Characterization of phenolic and secoiridoid aglycons present in virgin olive oil by gas chromatography–chemical ionization mass spectrometry

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Abstract

Olive oil phenolic and secoiridoid compound derivatives were detected by mass spectrometry. Chemical ionization (CI) allowed us to obtain parent ions, that were not detectable in the electron impact mode. CI experiments were performed using either CH_4 or NH_3 as the reactant gas. Unlike CH_4-CI, which fails to give molecular mass information, NH_3-CI provided, in all the cases explored, an adduct ion $[M+18]^+$ that was sufficiently stable to be detected and recorded. Information about molecular masses was very useful for the complete characterization of aglycons from glycosides occurring in virgin olive oils Aglycons of ligstroside, of decarbomethoxyoleuropein and of oleuropein were detected. Each aglycon, because of several tautomeric equilibria involving the ring opening of secoiridoid, showed up as compounds with four main structures after derivatization with bis(trimethylsilyl)trifluoracetamide.

Keywords: Olive oil; Food analysis; Aglycons; Glycosides; Secoiridoid; Phenolic compounds; Oleuropeins; Ligstroside; Hydroxytyrosol; Tyrosol

1. Introduction

Over the last decade, phenolic compounds have attracted great attention in food quality because of their antioxidant property for food stability [1–3]. In virgin olive oil, complex phenolic aglycons, derived from oleuropein and ligstroside, represent the most considerable part of the total phenolic fraction [4,5]. However, in the literature most reports are concerned with determination and identification of simple phenols [6,7], while little data exists on phenols linked with other classes of compounds.

As their names imply, the aglycons arise from glycosides present in olive fruits; these may be hydrolysed by one of a number of endogenous β-glucosidases, possibly activated during the crushing and malaxing processes (Fig. 1). These newly formed substances are polar compounds that are partitioned between the oily layer and the vegetation water, being more concentrated in the latter fraction because of their functional groups. Their presence in the oil is well documented [8,9]. More complex aglycons and simple phenols, such as tyrosol and hydroxytyrosol, are also present. As expected, the concentration of the simple phenols in a virgin olive oil sample increases with time, with the concomitant

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decrease of the aglycon concentration [10]. Thus, in a previous paper, we reported on the characterization of complex phenolic compounds dissolved in olive oil by means of GC–MS analysis of their trimethylsilyl derivatives [8]. We found that tyrosol (Ty) and hydroxytyrosol (HTy) were the main simple phenols present in olive oil. Furthermore, it was observed that fragmentation of aglycons induced under 70 eV electron impact (EI) always brought about a main peak at m/z 192 [Ty trimethylsilyl (TMS) derivative] or m/z 280 (HTy TMS derivative). These findings were considered pieces of evidence for the structure of the parent aglycons, thought to be compounds of types II, III and IV, respectively (see Fig. 1). However, it seemed desirable to develop a method that could allow one to detect correctly and characterize the related parent ions.

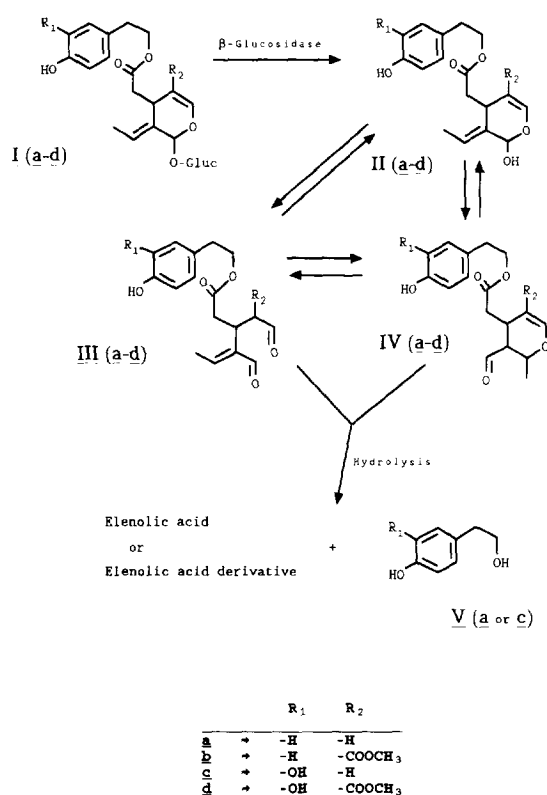By using soft ionization methods, it is possible to

impart relatively little internal energy to the analyte molecules and the ions detected in mass spectra are predominantly the molecular or adduct species rather than fragment ions. Chemical ionization (CI) is the most widespread soft ionization method applied in MS. It has been used to identify and quantify a range of analytes including sulphur vesicants [11], long-chain alkenones [12], trichothecene mycotoxins [13], sarin and soman [14], volatile terpenoid esters and phenylpropanoids [15] and other classes of compounds.

With the aim of improving our knowledge of aglycon structures of natural glycosides, we applied this technique to the study of the phenolic fractions of virgin olive oil.

2. Experimental

2.1. Materials and reference compounds

Virgin olive oil was extracted from fresh fruits of *Olea europaea* L., cv. *Leccino* grown in Abruzzo, by a conventional three-phase direct centrifugation plant fitted with a horizontal decanter (Rapanelli, Foligno, Italy). The operating conditions were as follows: deleafed olives were crushed with a moving metallic hammer crusher, and the paste was malaxed for 60 min at 22°C; 60–80 l of water per 100 kg of olives was added to the olive paste, and the oily must (olive oil–water mixture) was separated in an upright automatic-discharge centrifugal separator. All solvents, for organic residual analysis were of HPLC grade and were purchased from J.T. Baker (Deventer, Netherlands). β-Glucosidase from almonds, 3,4-dihydroxyphenylacetic acid and 2-(4-hydroxyphenyl)-ethanol (Ty) were purchased from Fluka (Buchs, Switzerland); 1,3-dihydroxybenzene and bis-(trimethylsilyl)trifluoracetamide (BSTFA) were from Merck (Darmstadt, Germany); oleuropein ($\geq$95% purity, tested by GC analysis of its TMS derivative) was from Extrasynthése (Z.I. Lyon-Nord, Genay, France); 2-(3,4-dihydroxyphenyl)ethanol (HTy) was synthesised according to the method described by Schöpf et al. [16]; oleuropein aglycon was prepared by β-glucosidase hydrolysis of pure oleuropein [17].

		R₁	R₂
a	→	-H	-H
b	→	-H	-COOCH₃
c	→	-OH	-H
d	→	-OH	-COOCH₃

Fig. 1. β-Glucosidase hydrolysis of glycosides into their corresponding aglycons and further evolution to simple phenols.

2.2. Extraction of phenolic compounds

The detailed methodology of extraction has been reported previously [8]. Briefly, 30 g of dried (over anhydrous Na_2SO_4) virgin olive oil was extracted using 3×30 ml of CH_3OH in an ultra-turrax apparatus (Model T 25; IKA Werke, Staufen, Germany). After removing methanol, the residue was taken up with 10 ml of CH_3CN. Washings with hexane (3×20 ml) were performed and the resulting CH_3CN solution was evaporated under vacuum, giving a residue that was dissolved in 7 ml of acetone. The resulting acetone solution was submitted to spectroscopic or chromatographic analyses. Derivatization was performed by the addition of 120 μl of BSTFA to 1 ml of the acetone solution; after 1 h the solution is ready for GC analysis.

2.3. Preparative HPLC analysis

The acetone solution was evaporated under a N_2 current and taken up with 1 ml of CH_3CN. The resultant solution was injected onto a high-performance liquid chromatograph (50 μl at a time). The HPLC system was composed of a Thermo Separation Products (TSP) liquid chromatograph, Model P 2000 coupled to a TSP membrane degasser and equipped with a Spherisorb semi-prep S5 ODS2 column, of 250 × 10 mm I.D. The mobile phase was acetonitrile (A)–water (B) at a flow-rate of 4 ml/min. The solvent gradient was changed according to the following conditions: isocratic at 20% (v/v) A for 1 min; from 20 to 100% A in 7 min, isocratic at 100% A for 6 min. The eluate was detected at 278 nm using an UV–Vis detector (Perkin Elmer Lambda 1) coupled to a TSP integrator (Model SP 4400).

2.4. GC–MS instrumental analysis

Analyses were performed using a Finnigan Mat 8222 mass spectrometer coupled to a Varian 3400 gas chromatograph. Suitable separation of the analytes was achieved using a DB5 MS capillary column (J & W Scientific), 30 m×0.25 mm I.D., 0.25 μm film thickness. Helium was employed as the carrier gas with a linear velocity of 38 cm/s. Sample aliquots of 1 μl were injected. The oven temperature program

was as follows: 2 min at 40°C, from 40 to 180°C at 20°C/min, 0 min at 180°C, from 180 to 320°C at 4°C/min, 20 min at 320°C; GC injector temperature was 310°C with a split ratio of 1:30; the transfer line temperature was held at 320°C.

The conditions used in the EI mode were as follows: ion source temperature, 250°C; electron energy, 70 eV; filament current, 500 μA and electron multiplier voltage, 1200 V.

CIs were performed with high-purity methane (Matheson res. pur. >99.9995%) and ammonia (Air-Liquide >99.995%). The pressure inside the ion source, as indicated by a remotely positioned ionization gauge, was about 60 Pa for methane, 66 Pa for ammonia and the temperatures were 200 and 150°C, respectively. Acquisition mass ranges were 30–620 u for EI and 100–820 u for CI and the scan time was 1 s.

2.5. NMR analysis

^{1}H and ^{13}C NMR Spectra (Bruker 300 MHz) were recorded in a solution of either $CDCl_3$ or CD_3OD. The chemical shifts for compounds IIIa, IVa, IVd, Va and Vc (Fig. 1) were identical to those reported previously [9].

3. Results and discussion

Methane (CH_4-CI) and ammonia (NH_3-CI) were the two different reactant gases used in the CI mode. Results from both experiments were compared with data obtained by 70 eV EI. The chromatogram of a polar fraction extracted from virgin olive oil recorded in the EI mode is reported in Fig. 2. With the exception of some peaks that could not be assigned (nos. 2, 4–10, 15, 16), and peaks 11, 12, 13, 14 and 22, attributed to the TMS derivatives of $C_{16:0}$, $C_{18:2}$, $C_{18:1}$, $C_{18:0}$ and monooleilglycerol, respectively, the others were attributed to Ty (no. 1), HTy (no. 3) and their corresponding monoterpenoic derivatives (nos. 17–21, 23–28).

The first observation is that the detection sensitivity of single compounds in the resulting chromatograms decreases in the following order: EI>CH_4-CI>NH_3-CI (Fig. 3). In CH_4-CI mode, it was
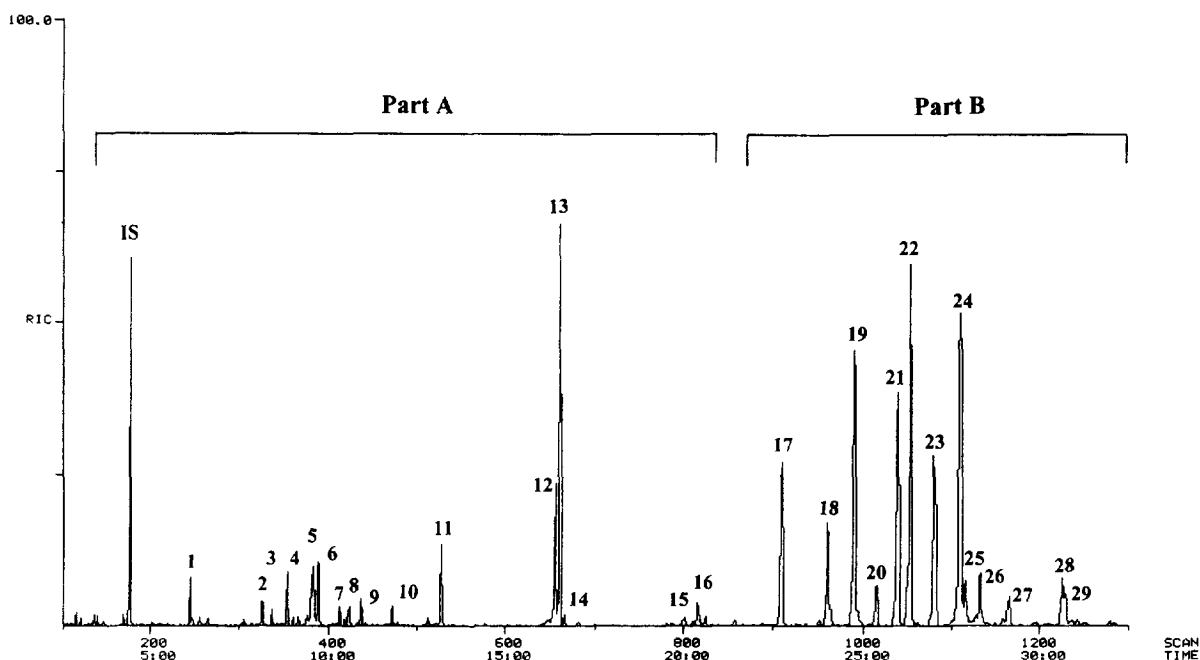
Fig. 2. GC–MS chromatogram in EI mode of derivatized (TMS) methanolic extract of a virgin olive oil sample (cv. *Leccino*). Peaks: IS (internal standard), 1,3-dihydroxybenzene; 1, tyrosol; 2, unknown; 3, hydroxytyrosol; 4–10, unknowns; 11, $C_{16:0}$; 12, $C_{18:2}$; 13, $C_{18:1}$; 14, $C_{18:0}$; 15–16, unknowns; 17, IIIa (IVa); 18–20, IIa and other two enolic forms of IIIa or IVa; 21, IIIc (IVc); 22, monooleilglycerol; 23–24 and 26, IIc and two other enolic form of IIIc or IVc; 25, two unknown aglycons; 27, unknown aglycon; 28, IIId (IVd); 29, unknown aglycon.

possible to detect correctly the quasi-molecular ion $[M+H]^+$; however, the relative abundance of parent ion peaks was too low. Apparently, the high internal energy provided to the analyte from protonated methane induces a fast fragmentation of the analyte [18].

When comparing phenolic compound mass spectra recorded in NH_3-CI mode with those obtained by EI and CH_4-CI, an abundant peak, corresponding to the molecular ion plus 18 that is $[M+NH_4]^+$, was observed in all cases. The $[M+H]^+$ peak was present only for a few compounds and where it was detectable, its relative abundance was very low (11% of peak 19 was the max. value; Table 1) because the dominant ionization mechanism was adduct formation to give $[M+NH_4]^+$ species [19].

The peaks appearing in part A of the chromatogram reported in Fig. 2 confirmed the presence of Ty and HTy. Comparison of EI and CH_4-CI spectra showed evidence of different fragmentation patterns for both Ty and HTy. For Ty, m/z 193 was the main peak corresponding to the loss of TMS-OH from the

quasi-molecular ion (Table 1) rather than m/z 179 derived from benzylic fragmentation favoured in EI mode [8]. HTy shows a similar trend; the only difference was that m/z 281 $[(M+H)-TMS-OH]^+$ did not appear as a main peak (Table 1). NH_3-CI spectra of both compounds clearly showed the above-mentioned formation of adduct $[M+NH_4]^+$.

Peaks arising from aglycons appeared on part B of the chromatogram (Fig. 2); an expanded view of that part in the different acquisition modes has been reported in Fig. 3. Their elution order, as expected, was dependent on the structure and molecular mass of the silylated derivatives. As mentioned in a previous study [9], it was important to search for the molecular moieties making up ligstroside and/or decarbomethoxyoleuropein.

The EI data provided evidence to support the presence of compound IIIa, or its isomeric structure IVa (Fig. 1; peak 17); in fact, from the EI mass spectrum, it was only possible to detect the m/z 192 derived from a Mc Lafferty rearrangement and a very small parent ion at m/z 376. CH_4-CI confirmed
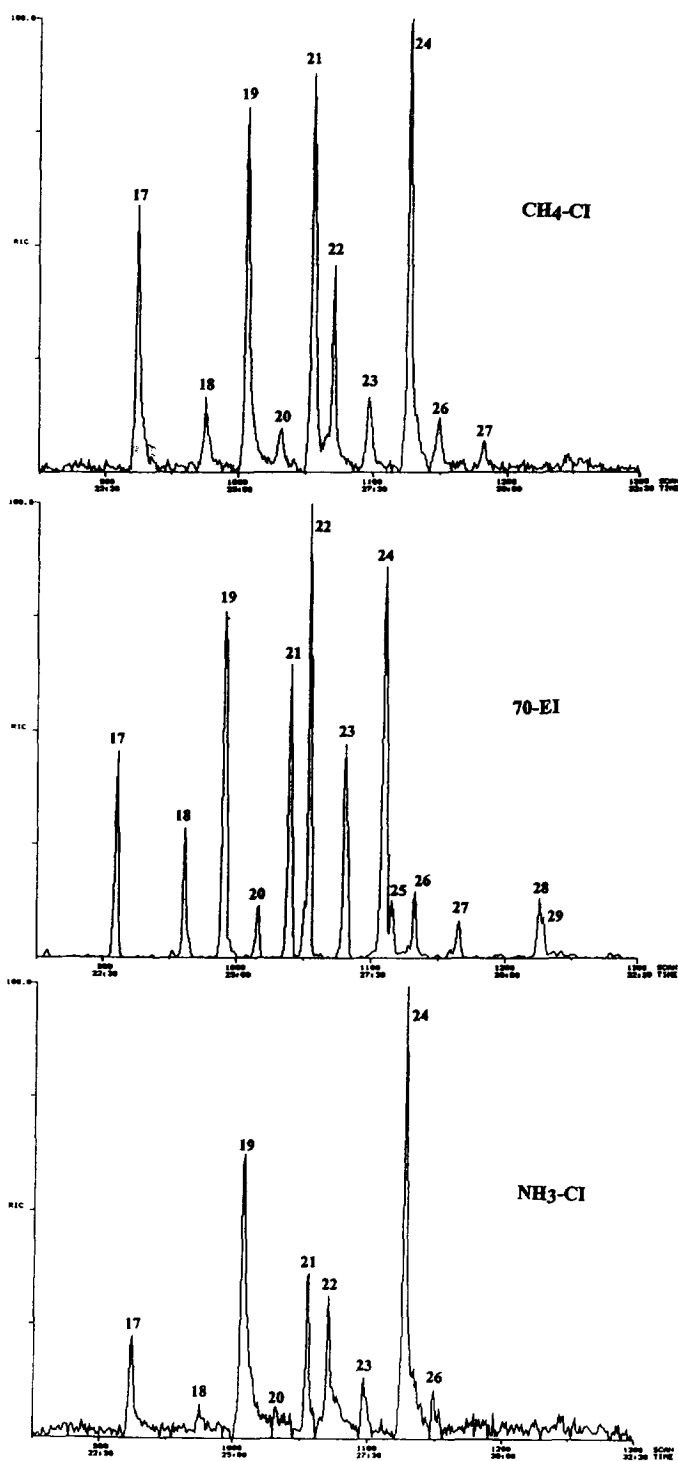
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Fig. 3. Expanded view of part B of the chromatogram reported in Fig. 2: zone of aglycons in different acquisition modes.

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Table 1
Summary of mass spectral data. Molecular ion adducts and fragment ions are reported along with their relative abundance (in brackets)

EI			CH$_4$-CI			NH$_3$-CI		
Peak no.	Molecular ion	Fragment ions	Peak no.	Quasi-molecular ion	Fragment ions	Peak no.	Adduct ion	Fragment ions
1	282 (22)	179(100) 193 (10) 267 (11)	1	283 (20)	193(100) 221 (12) 267 (56)	1	300 (100)	182 (2.5) 193 (13)
3	370 (49)	193 (16) 267(100) 355 (5)	2	371 (22)	281 (69) 309 (10) 355(100)	xxx	388 (100)	267 (20) 281 (22) 370 (37)
17	376 (1.5)	192(100) 177 (43)	17	377 (0.7)	193 (100)	17	394 (100)	270 (2.5) 228 (8)
18	448 (6)	192(100) 193 (99) 177 (16)	18	449 (<0.5)	193(100)	18	466 (100)	449 (3.5) 316 (7)
19	448 (7)	193(100) 192 (99) 177 (33)	19	449 (4)	193(100)	19	466 (100)	449 (11) 228 (25)
20	448 (1.5)	193(100) 192 (92) 177 (15)	20	449 (3)	193(100)	20	466 (100)	449 (7)
21	464 (25)	280(100) 193 (50)	21	465 (5.5)	280(100)	21	482 (100)	358(1.3) 272 (3)
22	500 (7)	103(100) 129 (90) 410 (36)	22	501 (91)	131(100) 485 (95) 411 (88)	22	518 (100)	501 (23) 489 (6)
23	536 (6)	280(100) 397 (18) 193 (28)	23	537 (2.7)	281(100)	23	554 (100)	518 (49)
24	536 (23)	280(100) 193 (64)	24	537 (7)	281(100)	24	554 (100)	537 (4) 258 (11)
25	2 Products	280 192	–	–	–	–	–	–
26	536 (7)	280(100)	26	N.D.	281(100)	26	554 (100)	537(1.4)
27	N.D.	280 (100)	27	N.D.	281(100)	–	–	–
28	522 (9)	280(100) 193 (20)	yyy	523 (13)	281(100)	yyy	540 (100)	232(41) 316(80) 512(12)
yyy	594 (4)	280(100)	yyy	595 (5)	281(100)	yyy	612 (100)	–

xxx: Mass spectrum recorded from a synthetic sample of silylated hydroxytyrosol.
yyy: Mass spectrum recorded from a synthetic sample of silylated oleuropein aglyon.
N.D.: Not detectable

the presence of a molecular ion by its $[M+1]^+$ ion (m/z 377), but with a very low relative abundance (0.7%; Table 1), while NH$_3$-CI, showing an abundant adduct ion at m/z 394, confirmed the presence of IIIa (IVa) (Fig. 4).

NMR investigation of a pure sample of decarbomethoxylated ligstroside aglycon (obtained by preparative HPLC; see Section 2) gave some interesting data. In chloroform, the ratio between the

sufficiently stable isomeric structures IIIa and IVa, calculated by the ratio of the aldehydic signals at 9.24 or 9.64 ppm of IIIa, and the aldehydic signal at 9.48 ppm of IVa, is about three. Combining NMR data with GC analysis, it was possible to reach the following conclusion. The most stable isomeric structure was IIIa, even if the isomer IVa was also present. On the other hand, using GC–MS it was difficult to discriminate between the two silylated
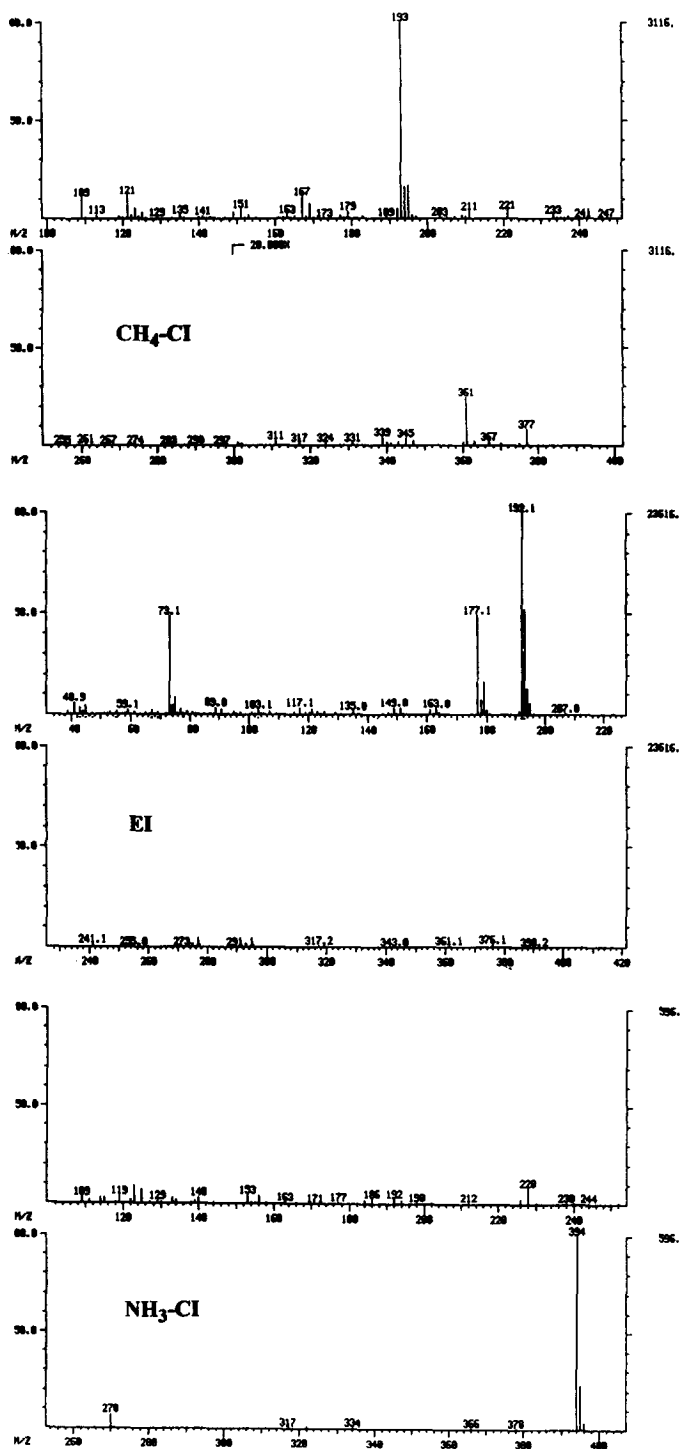
Fig. 4. Mass spectra of TMS derivative of decarbomethoxyligstroside aglycon (structure type IIIa or IVa).

structures because of their identical fragmentation patterns. We can only say that, at the temperature conditions used during GC analysis, either only one isomer was present in the chromatogram, or there were two isomers with identical retention times.

Although peak 17 is attributable to the silylated form of decarbomethoxyligstroside aglycon, other possible molecular structures should be taken into consideration. It is known from the pertinent literature that aglycon molecules of type III may enter a number of equilibria through ring opening of secoiridoid [8,20]. This was the reason why we found, together with IIIa (IVa), three other peaks (18, 19 and 20) all of which had similar mass spectra (Table 1), probably corresponding to a structure of type IIa (Fig. 1) and to two other silylated enolic forms of mono- or dialdehydic forms of the elenolic acid moiety (Fig. 5).

A careful analysis of the remaining part of chromatogram (Fig. 3) allowed us to confirm that each linked phenol was present in four isomeric structures. Peak 21 (EI), with m/z 464, fully confirmed by m/z 465 in CH_4-CI mode and m/z 482 in NH_3-CI mode, was attributed to the silylated form of IIIc

(IVc) (Table 1). On the other hand, peaks 23, 24 and 26, having m/z 536 (464 plus 72), were attributed to the silylated form of IIc and to two other silylated enolic isomers represented in Fig. 5, respectively (Table 1).

Because of the low abundance of oleuropein aglycon in the polar fraction of the analyzed oil, we obtained an aglycon sample by β-glucosidase hydrolysis of commercial oleuropein. EI–MS spectral data of oleuropein aglycon showed the presence of several isomeric forms and, as expected, mainly four structures were predominant: one m/z 522 and three m/z 594. Peak 28 was the only peak in the chromatogram of our olive oil extract with an appreciable concentration for recording mass spectra in the EI mode only. According to the above reasoning, the peak with m/z 522 was attributed to silylated oleuropein aglycon (IIId or IVd; Table 1) while peaks with m/z 594 were attributed to its silylated isomers. NMR investigation of oleuropein aglycon in chloroform showed the only presence of aldehydic signal around 9.5 ppm and the total absence of signals at 9.28 and 9.64 ppm showing that the predominant structural isomer in chloroform was IVd (Fig. 1). These findings are in agreement with previous reports [20]. When derivatization is performed, several structures, present only in trace concentration at the equilibrium, are blocked as evidenced by the formation of TMS derivatives of enolic forms.

This is in contrast with the results found on the structure of decarbomethoxylated ligstroside which, in chloroform, was predominant as the dialdehydic form. This seems to suggest that the carbomethoxy group plays an important role in determining the structure of the detected aglycons. Its presence clearly shifts the equilibria represented in Fig. 1 and Fig. 5 towards the formation of the monoaldehydic form (IV), being more stable because of the conjugation between the carboxylic moiety and the double bond of the dihydropyrane ring.

Fig. 5. Possible structures related to several equilibria always present in molecules of the glycoside aglycon type.

4. Conclusion

Phenols and secoiridoid compounds of the virgin olive oil extracted from *Olea europaea* L., cv *Leccino*, were detected by MS.

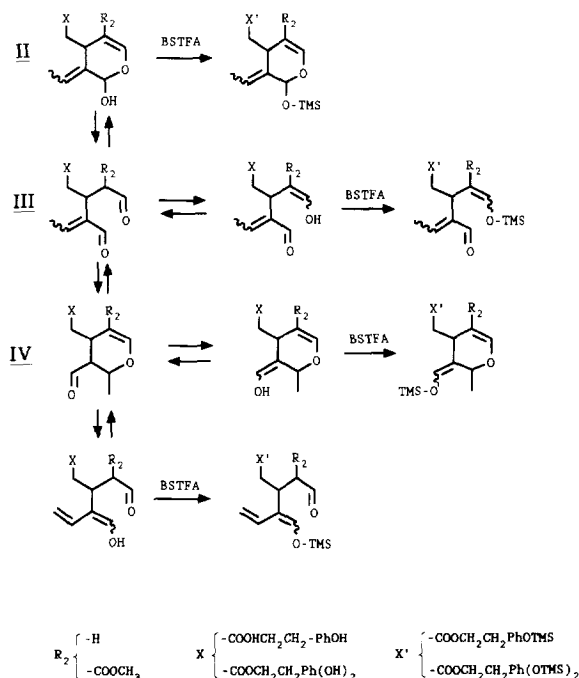The low abundance of molecular ions in the 70 eV
EI mode of linked phenols moved us to apply soft
ionization methods, using CH_4-CI and NH_3-CI,
despite the fact that total ion currents were lower.
CH_4-CI did not give good information about molecular masses since $[M+H]^+$ was still not sufficiently
abundant and fragmentation was the favoured process. On the contrary in NH_3-CI, the formation of
adduct ions $[M+NH_4]^+$ was very useful for the
correct assignment of molecular masses to the
studied compounds.

Among simple phenols, Ty and HTy were observed. Spectroscopic data provided pieces of evidence that all linked phenolic compounds were
aglycons from ligstroside, oleuropein and their corresponding decarbomethoxylated glycosides. The large
number of peaks attributable to aglycons from
glycosides naturally occurring in olive fruits (part B
reported in Fig. 2) were due to several keto-enolic
tautomeric equilibria that involve the ring opening of
secoiridoids.

NMR investigation of purified and underivatized
aglycons showed that the favoured structure of
decarbomethoxylated aglycons was the dialdehydic
form while monoaldehydic structure was favoured
for ligstroside and oleuropein aglycons.

Acknowledgments

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