Quantitation of Oleuropein in Virgin Olive Oil by Ionspray Mass Spectrometry−Selected Reaction Monitoring

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This work describes the unambiguous evaluation of the presence of oleuropein in virgin olive oils by ionspray tandem mass spectrometry (ISI-MS/MS). The oil samples obtained from different cultivars, such as Carolea, Cassanese, Coratina, Dolce di Rossano, Roggianella, and Tonda di Strongoli, grown in different geographical areas of Calabria, Italy, have shown an average content of oleuropein ranging from 1 ppb to 11 ppm. Commercial virgin oil samples, blended in some cases, contain significant amounts of this pharmacologically important antioxidant. The MS/MS methodology was applied in a triple-quadrupole instrument, through continuous scanning of the third analyzer to detect oleuropein in methanol extracts and in selected ion monitoring (SRM) for its quantitative assay.

Keywords: *Oleuropein; virgin olive oil; quantitative assay of oleuropein; ionspray mass spectrometry; selected reaction monitoring (SRM)*

INTRODUCTION

Virgin olive oil is a typical component of the so-called "Mediterranean diet" that, unlike other vegetable oils, is consumed unrefined and, consequently, rich in phenolic compounds whose content in fresh olive oils ranges up to 1000 mg/kg (Montedoro et al., 1993). The radical scavenger activity of phenols protects the oil during its aging and preserves some of its organoleptic properties. One of the main vectors of phenols in olive drupes and leaves is represented by oleuropein (**1**), whose presence in fresh olive oils has been only hypothesized.

The so-called phenolic component of oils, a parameter normally used to assess the quality of the product, is basically due to the presence of hydroxytyrosol [(3,4-dihydroxyphenyl)ethanol], tyrosol [(4-hydroxyphenyl)-ethanol], and some other molecules containing phenol groups. The latter partially derive from enzymatic or chemical degradation of secoiridoid glycosides such as **1**, which carrier the phenol moieties inside the cells (Montedoro et al., 1993; Gariboldi et al., 1986).

Although the detection of phenols is a means for assessing the quality of this edible oil of high nutritional value, an indirect proof of the accuracy of the procedure employed during its manufacturing from drupes is certainly represented by the detection and quantitation of intact secoiridoid glucosides such as **1**. Oleuropein, in fact, undergoes facile hydrolysis (Gariboldi et al., 1986; Limiroli et al., 1995), and its presence in the oil is an indication of the mildness of the extraction procedure. Moreover, the number of studies that report on the biological and pharmacological activity of oleuropein (Chimi et al., 1991; Panizzi et al., 1960; Fleming et al., 1973; Visioli et al., 1998) allow us to assume that its presence confers to olive oil, whose nutritional value is widely recognized, a preventive action toward a number of pathologies generated by free-radical action.

MATERIALS AND METHODS

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

Mass Spectrometry. The ionspray (pneumatically assisted electrospray, ISI) mass spectra were acquired on a Perkin-Elmer Sciex API III Plus mass spectrometer (Sciex Co, Thornhill, ONT, Canada) equipped with a Perkin-Elmer Series 200 dual solvent delivery system (Perkin-Elmer Co, Norwalk, CT). The ionspray spectra in the positive ion mode were obtained under the following conditions: ionspray voltage, 5.5 kV; orifice voltage, 60 V; scan range, m/z 100−900; scan rate, 5.3 ms/u; no interscan delay; resolution > 1 u. The formation of product ions was induced by collision (CID) of selected precursors with argon in the improved collision cell of the PE Sciex API III Plus instrument and mass-analyzed using the second analyzer of the instrument under the same experimental condition described above for the ionspray spectra. Other experimental conditions for CID included the following: collision energy, 20 eV; collision gas thickness (CGT), 253 × 1013 molecules/cm^2; scan range, m/z 20−900. For the confirmation of oleuropein in the extracts, selected reaction monitoring (SRM) was used with a dwell time of 300 ms. The data reported in Table 3 derive from an analysis of variance according to LSD test.

Oleuropein standard and the stock extract of each sample in acetonitrile was further diluted with 50% acetonitrile containing 0.1% formic acid (FA) and 5 mM ammonium acetate solution. This solution was delivered to the ionspray source by flow injection analysis (FIA) by a PE series 200 autosampler. The flow rate was 100 μL min^{-1} of 50% acetonitrile containing 0.1% FA and 5 mM ammonium acetate.

Plant Material. Olive (*Olea europaea* L.) oils were obtained from handpicked olive drupes harvested from the end of September to the end of December 1996 and 1997, respectively.

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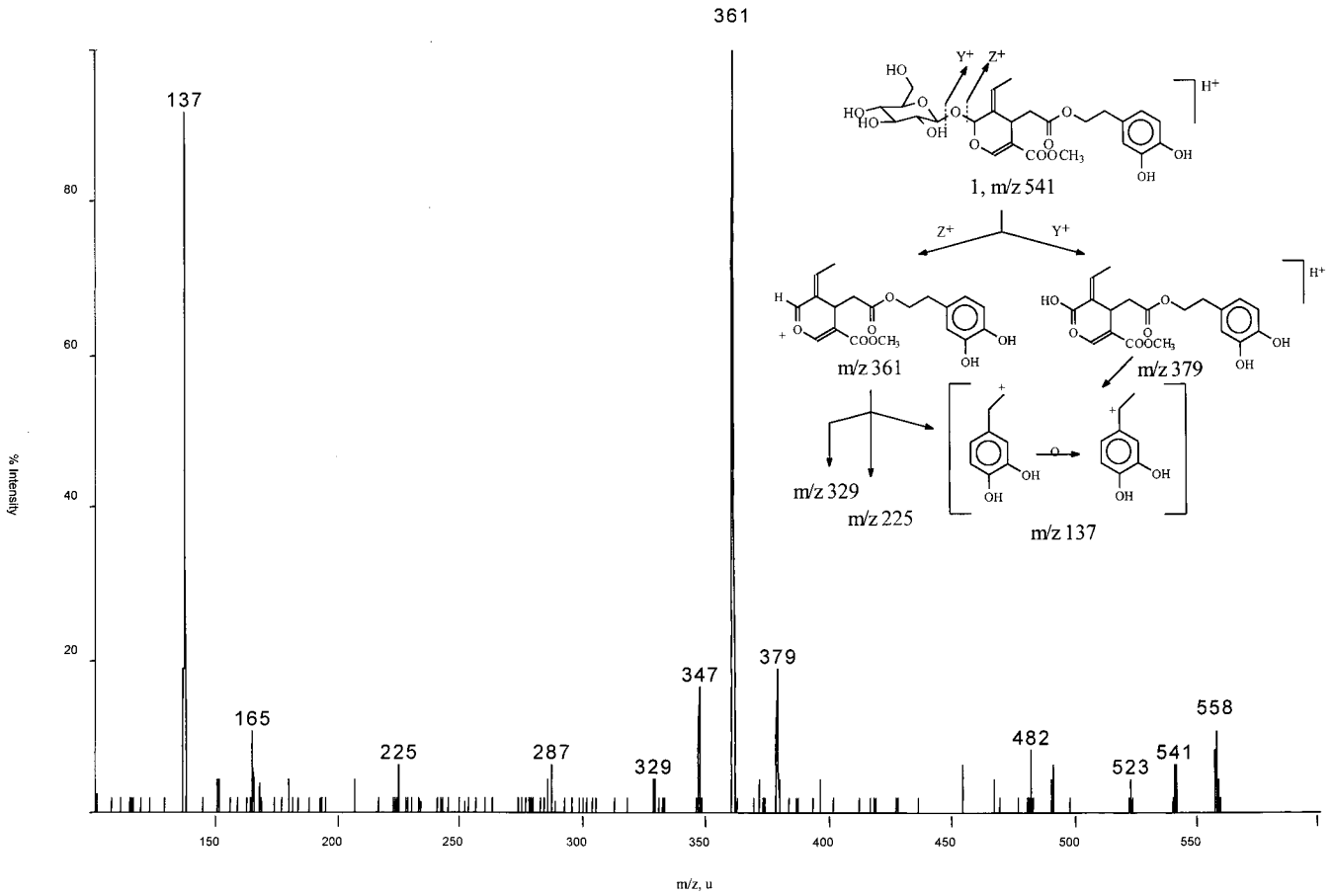

Figure 1. ISI-MS/MS spectrum of oleuropein **1** and the structure of the main fragment ions.

In 1996, the olive drupes were collected from the same olive trees of Carolea and Cassanese cultivars growing at experimental field of Istituto Sperimentale per l'Olivicoltura (I.S.Ol.). In 1997, the olive drupe samples came from different cultivars growing in different geographical areas of Calabria, Italy.

Workup of Plant Material. Olive Oils from the 1996 Crop. The oil was obtained from olive drupes (5 kg) crushed with a hammer mill after hydraulic pressing and centrifugation and 20 min of kneading at room temperature.

Olive Oils from the 1997 Crop. Olive drupes (5 kg) were crushed with a hammer mill, and the oil was obtained by continuous centrifugation process after 15 min of kneading at room temperature.

The olive oil samples from commercial sources were produced by the two-phase continuous centrifugation system.

The phenolic extract of virgin olive oil was obtained as follows: 10 g of virgin olive oil added to 25 mL of methanol was mixed with an Ultraturrax at 3500 rpm for 2 min (×3). The combined methanolic extract was concentrated in a vacuum under a stream of nitrogen at <35 °C until it reached a syrupy consistency and partitioned in acetonitrile/hexane 4/6 v/v (50 mL). Solvent evaporation to dryness afforded a yellowish foam that was dissolved in 2 mL of acetonitrile.

RESULTS AND DISCUSSION

The detection and quantitation of oleuropein in virgin olive oils aims at evaluating the presence of those micro components that could be considered as markers of quality and origin.

We have recently demonstrated that the presence of this secoiridoid in crude extracts of olive leaves (De Nino et al., 1997) and olive oils (Perri et al., 1996) can be ascertained by ionspray mass spectrometry (Bruins et al., 1987; Huang et al., 1990). The latter is used to nebulize and ionize solutions containing the analyte of interest in the atmospheric pressure source of the instrument, where the ionization of the analytes is driven by the field at the needle tip that disperses the energy-charged droplets (Mann et al., 1992). The method has permitted also the identification of an unknown compound present in trace amounts in the crude extract of *Carolea* cv leaves (De Nino et al., 1999). The evaluation of the structure of a component in a complex matrix can be conveniently done by means of the classic tools of mass spectrometry/mass spectrometry (MS/MS) methodology in tandem instruments (McLafferty, 1980; Busch et al., 1988).

An acetonitrile solution of standard oleuropein ionized as previously described produces mainly the species $[M + NH_4]^+$ at *m/z* 558. The latter, transmitted by the first mass filter into the second quadrupole of a triple quadrupole instrument and allowed to react there with an inert gas, produces the MS/MS spectrum of Figure 1, through the scanning of the last mass analyzer.

A detailed study of the mechanism of formation of the main daughter ions at *m/z* 137 and 361 has shown (De Nino et al., 1997) that they are due to a series of consecutive and competitive unimolecular fragmentations of the initially formed ammoniated species. These two fragments are eligible as reference peaks for a quantitative determination of oleuropein in olive oil.

The spectrum reported in Figure 1 was obtained by continuous scanning of the third quadrupole of the instrument, by keeping fixed the first one for the transmission of the parent ion only. In a typical mass spectrometric analytical application known as selected reaction monitoring (SRM), it is possible to tune the last

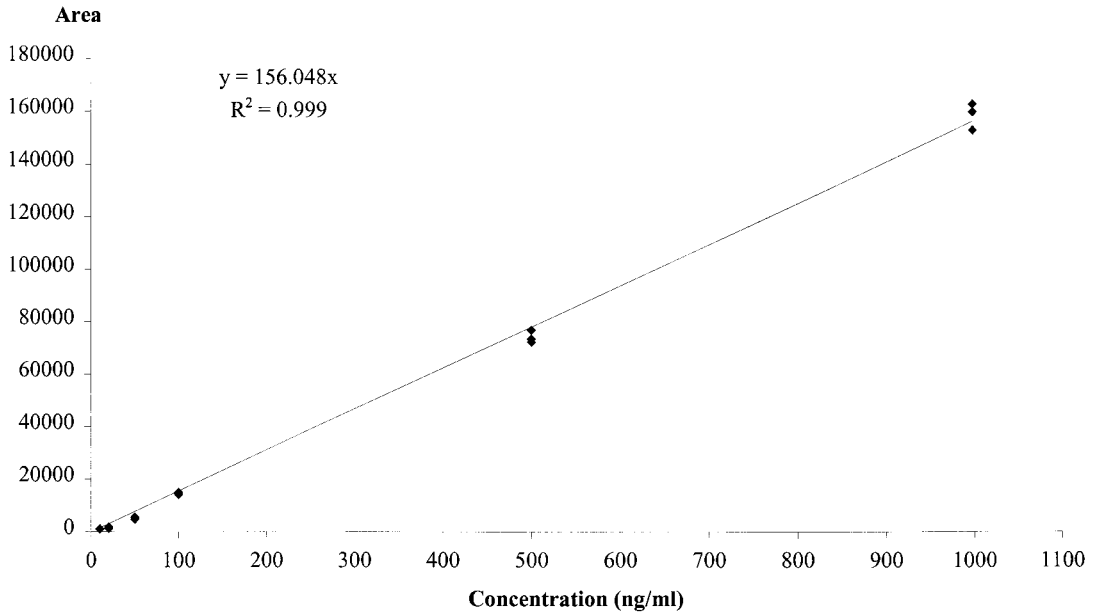

Figure 2. Standard calibration curve generated from triplicate injections of 10 μL of oleuropein using the ionspray ionization−SRM method. Regression coefficient at the 95% confidence interval was 0.999.

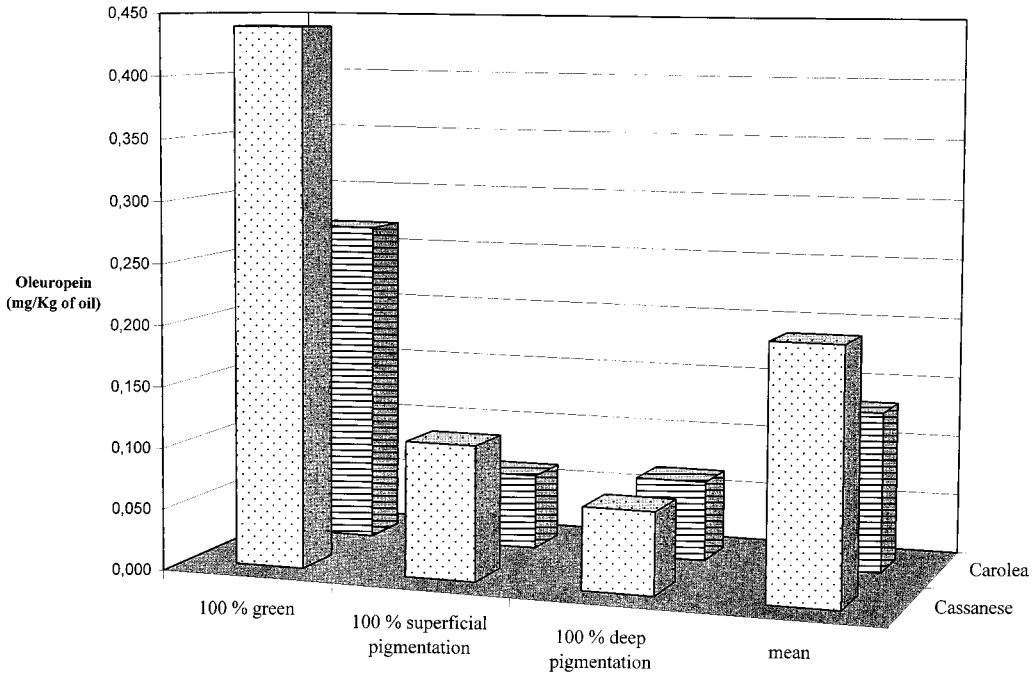

Figure 3. Ripening-phase-dependent oleuropein content in virgin olive oils of Cassanese and Carolea cultivars. Oleuropein assay in olive oil.

analyzer for the transmission of a single daughter ion. A computer-assisted scanning of the same analyzer can concurrently monitor multiple transitions. Under these conditions, the quantitative assay of a given analyte does not rely on the relative intensity of a peak present in the mass spectrum but depends on specific transitions linking precursor and daughter ions without any ambiguity. In the case of oleuropein, the transitions 558 → 361 and 558 → 137 were chosen as representative of the molecule. This is a very sensitive and selective means of determining oleuropein contents in solution as well as in a natural mixture. As little as 0.18 pg/μL of oleuropein can be detected. A standard calibration curve (Figure 2) with a linear coefficient of 0.999, generated from triplicate 10 μL injections of oleuropein solutions at different concentrations, shows a linear range from 10 ng/mL to 1 μg/mL, with an exceptional reproducibility.

A first assay of the oleuropein content, if any, in virgin olive oils was conducted on samples of Cassanese and Carolea cultivars. The oil was produced from drupes collected in 1996 from the same olive trees in the orchard of I.S.Ol. at different harvest times.

The first important result achieved was the unambiguous detection of oleuropein, which was identified by matching its MS/MS spectrum with that obtained from a standard sample. Moreover, the content of **1** was also determined by applying the SRM method. From the histogram reported in Figure 3, it appears that the content of **1** varies with the aging of the drupes even though a quantitative correlation between the ripening phase and the bioavailability of **1** cannot be drawn at

Table 1. Recovery Test of Oleuropein (1) (The Artificial Samples Were Obtained by Spiking a Commercial Seed Oil with Known Amounts of 1)

samples (μg/kg)	calcd concn	standard deviation	recovery (%)
100	17.613	1.346	17.613
200	21.960	6.052	10.980
1000	100.750	3.102	10.075

the present stage of knowledge. The maximum amount of oleuropein detected in Cassanese and Carolea cultivars was 440 and 268 μg/kg, respectively, while the mean content of oleuropein in Cassanese and Carolea cultivars was 206 and 132 μg/kg, respectively.

These values, however, do not necessarily represent the real amount of the searched analyte in the given samples. A reliable recovery test was therefore conducted on artificial samples where the analyte was in a chemical environment similar to olive oil in terms of solubility and partition coefficient with the solvent used for its extraction. Stock solutions of commercial seed oil, spiked with different amounts of oleuropein ranging from 100 to 1000 μg/kg, were therefore prepared. The results obtained by applying the SRM method showed that no more than 20% of the original content was really evaluated (Table 1). It should be pointed out, however, that the acetonitrile solutions of the same analyte, tested for the calibration curve, gave a nearly quantitative recover of material.

It can be suggested, therefore, that the different behavior is due to differences in the physical properties of the hydrophilic analyte in a lipophilic matrix that does not allow a complete partitioning of oleuropein during the extraction procedure. Nevertheless, two main goals have been fulfilled: (i) the unequivocal determination of oleuropein in virgin olive oils by MS/MS and (ii) its quantitative assay by SRM, which is reproducible in a broad range of concentrations, even though it is likely the real amount of antioxidant present could be underestimated.

The method was extended to a number of virgin olive oil samples coming from different geographical areas of Calabria produced either in the laboratory of the I.S.Ol. or obtained from different commercial sources (Table 2).

Oleuropein is always present, even in the 1-year-aged samples stored at 5−10 °C, and its content ranges up to the interesting value of 11.23 mg/kg, which, as previously mentioned, could be underestimated. In general, from the examination of data given in Table 2, it is difficult to correlate the oleuropein content of olive oil samples to the genotypes. However, the mean content of oleuropein in Cassanese cultivar, obtained from the data reported in Table 2 excluding the outlying values, is significantly different at the 1% level (LSD test, Table 3.

Therefore, a more rigorous study of the factors affecting its content in olive oils by the knowledge of agronomic, technological, and enzymatic variables is in progress.

CONCLUSIONS

It has been unambiguously demonstrated that oleuropein is present in virgin olive oils and that its concentration could be related likely to the different types of cultivars. Ionspray ionization in connection with SRM methodologies provides a highly reproducible and fast method for the quantitative assay of this secoiridoid in olive oils.

Table 2. Oleuropein Content in Experimental and Commercial Virgin Olive Oil Samples

source	cultivar	harvest date	oleuropein (μg/kg of oil)
I.S.Ol.[a]	Carolea	9/22/97	3.82 + 0.25
I.S.Ol.	Carolea	9/24/97	1.52 + 0.23
I.S.Ol.	Carolea	9/24/97	4.77 + 0.14
I.S.Ol.	Carolea	10/01/97	2.03 + 0.12
I.S.Ol.	Carolea	10/01/97	44.41 + 0.37
I.S.Ol.	Carolea	10/06/97	5652.29 + 234.05
I.S.Ol.	Carolea	10/08/97	2.57 + 0.37
I.S.Ol.	Carolea	10/13/97	11.88 + 1.47
I.S.Ol.	Cassanese	9/29/97	44.00 + 1.18
I.S.Ol.	Cassanese	10/06/97	174.73 + 4.65
I.S.Ol.	Cassanese	10/10/97	37.70 + 1.03
I.S.Ol.	Cassanese	10/20/97	3151.92 + 114.57
I.S.Ol.	Coratina	11/06/97	12.74 + 1.38
I.S.Ol.	Coratina	11/06/97	7.23 + 0.43
I.S.Ol.	Coratina	11/06/97	3.58 + 0.11
I.S.Ol.	Dolce di Rossano	9/30/97	8.44 + 0.64
I.S.Ol.	Dolce di Rossano	9/30/97	29.61 + 0.38
I.S.Ol.	Dolce di Rossano	10/13/97	4.03 + 0.95
I.S.Ol.	Tondina	9/29/97	11.3 + 1.07
I.S.Ol.	Tondina	9/30/97	7.61 + 0.51
I.S.Ol.	Tondina	10/12/97	1.27 + 0.30
I.S.Ol.	Tondina	10/27/97	11226.33 + 1243.67
I.S.Ol.	Tonda di Strongoli	9/15/97	289.08 + 5.73
I.S.Ol.	Tonda di Strongoli	9/26/97	15.08 + 0.67
commercial	Carolea + Dolce di Rossanob	11/10/96	136.46 + 2.59
	Carolea + Dolce di Rossanob	11/10/96	1.88 + 0.15
	Carolea	12/01/96	3.09 + 0.30
	Dolce di Rossano	12/10/96	5.2 + 1.02

[a] I.S.O., Istituto Sperimentale per l'Olivicoltura. [b] Carolea + Dolce di Rossano, mixture of olive oils from two cultivars.

Table 3. Statistical Analysis of Variance Applied to the Data Reported in Table 2 (The Outlying Data and Those of Tonda Di Strongoli Have Not Been Considered)

type of cultivar	mean content[a] of oleuropein (μg/kg oil)
Carolea	10.14A
Cassanese	85.33B
Coratina	7.85A
Dolce di Rossano	14.03A
Roggianella	6.73A
Commercial oils	3.99A

[a] Means in column followed by the same letter are not significantly different at $p \leq 0.01$.

The proposed method could be employed for quality assessment of virgin olive oil.

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