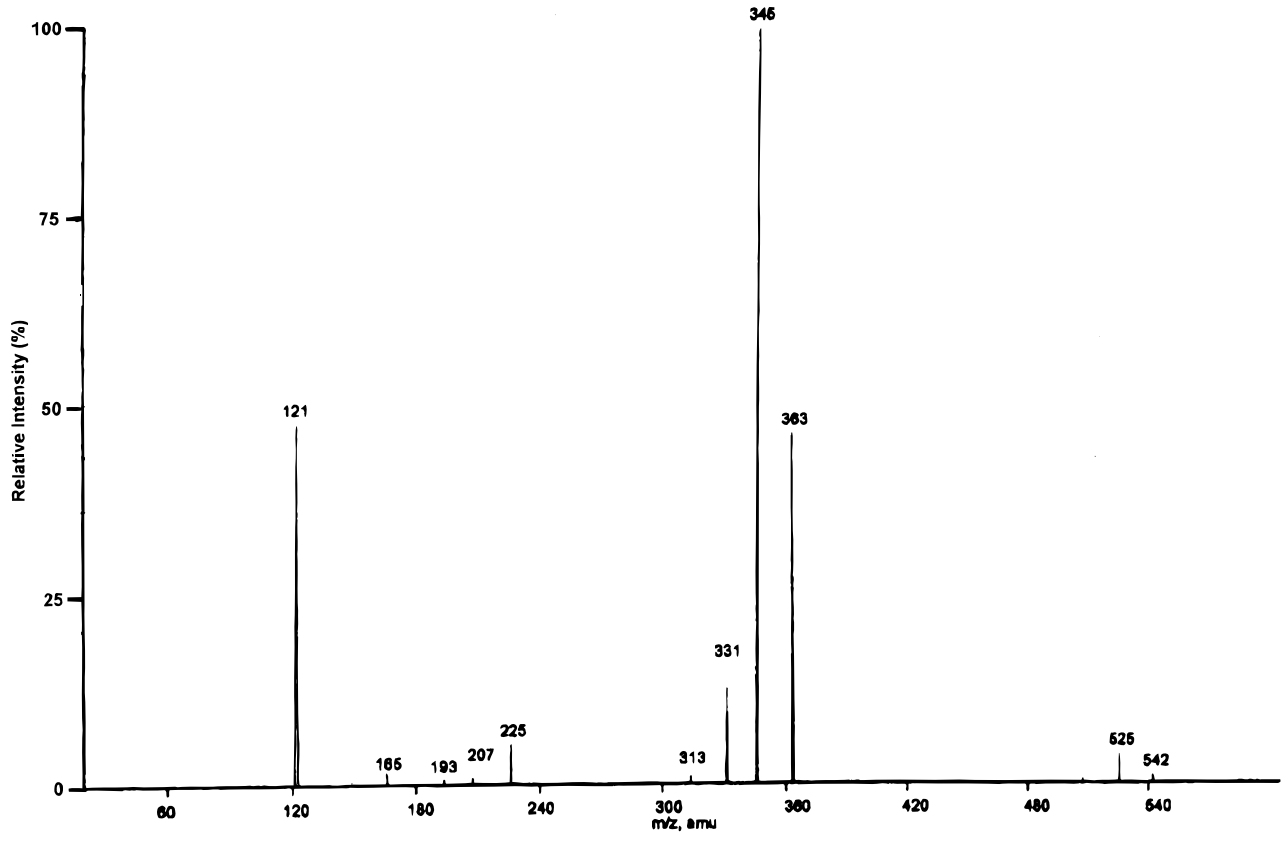


Figure 4. ISI tandem mass spectrum of ligstroside (**2**).

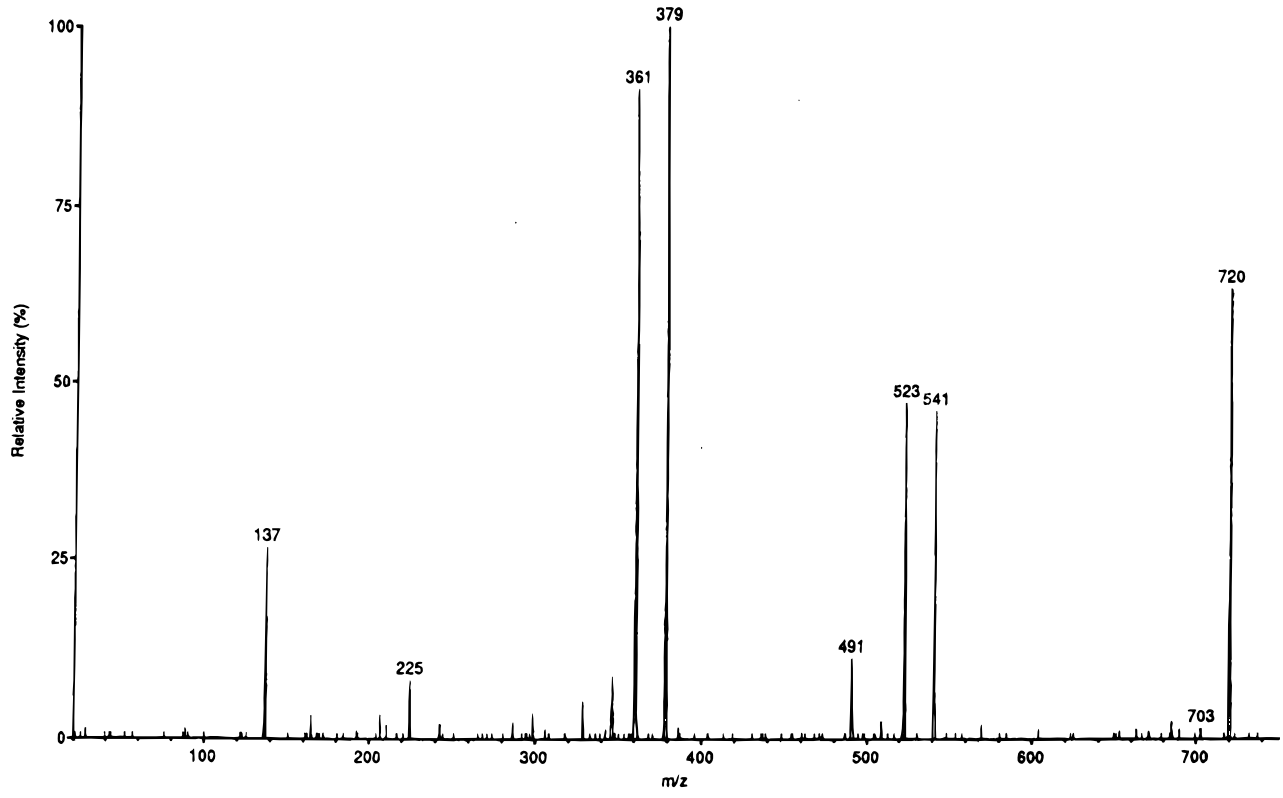


Figure 5. ISI tandem mass spectrum of the *m/z* 720 ion from the extracted ion chromatogram shown in Fig. 1(D).

and Z fragments involving cleavage of the glycosyl linkage of ring A, by analogy with the behaviour of oleuoropein, but there are no means to explain the formation of the other pair of ionic species at *m/z* 379/361. The protonated disaccharide **6** might reasonably form the pairs of peaks mentioned above by consecutive and/or competitive glycosyl bond cleavage (Scheme 1) as found for the $[M + NH_4]^+$ of angustifolioside A (**4**). Nevertheless, assuming the Z'^+ species (Scheme 1) cannot be formed since they require the cleavage of the phenolic carbon–oxygen bond, it can be suggested that the formation of the product ions due to glycosyl bond cleavage corresponds to competitive and consecutive Y- and Z-type reaction. The formation of the other product ions can be easily interpreted as being due to consecutive processes of the intermediate oleuropein species at *m/z* 541.

However, comparison of the tandem mass spectra in Figs 2 and 5 shows that the *m/z* 361/379 relative ratio changes significantly. If the expected fragmentation pathways of ionized **4** and **6** are considered, it appears that there are more reaction channels leading to *m/z* 379 in the case of **4**, whereas the situation is reversed in the case of the hypothetical disaccharide **6**. It might be suggested that the unknown diglucoside found in olive leaves corresponds to angustifolioside A, whose natural origin has been proved in other genera of Oleaceae;[48] however, the experimental data obtained so far do not allow a straightforward structural assignment.

CONCLUSIONS

The identification of the phenolic components of olive leaves has attracted interest in the last few years owing to their biological and pharmacological activity. The LC/MS/MS method described here provides unique opportunities for the fast and reliable identification of these species in crude methanolic extracts without any purification step. The sensitivity of the method is suitable for the satisfactory characterization of unknowns present in trace amounts which cannot readily be isolated and purified.

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