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ScienceDirect
Food Chemistry 105 (2007) 761–770
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Characterization of phenolic extracts from olives (*Olea europaea* cv. Pisciottana) by electrospray ionization mass spectrometry

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Received 26 April 2006; received in revised form 17 January 2007; accepted 21 January 2007

Abstract

Olives are a potential source of natural anti-oxidants; unfortunately, a complete characterization of olive fruit phenolic profile is still to be achieved, as it is characterized by great complexity and variability.

In this study, electrospray ionization mass spectrometry (ESI-MS) was used to characterize phenolic extracts from olive fruits, establishing the most suitable conditions of analysis in order to detect phenolic derivatives. This technique proved to be very useful in the identification and in the structure elucidation of olive phenolic compounds. The olive pulp phenolic fraction proved to be dominated by phenolic glucosides, mainly hexose derivatives of phenolic alcohols, flavonoids, secoiridoids and oleosides. Several oleuropein derivatives were detected, proving that they are already present in olive pulp and not, as previously suggested, originated during oil extraction.

With this study an effective method to characterize olive phenolic compounds was offered and it was possible to confirm the presence of several biophenols in olive extracts, thus giving a contribution to the characterization of olive pulp phenolic fraction.
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Keywords: Phenolic compounds; Olive; Anti-oxidants; Electrospray ionization; Mass spectrometry

1. Introduction

Over the last years, researchers have become increasingly interested in dietary phenolic compounds because of free radical scavenging activity and other potential beneficial effects on human health associated with their assumption (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004). Olive (*Olea europaea* L.) fruits and derived products represent a recognized valuable source of several natural compounds with important bioactivity (Bouaziz, Grayer, Simmonds, Damak, & Sayadi, 2005), including anti-oxidants such us carotenoids, tocopherols, flavonoids and phenolic compounds, among which the most abundant are the secoiridoid derivatives such us oleuropein and demethyloleuropein (Bianco & Uccella, 2000; Ryan & Robards, 1998).

Many activities have been reported for most of the phenolic compounds from olives: they act as anti-oxidant, anti-inflammatory, anti-viral, anti-cancerogenic agents (Aruoma et al., 1998; Visioli, Poli, & Galli, 2002).

Olives and their derivatives may be considered, therefore, as a potential source of natural anti-oxidants which could be used in food and pharmaceutical industries.

The importance of olive phenolic compounds is due not only to their nutritional properties but also to their influence on oil taste and, thanks to their radical-scavenging activity, on oil shelf-life, preventing its loss of sensory and nutritional quality during storage.

Many studies have focused on the phenolic composition of olive oil rather than olive pulp, so a complete characterization of olive fruit phenolic profile is still to be achieved. Difficulties arise from being it characterized by a great complexity and a wide variability, both qualitatively and

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0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.01.037

quantitatively, depending on geographical, varietal, seasonal, agronomic factors and fruit ripening degree (Ryan & Robards, 1998), but also from the variability in the extraction and quantification methods. For these reasons, many of the molecules present in olive pulp are still unidentified and it is difficult to compare data within the literature.

Different high-resolution techniques have been used to identify olive phenolic compounds; among these, Liquid Chromatography–Mass Spectrometry (LC–MS) technique is of primary importance: coupling chromatrographic separation to mass spectrometry represents a very powerful tool for the analysis of natural products since the mass spectrometer is a universal detector, able to achieve very high sensitivity and to provide information on molecular weights as well as structural information (Ryan, Robards, Prenzler et al., 1999).

Various options of LC–MS interfaces have been used. Particularly, electrospray ionization mass spectrometric technique (ESI-MS) provides a useful tool to define the main chemical structures present in a biophenolic extract.

In this study, ESI-MS was used to characterize phenolic extracts from olive (*Olea europaea* L.) fruits cv. Pisciottana, a typical cultivar of the southern part of Campania region (Italy), establishing the most suitable conditions of analysis in order to detect and characterize phenolic derivatives and other compounds present in olive extracts.

The purpose of the current work was to contribute to the knowledge of olive pulp phenolic profile and to develop an analytical procedure based on electrospray ionization-mass spectrometry (ESI-MS) useful to identify, characterize and eventually quantify these important compounds.

A comprehensive knowledge of the phenolic composition of olive fruits is fundamental to better understand the biochemical pathways in which they are involved and, ultimately, to shed more light on the physiology of olive trees.

2. Materials and methods

2.1. Plant material

Olea europaea L. fruits (Pisciottana cultivar) were obtained from an olive orchard in Camerota, Salerno, Italy. Samples were collected in October 2005, at the green maturation period: ripeness index, determined according to the method developed by the Agronomic Station of Jaén (Spain) (Sacchi, Della Medaglia, Ambrosino, Paduano, & Spagna Musso, 2001), was 4.0. Fruits (1000 g) were selected randomly from around the trees, were immediately frozen and stored at −20 °C until the analysis.

2.2. Chemicals

Methanol, water, hexane, ethyl acetate and formic acid were HPLC-grade solvents purchased from Carlo Erba (Milan, Italy). Caffeic acid and tyrosol were purchased from Fluka Co. (Buchs, Switzerland); oleuropein, luteolin-7-glucoside, verbascoside, luteolin and apigenin were obtained from Extrasynthese (Genay, France).

2.3. Extraction of phenolic compounds

The procedure followed to extract phenolic compounds from olive fruits was based on the method described by Brenes, Rejano, Garcia, Sanchez, and Garrido (1995), to which some modifications had been introduced. Olive pulp (10 g) was mixed in a blender with 30 ml of methanol:water 80/20 (v/v), the mixture was centrifuged for 5 min at 3500 rpm and filtered. This extraction was repeated successively three times, the extracts were collected and organic solvent was evaporated under vacuum. The water phase was washed with hexane (3 × 15 ml), in order to remove the lipid fraction, and the phenolic compounds were extracted with ethyl acetate (5 × 20 ml). This solvent is often used to extract phenolic compounds from aqueous matrices (Obied et al., 2005) as it shows the best recovery efficiency among solvents who are not mixable with water. After evaporation of the organic solvent under vacuum, the dry residue was dissolved in 2 ml of methanol and filtered on a polytetrafluoroethylene (PTFE) syringe filter (0.2 μm pore size); this solution was used for HPLC analysis.

2.4. Reversed-phase high-performance liquid chromatography

HPLC–UV analysis was performed both to acquire UV spectra of each phenolic compound and to collect a fraction (corresponding to the dialdehydic form of decarboxymethyl oleuropein aglycon plus luteolin glucoside) for the following identification by HPLC–MS.

Reversed phase HPLC analysis was performed on a LC-10ATvp Shimadzu (Milan, Italy) liquid chromatograph equipped with a SPD-M10Avp diode array detector (Shimadzu). The chromatographic separation was achieved on a 5 μm ODS-3 Prodigy (250 mm length × 4.6 mm i.d.) reversed-phase column (Phenomenex, Macclesfield, UK). The solvent system used was a gradient of solvent A (water + formic acid 0.5%) and solvent B (acetonitrile:methanol 50/50 v/v + formic acid 0.5%). A step gradient from 15% to 55% B (35 min) was applied at a flow rate of 1.2 ml min^{-1}.

The methanol extract (20 μl) was injected, after dilution 1:8 with water.

2.5. Liquid chromatography with electrospray ionization mass spectrometry

The LC instrumentation consisted of two Shimadzu LC-10ADvp pumps (Shimadzu, Milan, Italy) equipped with a multisolvent delivery system. A Discovery HS C18 column (5 μm, 150 mm length × 2.1 mm i.d., Supelco, St. Louis, MO, USA), at a flow rate of 0.35 ml min^{-1}, was used. The solvent system used was a gradient of solvent A

(water + formic acid 0.25%) and solvent B (methanol + formic acid 0.25%), with a step gradient from 5% to 55% B (45 min). 20 μl of each methanol extract was injected, after dilution 1:8 with water. The mass spectrometry system consisted of a mass spectrometer Shimadzu LCMS-2010EV (Shimadzu, Milan, Italy) equipped with an ESI source; the Microsoft Windows-based software LCMS Solution version 3.20 (Shimadzu, Milan, Italy) was used for data acquisition and processing. Qualitative analyses were performed in negative ion mode with the following operating parameters: interface voltage 4 kV; nebulizer gas flow 1.5 l min^{-1}; block heater temperature 250 °C; curved desolvation line temperature and voltage 300 °C and −5 V, respectively; Q-Array voltage 0 V DC and 150 V RF; detector voltage 1.5 kV; scan range *m/z* 60–900.

Some operating parameters (interface voltage and Q-Array voltage) were then modified in order to obtain a moderate fragmentation of the deprotonated molecular ions: an interface voltage of 5 kV and a Q-Array voltage of −50 V DC and 150 V RF were used.

3. Results and discussion

Phenolic compounds give peaks of lower intensity in negative than in positive ion mode; nevertheless, in this study analyses were performed in negative mode because cleaner spectra were obtained. Operating parameters were optimized by direct injection of solutions of standard phenolic compounds into the ion source, in order to obtain mass spectra dominated by the pseudomolecular ion $[M–H]^-$ for each of the compounds of interest.

Moreover, in order to confirm the structural information obtained from the deprotonated molecular ion, the ion source operating parameters were modified to generate a moderate dissociation–fragmentation induced by the collisions in the ionic transport region.

The efficiency of the ESI-MS analysis is affected by the mobile phase employed: a suitable solvent should permit the formation of ions in solution and allow an easy nebulization and desolvation (Abian, Oosterkamp, & Gelpi, 1999). The pH is also important: in the LC analysis of phenolic compounds, acidification of the mobile phase allows the best separation, because the hydroxyl groups are kept in their acidic form, thereby increasing their retention on the column and decreasing peak broadening caused by formation of the deprotonated form. Attention must be paid to select the suitable acid to use, giving preference to those which are weaker ion-pairing agents (Cuyckens & Claeys, 2002). In this study the highest sensitivity and the best separation in LC–ESI-MS analysis were obtained with water and methanol as solvents acidified with formic acid (0.25%).

A representative total ion current (TIC) chromatogram of an olive methanol extract is shown in Fig. 1.

Identification of phenolic compounds was based on the search for pseudomolecular $[M–H]^-$ ions, using extracted ion mass chromatograms (computer-generated plots of the abundance of a specific ion extracted from the total ion current chromatogram), together with the interpretation of its collision-induced dissociation (CID) fragments. When authentic standards were available, identification was carried out by comparing retention times and mass spectra with those of standards.

Data obtained from the LC–MS analysis of olive extracts are summarized in Table 1.

3.1. Peaks 1; 2

The two first eluted compounds (retention time 1.3 and 2.5 min, respectively) were characterized by a spectrum with an intense ion at *m/z* 191. Other ions were observed which, however, did not allow the identification.

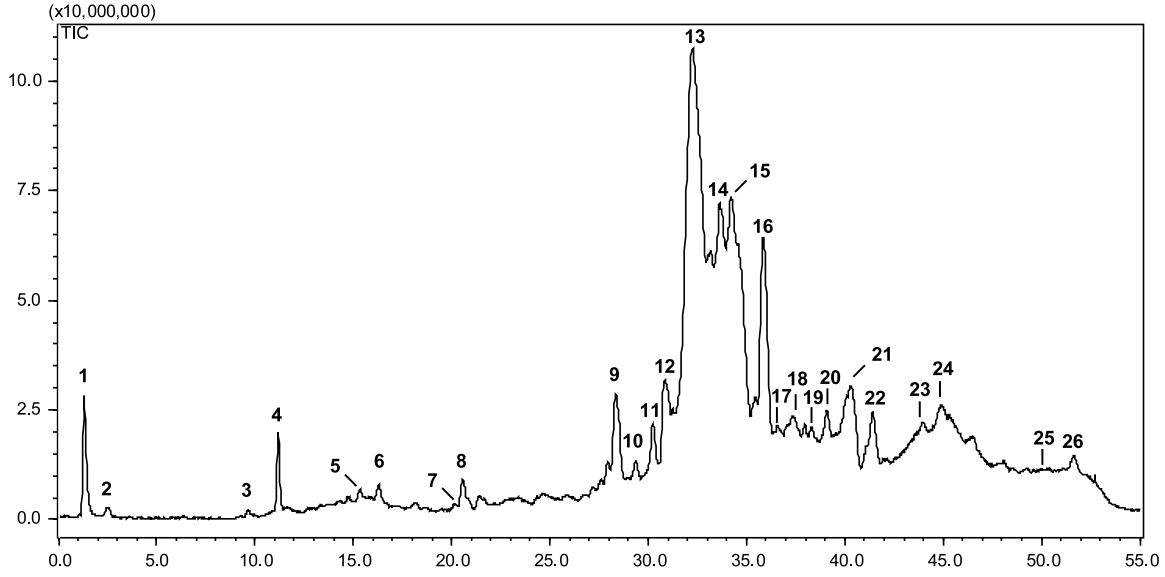

Fig. 1. HPLC–ESI-MS chromatogram (total ion current – TIC) of phenolic extract from olive pulp. See Section 2 for operating conditions. See Table 1 for peak identification.

Table 1
Phenolic compounds identified in olive pulp phenolic extracts

Peak no.	Approximative r.t. (min)	Pseudo molecular ion $[M–H]^-$	Main fragments	Compound
1	1.3	NI[a]	191	–
2	2.5	NI	191, 405	–
3	9.6	153	123	Hydroxytyrosol
4	11.2	315	153, 123	Hydroxytyrosol glucoside
5	15.4	NI	241	Elenolic acid derivative
6	16.3	NI	241	Elenolic acid derivative
7	19.5	179	135	Caffeic acid
8	20.6	403	–	Elenolic acid glucoside
9	28.4	525	389, 319, 183	Demethyloleuropein
10	29.4	NI	653, 477	–
11	30.2	NI	485, 599	–
12	30.9	623	461, 161	Verbascoside
13	32.2	447	285	Luteolin-7-glucoside
13	32.3	319	183, 139	OHTY-DEDA
14	33.4	609	301	Rutin
15	34.2	377	307	Oleuropein aglycon
16	35.8	539	377, 307, 275, 225	Oleuropein
17	36.3	447	301	Quercetin-3-rhamnoside
18	37.3	447	285	Luteolin-4-glucoside
19	38.0	551	507, 389, 341	6′-β-Glucopyranosyl oleoside
20	39.0	539	377, 307, 275	Oleuroside
21	40.3	377	307	Oleuropein aglycon
22	41.4	535	491, 325	6′-Rhamnopyranosyl oleoside
23	44.0	523	361, 291, 259, 101	Ligstroside
24	44.9	285	–	Luteolin
25	50.1	269	–	Apigenin
26	51.6	NI	577	–

[a] Not identified.

3.2. Hydroxytyrosol and tyrosol

The examination of the chromatograms in full scan mode revealed the presence of hydroxytyrosol. The spectra generated for this phenolic alcohol in negative ion mode gave the deprotonated molecule $[M–H]^-$ and the ion fragment at m/z 123, which is due to the loss of the CH_2OH group (De la Torre-Carbot et al., 2005).

Tyrosol was not revealed, in MS analysis, probably because of its acidic properties and of operating parameters (solvent and MS conditions) unsuitable to its ionization. However, the ion corresponding to the deprotonated molecule $[M–H]^-$ at m/z 137 was detected in Selected Ion Monitoring (SIM) mode analysis. Furthermore, this compound was identified in the olive extracts by HPLC–UV analysis comparing UV spectrum and retention time with those of the standard.

These two compounds are frequently detected in olive pulp and have been widely studied, as regards in particular their anti-oxidant activity and their health-beneficial properties, which result much higher for hydroxytyrosol as compared to tyrosol (Obied et al., 2005; Ryan & Robards, 1998).

3.3. Hydroxytyrosol glucoside

The ESI-MS spectra of the compound eluted at 11.2 min showed an intense ion at m/z 315, which formed two major fragments by CID, one at m/z 153 and the other at m/z 123 (Fig. 2). This evidence suggests that the compound could be identified as a hydroxytyrosol hexoside. Three isomers of hydroxytyrosol glucoside have been reported (Bianco et al., 1998; Cardoso et al., 2005; Ryan et al., 2002): Bianco et al. (1998) found hydroxytyrosol-4-β-glucoside to be the most abundant isomer in olive pulp, while Cardoso et al. (2005), according to that published by De Nino et al. (1999), identified hydroxytyrosol-1′-β-glucoside in olive pulp and olive pomace.

The fragmentation profile of the pseudomolecular ion at m/z 315 showed a great similarity to that reported by De Nino et al. (1999) for hydroxytyrosol-1′-β-glucoside, allowing us to identify this compound as this glucoside isomer.

3.4. Peaks 5; 6

At retention times 15.4 and 16.3 min two compounds were observed which ESI-MS spectra were characterized by an intense ion at m/z 241, corresponding to the deprotonated molecule of elenolic acid. These observation, in addition to the consideration that these compounds could be detected only at 240 nm in HPLC–UV analysis, allowed us to infer that they should be identified as elenolic acid or derivatives.

3.5. Caffeic acid

Identification of caffeic acid was achieved by comparing the ESI-MS spectra with those of the pure standard. The spectra generated for this phenolic acid in negative ion mode gave the deprotonated molecole $[M–H]^-$ at m/z 179 and a characteristic fragment at m/z 135 $[M–H\text{-}44]^-$ due to the loss of CO_2.

3.6. Elenolic acid glucoside

The compound that eluted at 20.6 min showed an intense ion at m/z 403, corresponding to the characteristic molecular ion $[M–H]^-$ described in literature for 11-methyloleoside (elenolic acid glucoside), a degradation product of oleuropein which accumulates during ripening. This compound has been already identified in olives (Cardoso

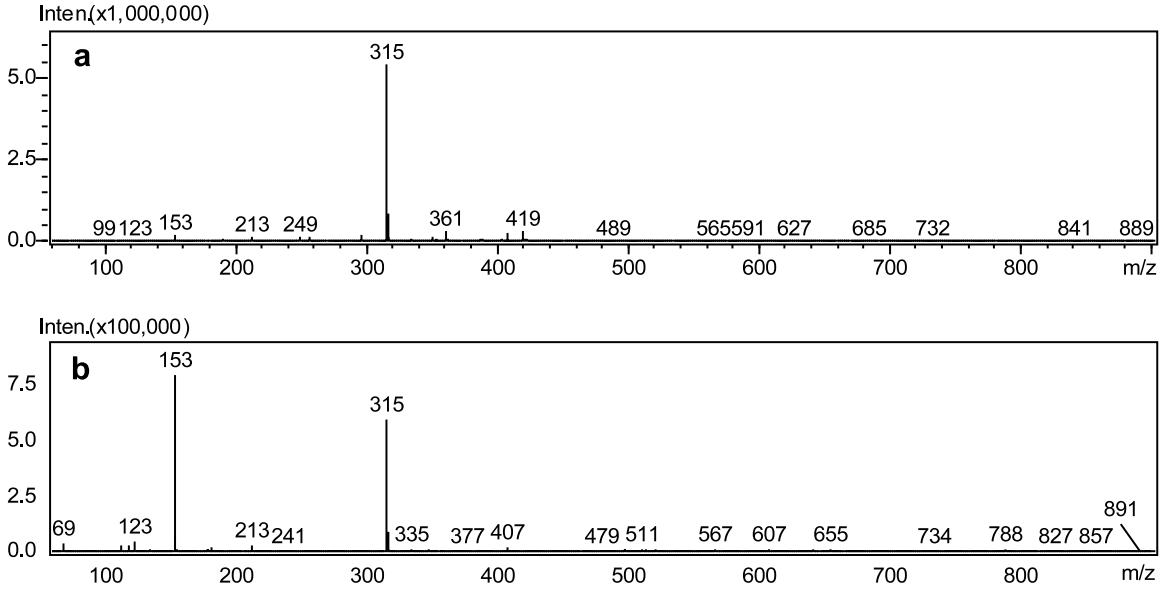

Fig. 2. Mass spectra obtained in full scan mode acquisition for hydroxytyrosol glucoside: (a) initial MS conditions; (b) modified MS conditions in order to obtain better fragmentation (see Section 2).

et al., 2005; Ryan, Robards, Prenzler et al., 1999; Ryan et al., 2002), such as in other species belonging to the Oleaceae family (Soler-Rivas, Espín, & Wichers, 2000).

3.7. Demethyloleuropein

Study of the mass spectrum and of the fragmentation profile (Fig. 3), together with their comparison with data reported in literature (Ryan et al., 2002; Soler-Rivas et al., 2000), allowed the identification of demethyloleuropein in olive phenolic extracts. The mass spectrum of this secoiridoid glycoside showed an intense peak at *m/z* 525, corresponding to the deprotonated molecule (MW 526 amu). The fragmentation profile obtained by CID was characterized by three major fragments at *m/z* 389, at *m/z* 319 and at *m/z* 183, respectively. The ion at *m/z* 389 indicates the hydroxytyrosol release (Fig. 3b) from the molecule, confirmed by the observation in positive ion mode of a fragment at *m/z* 137, characteristic of hydroxytyrosol derivatives (De Nino et al., 1999). As shown in Fig. 3b, the fragment at *m/z* 319 is justified by the loss of a CO_2molecule from the carboxylic group of the aglycon (Fig. 3b, fragmentation C), resulting in its turn from elimination of the glucose moiety (fragmentation B). Further loss of hydroxytyrosol (fragmentation A) from the latter structure (*m/z* 319, corresponding to decarboxymethyl oleuropein aglycon) originates a fragment at *m/z* 183, corresponding to decarboxymethyl elenolic acid.

Presence of demethyloleuropein in olive fruits has been already reported (Ryan & Robards, 1998); such as elenolic acid glucoside, this glycosylated derivative of oleuropein accumulates during fruit ripening, reaching its maximum during black maturation (Soler-Rivas et al., 2000). It is possible that both these compounds are originated from oleuropein by the action of esterases during olive ripening (Amiot, Fleuriet, & Macheix, 1989).

3.8. Verbascoside

Olea europaea fruits contain, besides oleuropein, ligstroside and demethyloleuropein, other phenolic glucosides such us verbascoside (a heterosidic ester of caffeic acid and hydroxytyrosol) and other minor components with analogous structure (isoacteoside, β-hydroxyacteoside) (Ryan, Robards, Prenzler et al., 1999; Soler-Rivas et al., 2000). Both in full scan and in SIM mode, only a compound (RT 30.9 min) characterized by an ion at *m/z* 623 (deprotonated verbascoside molecule) was detected in olive extracts from Pisciottana cultivar. The compound was identified as verbascoside, by comparing mass spectrum, retention time and HPLC–UV spectrum with those of the standard compound. The base peak in negative ion mode was represented by the pseudomolecular ion (*m/z* 623) with few other fragments of lower intensity which were useful for the identification. Particularly, two ions characteristic of verbascoside in MS fragmentation, at *m/z* 461 and 161, were observed. The loss of caffeic acid produced an ion at *m/z* 461 and a ketene as a neutral fragment, while the peak at *m/z* 161 resulted from a proton transfer and the production of an anionic ketene (Ryan, Robards, Prenzler et al., 1999).

3.9. Dialdehydic form of decarboxymethyl oleuropein aglycon (OHTy-DEDA)

Examination of the chromatograms of olive extracts in full scan mode revealed the presence of the deprotonated molecule $[M–H]^-$ of OHTy-DEDA (at *m/z* 319), that coelutes with a flavonoid (luteolin glucoside) in the HPLC

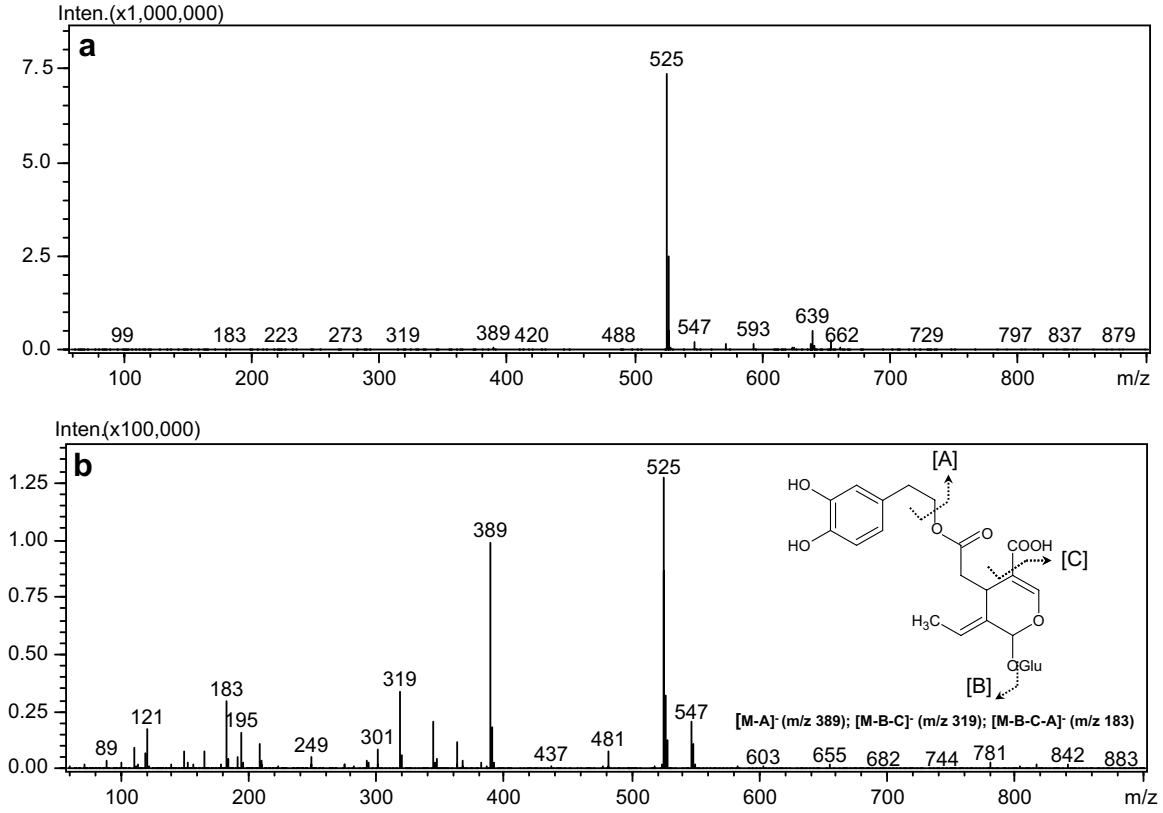

Fig. 3. Mass spectra obtained in full scan mode acquisition for demethyloleuropein: (a) initial MS conditions; (b) modified MS conditions in order to obtain better fragmentation (see Section 2). Proposed scheme for demethyloleuropein fragmentation.

conditions used. The identification was achieved by collecting fractions corresponding to this compound in HPLC–UV analysis and then subjecting these fraction to ESI-MS analysis.

The fragmentation spectra showed the pseudomolecular ion $[M–H]^-$, a fragment at *m/z* 183 which can be explained by the loss of hydroxytyrosol, and a fragment at *m/z* 139 originated by elimination of a CO_2 molecule from the elenolic derivative fragment (*m/z* 183).

The presence of OHTy-DEDA in olive fruits of Pisciottana cultivar confirms what suggested by Ryan et al. (2002): this compounds has to be considered an intermediate in the biosynthesis of oleuropein rather than a breakdown product resulting from enzymatic activity during the oil extraction process, as previously suggested by Rovellini and Cortesi (2002) and Servili et al. (1999).

3.10. Rutin

By means of LC–MS analysis it was possible to reveal the presence of quercetin-3-*O*-rutinoside (rutin) in the olive extracts. Structure assignment was based on a systematic search for molecular ion using extracted ion mass chromatograms and on comparison with data reported in literature (Bouaziz et al., 2005; Ryan et al., 2002; Vlahov, 1992). The ESI-MS spectrum acquired in full scan mode displayed an intense pseudomolecular ion at *m/z* 609, a fragment at *m/z* 301, which is diagnostic of quercetin derivatives, and a fragment at *m/z* 463, due to the loss of 146 amu corresponding to rhamnose. The presence of rutin has been reported in other olive cultivars (Bouaziz et al., 2005; Cardoso et al., 2005; Ryan et al., 2002; Vlahov, 1992); many biological effects have been attributed to this flavonoid, which shows anti-oxidant, anti-inflammatory, anti-thrombotic, cytoprotective, vasoprotective and antimicrobial activities (Raj Narayana, Sripal Reddy, Chaluvadi, & Krishna, 2001).

3.11. Oleuropein aglycon

Oleuropein aglycon in its various tautomeric forms was detected in the olive extracts: at retention times 34.2 and 40.3 min two compounds eluted which showed MS fragmentation patterns similar to those reported for oleuropein derivatives (Caruso, Colombo, Patelli, Giavarini, & Galli, 2000; De la Torre-Carbot et al., 2005). In addition to the pseudomolecular ion at *m/z* 377, in the mass spectra it was possible to observe a fragment at *m/z* 307, due to the loss of C_4H_6O (Caruso et al., 2000).

3.12. Oleuropein

As expected, oleuropein, one of the major components of olive phenolic fraction, was identified in the extracts

from olive pulp. This secoiridoid is able to confer resistance to disease and to insect infestation of the tree (Soler-Rivas et al., 2000); furthermore, it is responsible for the bitter taste of olives (Gutierrez-Rosales, Rios, & Gomez-Rey, 2003) and shows anti-oxidant properties due to its catechol moiety (Visioli & Galli, 1994).

Identification was carried out by LC–MS and HPLC–UV analysis. The ESI-MS spectrum showed a pseudomolecular ion at *m/z* 539 with fragments consistent with the reported fragmentation scheme: the ion at *m/z* 377 arises from cleavage of the glycosyl bond (Ryan, Robards, Prenzler et al., 1999); the ion at *m/z* 307 is justified by the loss of a C_4H_6O fragment (Caruso et al., 2000), while the fragment at *m/z* 275 may derive from rearranged fragments (De la Torre-Carbot et al., 2005); an ion at *m/z* 223 was also observed and may arise from a McLafferty-type rearrangement of the ester function (Ryan, Robards, Prenzler et al., 1999).

3.13. Luteolin glucoside

Two compounds (RT 32.2 and 37.3 min) with mass spectra corresponding to that of luteolin glucoside have been detected in olives of Pisciottana cultivar. For the first isomer, which coelutes with the dialdehydic form of decarboxymethyl oleuropein aglycon (OHTy-DEDA), the identification was carried out by collecting fractions corresponding to this compound in HPLC–UV analysis, which were then subjected to ESI-MS analysis.

These two luteolin-glucosides showed spectra dominated by the pseudomolecular ion (at *m/z* 447); after fragmentation (induced by modification of some operating parameters of the ion source, see Section 2), a strong peak at *m/z* 285 was observed, corresponding to a loss of 162 amu (loss of glucose), as shown in Fig. 4a.

The hypothesis that one of these peaks corresponded to quercetin-rhamnoside was rejected from a consideration that the mass spectrum of this compound would have shown a peak at *m/z* 301 (corresponding to the aglycon moiety), rather than the observed peak at *m/z* 285. The first eluted compound (RT 32.2 min) was identified as luteolin-7-glucoside by comparing retention time and spectra with those of the authentic standard. In olives two other luteolin glucosides have been detected: luteolin-4′-glucoside (Bouaziz et al., 2005; Ryan et al., 2002) and another isomer which has been identified neither as luteolin-6-glucoside nor as luteolin-8-glucoside (Ryan et al., 2002). These findings, together with the consideration that luteolin-4-glucoside was the only isomer eluting after luteolin-7-glucoside in reversed phase HPLC analysis, suggested that the second

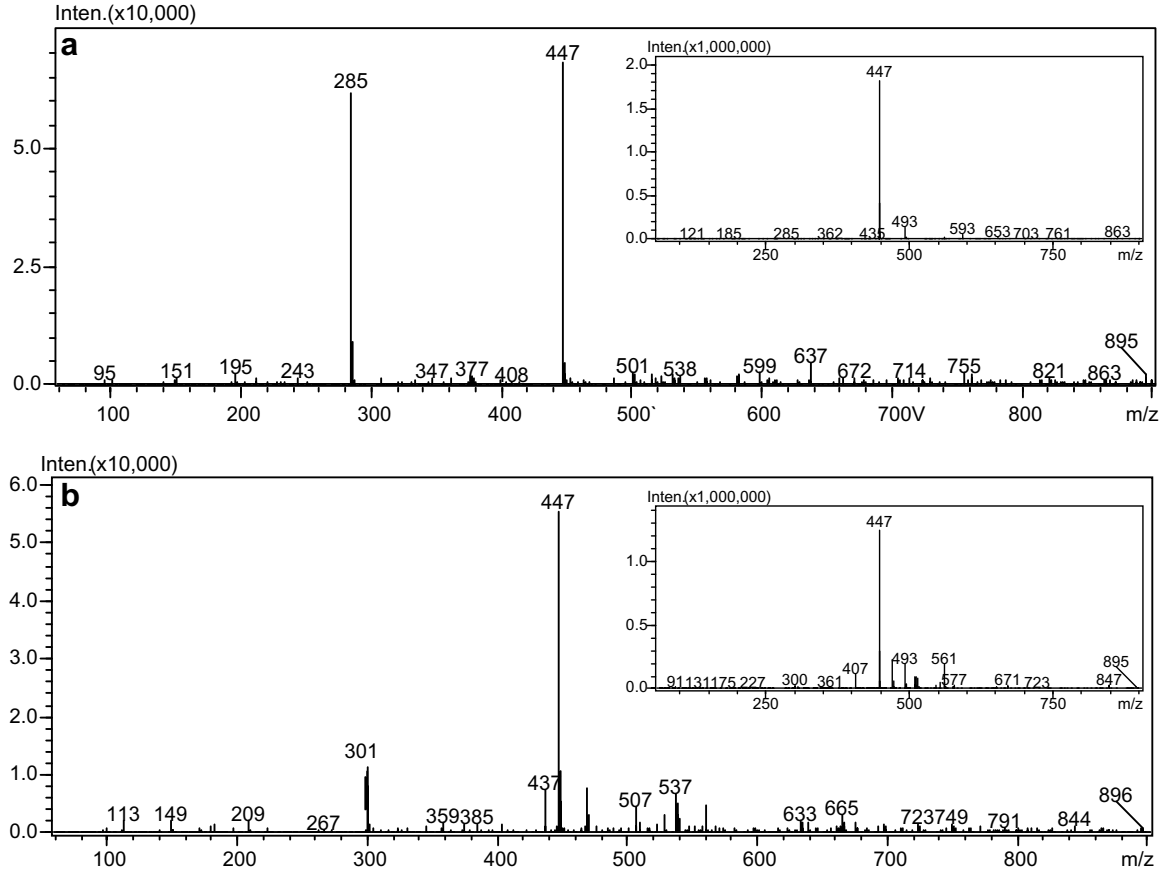

Fig. 4. Mass spectra obtained in full scan mode acquisition for luteolin-7-glucoside and for quercetin-3-rhamnoside. Modified MS conditions in order to obtain better fragmentation (see Section 2). In the inset: mass spectra obtained for the same compounds with initial MS condition (see Section 2).

eluting compound (RT 37.3 min) might be identified as luteolin-4′-glucoside.

3.14. Quercetin-3-rhamnoside

The spectrum generated by ESI-MS in negative acquisition mode for the compound eluting at 36.3 min showed an intense ion at *m/z* 447 and a fragment at *m/z* 301 (Fig. 4b), which could correspond to the pseudomolecular ion of quercetin-3-rhamnoside (quercetrin) and to its aglycon (quercetin), respectively. Quercetin-3-rhamnoside has been rarely reported among the flavonoids identified in *O. europaea*, although it has been already detected in olive pulp and leaves (Ryan, Robards, & Lavee, 1999; Vlahov, 1992). For this flavonoid several biological properties have been reported: it shows anti-oxidant effect on oxygen radical-generated DNA damage, anti-viral and anti-inflammatory activity (Camuesco et al., 2004; Raj Narayana et al., 2001); its aglycon (quercetin) has been reported to have cardiotonic, anti-neoplastic, anti-microbial and anti-ulcerogenic activity, to protect low-density lipoproteins (LDL) against oxidative modifications and to be one of the most effective flavonoids in scavenging free radicals (Raj Narayana et al., 2001).

3.15. 6′-β-glucopyranosyl oleoside

An oleoside derivative (6′-β-glucopyranosyl-oleoside), recently detected for the first time in *O. europaea* by Cardoso et al. (2005), was identified in the olive extracts (RT 38.0 min). The ESI-mass spectrum of this compound showed an intense pseudomolecular peak at *m/z* 551. Moreover, the fragmentation pattern was in agreement with the scheme suggested by Cardoso et al. (2005).

3.16. Oleuroside

The compound which eluted at 39.1 min exhibited the same mass spectrum as that reported for oleuropein, with the presence of an intense ion at *m/z* 539 and several other fragments (*m/z* 377, 307 and 275) suggesting that it could be identified as oleuroside. This glucoside is a structural isomer of oleuropein differing in the position of the olefinic double bond; it was shown to elute after oleuropein under reversed phase conditions (Savournin et al., 2001).

Oleuroside is a phenolic derivative of secologanins; among this kind of secoiridoids, it is the only to have been identified in olive leaves and pulp (Kuwajima, Uemura, Takaishi, Inoue, & Inouye, 1988; Ryan et al., 2002).

3.17. 6′-β-rhamnopyranosyl oleoside

The MS spectrum of this compound revealed an intense ion at *m/z* 535, which could correspond to the deprotonated molecule $[M–H]^-$. Cardoso et al. (2005) detected in olive pomace and in olive pulp an oleoside derivative with molecular mass of 536 amu, which was identified as 6′-β-rhamnopyranosyloleoside. The fragmentation pattern shown by this compound was in agreement with that reported by Cardoso et al. (2005). However, further study is needed for an ultimate structure assignment.

3.18. Ligstroside

Identification of ligstroside, another phenolic glucoside, was carried out by examination of the fragmentation spectra, which were characterized by the pseudomolecular ion at *m/z* 523 $[M–H]^-$ and by two other fragments at *m/z* 361 and *m/z* 291. As reported for oleuropein, these fragments correspond, respectively, to the cleavage of the glycosyl bond (*m/z* 361) and to the loss of a C_4H_6O fragment (Caruso et al., 2000). Ions at *m/z* 259 and 101 were also observed, which may be due to rearrangement fragments, as previously reported (De la Torre-Carbot et al., 2005).

3.19. Luteolin and apigenin

The peak revealed at a retention time of 44.9 min showed an ESI mass spectrum characterized by an intense ion at *m/z* 285, indicating the presence of luteolin. Apigenin was also detected at a retention time of 50.1 min. The structure assignment was based on the presence of an intense pseudomolecular ion at *m/z* 269 in the mass spectrum and was confirmed by HPLC–UV analysis. In several studies these flavonoids have been detected in olive fruits of different cultivars (Bouaziz et al., 2005; Owen et al., 2003; Ryan et al., 2002) and have proved to possess important biological properties, such us anti-oxidant, anti-inflammatory, anti-microbial and cardiotonic activity, ability to scavenge free radicals and to inhibit low-density lipoprotein oxidation (Raj Narayana et al., 2001).

3.20. Peak 26

The compound which eluted at 51.6 min exhibited a mass spectrum with the main ion at *m/z* 557, which may correspond to the pseudomolecular ion $[M–H]^-$. A fragment at *m/z* 513 was also detected, suggesting the presence of a COOH group easily removed. These evidences, however, were not enough to suggest a plausible structure for this compound.

4. Conclusions

This work is the first report on the identification and characterization of phenolic compounds in olive pulp of the old cultivar Pisciottana, a widespread traditional cultivar of the southern part of Campania region. The ESI-MS analysis allowed the characterization of olive pulp phenolic profile, proving to be a very useful technique in the identification and in the structure elucidation of phenolic compounds. All operating parameters were optimized in

order to obtain very clear and easy to interpret mass spectra, dominated by the pseudomolecular ion for each of the compounds of interest; the same technique, with some modifications in the operating parameters, allowed to obtain a moderate fragmentation, thus confirming the structural information obtained from the deprotonated molecular ion.

The olive pulp phenolic fraction proved to be dominated by phenolic glucosides, mainly hexoses derivatives of phenolic alcohols, flavonoids, secoiridoids and oleosides. Several oleuropein derivatives were detected, proving that they are already present in olive pulp and not, as previously suggested for the dialdehydic form of decarboxymethyl oleuropein aglycon, originated only during oil extraction.

With this study an useful and effective method to characterize olive phenolic compounds was offered and it was possible to confirm the presence of several biophenols in olive phenolic extracts, some of which have been not frequently reported in olive tissues, thus giving a contribution to the characterization of olive pulp phenolic fraction. Many compounds, among those identified, contribute significantly to the health benefits associated with the consumption of olives and virgin olive oils; moreover, they could be extracted from olive tissues and used as natural anti-oxidants, substituting synthetic anti-oxidants in food, cosmetic or pharmaceutical industries. In addition, the complete characterization of olive minor components is needed to better understand the plant responses to biotic and abiotic stresses and, ultimately, contributes to the knowledge of olive physiology and metabolism.

Acknowledgements

This work was supported by the Italian Ministry of University and Research and by Industria Olearia Biagio Mataluni within the project "Controllo Qualità ed Innovazione Tecnologica nell'Industria Olearia" (DM 593-8/08/2000, Prot. MIUR 1866-18/02/2002). A special thanks to Dr. Antonio Vassallo for his kind help in collecting samples.

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