GC–MS Evaluation of Phenolic Compounds in Virgin Olive Oil

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A new gas chromatographic method for detection of phenolic compounds in virgin olive oils was developed. Identification of chromatographic peaks was made by mass selective detection. The presence of a main peak at *m/z* 192 or at *m/z* 280, related only to tyrosol and hydroxytyrosol, evident in the mass spectra of linked phenols, was very profitable for assigning the phenolic nature to minor polar compounds extracted by methanol from virgin olive oil. Twelve structures are possible, and some of them are deemed more likely on the basis of the chemical behavior of the compounds studied. The presence of a ligstrosid aglycon containing no carbomethoxy group and of oleuropein aglycon derivatives was evidenced.

Keywords: *Phenolic compounds; virgin olive oil; mass spectroscopy*

INTRODUCTION

Phenolic compounds are of great importance in biological systems and, in particular, in the vegetable kingdom. They are present principally in the fruits but also, in a minor amount, in leaves, flowers, and other vegetable organs (Vazquez Roncero et al., 1974; Gutfinger, 1981; Bianchi and Pozzi, 1994).

Virgin olive oil contains phenolic derivatives, in contrast to edible seed oils that lose this important group of compounds in the various refining stages (Cantarelli, 1961; Montedoro and Cantarelli, 1969). Their content is heavily affected by the variety, location, maturity degree, and oil extraction procedures (Solinas et al., 1978; Montedoro and Garofolo, 1984; Amiot et al., 1986; Solinas, 1987).

The level of these substances is a very important parameter in the evaluation of virgin olive oil quality since phenols are strictly related both to the oil's resistance to oxidation because of their antioxidative properties (Montedoro, 1972; Papadoupoulos and Boskou, 1991) and to the typical bitter taste of olive oil (Angerosa and Solinas, 1990; Olias, 1992). Furthermore, some studies showed that the amount of phenolic substances present, just as the fatty acid composition, is related to the health beneficial effects that make virgin olive oil a very valuable and appreciated commodity (Panizzi et al., 1960; Galli et al., 1992).

Therefore, it is very important to assay phenols in oil both qualitatively and quantitatively. Several methods were proposed: their direct quantification on the methanolic fraction by means of Folin–Ciocalteu reagent had been earlier modified by adding to the procedure a purification by paper chromatography or TLC on silica gel or cellulose, before spectrophotometric analysis (Ragazzi and Veronese, 1973). Subsequently, GLC (Janer del Valle and Vazquez Roncero, 1980; Solinas and Cichelli, 1982; Solinas, 1987) and HPLC (Solinas and Cichelli, 1982; Amiot et al., 1986; Montedoro et al., 1992; Tsimidou et al., 1992; Akasbi et al., 1993) methods were developed. HPLC methods, which generally use UV detection, are more successful, since they, in contrast to GLC methods, do not require derivatization prior to the quantitative analysis. An excellent series of experiments was performed by Montedoro and his group. By using HPLC and NMR techniques, they separated and identified some aglycon derivatives present as the dialdehyde forms of elenolic acid linked to both hydroxytyrosol and tyrosol in the olive oil (Montedoro et al., 1993).

Recently HPLC was coupled with an amperometric detector with interesting results (Mannino et al., 1993); phenols in olive oils were also measured by using organic phase biosensors (Wang et al., 1993). Nevertheless, these new methods for the determination of phenolic substances did not allow their identification.

In 1987 an HRGC method was developed by Solinas for the quantitative evaluation of phenolic substances. The methodology needs (i) purification of the methanolic extract by absorption of the mixture on Celite–Polyclar AT; (ii) removal of, first, the largest part of methanol and then the last traces of solvent by means of pentane–acetone–methanol azeotropic distillation; (iii) GC analysis after BSTFA derivatization.

Although Solinas was able to characterize the simplest compounds such as tyrosol and hydroxytyrosol, using this method he could only offer speculations about the identities of the other linked phenols occurring in large quantities in the second part of chromatogram.

Therefore, our aims were, first, to simplify the extraction and purification procedures to have at our disposal a rapid, more effective, and accurate HRGC method of phenol dosage, which could be suitable for routine determinations; and, second, to characterize the more complex molecules by means of GC–MS and spectroscopic techniques.

EXPERIMENTAL PROCEDURES

Materials and Reference Compounds. Virgin olive oil extracted from olives of Coratina variety cultivated in Abruzzo was used.

4-Hydroxyphenyl acetic acid, *p*-coumaric acid, vanillic acid, 4-hydroxybenzoic acid, syringic acid, caffeic acid, 3,4-dihydroxyphenylacetic acid, protocatechuic acid, ferulic acid, β-glucosidase from almonds, and 2-(4-hydroxyphenyl)ethanol (Ty) were purchased from Fluka (Buchs, Switzerland); and resorcin, cinnamic acid, omovanillic acid, and bis(trimethylsilyl)trifluoracetamide (BSTFA) from E. Merck (Darmstadt, Germany).

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Table 1. Response Factors and Percent Yields of Some Reference Phenolic Compounds

phenolic compound	response factors	yield % for neat methanol	yield % for methanol–water 80:20 (v/v)
resorcin	1.00	77.3	77.3
tyrosol	0.87	74.2	74.2
hydroxytyrosol	0.60		
4-hydroxybenzoic acid	0.76	73.1	69.0
4-hydroxyphenylacetic acid	0.70	61.8	68.1
vanillic acid	0.60	71.1	72.1
omovanillic acid	0.58	58.7	65.9
protocatechuic acid	0.89	68.0	69.0
syringic acid	0.49	70.0	70.2
p-coumaric acid	0.65	63.9	56.7
ferulic acid	0.51	90.6	36.1
caffeic acid	0.70	51.5	36.1

Oleuropein was from Extrasynthése (Z. I. Lyon-Nord, Genay, France; ≥95% purity, tested by GC analysis of its TMS derivative).

2-(3,4-Dihydroxyphenyl)ethanol (HTy) was synthesized according to the literature method (Schöpf et al., 1949). Elenolic acid was obtained by acid hydrolysis of the pure oleuropein glycoside according to the procedure of Walter et al. (1973); oleuropein aglycon was prepared by enzymatic hydrolysis of pure oleuropein by use of β-glucosidase according to the method of Walter et al. (1973).

Extraction of Phenolic Compounds. Thirty grams of dried (over anhydrous Na_2SO_4) virgin olive oil was added to 1 mL of resorcin solution (internal standard; diethyl ether solution 5 g/dm^3). Extraction of phenolic compounds was performed by means of 3 × 30 mL of CH_3OH (or alternatively H_2O–CH_3OH 20:80) in an Ultra-Turrax apparatus. To remove most of the residual oil, the methanolic solution was kept overnight at −20 °C; after filtration, the solution was concentrated in vacuum, keeping the bath temperature under 35 °C, and the syrupy residue was taken up with 10 mL of CH_3CN. To eliminate the residual traces of glyceride, three 20 mL washings with hexane were performed, and the resulting CH_3CN solution was evaporated in vacuum, keeping the temperature under 35 °C, giving a residue that was dissolved in 7 mL of acetone. The resulting acetone solution was submitted to chromatographic or spectroscopic analyses.

HRGC and GC–MS Analyses. Two different derivatization conditions were adopted: (i) 150 μL of BSTFA was added to 1 mL of acetone solution, and (ii) 1 mL of acetone solution was evaporated under N_2 current and taken up with 0.5 mL of BSTFA.

HRGC was carried out with a Carlo Erba Mega Series 5160, equipped with an on-column injection system and a FID detector, on a Nordion silica capillary SE-54 column (25 m length; 0.32 i.d.; 0.1 μm film thickness). The oven temperature program was as follows: from 70 to 135 °C at 2 °C/min, 10 min at 135 °C, from 135 to 220 °C at 4 °C/min, 10 min at 220 °C, from 220 to 270 °C at 3.5 °C/min, 20 min at 270 °C; the temperature of the detector was held at 315 °C. The carrier gas was H_2, and the carrier pressure on the head of the column was 35 kPa. Quantification was done by peak area integration with a Carlo Erba Mega Series integrator.

GC–MS was performed with a HP Model 5890A, equipped with an on-column injection system and a mass selective detector Model HP 5970B, on a Nordion silica capillary SE-54 column (25 m length; 0.32 i.d.; 0.1 μm film thickness). The carrier gas was He, and the carrier pressure on the head of the column was 10 kPa. The oven temperature program was the same used for HRGC determination; the transfer line temperature was held at 300 °C.

HPLC Analysis. One milliliter of acetone solution was evaporated under N_2 current and taken up with 1 mL of CH_3OH. Ten microliters of the obtained solution was injected in a high-performance liquid chromatograph. The HPLC system was composed of a Spectra-Physics liquid chromatograph Model SP 8800 equipped with an C_{18} Erbasil column (10 μm; 250 mm × 4.6 mm), coupled with a Spectra-Physics UV–vis detector Spectra FOCUS model, able to perform automated peak purity and spectral analysis. The mobile phase was methanol (A)–acetic acid in water (B) (pH 3.2) at a flow rate of 1.4 mL/min. The solvent gradient changed according to the following conditions: from 5% A to 50% A in 11 min, from 50% A to 75% A in 11 min, from 75% A to 100% A in 9 min, isocratic at 100% A for 19 min. Eluates were detected at 232 and 278 nm.

NMR Analysis. ^1H and ^{13}C NMR spectra (Bruker 300 MHz) were recorded in $CDCl_3$ or CD_3OD solution.

Chromatography Column. The phenolic extract obtained from 90 g of oil was dissolved in 2 mL of 4:6 chloroform–ethyl

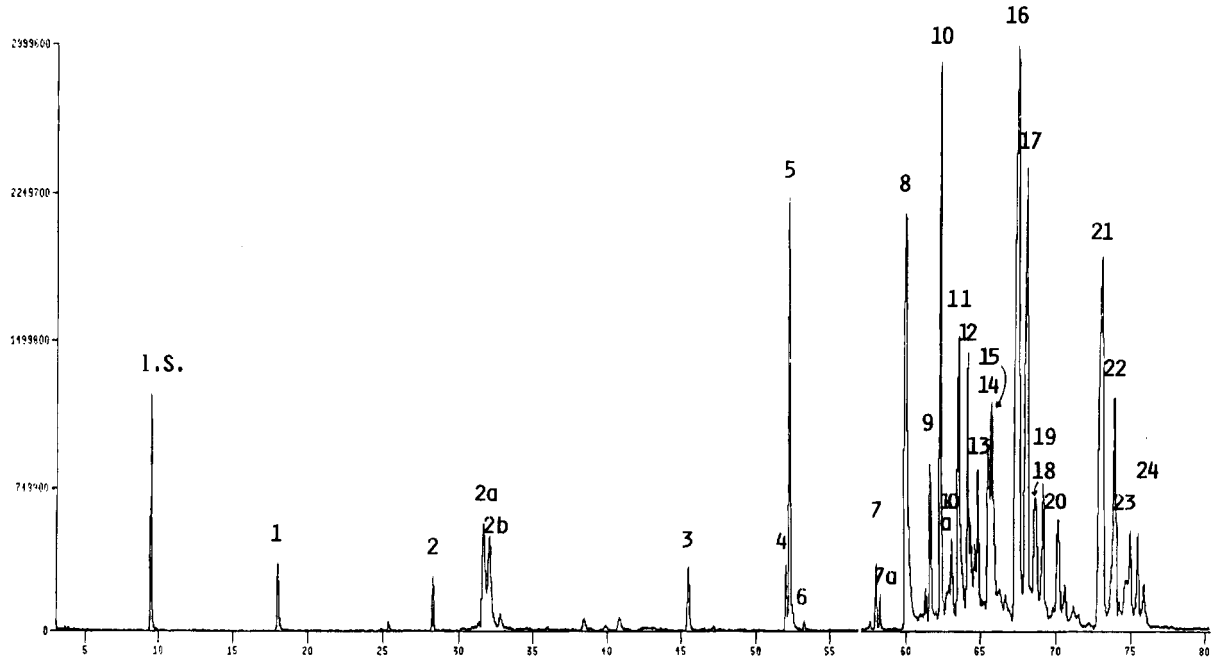


Figure 1. Chromatographic profile of derivatized olive oil methanolic extract. Peaks: 1, tyrosol; 2, hydroxytyrosol; 2a and 2b, unknown; 3, palmitic acid; 4, linoleic acid; 5, oleic acid; 6, stearic acid; 7 and 7a, unknown; 8, dialdehydic form of ligstrosid aglycon containing no carbomethoxy group (**1aT**); 9, 10, 10a, 14, 16, 17, 18, and 19, linked phenols containing tyrosol; 11, 13, 15, 20, 21, 22, 23, and 24, linked phenols containing hydroxytyrosol; 12, monoglyceride.

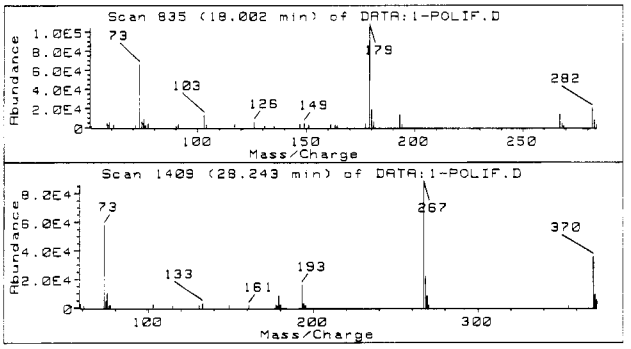
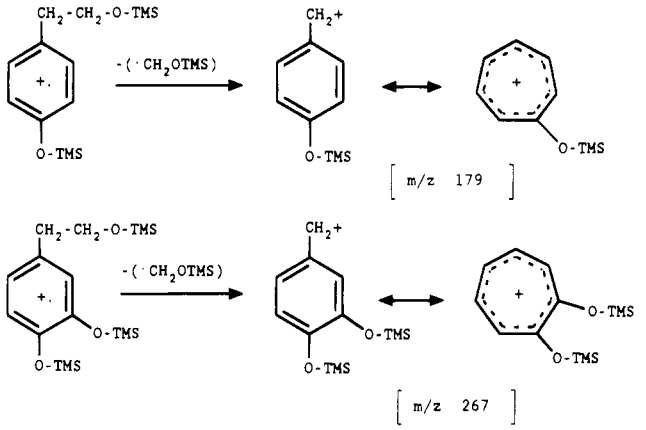
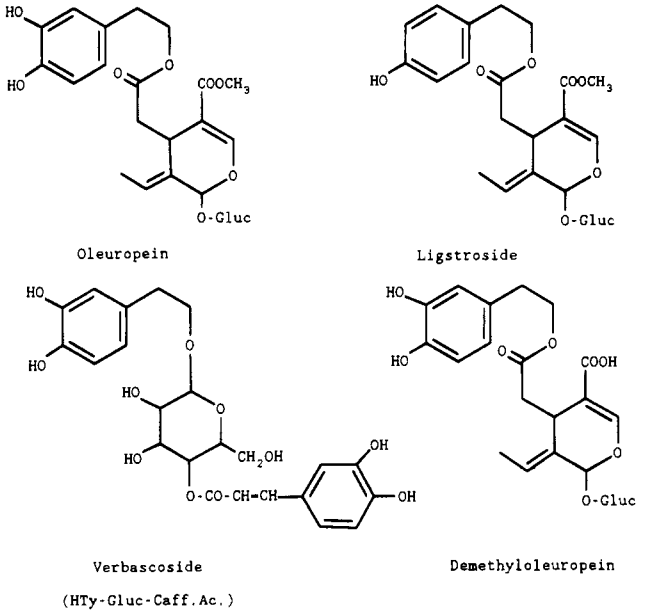


Figure 2. Mass spectra of tyrosol and hydroxytyrosol.

Scheme 1. Fragmentation Mechanism of Tyrosol and Hydroxytyrosol



Scheme 2. Main Glycosides Present in Olive Fruit



acetate and passed through a chromatographic column (50 mm i.d.; 800 mm length) filled with 100 g of Merck EM type 60 (230–400 mesh) silica gel. First, 500 mL of 4:6 chloroform–ethyl acetate was used, followed by 500 mL of pure ethyl acetate; 70 fractions were collected, concentrated, and submitted to TLC and GC detection and, when it was possible, to spectroscopic analyses.

Hydrolysis. Two different hydrolysis procedures were used, viz. strong and mild conditions.

Five milliliters of acetone phenolic solution was added to 50 mL of 2 N HCl solution. The mixture was heated at 70 °C for 15 h, and the reaction products were extracted with 3 × 20 mL of ethyl acetate. After drying and concentration in vacuum, the residue was dissolved in 5 mL of acetone; 1 mL of this solution was submitted to derivatization for HRGC analysis.

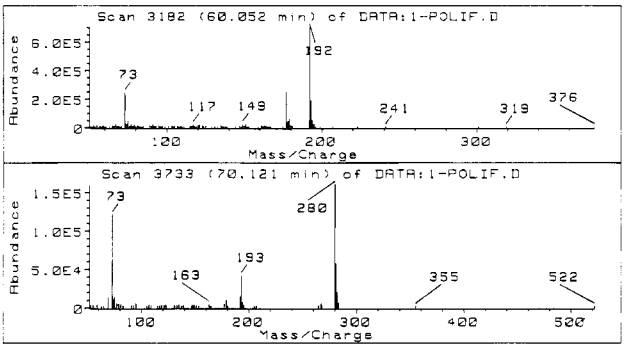


Figure 3. Mass spectra of one representative compound containing tyrosol (peak 8) and one containing hydroxytyrosol (peak 20).

For the mild condition the procedure was modified only in the HCl concentration (10^{-2} N), temperature of reaction (25 °C), and reaction time (20 min).

RESULTS AND DISCUSSION

The methodology described under Experimental Procedures is faster than the one developed by Solinas, since it avoids chromatography column purification of phenolic substances (requiring 3–4 h) as well as removal of methanol traces by means of the pentane–acetone azeotrope. Furthermore, washings of the Polyclar–Celite mixture with methanol can introduce some impurities subsequently found in the chromatogram.

Quantitative extraction of phenolic compounds from natural matrices is difficult. Therefore, tests of recovery were carried out to ensure the effectiveness of the extraction procedure of phenolic compounds from the oily matrix. Thus, to two samples of refined olive oils were added known amounts of 10 pure simple phenols and then the extraction was simulated. To extract phenols from the oil we chose and compared neat CH_3OH with CH_3OH-H_2O 80:20 (v/v). The latter, aqueous methanol, was previously reported as the most efficient extraction solvent (Montedoro, 1992). Extraction yields, evaluated for most of the phenols present in the oil and shown in Table 1, were similar when neat methanol was used; in the case of ferulic and caffeic acids when 80:20 methanol–water was used, the yields were poor. These results were in contrast with previously reported data whose quantification was carried out according to a colorimetric procedure (Montedoro, 1992). The incomplete recovery of some components and the formation of considerable emulsions between the oil and the methanol–water layer suggested that we choose neat methanol as extraction solvent.

A great number of simple phenolic compounds have been found in virgin olive oils by several researchers (Janer del Valle and Vazquez Roncero, 1980; Solinas and Cichelli, 1982; Montedoro et al., 1992; Tsimidou et al., 1992; Akasbi et al., 1993), but in our chromatograms only tyrosol (Ty) and hydroxytyrosol (HTy) were observed, while all of the other simple phenols reported in the literature are not evidenced. Therefore, these simple phenols, if present in our samples, have levels too low to be detected in our working conditions (see recoveries and response factors in Table 1). Furthermore, the used methodology has a good repeatability, since several analyses carried out on the same sample produce a repeatability coefficient of variation (CVr) of $\pm 6 \div 7\%$.

Scheme 3. McLafferty Rearrangement Proposed for Linked Phenolic Compounds

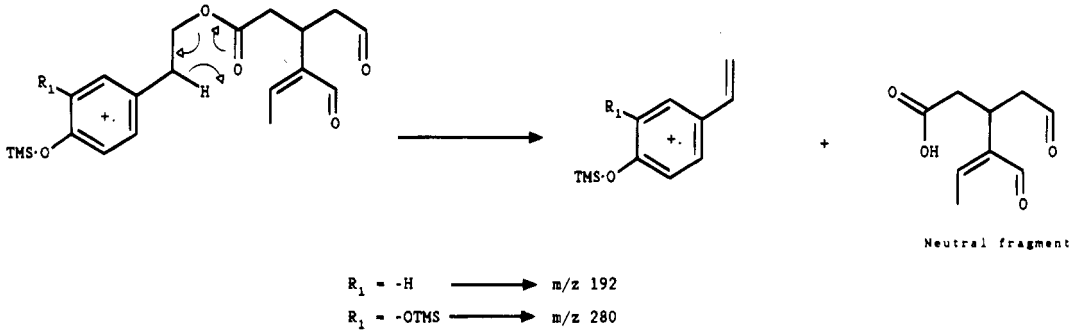


Table 2. Parent Ions of Peaks Present in the Chromatographic Profile of Derivatized Olive Oil Methanolic Extract

peak no.	molecular ion mass	peak no.	molecular ion mass
1	282	11	464
2	370	12	
2a	314	13	
2b	314	14	
3	328	15	536
4	352	16	432
5	354	17	432
6	356	18	
7		19	
7a		20	522
8	376	21	520
9		22	594
10	448	23	
10a		24	

The identification of peaks present in the gas chromatogram has been mainly carried out by GC–MS; furthermore, some peak identification, after their separation by preparative column chromatography, has been confirmed by GC–MS, NMR, and HPLC analyses of the single-column chromatography fractions. In Figure 1 is reported a typical gas chromatogram of the phenolic fraction in a virgin olive oil. The first part of the chromatogram shows only the presence of Ty and HTy, fully identified by their mass spectra shown in Figure 2.

The fragmentation mechanism of simple phenols (Scheme 1) in GC–MS, in fact, has been studied by other researchers (Cortesi and Fedeli, 1983); according to their conclusions, the mass spectrum of Ty shows a low abundance molecular ion (M^+; *m/z* 282; 20%) and the main peak at *m/z* 179 (deriving from the benzylic fragmentation $M^+ - 103$). HTy presents a similar trend with a molecular ion slightly more abundant than with Ty (M^+; *m/z* 370; 30%) and the higher peak at *m/z* 267 ($M^+ - 103$).

The second part of the chromatogram is very complicated because of the presence of a great number of peaks, some among them related to phenols with high molecular weights. Their identification is difficult, since the fragmentation of complex phenolic compounds has been incompletely studied in the literature.

It is well-known that in olives phenolic compounds are present as glycosides. Particularly, oleuropein, demethyloleuropein, and ligstroside are considered to be the main natural glycosides (Panizzi et al., 1960; Ragazzi. et al., 1973; Kubo and Matsumoto, 1984; Gariboldi et al., 1986), but some researchers also found verbascoside (Fleuriet et al., 1984). Interesting changes of their contents were monitored during the harvesting of olives. These compounds are soluble in the aqueous phase, and only their corresponding aglycons, shown in Scheme 2, can be partly dissolved in the oil; consequently, it could be assumed that phenolic substances occurring in the virgin oils are derivatives of the abovementioned aglycons.

Mass spectra of all peaks present in the gas chromatogram were recorded. With the exception of some spectra (referred to as peaks 3–6 and 12, which have been assigned to free fatty acids and monoglycerides), all of the other mass spectra were characterized by the presence of a main peak at *m/z* 192 or at *m/z* 280, even masses obviously deriving from rearranged fragments (two representative examples are reported in Figure 3).

These masses could be justified by the existence of the fragments that can be formed through a McLafferty rearrangement (Spiteller, 1966; Beynon et al., 1968), typical of β-phenyl ethyl esters, shown in Scheme 3. Previous literature shows that the molecular ion is weak or absent. In our experiment a low-intensity peak (ca. 1%) has always been revealed and the base peak is always due to the corresponding styrene derivative. This kind of fragmentation is favored over the benzylic fragmentation because it occurs through a six center stabilized intermediate that, after hydrogen transfer, leads to a very stable styrene ion derivative.

The same fragments could be obtained from cinnamic esters, but the total absence of cinnamic derivative compounds in the product mixture deriving from complete hydrolysis of phenolic extract via acid catalysis allows us to exclude, with a high degree of confidence, the presence of aglycons containing cinnamic acid derivatives such as caffeic acid. Therefore, the *m/z* 192 and 280 fragments have to be related to β-phenyl ethanol derivatives (Ty and HTy) contained in the structures of natural aglycons that are soluble in the oil.

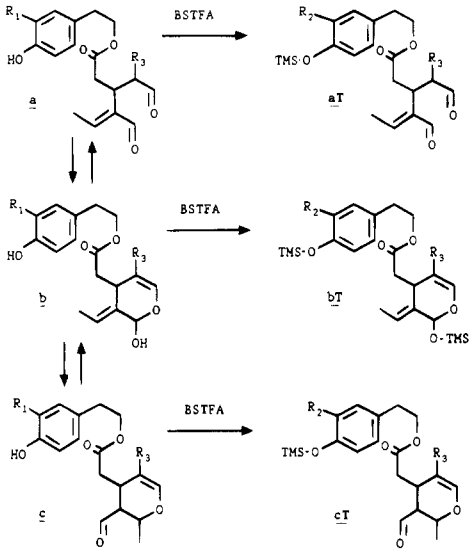
This characteristic fragmentation of β-phenyl ethyl esters was very useful for interpretation since the presence of *m/z* 192 or 280 peaks in the spectrum allows unambiguous structure assignments of the analyzed compounds as phenol derivatives and, furthermore, enables us to establish if they are Ty or HTy derivatives.

To obtain further knowledge of the structures of the high molecular weights phenols, some further experiments were carried out.

Comparison between reference mass spectra of purified enzymatic hydrolysate of pure oleuropein and those of the substances present in the phenolic extract allowed us to establish that peaks 20–24 are related to oleuropein aglycon. Moreover, mass spectra showed the molecular ion at *m/z* 594 for peak 22 and at *m/z* 522 for peak 20.

The fractionation of the methanolic extract via silica gel column chromatography gave further important information. The main product collected with high

Scheme 4. Chemical Structures of Linked Phenolic Compounds and Their Hypothesized Interconversion



(a)

1a	R_1= R_3= -H	1aT	R_2= R_3= -H
1a'	R_1= -H; R_3= -$COOCH_3$	1a'T	R_2= -H; R_3= -$COOCH_3$
2a	R_1= -OH; R_3= -H	2aT	R_2= -OTMS; R_3= -H
2a'	R_1= -OH; R_3= -$COOCH_3$	2a'T	R_2= -OTMS; R_3= -$COOCH_3$

(b)

1b	R_1= R_3= -H	1bT	R_2= R_3= -H
1b'	R_1= -H; R_3= -$COOCH_3$	1b'T	R_2= -H; R_3= -$COOCH_3$
2b	R_1= -OH; R_3= -H	2bT	R_2= -OTMS; R_3= -H
2b'	R_1= -OH; R_3= -$COOCH_3$	2b'T	R_2= -OTMS; R_3= -$COOCH_3$

(c)

1c	R_1= R_3= -H	1cT	R_2= R_3= -H
1c'	R_1= -H; R_3= -$COOCH_3$	1c'T	R_2= -H; R_3= -$COOCH_3$
2c	R_1= -OH; R_3= -H	2cT	R_2= -OTMS; R_3= -H
2c'	R_1= -OH; R_3= -$COOCH_3$	2c'T	R_2= -OTMS; R_3= -$COOCH_3$

purity was that related to peak 8 in the chromatogram. Complete characterization of this product was obtained through mass spectrometry (main peak at *m/z* 192; parent ion of TMS derivative M^+; *m/z* 376; parent ion of unsilylated M^+; *m/z* 232) and UV analysis and by ^1H and ^{13}C NMR spectra. NMR data were identical to those reported by Montedoro et al. for the dialdehydic form of ligstrosid aglycon not containing the carbomethoxy group.

The mass spectra of the remaining peaks of the methanolic extract chromatogram were compared with those recorded for compounds present in the other chromatography column fractions. This comparison confirmed that peaks 8–10, 10a, 14, and 16–19 contain, as the most abundant peak, the fragment *m/z* 192, while for peaks 11, 13, 15, and 20–24 the most abundant fragment is *m/z* 280. Molecular ion values, when it was possible to evaluate them despite their low abundance, are reported in Table 2.

Since only *m/z* 192 or 280 fragments were present, other derivatives different from Ty or HTy were completely absent, and it could be considered that some complex phenols detected by gas chromatography had structure types **a**, **b**, and **c** (Scheme 4).

We suggest that products **a**–**c** are in equilibrium as shown in Scheme 4. This was fully supported by results obtained from acid-catalyzed hydrolysis of the phenolic extract carried out in mild condition such as cold 10^{-2} N HCl solution. In fact, a relevant increase of peaks 8, 10a, 11, 15, and 20 was observed as were important decreases for peaks 10, 21, and 22.

On the basis of these results, chromatographic peak 10, showing in addition to the *m/z* 192 fragment also the molecular ion at *m/z* 448, can be attributed with high probability to the aglycon of ligstrosid without a carbomethoxy group (**1bT**), the only isomer containing two TMS groups. Furthermore, this assignment is confirmed by the total suppression of peak 10 under mild acidic hydrolysis conditions and by the corresponding increase of peak 8 under this condition. This behavior explains also why it was not possible to obtain compound **1bT** by silica gel column chromatographic fractionation: the acidic sites of silica gel changed the equilibrium toward the corresponding dialdehyde form (**1aT**).

Similar trends were noted for peaks 11 and 15, containing HTy, as well as for peaks 20 and 22, even if in a lower proportion; consequently, formulas **2aT** and **2bT** were assumed for peaks 11 and 15 while **2a′T** and **2b′T** for peaks 20 and 22.

A further comment is needed to follow and to understand some results of our experiments. When BSTFA was used both as silylating agent and as solvent, peaks 8 and 11 drastically decreased, while peaks 10 and 15 showed a great increase. This can be attributed to removal of unsilylated **b** form by silyl agent; as a consequence, the equilibrium is shifted toward a formation of the other **b** form.

Although peaks 16, 17, and 21 are very abundant in the chromatogram and their molecular weights are available, at this moment it is possible to say only that they are Ty or HTy derivatives; further data are required for a satisfactory structure assignment. Their identification as well as that of remaining products is in progress, through different approaches.

ABBREVIATIONS USED

BSTFA, bis(trimethylsilyl)trifluoracetamide; Ty, 2-(4-hydroxyphenyl)ethanol, tyrosol; HTy, 2-(3,4-dihydroxyphenyl)ethanol, hydroxytyrosol.

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