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Effect of freeze injuries in olive fruit on virgin olive oil composition

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Abstract

The aim of this work was to study the effect of freeze damage during the harvest period on the composition and quality indices of olive oil from the Arbequina cultivar. No differences were found in the quality indices of oils extracted from olives affected by freeze injuries. On the other hand, slight differences were observed in the carotenoid and chlorophyll contents. These were reflected in higher luminosity values (L^*), and an important decrease in the concentrations of secoiridoid derivatives and 4-(acetoxyethyl)-1,2-dihydroxybenzene (3,4-DHPEA-AC), giving rise to a lower oil stability and sensory changes, such as the absence of bitter taste, and less pungent taste of oils extracted from olives after frost damage. Slight rises were also observed in the concentrations of simple phenols, such as vanillic acid and vanillin, that gave rise to sweeter oils.
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Keywords: Arbequina cultivar; Freeze injuries; Virgin olive oil; Phenolics

1. Introduction

In developed countries, consumer needs are generally met in terms of qualitative food attributes related to nutritional balance and quality. This has generated a market orientation towards high-quality products that can satisfy growing demands and, at the same time, encourage the diversification of agricultural products, as part of rural development.

A Protected Designation of Origin (PDO) is the term used to describe foodstuffs produced, processed and prepared in a given geographical area. It implies a close link between the product, the soil and climatic factors, as well as the human factors that shape a particular character of a region in relation to its agricultural products.

The virgin olive oils with protected designation of origin are regulated by administrative norms that define several requirements. These include the existence of an olive-growing area with specific climatic and edaphological characteristics, where oils with similar features have traditionally been obtained. These areas have defined agronomic practices, the use of particular olive cultivars for oil production and uniform methodologies for first quality virgin olive oil extraction. All this leads to obtaining oils that correspond to the distinctive sensorial quality of the area (Civantos, Contreras, & Grana, 1992).

As the leading olive oil-producing country, Spain protects its oil types and, at present, there are nine olive oils with protected designation of origin, two of them ("Les Garrigues" and "Siurana") being located in Catalonia. Most of the oil produced in the south of the province of Lleida (Catalonia, Spain) is protected by the designation of origin "Les Garrigues". This PDO occupies about 35,715 ha with an average of 120 trees per ha and is recognised by the Spanish Ministerial Orders of the 28 October 1975 and the 22 January 1994 (BOE, 1977, 1994) and European Community recognition corresponds to EEC Regulation 1107/96.

The climate of the area is Mediterranean with a continental influence. It is characterised by hot, dry summers and long, cold winters. The important daily and annual temperature variations distinguish this area from other Mediterranean olive-growing areas whose more temperate climate is due to their proximity to the sea. Rainfall is scarce and irregular, mostly falling in spring.

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The olive harvest period in the area covers the three months from November to January, with the aim of finishing harvesting before the arrival of the autumn frosts that are very common in this region.

The harvest period for the 2001–2002 crop season was atypical in the ‘‘Les Garrigues’’ region. A cold front from Siberia moved to the south of Europe and arrived in Catalonia (Spain) on 15 December bringing snowfalls, together with an important drop in temperature. Trees were covered by snow for more than 10 days because of the persistent cold. Climatic data obtained from the weather station situated at La Granadella, in the geographical centre of ‘‘Les Garrigues’’ region, showed that maximum temperatures were below −5 °C for more than 110 consecutive hours, reaching minimum values of −12.5 °C. As a consequence of these exceptional weather conditions, about 40% of *Arbequina* olive oil production in the PDO ‘‘Les Garrigues’’ was affected and negative effects have also been forecast for the coming crop seasons as a result of the high number of olive trees damaged by the extreme temperatures.

The major processes involved in fruit ripening, such as softening, respiration, ethylene production and the activity of pectic enzymes, are enhanced in freeze-injured fruit (Fuchs, Zauberman, & Yanko, 1975, 1981). As a result of those processes, fruits may shrivel easily, ripen earlier, lose water, and become soft. Several studies have been carried out on the nature of freeze damage in olive trees from a botanical and horticultural point of view (Denney et al., 1993; Fiorino & Mancuso, 2000). In olive tissues, temperatures below 0 °C are accompanied by the freezing of extracellular water. Thermodynamic equilibration is achieved, either by cellular dehydration and continued extracellular ice formation, or by intracellular ice formation. The manner of equilibration is influenced by the rate at which the cell is cooled and the minimum temperature to which it is exposed relative to the efflux of water from the cell (Steponkus, 1981). In fact, freeze injuries are a consequence of cell dehydration and important cell destruction caused by ice crystals forming inside parenchyma cells. These cause serious destruction of cell membranes, leading to cell death and a high oxidation of cell contents as a result of contact between enzymes and their respective substrates. All those changes observed in fruit may have an effect on composition, since the quality of virgin olive oils is directly related to the physiological state of the fruit from they are extracted.

The aim of this work was to study the effect of freeze damage in olive fruit during the harvest period, which is a cause of concern in this area where a high-quality oil is produced, on virgin olive oil composition and quality indices, as well as the effect on the oil sensory attributes. Of special relevance are the changes in minor components, such as chlorophylls, carotenoids, α-tocopherol and phenolic compounds and the sensory attributes that play an important role in the organoleptic characteristics and antioxidant properties of virgin olive oils.

2. Materials and methods

2.1. Materials

Twenty olive oil samples from various olive oil mills from all over the region of ‘‘Les Garrigues’’ (Catalonia, Spain) were obtained in the 2001–2002 crop season. Oils were taken directly from the production line on the basis of a protocol established by the Regulator Organism of the Protected Designation of Origin ‘‘Les Garrigues’’.

Oils collected between the 7 and 14 December were classified as *Before frost* (10 samples) and oils collected between the 28 December to 18 January were classified as *After frost* (10 samples).

2.2. Physico-chemical parameters

Determinations of the free fatty acid content, peroxide value, and ultraviolet absorption characteristics at 270 nm were carried out following the analytical methods described in the Regulation EEC/2568/91 of the European Union Commission. Results were expressed as percentage of oleic acid, milliequivalents of active oxygen per kilogramme of oil (meq O_2/kg), and absorbance at 270 nm, respectively.

2.3. Determination of pigments and chromatic ordinates

2.3.1. Pigment content

The chlorophyll fraction at 670 nm and the carotenoid fraction at 470 nm were evaluated from the absorption spectrum of each virgin olive oil sample (7.5 g) dissolved in cyclohexane (25 ml) (Mínguez-Mosquera, Rejano, Gandul, Sánchez, & Garrido, 1991). The chlorophyll and carotenoid content are expressed as mg of pheophytin ‘‘a’’ and lutein per kg of oil, respectively.

2.3.2. Oil colour

A colorimeter (chromometer type Color-Eye 3000, Macbeth) was used to assess the oil colour with the Optiview 1.1 computer program, and the CIELAB colorimetric system was applied. Oil samples were examined without dilution to avoid colour variation and the tristimulus values X, Y, and Z were calculated for illuminant C from the absorption spectrum. The oil colour is expressed as chromatic ordinates a^*, b^* and L^*.

2.4. α-Tocopherol determination

α-Tocopherol was evaluated by high-performance liquid chromatography (HPLC) with direct injection of

an oil-in-hexane solution. The HPLC system consisted of a Waters 600 pump, with a column heater and a Waters 717 plus Autosampler included, the detection being carried out with a Waters 996 photodiode array detector. The system was controlled with Millenium software (Waters Inc., Mildford, MA). A 25 cm × 4 mm i.d., 5 μm, Supelcosil LC-NH2 (Supelco Inc., Bellefonte, PA) column was used. Separation was achieved by isocratic elution, the mobile phase being hexane/ethyl acetate (70:30). An injection volume of 20 μl, and a flow rate of 1 ml/min were used. Chromatograms were recorded at 295 nm. α-Tocopherol was quantified by the external standard method. The linearity of the response was verified by the α-tocopherol analysis of six standard solutions with known concentrations. Results are given as mg of α-tocopherol per kg of oil.

2.5. Fatty acid determination

The fatty acid composition of the oils was determined by gas chromatography (GC) as fatty acid methyl esters (FAMEs). FAMEs were prepared by saponification/methylation with sodium methylate according to the European Union Commission modified Regulation EEC 2568/91 (León-Camacho & Cert, 1994). A chromatographic analysis was performed in a Hewlett Packard 5890 Series II gas chromatograph, using a capillary column (SP 2330, Supelco). The column temperature was isothermal at 190 °C and the injector and detector temperatures were 220 °C. Fatty acids were identified by comparing retention times with standard compounds. Six fatty acids were considered in this study. These were palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic (18:3) acids, expressed as percentages of fatty acid methyl esters.

2.6. Total phenol content

Phenolic compounds were isolated using the modified method described by (Vázquez Roncero, Janer del Valle, & Janer del Valle, 1973) with triple extraction of an oil-in-hexane solution with a 60% vol/vol water/methanol mixture. The concentration of total polyphenols was estimated with Folin–Ciocalteau reagent at 725 nm. Results were expressed as mg of caffeic acid per kg of oil.

2.7. Bitter index

The bitter index (K_{225}) was evaluated by extraction of the bitter components of a sample of 1.0±0.01 g oil dissolved in 4 ml hexane passed over a C18 column (Waters Sep-Pack Cartridges), previously activated with methanol (6 ml) and washed with hexane (6 ml). After elution, 10 ml hexane were passed to eliminate the fat, and then the retained compounds were diluted with methanol/water (1/1) to 25 ml. The absorbance of the extract was measured at 225 nm against methanol/water (1/1) in a 1-cm cuvette (Gutiérrez, Perdiguero, Gutiérrez, & Olías, 1992; Gutiérrez-Rosales, Garrido-Férnandez, Gallardo-Guerrero, Gandul-Rojas, & Mínguez-Mosquera, 1992).

2.8. Stability

Stability is expressed as the oxidation induction time (h), measured with a Rancimat 679 apparatus (Metrohm Co., Switzerland), using an oil sample of 3 g warmed to 120 °C, and 20 l h^{-1} air flow. The time taken to reach a fixed level of conductivity was measured (Laübli & Bruttel, 1986).

2.9. HPLC analysis of phenolic compounds

2.9.1. Phenolic extraction

Phenols were extracted from virgin olive oil following the procedure of Montedoro, Servili, Baldioli, and Miniati (1992). 2 × 20 ml of methanol/water (80:20 v/v) were added to 45 g of virgin olive oil and homogenised for 2 min with a Polytron. The two phases were separated by centrifuging at 3000 rpm for 10 min. Hydroalcholic extracts were then combined and concentrated in a vacuum at temperatures below 35 °C until a syrupy consistency was reached. 5 ml of acetronitrile were added to the extract and it was washed with 3 × 20 ml of hexane. The apolar phases were also purified with 5 ml of acetonitrile. The resulting acetonitrile solution was evaporated under vacuum and the residue dissolved in 5 ml of acetonitrile. Finally, an aliquot of 2 ml was evaporated under a stream of nitrogen.

2.9.2. HPLC analysis of phenolic compounds

The phenolic fraction extracted was dissolved in 1 ml of methanol and analysed by HPLC. The HPLC system consisted of a Waters 717 plus Autosampler, a Waters 600 pump, a Waters column heater module and a Waters 996 photodiode array detector managed by a Millenium 2000 software (Waters Inc., Milford, MA). The column was a Inertsil ODS-3 (5 μm, 15 cm × 4.6 mm i.d., GL Sciences Inc.) equipped with a Spherisorb S5 ODS-2 (5 μm, 1 cm × 4.6 mm i.d., Technokroma, Barcelona, Spain) precolumn. HPLC analysis was performed, following the same procedure as Montedoro et al. (1992). The eluents were 0.2% acetic acid (pH 3.1) and methanol and the flow rate was 1.5 ml/min and the injection volume 20 μl. The total run time was 60 min, the initial composition was 95% acetic acid, 0.2 and 5% methanol and the gradient changed as follows. The concentration of methanol was maintained for 2 min, then it was increased to 25% in 8 min and finally, the methanol percentage was increased to 40, 50 and 100%

in 10 min periods. It was maintained at 100% for 5 min. Initial conditions were reached in 15 min. Chromatograms were obtained at 280 and 339 nm.

2.9.3. Reference compounds

Tyrosol and *p*-coumaric acid were obtained from Extrasynthèse Co. (Genay, France). Vanillic acid, vanillin and ferulic acid were obtained from Fluka Co. (Buchs, Switzerland). Hydroxytyrosol was kindly donated by Professor Montedoro (University of Perugia, Italy). The rest of the phenolic compounds were obtained using a semi-preparative HPLC column Spherisorb ODS-2 (5 μm, 25 cm × 10 mm i.d., Tecknokroma, Barcelona, Spain) and a flow rate of 4 ml/min. The mobile phases and gradient were described elsewhere (Tovar, Motilva, & Romero, 2001). Sample extracts were analysed using a ZMD mass spectrophotometer (Waters Inc.) equipped with an electrospray ionization ion source (ESI). The ion spray mass spectra in the negative-ion mode were obtained under the following conditions: capillary voltage, 2.5 kV; cone voltage, 10 V; desolvation temperature, 400 °C and source temperature, 120 °C.

Individual phenols were quantified by a four-point regression curve on the basis of the standards obtained from commercial suppliers or from preparative HPLC as described earlier. Quantification of the phenolic compounds was carried out at 280 nm.

2.10. Sensory analysis

Sensory analysis of the samples was carried out by trained panellists from PDO ‘‘Les Garrigues’’, according to the method described in Annex XII of European Union Commission (EEC 2568/91). Panellists are trained to answer a special questionnaire to evaluate the intensity of predetermined sensory attributes on a scale from 0 to 5 and the overall-grading.

2.11. Statistical analysis

The data were subjected to an analysis of variance using the SAS version 8.02 (SAS Institute Inc., Cary, NC, USA). Separation of the means was obtained using the least square means test and significant difference was defined at $P \leqslant 0.05$.

3. Results and discussion

The values for the quality indices, free fatty acid content and peroxide value of after-frost oils showed slightly higher values (Table 1). In fact, they are related to the degree of deterioration of an oil, determined by the state of the fruit and its manipulation before and during oil processing. However, it is not significant because the average values of free fatty acid content, peroxide value and K_{270} were considerably below the limit established by EEC legislation for virgin olive oil. A possible explanation for the low increase observed in the peroxide value of oils obtained after frost could be that the olives were harvested and processed within 24 h immediately after freeze damage, as soon as it was possible to harvest them.

Table 1 shows the chlorophyll and carotenoid concentration and chromatic ordinates of oils obtained before and after frosts. Oil pigment content decreased slightly after freeze damage ($P<0.05$). The chlorophyllase and lypoxygenase enzymes could be involved in this loss of chlorophyllic and carotenoid pigments, favoured by deterioration of the olive fruit (Mínguez-Mosquera, 1997). The chromatic ordinates a^* and b^* were not affected by the low temperatures reached while luminosity (L^*), negatively correlated with total pigment content, and increased in oils obtained after freeze damage.

There were no significant differences in the α-tocopherol contents between oils obtained before and after freeze damage (Table 1). Tocopherol is more dependent on the olive lipid content as it is a lipid-soluble compound.

A remarkable difference between oils obtained before and after frosts was the total phenol content (Table 1), showing significant decrease in those oils obtained from fruits under freeze conditions. The olive tissue destruction caused by the ice crystals formed inside parenchyma cells may encourage the oxidative degradation of phenolic compounds in PPO-catalysed reaction. It has been reported that the main agent responsible for enzymatic browning in fruits and vegetables is polyphenol oxidase (PPO; 1.14.18.1). It catalyses two differ-

Table 1
Quality indices, pigment content and chromatic ordinates of virgin olive oils obtained from olive fruits harvested before and after frost damage ($n=10$)

	Frost damage[a]	
	Before	After
Acidity (% w/w of oleic acid)	0.11	0.14 NS
Peroxide value (meqO_2/kg)	6.7	8.0 NS
K_{270}	0.096	0.096 NS
Chlorophylls (mg/kg)	5.5	4.2 *
Carotenoids (mg/kg)	7.1	6.8 *
Chromatic ordinates		
L^*	87.3	89.1 **
a^*	−2.4	−1.7 NS
b^*	94.9	94.7 NS
α-Tocopherol (mg/kg)	205	173 NS
Total phenols (ppm)	99	61 *
Stability (h)	8.2	5.8 *
K_{225}	0.123	0.093 **

[a] Significance level. NS, not significant ($P>0.05$); * $P<0.05$; ** $P<0.01$.

Table 2
Oil fatty acid composition (expressed as percentages) of virgin olive oils obtained from olive fruits harvested before and after frost damage ($n = 10$)

		Frost damage[a]	
		Before	After
Palmitic acid	(C16:0)	13.9	13.8 NS
Palmitoleic acid	(C16:1)	1.33	1.28 NS
Estearic acid	(C18:0)	2.17	2.17 NS
Oleic acid	(C18:1)	71.4	71.7 NS
Linoleic acid	(C18:2)	10.6	10.4 NS
Aracidic acid	(C20:0)	0.38	0.39 NS
Linolenic acid	(C18:3)	0.25	0.23 NS

[a] Significance level. NS, not significant ($P > 0.05$).

ent reactions in the presence of molecular oxygen: the hydroxylation of monophenols to o-diphenols and the oxidation of *o*-diphenols to *o*-quinones which polymerise non-enzymatically and give rise to heterogeneous black, brown or red pigments, commonly called melanins (Tomás-Barberán & Espín, 2001). The bitter index (K_{225}) and oxidative stability of oils followed the same pattern as total phenol content. In fact, phenols improve oil resistance to oxidation and, to a certain extent, are responsible for its sharp bitter taste.

No significant differences were found in the fatty acid composition of oils obtained before and after frost (Table 2).

Fig. 1 shows the chromatographic profile of the phenolic extracts from Arbequina virgin olive oils from the

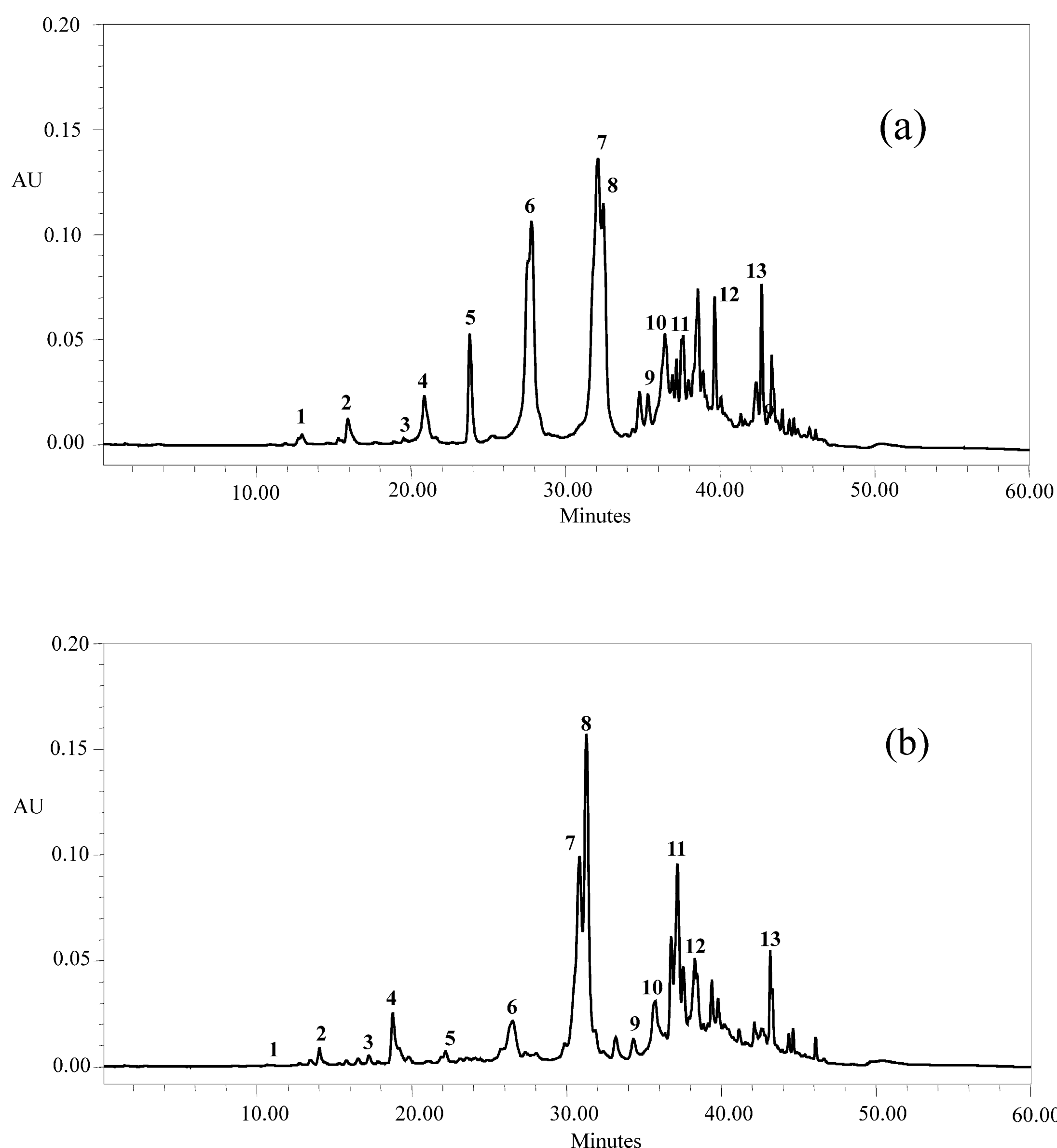

Fig. 1. HPLC chromatograms (at 278 nm) of phenolic extracts from olive oil. (a) Before frost damage. (b) After frost damage. See Materials and Methods for chromatographic conditions. See Table 3 to identify the peaks.

same mill but obtained before and after frost, respectively. Table 3 shows the average concentrations of the quantified phenolic compounds in the oils examined in this study.

Peaks 1–8 and Peak 10 were identified according a previous paper (Tovar et al., 2001). There is a larger number of compounds in the phenolic fraction than in those already defined. Peaks 9, 11 and 12 represent unknown complex phenolic compounds found in all olive oils analysed, with a spectrum similar to that of secoiridoid derivatives, showing two maxima at 210 and 278 nm. Peak 13, however, shows a UV spectrum similar to that of *trans*-cinnamic acid, with a maximum at 276 nm. Studies are in progress to isolate and identify these peaks.

The main phenolic compounds found in oils from PDO ‘‘Les Garrigues’’ analysed in this trial were the secoiridoid derivatives, a dialdehydic form of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA), oleuropein aglycone (3,4-DHPEA-EA) and the dialdehydic form of elenolic acid linked to tyrosol (*p*-HPEA-EDA), followed in order of quantitative importance by lignans and the phenolic compound 4-(acetoxyethyl)-1,2-dihydroxybenzene (3,4-DHPEA-AC) (Table 3).

Low levels of the simple phenols 3,4-DHPEA, *p*-HPEA, vanillin and vanillic acid were found, while ferulic and *p*-coumaric acids were identified but could not be quantified.

The concentrations of most olive oil phenolic compounds were affected by the freeze conditions. The three secoiridoid derivatives and the compound 3,4-DHPEA-AC decreased in the oils obtained after the freeze damage suffered by the olive fruit. Secoiridoid derivatives are compounds of major interest since their antioxidant activity has already been evaluated and has been shown to extend the shelf-life of olive oil (Baldioli, Servili, Perretti, & Montedoro, 1996; Gennaro, Piccioli-Bocca, Modesti, Masella, & Coni 1998). Moreover, they have been related to the bitter attribute of oils. Correlations have also been found between them and the bitter index (Tovar et al., 2001). The concentration of the unknown phenolic compounds, Peak 9 and Peak 12, decreased, as did the earlier-mentioned compounds, which may indicate that they are bio-chemically related.

Wounding of olive fruit, caused by the formation of ice crystals, induces cellular decompartmentalisation which would allow mixing of phenolic substrates and PPO. Thus, the decrease in the level of those compounds could be due to an enzymatic oxidation that would result in the browning of the olive fruit.

Despite showing a UV spectrum similar to that of Peaks 9 and 12, Peak 11 followed a different pattern. Its content in the oils increased significantly after freeze damage, as it was a compound resulting from the oxidative process.

The contents of lignans and Peak 13 remained practically unchanged while the 3,4-DHPEA, vanillic acid and vanillin concentrations increased in oils obtained after freeze damage. The increase in 3,4-DHPEA content may be a consequence of the degradation of secoiridoid derivatives in their structure. On the other hand, vanillic acid and vanillin are considered to be lignin degradation compounds (Fernández-Bolaños, Felizón, Brenes, Guillén, & Heredia, 1998).

One of the aims of this study was also to explore the influence of freeze conditions on the virgin olive oil sensory attributes and relate these to the chemical composition. Differences are detected between oils in aroma, and mouthfeel perceptual differences are detected between oils. The sensory notes of artichoke, tomato and almond were perceived in before-frost oils, and no unpleasant aroma or flavour was detected (Table 4). After-frost olive oils were qualified as non-extra virgin olive oil. Defects were defined as frozen olives by some panellists, while others defined these oils as thicker, softer and with the term rancid tallow.

Table 3
Phenolic compounds (mg kg^{-1}) of virgin olive oil obtained from olive fruits harvested before and after frost damage ($n=10$)

Peak	Phenolic compounds[a]	Frost damage[b]	
		Before	After
1	3,4-DHPEA	0.12	0.21 **
2	p-HPEA	0.95	0.42 NS
3	Vanillic acid	0.08	0.15 *
4	Vanillin	0.17	0.42 **
5	3,4-DHPEA-AC	22	1 **
6	3,4-DHPEA-EDA	116	17 **
7	*p*-HPEA-EDA	44	28 NS
8	Lignans	80	80 NS
9	Peak 9	5.21	1.20 *
10	3,4-DHPEA-EA	79	33 **
11	Peak 11	6.50	22 *
12	Peak 12	9.30	5.24 **
13	Peak 13	7.36	4.54 NS

[a] 3,4-DHPEA, hydroxytyrosol; *p*-HPEA, tyrosol; 3,4-DHPEA-AC, 4-(acetoxyethyl)-1,2-dihydroxybenzene; 3,4-DHPEA-EDA, dialdehydic form of elenolic acid linked to hydroxytyrosol; *p*-HPEA-EDA, dialdehydic form of elenolic acid linked to tyrosol; 3,4-DHPEA-EA, oleuropein aglycone.

[b] Significance level. NS, not significant ($P>0.05$); * $P<0.05$; ** $P>0.01$.

Table 4
Intensities of attributes perceived by panellists for oils before and after frost damage ($n=10$)

	Frost damage	
	Before	After
Bitter	2	Absence
Pungent	2–3	1–2
Sweet	2–3	4

A less bitter and pungent taste should be related to the noticeable reduction of oil phenolic compounds, especially secoiridoid compounds.

4. Conclusions

No differences were found in the quality indices of oils affected by freeze injuries, since olives were harvested and processed in a short-time period. The most important changes were observed in the minor components of olive oil, i.e. pigments and the phenolic fraction. In conclusion, frost damage caused two main changes in virgin olive oil composition. These were slight decreases in chlorophyll and carotenoid contents, reflected in higher values of luminosity (L^*) and an important decrease in the concentration of secoiridoid derivatives and 3,4-DHPEA-AC which play an important role in oil stability and the sensory attributes. Therefore, after-frost oils showed lower stability and suffered important changes in their sensory attributes, leading to absence of the bitter taste and less pungent taste. On the other hand, after the freeze conditions, there were slight rises in the concentrations of simple phenols, such as vanillic acid and vanillin, giving rise to sweeter oils.

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