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Oleuropein expression in olive oils produced from drupes stoned in a spring pitting apparatus (SPIA)

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Abstract

A new device for the preparation of stoned olive oils, called SPIA, has been developed, aiming at fulfilling the targets of: (i) employing a less powered engine, (ii) reducing the size of the machine and (iii) reaching a good efficiency in terms of oil yields. Oleuropein expression was used as a biochemical parameter to distinguish stoned oils from oils produced by conventional milling systems. *In vitro* experiments performed by exposing oleuropein to pit enzymes, showed an exponential decay of the substrate.

Keywords: Oleuropein; Stoned olive oil; Spring pitting apparatus; Tandem mass spectrometry; Isotope dilution

1. Introduction

The widespread use of olive oil as a foodstuff of high nutritional value sharing many beneficial effects (Evangelista, Antunes, Francescato, & Bianchi, 2004; Kiritsakis, Brintaki, & Spentzouris, 2007; Manna et al., 2004; Markin, Duek, & Berdicevsky, 2003; Somova, Shode, Ramnanan, & Nadar, 2003) has favoured the development of both basic and technological research (Servili et al., 2004; Soler-Rivas, Espin, & Wirkers, 2000) aiming at implementing the quality of the aliment and the methodologies for assaying, at the molecular level, the content of the important nutraceuticals, such as oleuropein, present there (De Nino et al., 2005; De Nino et al., 1999; Perri, Raffaelli, & Sindona, 1999). A technological improvement in the industrial production of olive oil seems to be represented by the manufacturing procedure which considers the use of stoned olives (Frega, Caglioti, & Mozzon, 1999). A part from the observations on the tiny differences in the yield of oil when conventional and stoning procedures are considered, it seems that a general agreement on the quality improvement when stoneless drupes are processed has not yet been reached. One recent report claims, in fact, that "no obvious influence of stoning on oil quality" can be evaluated (Patumi, Terenziani, Ridolfi, & Fontanazza, 2003).

Some negligible differences were noted by other authors (Lavelli & Bondesan, 2005), conversely, higher antioxidant capacity (Mulinacci et al., 2005) and higher amounts of volatile compounds (Amirante, Clodoveo, Dugo, Leone, & Tamborrino, 2006) were reported for stoned oils produced by means of a commercially available apparatus. In 2005, a project was launched by the southern Italian, Calabria region, aiming at developing new technologies in olive oil production based on the implementation of the stoning procedure in the framework of the European Union POR projects based on knowledge transfer from academic to pre-competitive research. A pilot plant was devised, in cooperation with MdB Company, to produce stoned olive oil to be compared with conventional one.

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This work aims at evaluating, by atmospheric-pressure-chemical-ionization tandem mass spectrometry (APCI-MS/MS), the relative amount of those biomarkers that could distinguish the differently produced oils at the molecular level

2. Materials and methods

2.1. The spring pitting apparatus (SPIA)

The stoned oil was prepared by means of the spring pitting apparatus (SPIA) described in other part of this work. A battery of seven sets of seven springs, mounted on a cylindrical holder, was placed on the machine shaft. The rotation per minute was ranging from 1000 to 3000 rpm in order to achieve good yields of olive pulp and avoiding stone breaking which could reduce the oil quality. The measured productivity was about 2000–2500 kg per hour utilizing a 5 kW power electrical engine, with a performance of about 18% in oil.

2.2. Chemicals

Solvents and reagents were commercially available (Sigma–Aldrich, St. Louis, MO). d_3-Oleuropein (**1B**) was synthesized in our laboratory (De Nino et al., 2005).

2.3. Oil samples

Twelve pairs of olive oils, obtained from whole fruit and stoned olives, were investigated. Each pair was obtained from the same batch of olives. The olive oil yield decreased by 20% from the whole fruit (6% in average) compared to the stoned olive oil. The oil was produced from the three different cultivars, Carolea, Cassanese and Dolce di Rossano, harvested in the period October 24–November 22, 2005, in three different geographic areas of Calabria, i.e., Cosenza (CS), Catanzaro (CZ) and Reggio di Calabria (RC).

2.4. Pit enzymes

The evaluation of the metabolic activity of water extracts of crushed pits was carried out by following, by APCI-MS/MS, the kinetic of oleuropein (**1A**) modification in that environment. **1A** (0.5 mg) and 20 mL of 0.1 M aqueous acetate buffer at pH 4.5 were added to 100 mg of crushed pits. The kinetic was monitored in the first 10 min of exposure of **1A** to the enzymes likely present in the extracts. At each programmed step, to 1 mL of the solution was added 2 mL of MeOH (to block the enzyme activity). The resulting solution (1 mL), after the addition of 2 mg/kg of the labeled oleuropein (**1B**) as internal standard, was submitted to mass spectrometric analysis. Similar conditions were used when oleuropein (**1A**) was completely digested and its metabolites were identified in the resulting solution by ESI-LC–MS in a *Fractionlynx* apparatus (see below).

2.5. Stone protein separation by gel electrophoresis

Method 1 (Murtaza, Kitaoka, & Muhammad Ali, 2005). The olive seeds from Carolea cultivar were ground with a mortar and pestle to produce fine powder in liquid nitrogen. The powder of seeds (0.6 g) was suspended in 6 mL of HPLC grade water. The suspension was stirred for 30 min in a shaker at room temperature and the suspension was centrifuged at 10,000 rpm (7400*g*) for 15 min. The supernatant was then filtered through No. 5A filter paper. The filtrate were precipitated with two volumes of cold acetone and re-suspended in gel loading buffer.

Method 2 (Wang, De-Dios-Alche, Castro, & Rodriguez-Garcia, 2001). Ten olive seeds were directly homogenized in a mortar cooled on ice using a buffer containing: 125 mM Tris–HCl, pH 6.8, 0.2% SDS, 1% 2-mercaptoethanol (denaturing, reducing conditions). After centrifugation at 10,000 rpm (7400*g*) for 5 min, the supernatant was boiled for 5 min and centrifuged again. Proteins in the supernatant were precipitated with two volumes of cold acetone and re-suspended in gel loading buffer.

2.6. Oil parameters

Acidity, peroxide value, spectroscopic indices K232 and K270 and fatty acid composition were determined according to the EU official method Commission Regulation, 1991; Commission Regulation, 2002, and to AOAC official methods (965.33, 940.28); total phenolic compounds was determined as reported in the literature (Gutiérrez, Albi, Palma, Rios, & Olías, 1989). Briefly, 1 g of olive oil is dissolved in 5 mL of hexane; the solution is loaded on a C_{18} cartridge (1 g × 6 mL) and washed twice with 5 mL of hexane. Phenols are eluted with 10 mL of MeOH. Final solution (1 mL) is submitted to the Folin Ciocolteau assay.

2.7. Tocopherols

Samples were analyzed using an Agilent 1100 (Waldbronn, Germany) HPLC equipped with fluorescence detector (FLD); the column was a Purospher STAR NH2 (5 μm) (VWR International, Milan, Italy). Peak integration and quantitative calculations were performed with the relative software; calibration curve was obtained by injecting standard solutions of tocopherol at different concentrations. The HPLC analyses were performed using a mobile phase composed of *n*-hexane and ethyl acetate (8:2). The flow rate was 1 mL min^{-1}; the injection volume was 20 μL of a solution obtained by diluting 600 mg of olive oil in 10 mL of *n*-hexane. The fluorescence detector was set as follows: $\lambda_{ex} = 295$ nm, $\lambda_{em} = 323$ nm. The time of analysis was 20 min.

2.8. Instrumentation

The assay of oleuropein (OLP) in stoned and whole fruit olive oils was carried out using an MDS Sciex API 2000 tri-

ple quadrupole mass spectrometer equipped with an APCI source (Applied Biosystem, Faster City, CA), interfaced with an 1100 HPLC system (Agilent Technologies, Waldbronn, Germany). All data were acquired and analyzed using Analyst software, version 1.4.1. Aqueous parts-per-million solutions of the analyte were delivered to the heated nebulizer by flow injection analysis (FIA); the flow rate was 400 μL min^{-1} of acetonitrile/5 mM ammonium acetate aqueous solutions (50:50, v:v). The experiments were performed at a source temperature (TEM) of 450 °C and at curtain gas (CUR) and source gas (GS1, GS2) pressures of 45, 70, and 50 psi, respectively, while the nebulizer current (NC), the declustering potential (DP); the focusing potential (FP) and the entrance potential (EP) were set to 4, 70, 250 and 5 V, respectively. The collision energy (CE) value was 40 eV and the collision gas pressure (CAD) was set to 2. The MRM experiments were performed using a dwell time of 250 ms. The spectra were acquired at unit resolution. The ESI-LC–MS experiment were carried out using a Fractionlynx system (Waters corporation, Milford, MA, USA) composed by a autosampler/collector Waters 2767 Sample Manager, a 600E pump working in analytical mode, a 486 UV detector and a ZMD mass spectrometer equipped with an ESI source. The separation was performed using a 250 × 4.6 mm 5 μm reversed phase C_{18} Luna (Phenomenex, Torrance, CA, USA) column at a flow rate of 1 mL min^{-1}. The run time was 60 min and the linear gradient was performed using H_2O (solvent A) and MeOH (solvent B) as eluting phase. The MS spectra was acquired in negative mode with the following conditions: capillary voltage 3.1 kV, cone voltage 12 V, extractor 2 V, RF lens 0.3 V, source block and desolvation temperature 120 and 250 °C, respectively, ion energy 0.5 V, LM resolution 14.5, HM resolution 15.0 and multiplier 650 V, the nebulizer gas was set to 650 L/h.

3. Results and discussion

3.1. The spring pitting apparatus (SPIA)

The main drawbacks associated to the existent pitting machines are represented by the relevant electrical power needed to operate the apparatus and by their efficiency, which often reduces the yield of oil from about 20% to about 16% for a given mass of olives. Owing to the increasing interest in stoned olive oil and in the framework of the POR project mentioned above, we have developed the spring pitting apparatus (SPIA) (Patent pending), whose main characteristics are represented by a less powered engine and a better efficiency in terms of performance in oil (Fig. 1a).

The separation of the pulp is obtained by the action of spring whips which spin-around in the separator cylinder where the olives are introduced from the top of the machine. This cylinder is drilled regularly in order to ensure the simultaneous expulsion of olives paste and stones. Thus, the paste accumulates in the external cylindrical reservoir and stones are collected in different holders thanks to the action of a properly designed collector (Fig. 2a). Of course, the core of the machine is represented by the separation module, based on the action of the spring whips. The machine performance also depends on the typology, number and position of these elements. They

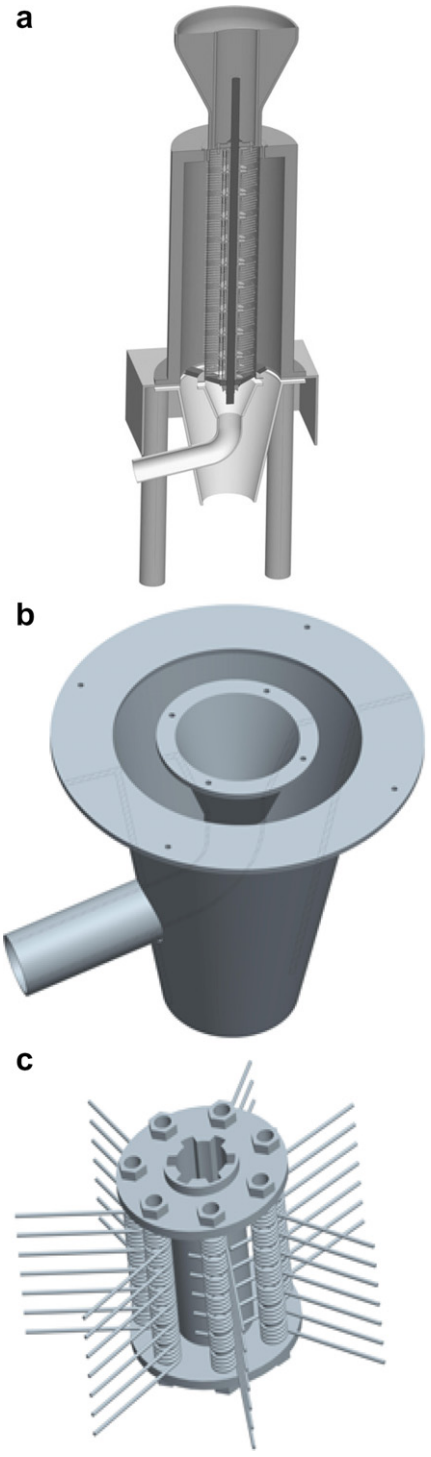

Fig. 1. Sketch of the machine (a). The paste and stones collector at the bottom of the machine (b). The spring whips holder (c).

have been mounted on a proper support as reported in Fig. 3A.

3.2. Macroscopic parameters of stoned and conventional oil

As previously mentioned (Amirante et al., 2006; Lavelli & Bondesan, 2005; Mulinacci et al., 2005; Patumi et al., 2003) most of the available evidence seems to indicate that the effect of stone removal before olive processing does not affect at a greater extent the quality of the oil. Table 1 reports the conventional parameters used to characterize the quality of olive oils, according to EU directives Commission Regulation, 1991; Commission Regulation, 2002.

Keeping all the parameters constant, the effect of stoning should be related, from a biochemical point of view, to the lack of activity of the endogenous enzymes present in the stones. To the best of our knowledge, a systematic study on the distribution and activity of endogenous olive stone enzymes has not yet been presented. It is, however, possible to rule out any particular effect of stone lipoxygenase (LOX) on the profiling of volatile components of stoned olive oil (Patumi et al., 2003). On the contrary, the action of both glucosidases and esterases, likely present in stones, might be more effective. The total phenol content should, in fact, be enhanced either when more lipophilic aglycones are formed, after the removal of the hydrophilic sugar moiety, and, likely, when oleocanthal (Beauchamp et al., 2005; De Nino et al., 2000; Montedoro et al., 1993) and its hydroxytyrosol homologue are obtained after deglycosylation and demethylation at position *11* of oleuropein. This effect, indirectly,

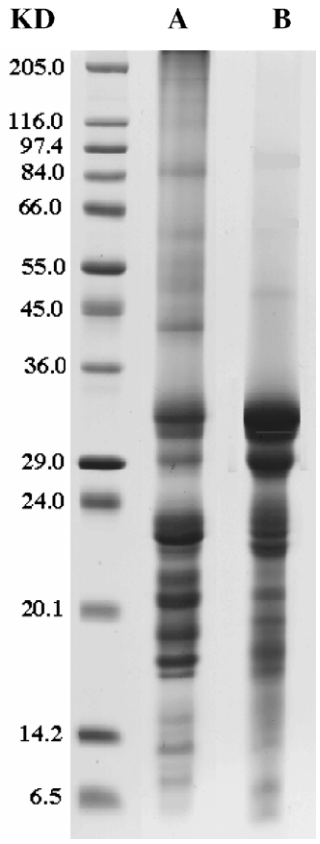

Fig. 3. SDS-PAGE separation of proteins from Carolea pits. Polyacrylamide gel (12.5%) was used and the spots were visualized with Coomassie blue. Protein extracted with method 1 (lane A) and 2 (lane B). Molecular weights (in kilodaltons) are shown on the left.

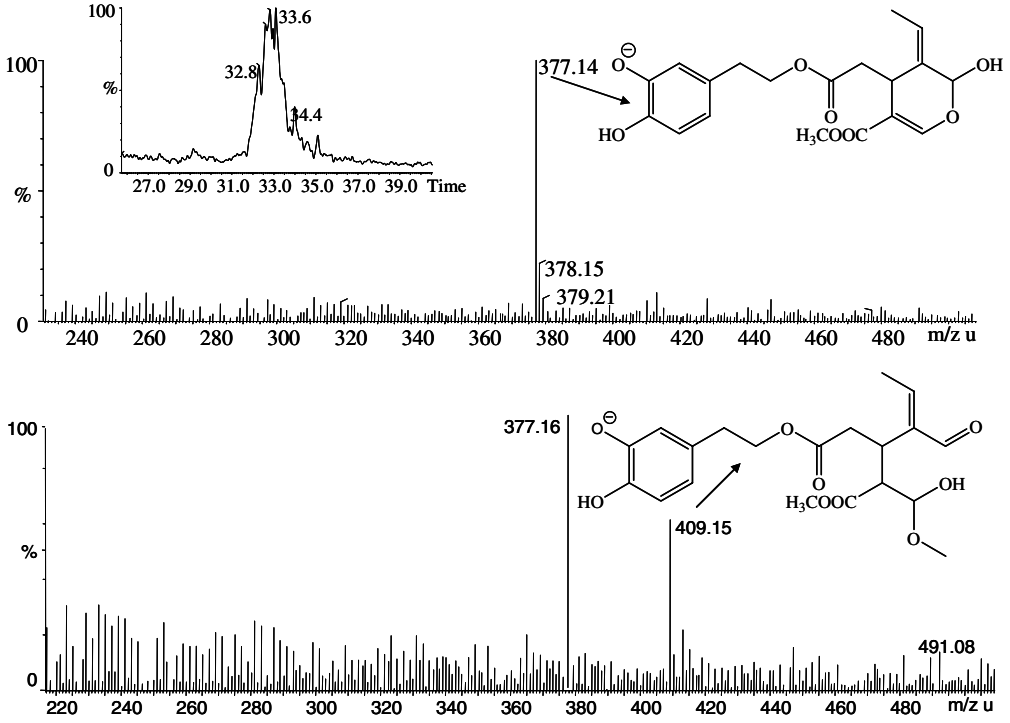

Fig. 2. ESI-MS spectra taken directly from the solution containing OLP (**1A**) and the water extracts of Carolea pits after 60 min of incubation.

Table 1
Conventional parameters to assess the quality of olive oils

Sample	Cultivar	District	Process	Acidity[a]	Peroxide value[b]	K232	K270	ΔK
1	Carolea	CZ	Conventional	0.20	4.80	1.56	0.11	−0.003
2	Carolea	CZ	Stoned	0.40	3.60	1.49	0.10	−0.002
3	Carolea	CS	Conventional	0.40	2.70	1.74	0.15	−0.003
4	Carolea	CS	Stoned	0.40	2.80	1.73	0.16	−0.002
5	Carolea	CS	Conventional	0.60	5.60	1.67	0.16	0.000
6	Carolea	CS	Stoned	0.60	3.60	1.40	0.11	−0.002
7	Carolea	CS	Conventional	0.40	5.00	1.63	0.13	−0.002
8	Carolea	CS	Stoned	0.40	4.80	1.48	0.15	−0.001
9	Carolea	RC	Conventional	0.40	6.40	1.42	0.12	−0.001
10	Carolea	RC	Stoned	0.40	4.40	0.93	0.05	−0.001
11	Carolea	CS	Conventional	0.70	11.60	1.61	0.10	0.000
12	Carolea	CS	Stoned	0.30	5.00	1.64	0.11	−0.002
13	Carolea	CS	Conventional	0.60	4.00	1.21	0.06	−0.001
14	Carolea	CS	Stoned	0.40	3.00	1.31	0.10	−0.001
15	Cassanese	CS	Conventional	0.40	4.40	1.77	0.16	−0.004
16	Cassanese	CS	Stoned	0.40	5.40	1.43	0.12	−0.001
17	Cassanese	CS	Conventional	0.40	7.20	1.49	0.11	−0.001
18	Cassanese	CS	Stoned	0.40	4.00	1.44	0.09	−0.001
19	Cassanese	CS	Conventional	0.40	6.60	1.51	0.12	−0.001
20	Cassanese	CS	Stoned	0.20	5.00	1.31	0.16	−0.002
21	Dolce di Rossano	CS	Conventional	0.60	5.00	1.48	0.15	−0.002
22	Dolce di Rossano	CS	Stoned	0.20	8.20	1.58	0.12	−0.001
23	Dolce di Rossano	CS	Conventional	0.60	8.40	1.80	0.15	−0.002
24	Dolce di Rossano	CS	Stoned	0.40	8.00	1.46	0.19	−0.001

[a] Expressed as % of oleic acid.
[b] Expressed as meq of O_2.

resembles the action of cell-wall-degrading enzymes added to the paste to improve the olive oil quality (Vierhuis, Servili, Baldioli, Schols, & Voragen, 2001). In agreement with recent findings (Amirante et al., 2006), differences in the total phenol content were observed between the oils produced with conventional and the SPIA procedure (Table 2), nevertheless, the detection of the volatile components, following a recently published method (Benincasa et al., 2003), did not show appreciable differences between the oils produced from the same type of drupes but with the two different methods described in Section 2.

It appears evident that the lack of consensus between the available data, including ours, on stoned olive oil is due to the unrefined parameters used to rationalize the analytical results. The archetype phenol compound of olive is represented by oleuropein (OLP, **1A**). We have demonstrated that the relative amounts of this secoiridoid in different tissues of olives can be easily determined by atmospheric-pressure-chemical-ionization tandem mass spectrometry (APCI-MS/MS) (De Nino et al., 2005). OLP can be considered a proper biomarker in the evaluation of the role likely played by stone enzymes. The elimination of the extra source of enzymes, during olive paste preparation should, in fact, enhance the content of **1A** in the oil thus produced.

3.3. Enzymatic activity of stones

The combined action of glucosidases and esterases on OLP (**1A**) leads to the major metabolites **2**, **3**, and **4** already found in olive oil (Chart 1) (Montedoro et al., 1993). The aglycone **3** undergoes fast rearrangements involving the cyclic semiacetal, open dialdehyde and their hydrated forms whose presence has been unequivocally ascertained by mass spectrometry (Beauchamp et al., 2005).

A preliminary check on the action of stone water extracts on pure samples of **1A** was therefore planned. Oleuropein was incubated with the water extracts from crushed stones obtained from Carolea cultivar, according to the procedure reported in Section 2. After 60 min of incubation both solutions, analyzed by electrospray mass spectrometry (ESI/MS, Fig. 2) showed that the extracted ion chromatogram centered at 33 min ca., corresponds to the oleuropein aglycon and its open chain isomers **3**. Hydroxytyrosol was not detected. It can be confidently assumed, therefore, that the effect of exposure of OLP to stone enzymes is essentially represented by a deglycosylation procedure.

Electrophoretic separation of the pull of protein present in olive stone water extracts, provided some clue for the existence of a β-glycosidase (Wang et al., 2001) in the region of 66 kDa, especially when the extraction method 1 (see Section 2) was used (Fig. 3).

After these encouraging preliminary results, the metabolization rate of OLP was followed in the first ten minutes, after its addition to the solution of stone water extracts (25 mg/kg), from Carolea and Cassanese cultivars (Fig. 4A and B), by mass spectrometry as described in Section 2. The experimental data fit well with an exponential decay with a correlation factors (R^2) of 0.9785. An appreciable difference in OLP decay rate was observed between

Table 2
Total phenols and α-tocopherol content in stoned and conventional oil

Sample	Cultivar	District	Process	Total phenols (mg/kg)[a]	α-Tocopherol (mg/kg)	Oleuropein (mg/kg)
1	Carolea	CZ	Conventional	172.3	310.6	0.023
2	Carolea	CZ	Stoned	207.4	252.3	0.062
3	Carolea	CS	Conventional	335.3	269.5	0.042
4	Carolea	CS	Stoned	225.9	231.7	0.176
5	Carolea	CS	Conventional	225.2	287.3	0.075
6	Carolea	CS	Stoned	290.8	279.2	0.085
7	Carolea	CS	Conventional	211.0	324.9	0.028
8	Carolea	CS	Stoned	220.2	230.8	0.114
9	Carolea	RC	Conventional	156.0	124.6	0.056
10	Carolea	RC	Stoned	307.1	247.2	0.069
11	Carolea	CS	Conventional	144.7	166.4	0.093
12	Carolea	CS	Stoned	199.0	102.9	0.128
13	Carolea	CS	Conventional	181.4	144.8	0.059
14	Carolea	CS	Stoned	182.1	182.1	0.063
15	Cassanese	CS	Conventional	40.8	301.6	0.045
16	Cassanese	CS	Stoned	76.0	272.4	0.072
17	Cassanese	CS	Conventional	59.2	167.1	0.067
18	Cassanese	CS	Destoned	185.6	164.7	0.074
19	Cassanese	CS	Conventional	35.6	223.6	0.056
20	Cassanese	CS	Stoned	57.8	224.3	0.089
21	Dolce di Rossano	CS	Conventional	84.7	148.1	0.041
22	Dolce di Rossano	CS	Stoned	176.4	180.6	0.083
23	Dolce di Rossano	CS	Conventional	46.0	298.4	0.071
24	Dolce di Rossano	CS	Stoned	72.9	214.9	0.203

[a] Expressed as mg/kg of caffeic acid.

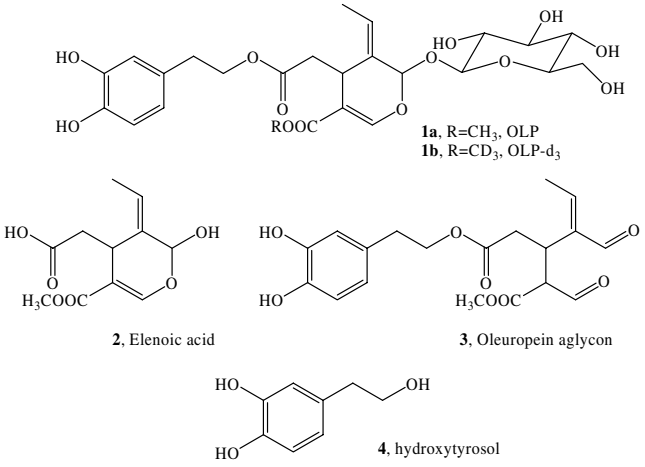

Chart 1.

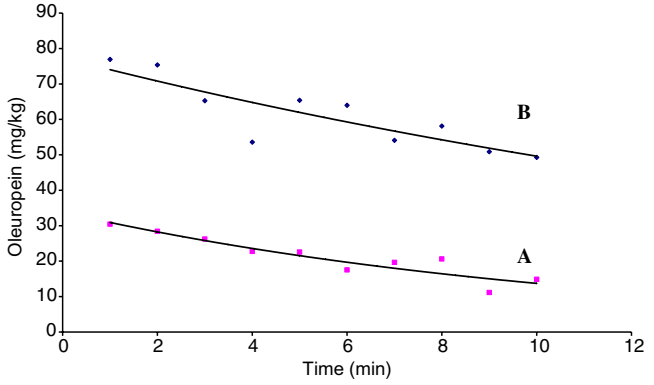

Fig. 4. Kinetic of OLP metabolism in water extract of Carolea pits (A) and Cassanese pits (B) followed by mass spectrometry in the first 10 min of exposure.

the two cultivars, since 51% and 36% of original glycoside were consumed, respectively, in the same 10 min period of time. The difference is too high to be attributed to experimental errors and uncertainties, and should be reflected in the total amount of oleuropein content of stoned oils produced by Carolea and Cassanese cultivars.

3.4. Oleuropein as a biomarker of stoned olive oil

A systematic investigation of the oleuropein content of stoned an conventional oils was therefore undertaken by means of the method developed in our laboratory (De Nino et al., 2005) whose reliability is guaranteed by the use of a proper deuterium labeled internal standard. The results (Table 2) show that **1A** is always present in larger amounts in stoned oils and that its relative concentration can be correlated with the cultivar.

The average distribution of OLP in the different cultivars (Fig. 5) reflects the kinetic of its metabolism, at least for the two investigated cases of Cassanese and Carolea cultivars.

The role of the presence of stones in olive oil making procedures is firmly established and unambiguously proved. A two to nearly fivefold increasing of oleuropein content was observed in olive oils produced from the same drupes on going from conventional to stoning procedures; this observation contributes to add value to the foodstuff

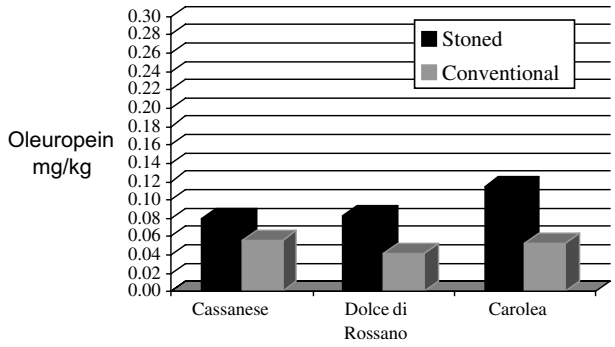

Fig. 5. Oleuropein expression in stoned and conventional olive oils from three different cultivars.

produced with the SPIA apparatus, because of the known and widely documented the nutraceutical effect played by oleuropein (**1A**).

3.5. Oleuropein in stoned olive oils

Nevertheless, it should be consider that the amount of **1A** in extravirgin olive oils hardly exceeds a couple of hundreds μg/kg (De Nino et al., 2005), therefore its variation does not contribute to appreciable macroscopic differences in the olive oils produced by the two different procedures.

4. Conclusions

The extra virgin olive oil produced from the pulp of drupes stoned by means of the SPIA machine here presented are of comparatively better quality than those produced from the intact drupe by means of conventional milling procedure. The macroscopic parameter which differentiates the two type of foodstuff is represented by the relative value of total phenol content, only, which is higher in stoned oils. The role of deglycosylation enzymes present in stones, evaluated by kinetic measurements, depends also on the examined cultivar. It should be stressed, however, that a clear-cut difference between experimental monovarietal olive oils produced by SPIA or conventional methods can only be established if the relative amount of oleuropein biomarker is considered as a reliable parameter.

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