Rapid Evaluation of Phenolic Component Profile and Analysis of Oleuropein Aglycon in Olive Oil by Atmospheric Pressure Chemical Ionization−Mass Spectrometry (APCI−MS)

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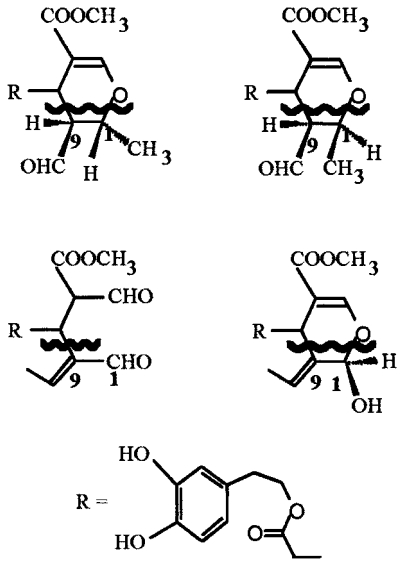
Epidemiological studies have linked the Mediterranean diet with a low incidence of cardiovascular diseases. Olive oil, the major fat component of this diet, is characterized by antioxidant properties related to their content in catecholic components, particularly oleuropein aglycon. Therefore quantification of these components in edible oils may be important in determining the quality, and consequently its commercial value. The present method allows us to obtain the profile of the phenolic components of the oil from the methanolic extracts of the crude olive oil. In particular tyrosol, hydroxytyrosol, elenolic acid, deacetoxyligstroside and deacetoxyoleuropein aglycons, ligstroside and oleuropein aglycons, and 10-hydroxy-oleuropein are clearly identified by atmospheric pressure chemical ionization−mass spectrometry (APCI−MS). Moreover, oleuropein and its isomers present in the oil are quantified by APCI−MS/MS analysis of the extracts without preliminary separation from other phenolic compounds.

Keywords: *Olive oil; APCI-MS and APCI-MS/MS; phenolic components; quantitative and qualitative analysis; oleuropein aglycon; catecholic compounds*

INTRODUCTION

Several epidemiological studies have demonstrated that the Mediterranean diet is associated with low incidence of cardiovascular diseases (Willett et al., 1995; Kushi et al., 1995). This property may depend on the presence of food rich in antioxidants, such as virgin olive oil which is an important component of the above dietary regimen. In particular, recent in vitro experiments suggested that components of virgin olive oil (VOO) prevent lipoprotein oxidation which is recognized to be a contributing factor in the development of atherosclerosis (Grignaffini et al., 1994; Visioli and Galli, 1998; Caruso et al., 1999). Recent reports have demonstrated that antioxidant properties of virgin olive oil (Visioli et al., 1998a) are related to their content in phenolic compounds (Damtoft et al., 1993) some of which are glycosides such as oleuropein and ligstroside (Montedoro et al., 1992). During crushing and malaxing processes, hydrolysis of the glycosidic bond occurs and the aglycons pass into the oil (Cortesi et al., 1995) while glycosides remain in the wastewater. The process also causes partial modification of oleuropein aglycon (OleA) due to the keto−enolic tautomeric equilibrium that involves the ring opening of secoiridoids. The generated isoforms of oleuropein (Scheme 1) show different chemical physical properties (Gariboldi et al., 1986; Angerosa et al., 1995) but maintain the catechol moiety thus contributing to the antioxidant properties of virgin olive oil. The concentration of these aglycons and other minor antioxidants in the oil depends on a number of factors including (a) variety of olive trees and their location; (b) fruit ripening; (c) process of olive crushing and extraction of oil, and (d) time and method of storage of olives and oil (for review see Ryan and Robards, 1998). Therefore, quantification of the oleuropein derived aglycons in edible oils may be important in determining the quality and consequently the commercial value of this nutrient (Tsimidou, 1998).

Scheme 1. Chemical Structure of Isomers Formed during Oleuropein Hydrolysis. R = Hydroxytyrosol Residue. Wave Lines Indicate the Fragment Lost by Collision of the *m/z* 377 in the APCI-MS/MS Analysis (see Figure 1c and d)



Presently, the two most widely used methods to measure phenolic substances in oil extracts employ either a colorimetric reaction (Favati et al., 1994) or a

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HPLC procedure (Cortesi et al., 1995). A major disadvantage of the former is its low specificity that does not allow us to identify the various phenolic compounds greatly differing from each other in terms of antioxidant quality. UV−HPLC gives rise to complex profiles of the phenolic fraction due to overlapping of various peaks. In addition, no single wavelength is ideal for the different phenols because they differ significantly from each other in this respect (Ryan and Robard, 1998). Therefore, UV−HPLC quantification is also poorly representative of the antioxidant quality of the oils. This prompted us to develop a fast and accurate analytical procedure based on atmospheric pressure chemical ionization−mass spectrometry (APCI−MS) with the aim of assigning to the olive oils their antioxidant power in terms of aglycons derived from oleuropein.

MATERIALS AND METHODS

Atmospheric Pressure Chemical Ionization−Mass Spectrometry (APCI−MS and APCI−MS/MS). An LCQ mass spectrometer (Thermoquest, USA) equipped with an APCI-ion trap operating in the negative mode was employed. The Microsoft Windows-NT-based software LCQ Navigator was used to control the instrument and for data acquisition and processing. The operating parameters were as follows: source voltage 1.7 kV; source current 5 μA; temperature and voltage of the capillary were 150 °C and 5 V, respectively; sheet gas flow rate 60 psi; vaporizer temperature 450 °C. For MS/MS analysis, collision energy was 32% of 5 V. Oil extracts (20 μL) were injected directly into the ion source through a rheodyne loop injector. Qualitative analysis was carried out in the APCI−MS scan mode (*m*/*z* 100−600), while quantitative analysis was performed by APCI−MS/MS monitoring OleA product ion.

Sample Preparation. The phenolic fraction of VOO samples was extracted by the method described by Montedoro (Montedoro et al., 1992) with minor modifications. Buthylated hydroxytoluene (BHT; 40 μM final concentration, Sigma, USA) was added to oil samples (3 mL) which were then extracted twice with methanol/water (80/20; v/v; 1 mL) for 20 min on a mechanical shaker. The collected organic phases were then taken to dryness under nitrogen. Extracts were stored at −20 °C until analysis. Storage for up to one month did not modify the concentration of the tested compounds.

Preparation of Oleuropein Aglycon. Authentic OleA was obtained according to Limiroli et al. (1995) from oleuropein glycoside (Extrasynthese, France). Briefly, the glycoside (130 mg) in 50 mM acetate buffer (pH 5.2) was incubated for 17 h at 32 °C under gentle shaking in the presence of 12 mg of β-glucosidase (EC 3, 2, 1, 21; 5, 7 U/mg; Sigma, USA) from almonds. Extraction of the aglycon was carried out with 20 mL of $CHCl_3/CH_3OH$ (2/1; v/v) and the organic phase was evaporated to dryness under nitrogen. Purity of the extract was checked by APCI−LC−MS using an RP-C18 column (250 × 4.6 mm, Alltech Associates Inc., Deerfield, IL) under the conditions described by Cortesi et al. (1995). As expected from the described keto−enolic isomerization of the secoiridoid moiety of oleuropein (Gariboldi et al., 1986: Angerosa et al., 1996), four peaks were present in the chromatogram (Figure 1a). As shown in the ion chromatogram obtained by loop injection of the aglycon (Figure 1b), all peaks were characterized by the ion at *m*/*z* 377, corresponding to the $[M-H]^-$ of OleA (Figure 1c). The presence of a significant amount of residual oleuropein glycoside was excluded because a trace signal was detected on the ion chromatogram at *m*/*z* 539 ($[M-H]^-$) at a retention time of oleuropein glycoside (28.83 min; Figure 1b). MS/MS by collision of the ion at *m*/*z* 377 (Figure 1c) was the origin of the ion at *m*/*z* 307 (Figure 1d) that was then used for quantification of OleA as described below. The latter ion is likely derived from the loss of a C_4H_6O fragment containing carbon atoms 1 and 9 (Scheme 1) thus indicating that it still contains the catechol moiety of OleA.

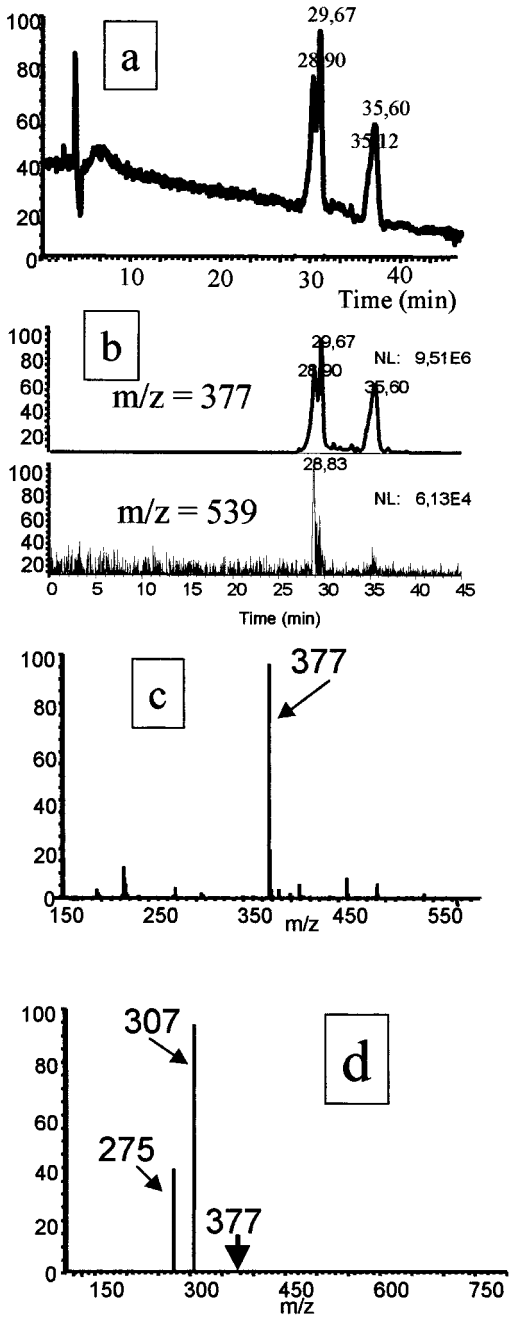

Figure 1. LC−APCI−MS of OleA prepared from oleuropein glycosides. (a) chromatogram of the total ion current (TIC) of OleA; (b) ion chromatogram of ions at *m*/*z* 377 (OleA) and 539 (oleuropein glycoside); (c) APCI−MS spectrum obtained by loop injection of OleA; (d) APCI−MS/MS spectrum obtained by collision of ion at *m*/*z* 377.

Quantitative Analysis of Oleuropein Aglycon. For quantitative analysis, VOO samples (3 mL) were spiked with phloretin (100 μg/sample, MW = 272) to be used as an internal standard and were extracted as described above. Dried extracts were dissolved in 1 mL of methanol and five aliquots (20 μL) of each extract were analyzed for APCI−MS/MS (loop injection) monitoring ions at *m*/*z* 307 and at *m*/*z* 167 derived from the collision of the $[M-H]^-$ ions of OleA (*m*/*z* 377) and phloretin (*m*/*z* 273), respectively. The mean peak area ratio (*m*/*z* 307/167) of the five injections was then calculated. Calibration curves for quantification were prepared using a sunflower oil (3 mL per sample) which did not contain oleuropein and OleA, as shown by preliminary APCI−MS/MS analysis. The oil was spiked with phloretin (100 μg/sample) and with various amounts of OleA (50, 100, 200, 500, and 1000 μg/sample corresponding to 16.6, 33, 66.7, 166.7, and 333.3 mg/L). Each

Table 1. Relative Abundance of Specific Ions in the APCI−MS Spectra of VOO Extracts

	oil A	oil B	oil C	oil D	oil E	oil F	oil G	oil H	oil I
m/z 137 tyrosol	0	0	3	0	0	8	0	0	0
m/z 241 elenolic acid	6	12	3	9	42	9	0	5	3
m/z 303 deacetoxyligstroside aglycon	0	11	0	0	0	0	0	0	0
m/z 361 ligstroside aglycon	35	65	87	64	72	39	0	19	14
m/z 153[a] hydroxytyrosol	0	0	7	6	0	12	0	7.5	5
m/z 319[a] deacetoxyoleuropein aglycon	3	30	8	14	58	9	93	0	9
m/z 377[a] oleuropein aglycon	100	100	100	100	100	100	100	100	100
m/z 393[a] 10-hydroxyoleuropein aglycon	2	0	6	37	28	9	0	12	0
OleA (mg/L)[b]	146	215	91	40	31	30	3	31	131
ratio of other catechols/OleA[c]	0.05	0.3	0.21	0.57	0.86	0.3	0.93	0.2	0.14

[a] Catecholic compounds. [b] Determined as described in the text. [c] Ratio of signal intensities *m/z* (153 + 319 + 393)/377.

Table 2. Inaccuracy (Bias %) and Imprecision (CV %) of OleA Quantification

OleA in spiked sunflower oil (mg/L)			
expected	found[a]	bias %	CV % ($n = 6$)
16.6	16.3	1.81	14.0
33.3	32.0	3.90	14.0
66.7	65.3	2.09	13.8
166.7	166.3	0.23	13.0
334.0	332.8	0.15	12.0

[a] Each found OleA concentration was obtained as the mean of 6 extracts prepared and analyzed in different days.

sample was extracted and analyzed. To calculate the extraction recovery, a series of sunflower oil samples (3 mL each) were spiked only with OleA as above while the internal standard was added after extraction. Ion ratios were then compared to those obtained analyzing mixtures of OleA and phloretin without preliminary extraction. Recovery percents were 98 ± 11 (mean ± SD; $n = 5$). This was considered to be the recovery obtained from olive oil because both matrixes are mainly acylglycerol mixtures. Linearity, imprecision, and inaccuracy of the method were determined by analyzing samples of spiked sunflower oil within a day and after different numbers of days.

RESULTS AND DISCUSSION

The analyses were performed on oils from different Italian cultivars (A−G), in particular homemade VOOs (A, B, and C), commercially available VOOs (D−F), and olive oil (G). One commercially available VOO from Spain (H) and one homemade VOO from a Greek cultivar (I) were also analyzed. The chromatogram of the total ion current (TIC) obtained in the LC−APCI−MS analysis of oil C (Figure 2a) showed several peaks in agreement with previous results on the GC−CI−MS identification of OleA isomers (Angerosa et al., 1996) and on LC−APCI−MS identification of polar compounds in VOO (Cortesi et al., 1995). As expected from the isomerization of the secoiridoid moiety of the oleuropein molecule (Scheme 1) described to occur during oil preparation from olives, the ion characteristic of OleA (*m/z* 377) was associated with a number of these peaks (Figure 2b). When oil extracts were analyzed by APCI−MS without LC separation, the ions detected were those reported in Figure 2c. The main bands of ions originated from aglycons derived from oleuropein (*m/z* 377) and from ligstroside (*m/z* 361), the latter containing a phenolic residue instead of the catecholic residue of oleuropein (Cortesi et al., 1995). Other phenolic components known to occur in oil extracts were identified (see the caption of Figure 2). Among those compounds only hydroxytyrosol, deacetoxy-oleuropein, and 10-hydroxy-oleuropein contain a catechol moiety and may therefore contribute to the antioxidant properties of the VOO. The pattern in the other tested VOO was similar

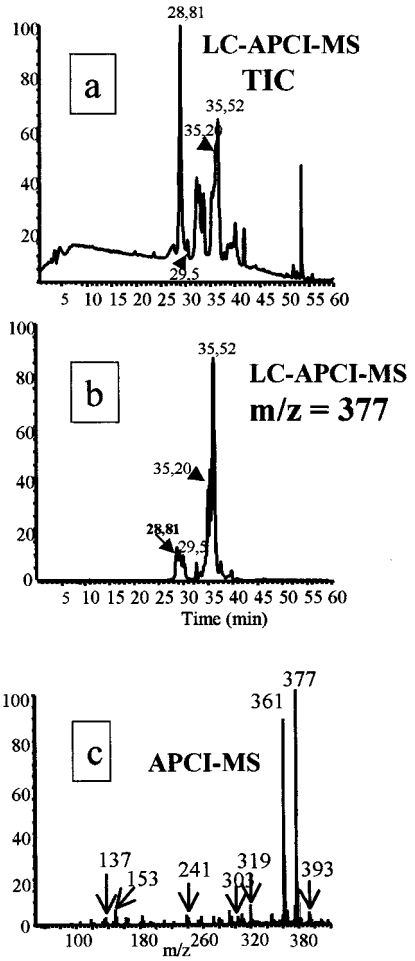

Figure 2. LC−APCI−MS of a VOO extract. (a) chromatogram of the total ion current (TIC); (b) ion chromatogram of the ion at *m/z* 377; (c) APCI−MS spectrum obtained by loop injection of the VOO extract; ion at *m/z* 137 = tyrosol; *m/z* 153 = hydroxytyrosol; *m/z* 241 = elenolic acid; *m/z* 303 = deacetoxy-ligstroside aglycon; *m/z* 319 = deacetoxyoleuropein aglycon; *m/z* 361 = ligstroside aglycon; *m/z* 377 = oleuropein aglycon; *m/z* 393 = 10-hydroxy-oleuropein.

(Table 1) with differences related only to the relative intensities of the detected ions.

Therefore determination of the OleA concentration combined with the ratios of the other catecholic compounds to OleA can be considered to represent the relative antioxidant power of the VOOs.

Linearity of OleA quantification was ensured by the calibration curves ($Y = a + bx$ where X represents OleA concentration (mg/L) and Y is the ion ratio *m/z* 307/167) which always gave $r^2 > 0.99$. The slope (b) of the calibration curves (mean ± SD of 6 preparations, prepared and analyzed in different days) was 0.052 +

0.016. Sameday imprecision was less then 7% expressed as CV% of analysis of the same sample at morning, midday and afternoon. Between-days imprecision (CV %) and inaccuracy (bias %) were as reported in Table 2. The limit of detection, with a signal-to-noise ratio of 3, was 0.05 mg/L.

The concentration of OleA and the ion ratio of other catechols/OleA reported in Table 1 for the tested samples indicate that homemade oils (A, B, C, and I) contain higher levels of antioxidants.

In conclusion, the described method allows us to obtain, from crude methanol extracts without any further analytical workup, a profile of the phenolic components of the nutrient as well as quantitative analysis of overall OleA, thus contributing to better characterize the commercial value of VOOs.

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