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LC-ESI-MS/MS IDENTIFICATION OF OLEUROPEIN AS MARKER OF *OLEA EUROPAEA* L. LEAVES USED AS A BULKING AGENT IN GROUND OREGANO AND SAGE

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ABSTRACT

The identification of the secoiridoid oleuropein by LC-ESI-MS/MS (negative) is demonstrated for the quality control directed toward confirmation of the presence of *Olea europaea* L. leaves used as bulking agent in ground oregano and sage. The described operating conditions are useful for the simple qualitative identification of this marker of *O. europaea* L. leaves. No tolerance limits are defined for the presence of the above mentioned extraneous vegetable material in ground oregano and sage, the herbs which have been identified in various cases as adulterated with *O. europaea* L. leaves. Despite the importance of quality control of aromatic herbs, only a limited number of papers have been published on the identification of phenolic compounds as markers of adulteration. The proposed method is simple and suitable for rapid routine analysis.

- Key words: aromatic herbs, adulteration, olive leaves, quality control, oleuropein, LC-ESI-MS/MS (negative) -

INTRODUCTION

While ground spices and herbs are often adulterated by the addition of bulking agents, few methods are available for screening commercial batches of some ground herbs such as oregano and sage for the presence of *O. europaea* L. leaves. In a recent paper we addressed this issue by the use of a stereo zoom microscope system to visualize the presence of the ground olive leaves (BONONI *et al.*, 2010a). However, an instrumental analytical method to obtain a specific and documentable confirmation would be useful. More recently we proposed a rapid GC/MS test based on the detection of two specific markers derived from the biophenol fraction of ground olive leaves to confirm the adulteration of ground oregano with this bulking agent (BONONI *et al.*, 2010b). In the present study, we have extended our earlier investigations adopting the phenolic compound oleuropein, a secoiridoid, as the more characteristic marker of *O. europaea* L. leaves and we have developed a method to confirm the presence of milled olive leaves in mixtures of ground oregano or sage. The secoiridoids, which have an oleosidic structure, are a specific group of coumarin-like compounds present in moderate amounts in *Oleaceae* and oleuropein in particular is an ester of 2-(3,4-dihydroxyphenyl) ethanol (known as hydroxytyrosol) and the elenolic acid glucoside (Fig. 1). In the present study, our aim is not to describe analytical parameters useful for quantitation of oleuropein, but rather to suggest and demonstrate the applicability of the simple operating conditions for the identification of this marker in the routine quality control of two very common aromatic herbs.

Oleuropein is present in the leaves of the olive tree and has been found in all constituent parts of the fruit (peel, pulp and seed) and various methods have been developed for the analysis of this compound (ANGEROSA *et al.*, 1995; JAPÓN-LUIÁN *et al.*, 2006; JAPÓN-LUIÁN and LUQUE DE CASTRO, 2008; JEMAI *et al.*, 2009; KIRITSAKIS *et al.*, 2010; LAGUERRE *et al.*, 2009; MARSILIO *et al.*, 2001; PAPOTI and TSIMIDOU, 2009; SAITTA *et al.*, 2002). The phenolic composition differs amongst different olive varieties, but oleuropein and ligstroside are the most common compounds present in the *O. europaea* L. cultivars and their concentrations are dependent on the season (BOUAZIZ and SAYADI, 2005; JEMAI *et al.*, 2009; KIRITSAKIS *et al.*, 2010; RANALLI *et al.*, 2006; RANALLI *et al.*, 2009; SOLER-RIVAS, 2000; TUCK and HAYBALL, 2002). Because of season variations in the content of these compounds and because of differences in drying conditions, their quantitative detection results would be difficult and essentially useless.

Herein we show the simple identification of oleuropein by LC-ESI-MS/MS in negative ion in samples of ground oregano leaves (or in ground sage leaves) containing ground olive leaves as a contaminant. We also demonstrate the fragmentation of the parent ion of oleuropein and describe the derived ions.

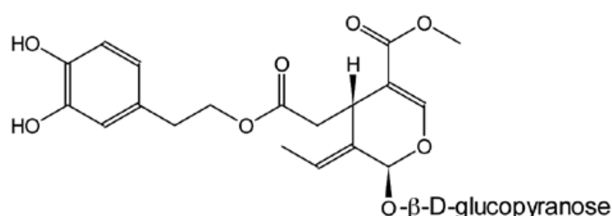

Fig. 1 - Structure of oleuropein (M.W. = 540 Da; CAS 32619-42-4), the main oleoside in Oleaceae.

MATERIALS AND METHODS

Reagents

Ethyl alcohol (99%) was purchased from Sigma-Aldrich (Milan, Italy). Filters GH Polypro (Hydrophilic Polypropylene Membrane Filter) 0.45 μm Pall were purchased from VWR (Milan, Italy). Acetonitrile, formic acid and water, all of chromatographic grade, were purchased from Merck (Darmstadt, Germany).

Samples

Authentic samples of oregano and sage were prepared from fresh plant material by drying at 40°C for ten days and crushing, following established industrial technologies. The samples were ground to a particle size diameter of 1.0 - 5.0 mm as usually found in the retail market. Other genuine samples, derived from other industrial drying procedures, have been also analyzed.

Dried leaves of *Olea europaea* L. of different origins, treated as previously described or collected in various regions of Italy and dried under both controlled and non-controlled conditions, were also studied. These leaves were ground to similar dimensions as for oregano.

Preparation of extracts

The vegetative samples (oregano or sage) subjected to the quality control analyses were treated as follows: ~ 1 g of each herb was mixed with 5 mL of ethyl alcohol at 20°-25°C. After magnetic stirring for 6 hours, the extract was centrifuged at 500 rpm for 3 min and the supernatant was filtered through a Pall 0.45 μm filter. For the LC-ESI-MS/MS (negative) analysis, an aliquot (20 μL) of the supernatant was injected in the HPLC system.

The efficiency of the extraction using ethanol/water (80/20) solutions was also evaluated, but the extraction results for oleuropein were less selective than those with pure ethanol.

HPLC/MS Equipment

The analytes were separated using a Shimadzu integrated HPLC system (Shimadzu, Milan, Italy) which consisted of a Shimadzu CBM 20 A system controller, two Shimadzu LC 20 AD XR pumps including a Shimadzu degasser DGU A3, a Shimadzu SIL 20 ACXR autosampler and a Shimadzu CTO 20 AC column oven. The analytes were detected using a triple quadrupole Applied Biosystems 2000 Mass Spectrometer with Analyst Software (Version 1.4.1) equipped with a Turbo Ion Spray Source operated in the ESI (negative ion) mode.

Chromatographic conditions

A stainless steel column Supelcosil™ 150 mm x 3 mm with 3 μm particle size and 120 Å pore diameter was used. Compounds were separated using as solvent A a mixture of water/formic acid (99.9:0.1 v/v) and as solvent B a mixture of acetonitrile/formic acid (99.9:0.1 v/v) at a flow rate of 0.4 mL/min. The elution was performed with a linear gradient from 6 to 20% B in 20 min, than isocratic 20% B for 20 min and finally returning to the initial conditions in 10 min.

MS analysis

To establish the appropriate conditions for the identification of oleuropein, an olive leaves ethanolic extract was produced. The extract was previously injected in the source of the mass spectrometer by continuous infusion at 10 μL/min.

The data acquisition was performed in the negative ion mode electrospray ionization (ESI-MS) and was achieved in Q1 mode. The same methodology was applied after fragmentation of the precursor ion (MS^2 mode). Analysis conditions to obtain the m/z 539.1 to 377.1 transition were fixed as follows: ionization voltage 4.5 kV, probe temperature 350°C, declustering potential (DP; -30 V), focusing potential (FP; -400 V), entrance potential (EP; -10V), cell entrance potential (CEP; 5 V), collision energy (CE; 16 V) and cell exit potential (CXP; 5 V). The m/z 377 ion, derived from the fragmentation of the pseudomolecular $[M-H]^-$ ion, corresponds to the oleuropein aglycone resulting from the loss of the glucose moiety.

RESULTS AND DISCUSSION

Using the analytical conditions described in Materials and Methods, various samples of oregano and sage from different origins were examined and the presence of oleuropein was not evident. On the other hand, various samples of olive leaves from Apulia and Tuscany regions of Italy were examined and in all cases oleuropein was identified as the most abundant compound.

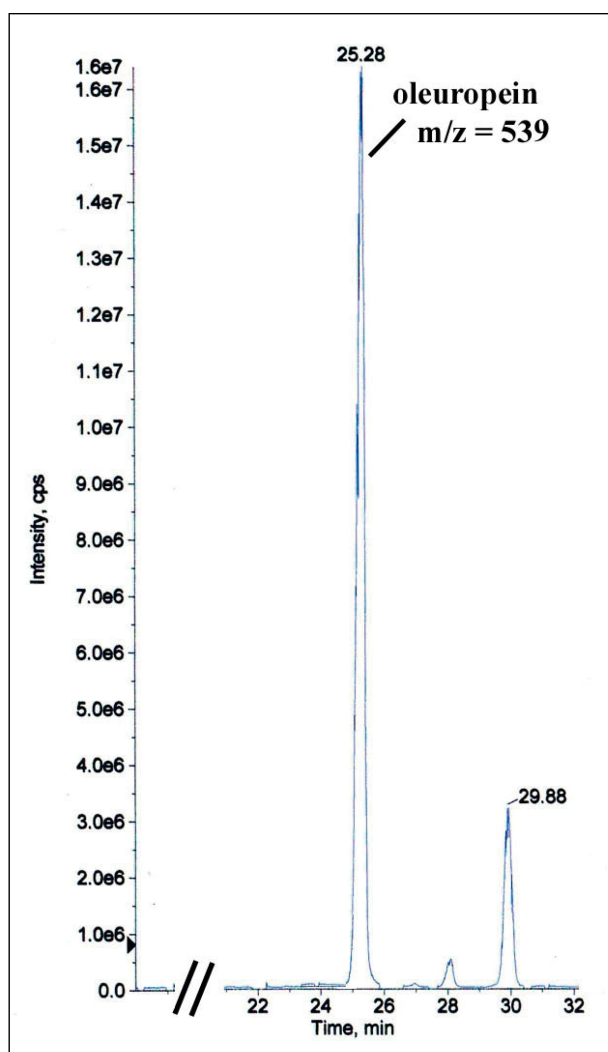

Fig. 2 - LC-ESI-MS/MS in negative ion mode chromatogram obtained from a sample of dried olive leaves ethanolic extract. The Q1 profile appearing at r.t. 25.3 min represents the precursor ion of oleuropein with m/z 539, corresponding to the pseudomolecular [M-H]- ion. The peak at r.t. 29.9 min is the isomer oleuroside, having the same molecular mass, 540 Da.

The Q1 chromatogram produced from the olive leaves ethanolic extract (Fig. 2) shows the profiles of the precursor ion (m/z = 539), which corresponds to the pseudomolecular $[M-H]^-$ ion of both oleuropein (major) and its isomer oleuroside (minor), whose molecular masses are 540 Da.

A comparison of the chromatograms of a sample of pure oregano (A) and a sample of oregano contaminated with *O. europaea* L. dried leaves (B) (Fig. 3) clearly shows that the Q1 chromatogram produced from the oregano ethanolic extract (Fig. 3A) did not contain the ion m/z = 539. The two peaks produced from oregano at r.t. < 25.0 min have m/z = 537 and their evidence is due only to the acquisition range (m/z = 539

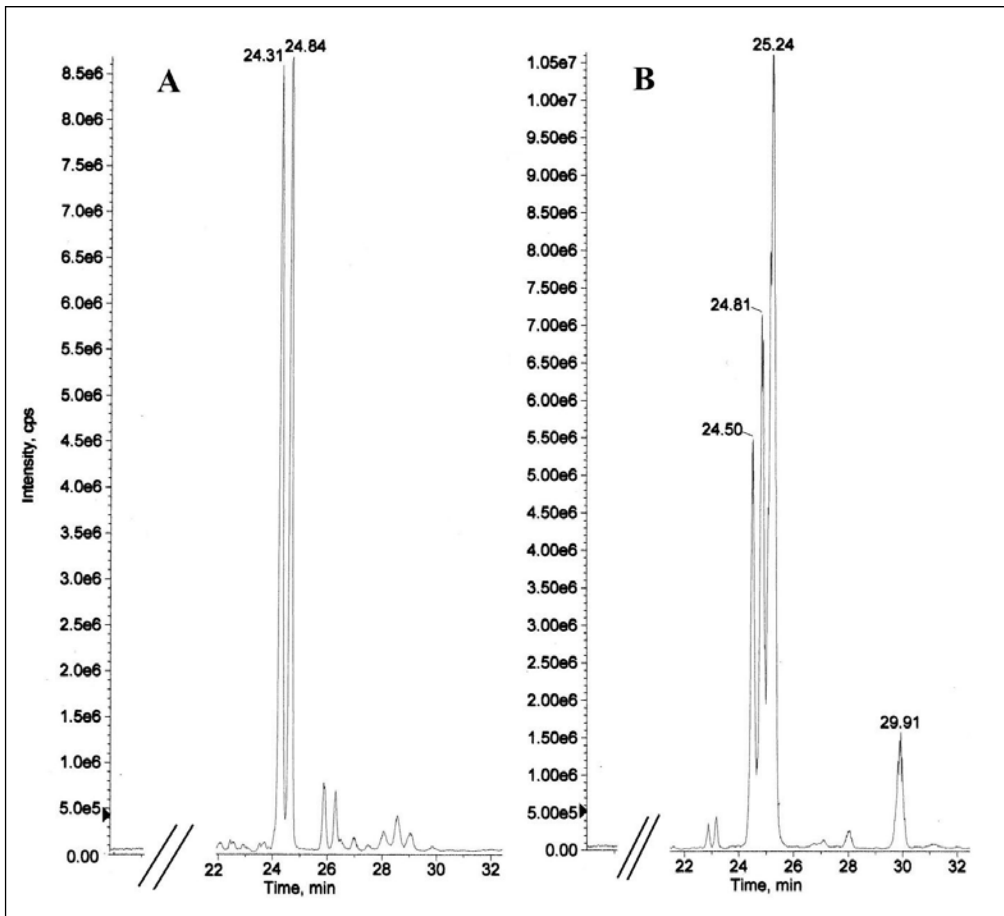

Fig. 3 - LC-ESI-MS/MS in negative ion mode chromatogram obtained from a sample of pure oregano (A) and a sample of oregano contaminated with ~ 20% of *Olea europaea* L. dried leaves (B). The peak at 25.2 min in (B) corresponds to the pseudomolecular [M-H]-ion of oleuropein (m/z = 539).

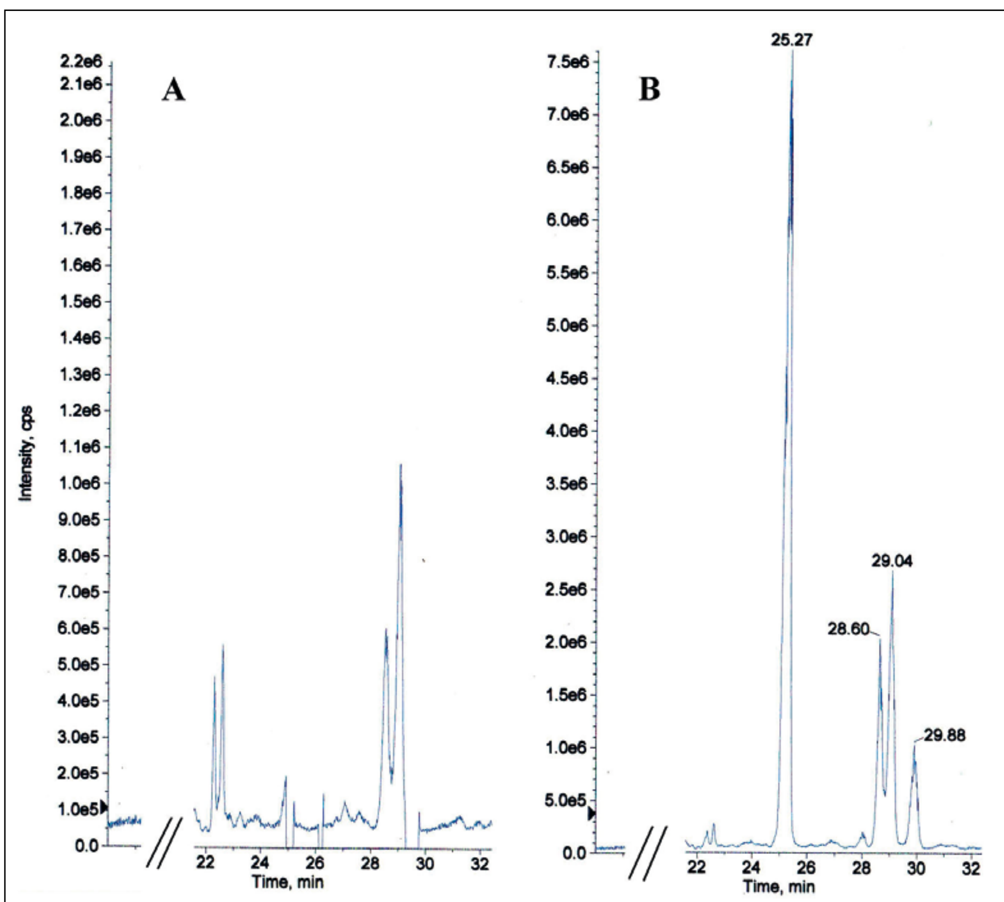

Fig. 4 - LC-ESI-MS/MS in negative ion mode chromatogram obtained from a sample of pure sage (A) and a sample of sage contaminated with ~ 10% of *Olea europaea* L. dried leaves (B). The peak at 25.3 min in (B) corresponds to the pseudomolecular [M-H]-ion of oleuropein (m/z = 539).

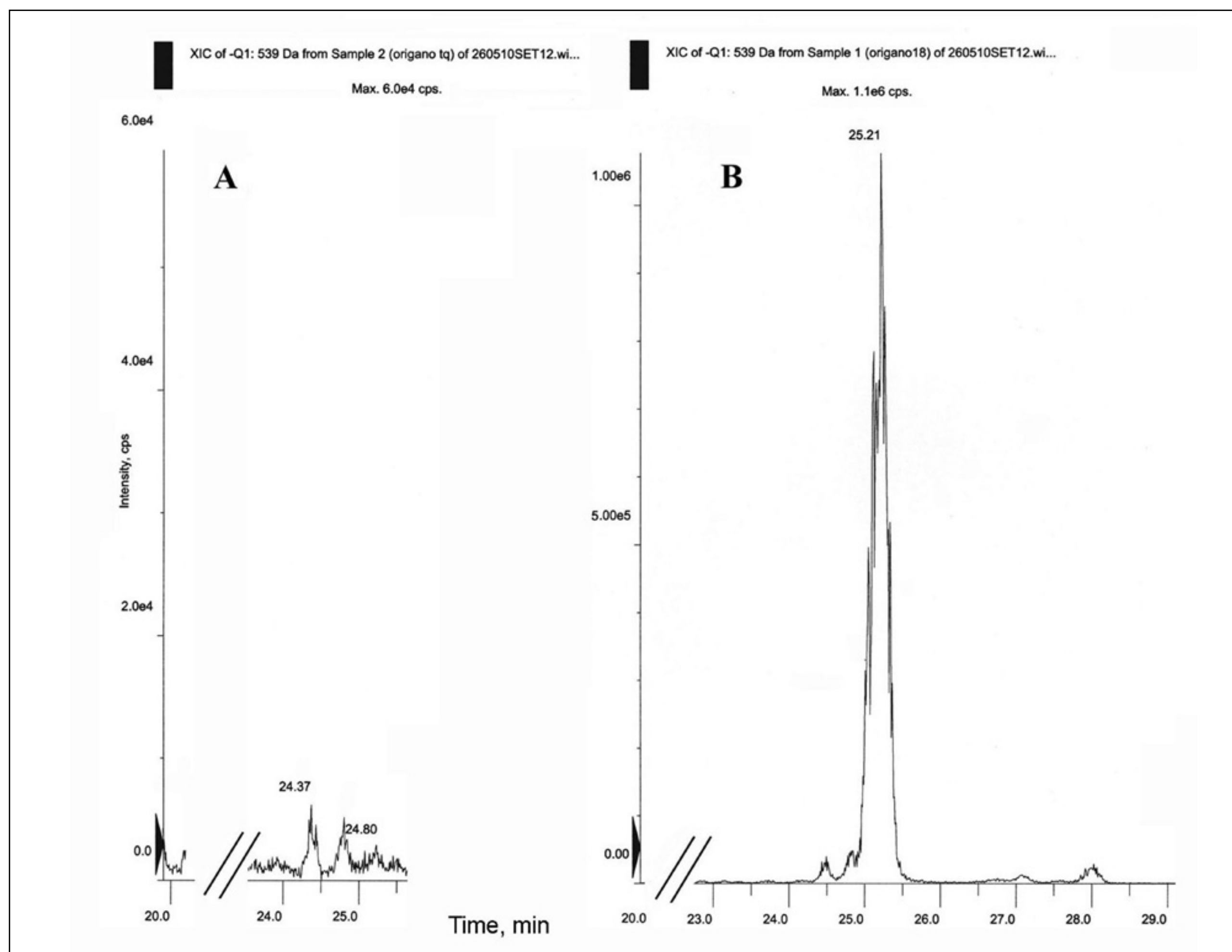

Fig. 5 - Extracted ion m/z = 539 detectable on LC-ESI-MS/MS in negative ion mode chromatogram obtained from a sample of pure oregano (A) and a sample of oregano contaminated with ~ 20% of *Olea europaea* L. dried leaves (B). The intensity values readable for pure oregano has not an analytical sense in the neighborhood of r.t. 25 min.

± 3). On the other hand, an extract of oregano containing ~ 20% of olive leaves shows a strong peak at r.t. 25.2 min having m/z = 539, i.e. the $[M-H]^-$ ion of oleuropein.

Fig. 4 compares a sample of pure sage (A) and a sample of sage contaminated with ~ 10% of olive leaves (B). The extract of contaminated sage shows, as in the case of contaminated oregano, the $[M-H]^-$ ion of oleuropein at r.t. 25.3 min.

For a better evidence Fig. 5 and 6 are reported which show, for the same samples of oregano and sage before considered, the extracted m/z = 539 ion chromatogram. The clear trace of m/z = 539 ion identified in the contaminated oregano and sage did not detectable at corresponding r.t. in the chromatograms of pure oregano and sage.

Operating in MS^2 mode the precursor ion at m/z 539 displayed the characteristic product ions at m/z 377, 307 and 275, as has been confirmed by others Authors (BAZOTI *et al.*, 2010; DEL BOCCIO *et al.*, 2003).

CONCLUSIONS

Even though the amounts of oleuropein in the collected olive leaves may vary to the age of the samples and due to genetic factors, this molecule will always be present in sufficient quantity to be identified in all dried samples derived from various origins and during various collecting periods. Therefore this secoiridoid phenolic compound may serve as a "marker" of the presence of ground and dried olive leaves in some herbs used in retail and industrial markets. In this paper, two examples (for ground oregano and sage) of the application of this LC-MS/MS method are shown for quality control. Considering that the olive leaves cannot be considered as a common extraneous vegetable material tolerable in aromatic herbs, no limits of their presence have been defined at the present time. Consequently, there is no finite quantity of olive leaves that can be tolerable in ground oregano or sage, which are the most common aromatic herbs used for food preparation. The quantita-

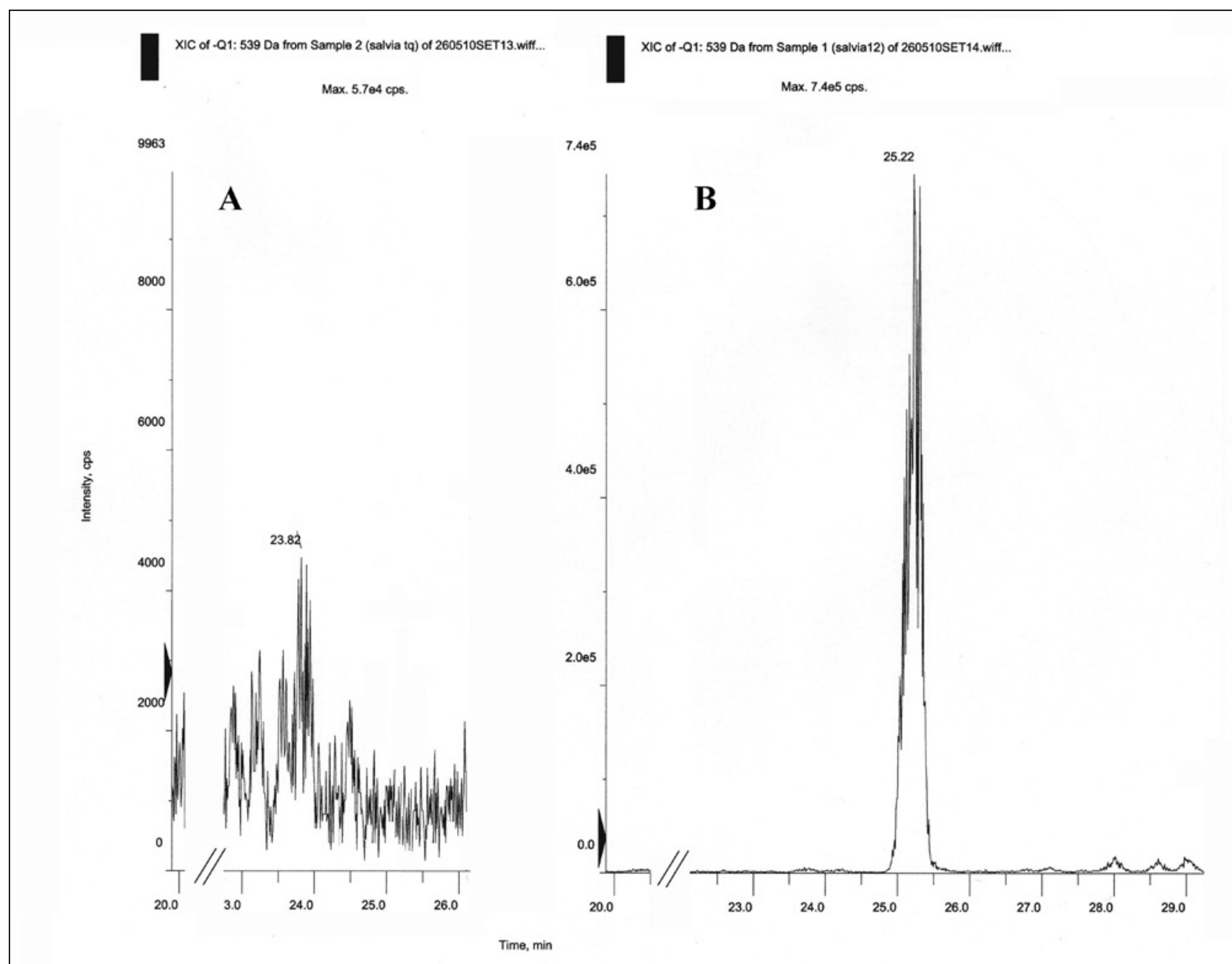

Fig. 6 - Extracted ion m/z = 539 detectable on LC-ESI-MS/MS in negative ion mode chromatogram obtained from a sample of pure sage (A) and a sample of sage contaminated with ~ 10% of *Olea europaea* L. dried leaves (B). The intensity values readable for pure sage has not an analytical sense in the neighborhood of r.t. 25 min.

tive evaluation of the presence of olive leaves, on the other hand, is not useful and is practically impossible, due to the highly variable content of oleuropein in dried olive ground leaves. The criterion for olive leaves identification presented in this paper is evidently useful for qualitative measurements, as specific confirmation of the adulteration evaluated by stereo zoom microscopy.

Therefore the suggested method has for the first time permitted a real identification of olive leaves for routine quality control and it has been adopted in the Analytical Research Laboratories (Di.Pro.Ve. - University of Milan, Italy).

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