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ORIGINAL PAPER

Changes in Quality Indices, Phenolic Content and Antioxidant Activity of Flavored Olive Oils during Storage

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Abstract The chemical characteristics, phenolic content and antioxidant activity of olive oils flavored with garlic, lemon, oregano, hot pepper, and rosemary were evaluated during 9 months of storage. At the end of the storage period, the unflavored and the garlic-flavored oils maintained their chemical parameters within the limits fixed for extra-virgin olive oils. After 9 months of storage, a noticeable decrease in phenolic content was observed in all the oils. The highest (35.0 ± 3.9 mg/kg oil) and the lowest (6.3 ± 0.4 mg/kg) phenolic contents were detected in the unflavored and garlic-flavored oils, respectively. Compounds such as 3,4-DHPEA-EDA (3,4-dihydroxyphenylethyl 4-formyl-3-formylmethyl-4-hexenoate, the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol) and *p*-HPEA-EDA (dialdehydic form of the decarboxymethyl elenolic acid linked to tyrosol) were the most abundant in both unflavored and lemon-flavored oils up till 6 months of storage. At the end of the storage period, increases in 3,4-DHPEA (hydroxytyrosol) and *p*-HPEA (tyrosol) were measured in almost all the oils. During storage, the antioxidant activity coefficients of the phenolic extracts, calculated according to the β-carotene bleaching assay, significantly decreased and, after 9 months, were in a decreasing order: rosemary (51.3 ± 4.2), hot pepper, lemon, oregano, unflavored, and garlic (8.5 ± 0.7).

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Introduction

Olive oil is widely used in the Mediterranean cuisine and is appreciated for its delicious taste and aroma, as well as for its nutritional benefits [1, 2] primarily related to its balanced fatty acid composition and the presence of considerable amounts of natural antioxidants [3].

A "flavored olive oil" can be defined as an olive oil (generally extra-virgin) processed with vegetables, herbs, spices or fruits in order to improve its sensory characteristics [4]. It is possible to choose among oils flavored with vegetables (garlic, onion, pepper, chilli, sun dried tomatoes), herbs (rosemary, oregano, basil, sage, thyme, fennel, juniper, estragon), spices (clove, nutmeg, ginger), mushrooms (truffles), fruits (lemon, orange, mandarin, apple, banana), nuts (almond, hazelnut, pine nuts) and aromas (for example, oils aromatized with vanilla are used to season shellfish, poultry and salads with vegetables, fruit and shellfish).

Aromatic plants and fruits have been used throughout the ages in many fields, from food flavoring to pharmaceutical, cosmetic and perfumery due to their content in essential oils and compounds [3] for which antimicrobial and antioxidant properties are usually ascribed [5]. The positive effects of antioxidants are determined by their ability to terminate radical chain reactions, scavenge active oxygen species, entrap electrophiles [6], and chelate metal ions [7]. Among antioxidants, the phenolic compounds exhibit a wide range of biological effects including antibacterial, anti-inflammatory, antiallergic, hepatoprotective,

antithrombotic, antiviral, anticarcinogenic, and vasodilatory actions [8]. Many of these biological functions have been attributed to their free radical scavenging and antioxidant activity. Thus, there is an increasing interest in herbs and spices as sources of natural antioxidants [9].

The oil flavoring can have an impact on the product shelf life. In fact, antioxidants have been widely used in fats and oils in order to prevent their oxidation and the production of undesirable flavors [10]. In addition, the presence of oxidized lipids reduces the nutritional value of foods and has undesirable effects on human health [11]. Damechki et al. [12] performed a study aimed to examine the presence of antioxidants and pro-oxidants in oils flavored with oregano and rosemary. They found the highest phenolic contents in the flavored oils rather than in the unflavored ones. Furthermore, according to Tsimidou [13], the oxidative stability is greater in flavored oils than in the unflavored ones. Baiano et al. [14] analyzed the effects of the addition of a mixture of garlic, laurel, and marjoram on selected chemical indices of olive oil from canned dried tomatoes. The addition of the herbs slowed polymerization reactions but did not inhibit the triacylglycerol oxidation and led to an increase in the kinetic constant of acidity, peroxide, and *p*-anisidine value. Antoun and Tsimidou [15] found that oregano and rosemary were able to slow the primary oxidation whereas the addition of garlic did not improve the oil stability. When added to sunflower oil, the extracts from *Ocimum basilicum* and *Origanum vulgare* L. did not improve the oxidation stability whereas the ethanolic extracts of *Satureiae hortensis* L., *Mentha piperita* L., *Melissa officinalis* L., and *Mentha spicata* L. appeared strongly active in retarding the oxidation reactions [9]. Concerning antioxidant activity, those essential oils showed good radical-scavenging properties according to the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay and the effectiveness order was: clove>>cinnamon>nutmeg> basil≥oregano>>thyme), at room temperature.

The present study investigated the effects of flavoring with garlic, lemon, oregano, hot pepper and rosemary on the quality indices, phenolic content and antioxidant activity of olive oils from the Italian cultivar *Peranzana* during 9 months of storage.

Materials and Methods

Oil Samples

Unflavored and flavored olive oils were obtained from healthy *Peranzana* olive fruits (*Olea europaea* L.) manually picked in olive groves located in the countryside near Torremaggiore (Apulia, Italy). Olive fruits were harvested in the year 2005 and the relative oils were obtained within 24 h by crushing the olives by a continuous processing system using a three-phase decanter and two vertical centrifuges. The organic lemons used for oil aromatization were picked in a grove located in the countryside near Rodi Garganico (Apulia, Italy). Spices and herbs such as garlic, oregano, Cayenne hot pepper, and rosemary were bought in a local market.

The flavored oils were obtained by adding the abovementioned flavoring agents to olives coming from homogeneous lots before pressing. The mixtures were then subjected to crushing and then to a malaxation treatment (30–35 °C) obtaining a malaxation mash. The flavored olive oils were separated from the malaxation mash by centrifugation. In this way, the flavors from the flavoring agents were very well absorbed into the oil, the residue of the flavoring ingredient was separated from the oil together with the olive residue and the aqueous fractions of the flavoring agents were removed along with the olive mill waste water.

The flavoring agents were added to 300 kg of olives in the following amounts: 60 kg of whole lemons; 5.5 kg of fresh rosemary; 30 kg of oregano powder; 10 kg of dehydrated Cayenne hot pepper; 10 kg of dehydrated garlic. The unflavored oil was used as a control. The production of each oil was replicated three times. The choice of the amounts of the added flavoring agents was made on the basis of the results of a research performed among the traditional producers of flavored olive oils in Apulia region.

In order to avoid the accidental contamination of each of the flavored oils with the residues of the flavored oil produced immediately before it, olives without addition of flavoring agents were submitted to crushing between the working cycles of two flavored oils and the oil obtained was discarded. Furthermore, after each working cycle, the decanter was completely washed with water to eliminate the residues of the flavored olive pastes.

Flavored and unflavored olive oils were stored in 1-L dark glass bottles at room temperature and three bottles of each replication were withdrawn and analyzed at production, 6, and 9 months of storage.

Quality Indices

Acidity (Cd 3d-63), peroxide value (Cd 8b-90), and spectrophotometric indices K_{232} and K_{270} (Ch 5–91) were determined according to AOCS methods [16]. The sensory analysis was performed according to Gambacorta et al. [17]. In this paper, the profile sheet suggested by the European Community Regulation 2568/91 for the sensory analysis of a virgin olive oil was modified taking into account those parameters ("fruity flavor different from olive" and "taste different from olive") able to conveniently describe a flavored olive oil. Briefly, a trained panel

composed of 12 judges was asked to evaluate the unflavored and flavored oils at production. Attributes such as olive fruity flavor and taste, fruity flavors and tastes different from olive, bitter, and sweet tastes were evaluated on a six-point (from 0 to 5) scale anchored with extremely low and extremely high related to the perception of the stimuli.

Extraction of Phenolic Compounds

Phenols were recovered from the oils by liquid–liquid extraction using methanol as the solvent and following the procedure reported in Montedoro et al. [18], slightly modified for its application to flavored oils. Two milliliters of methanol/water (70:30, v/v) and 2 mL of hexane were added to 5 g of virgin olive oil and mixed with a Vortex mixer for 10 min. The hydroalcoholic phase containing phenolics was separated from the oily phase by centrifugation (4,000*g*, 4 °C, 10 min). Hydroalcoholic phases were collected and submitted to another centrifugation (18,900*g*, at room temperature, 4 min). Finally, hydroalcoholic extracts were recovered with a syringe and then filtered though a 0.45 μm nylon filter (DISMIC-13NP, Advantec, Toyo Roshi Kaisha, Tokyo, Japan) before analysis.

The extracts prepared for HPLC analysis were obtained according to the same protocol, but with the addition to the oil of 0.5 mL of a solution of gallic acid (internal standard) at a concentration of 100 ppm, prepared in methanol/water (70:30, v/v).

Total Phenolic Content

The determination of the total phenolic compounds included the use of the Folin Ciocalteau reagent and the method was adapted from Di Stefano et al. [19]. In a test tube, 100 μL of phenolic extract or phenolic standard were mixed with the Folin Ciocalteau reagent (100 μL, 2 M) and, after 4 min, with an aqueous solution of Na_2CO_3 (800 μL, 5%). The mixture was heated in a water bath at 40 °C for 20 min and the total phenol content was determined colorimetrically at 750 nm. The standard curve was prepared using diluted solutions of gallic acid in a methanol:water solution (70:30, v/v). The total phenolic content was expressed as milligrams of gallic acid equivalents per kilogram of oil.

HPLC Phenolic Profile

The HPLC analysis of the phenolic extracts was carried out according to Gambacorta et al. [20], using an HPLC binary system (Agilent, model G1311A, Santa Clara, CA) equipped with a 7725 Rheodyne injector, a 20-μL sample loop, a diode array detector (Agilent, model G1315Bm Santa Clara, CA), and a ChemStation integrator (Agilent, Santa Clara, CA) for data acquisition. The stationary phase was a Nova-Pack C18 analytical column (150 × 3.9 mm i.d.) with a particle size of 4 μm (Waters, Milford, MA). The mobile phases for chromatographic analysis were: (a) water/acetic acid (98:2, v/v) and (b) methanol/acetonitrile (1:1, v/v) at a constant flow rate of 1 mL/min. The gradient program of solvent was as follows: 0–30 min 100% A; 30–45 min 70% A; 45–55 min 50% A; 55–65 min 40% A; 65–75 min 0% A. Detection of phenolic compounds was carried out at 280 nm. Spectra were recorded at wavelengths between 240 and 380 nm.

The identification of some phenolic components was carried out by comparing the peak retention times with those obtained by injection of the pure standards 3,4-dihydroxyphenylethanol or hydroxytyrosol (3,4-DHPEA) and *p*-4-hydroxyphenylethanol or tyrosol (*p*-HPEA), purchased from Extrasynthese (Genay Cedex, France), and vanillin, ferulic acid, coumaric acid, luteolin purchased from Sigma-Aldrich (Milan, Italy) and by analyzing the spectra obtained. The identification of compounds such as pinoresinol, 1-acetoxypinoresinol, 3,4-dihydroxyphenylethyl 4-formyl-3-formylmethyl-4-hexenoate, the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA), 3,4-dihydroxyphenylethanol-elenolic acid, an isomer of oleuropein aglycone (3,4-DHPEA-EA), 4-(acetoxyethyl)-1,2-dihydroxybenzene (3,4-DHPEA-AC), *p*-4-hydroxyphenylethanol-elenolic acid, an isomer of ligstroside aglycone (*p*-HPEA-EA) and dialdehydic form of the decarboxymethyl elenolic acid linked to tyrosol (*p*-HPEA-EDA) was made on the basis of studies found in the literature [21–25].

Quantification of phenolic compounds was performed according to the method of the internal standard (gallic acid, Extra-synthese, Genay Cedex, France) and on the basis of the response factors. The response factors were determined taking into account the recovery percentages of the phenolic compounds and the internal standard according to Escarpa et al. [26]. The recovery percentages were the following: 76.85% *p*-HPEA-EDA, 76.98% pinoresinol, 77.35% 3,4-DHPEA-EDA, 77.71% hydroxytyrosol, 79.19% tyrosol, 81.24% gallic acid.

Evaluation of the Antioxidant Activity

The antioxidant activity of the oil phenolic extracts was evaluated according to the *β*-carotene bleaching assay, in which the antioxidant activity was measured by the ability of a compound to minimize the loss of *β*-carotene in an emulsified aqueous system in the presence of oxygen at high temperatures (50 °C). Analyses were performed according to the method used by Obied et al. [27] with some modifications as described in the following. This test is based on the thermal autoxidation of linoleic acid and the

consequent formation of peroxyl radical that is able to scavenge hydrogen atoms from the β-carotene molecule, determining its bleaching. The β-carotene bleaching is detectable through the absorbance decrease, that is greater when the antioxidant content is low. Five milligrams of synthetic β-carotene type II (Sigma, St. Louis, MO) were dissolved in 50 mL of chloroform (J.T. Baker, Mallinckrodt Baker, Milano, Italy). Three milliliters of this solution were pipetted into a round-bottomed flask containing 40 mg of linoleic acid (Sigma, St. Louis, MO) and 400 mg of Tween 40 (Sigma, St. Louis, MO). After evaporation of chloroform to dryness under a vacuum at 40 °C, 100 mL of distilled water enriched with oxygen was added to the flask and the mixture was shaken to form a liposome solution. Aliquots of 1.5 mL of this solution were pipetted into test tubes containing 20 μL of phenolic extracts and immediately put into a water bath at 50 °C. The absorbance at 470 nm of samples and of a control containing an aqueous solution of methanol (70%, v/v) was monitored at regular intervals (15 min) on a Varian Cary 50 Scan UV-Visible spectrophotometer (Palo Alto, CA) until the complete β-carotene bleaching (after about 2 h). Absorbance decreased rapidly in the absence of antioxidants and slowly in sample extracts. The antioxidant activity was expressed as AAC (Antioxidant Activity Coefficient):

$$\mathrm{AAC} = \left(100 - \frac{\mathrm{Absof\ extract}_{0\,\mathrm{min}} - \mathrm{Absof\ extract}_{120\,\mathrm{min}}}{\mathrm{Absof\ control}_{0\,\mathrm{min}} - \mathrm{Absof\ control}_{120\,\mathrm{min}}}\right) \times 100$$

Statistical Analysis

Analyses were generally repeated at least three times for each sample. Mean values and standard deviations were determined. The discussion of the results was based on the one-way analysis of variance (ANOVA) and the Holm test at a confidence level of 95% performed by means of the Kaleidagraph Statistical Software (ver. 3.6.2; Synergy Software, Reading, PA).

Results and Discussion

Quality Indices

According to EC Commission Regulation 1989 [28], an extra virgin olive oil is a liquid fat that conforms to a series of chemical and sensory parameters (free fatty acid percentage ≤0.8 g oleic acid/100 g oil, peroxide value ≤20 mequiv O_2/kg, K_{232} ≤2.50, K_{270} ≤0.22, median of defects = 0, median of fruity >0), is free of defects. The chemical parameters measured on the flavored oils at production (Table 1) were included in the values mentioned above, with the exception of the K_{270} value referred to the rosemary-flavored oil. These data demonstrate the high quality of the oil tested though, by definition, flavored olive oils are not extra virgin oils. At production, significant differences were detected for each of the chemical parameters considered among the oils. Since the homogeneity of the starting olives, it is hard to formulate a hypothesis concerning these differences. Literature on this matter is poor but, on the basis of previous experiments [17], the anomalous behavior could be due to interactions taking place between olives and the components of the flavoring agents during the extraction phase and also to co-extraction of undesirable compounds. Storage determined increases in all the chemical indices, with the exception of acidity in garlic and oregano-flavored oils and K_{270} in unflavored oils and rosemary-flavored oils whose values remained substantially unchanged during the 9 months. During storage, the highest increases of acidity (about 36%) were detected in lemon and rosemary-flavored oils. Concerning the presence of primary oxidation products, unflavored and garlic-flavored oils showed the highest increases of peroxide values (17 and 19%, respectively), and the latter also suffered the highest increase (53%) in K_{232}. The highest increase in K_{270} (47%) was shown by the lemon-flavored oils. The best chemical parameters were always detected in the garlic-flavored olive oils. The highest acidity value was measured in the unflavored ones. The oils flavored with hot pepper showed the highest peroxide value (an index of the primary oxidation) whereas those flavored with rosemary had the highest values of K_{232} (another parameter related to the presence of primary oxidation products) and K_{270} (an index of secondary oxidation). Concerning sensory analysis, none of the samples showed defects. The unflavored oil showed the sensory attributes typical of the *Peranzana* cultivar: an intense olive fruity flavor (3.2 ± 0.1) with herbaceous and floral notes; a strong olive taste (4.5 ± 0.2); a sweet taste (3.0 ± 0.2) with a good balance between bitter and spicy (3.5 ± 0.3 and 3.2 ± 0.1, respectively). In all the flavored oils, instead, the sensory notes of the added herbs, spices and fruits hid these characteristics. In particular, the flavors and the tastes typical of the flavoring agents predominated (scores always higher than 3.5) and were accompanied by decreases in the olive fruity flavor and taste (score between 2.0 and 3.0 for both the parameters) and also in the sweet (between 2.2 and 2.8) and bitter tastes (between 2.6 and 2.8). This finding would suggest the opportunity of adding the flavoring agents in smaller quantities. Nevertheless, it is difficult to predict the intensity of the sensory characteristics of a flavored olive oil since it depends not only on the amount of the flavoring agents added but also on the cultivar and the maturation index of the starting olives, the variability in the strength of flavoring agents (due to

Table 1 Chemical parameters of unflavored and flavored oils during storage

Samples	Acidity (g/100 g)			P.V. (mequiv O_2/kg)			K_{232}			K_{270}		
	At production	After 6 months	After 9 months	At production	After 6 months	After 9 months	At production	After 6 months	After 9 months	At production	After 6 months	After 9 months
Unflavored oil	0.48 ± 0.04 a B	0.52 ± 0.08 a,b A,B	0.58 ± 0.02 a A	10.5 ± 0.9 d C	11.3 ± 0.3 d B	12.3 ± 0.1 e A	1.81 ± 0.01 e C	1.88 ± 0.04 d B	1.92 ± 0.00 e A	0.14 ± 0.01 c,d A	014 ± 0.02 c A	0.16 ± 0.01 c A
Garlic-flavored olive oil	0.25 ± 0.04 d A	0.26 ± 0.04 d A	0.28 ± 0.04 d A	7.5 ± 0.7 e C	8.4 ± 0.7 e B	8.9 ± 0.1 f A	1.34 ± 0.02 f C	1.77 ± 0.17 d B	2.05 ± 0.11 d A	0.13 ± 0.01 d B	0.16 ± 0.07 b,c A,B	0.17 ± 0.02 c A
Lemon-flavored olive oil	0.39 ± 0.01 b B	0.38 ± 0.08 b,c B	0.53 ± 0.04 a A	11.9 ± 0.1 c B	11.7 ± 0.1 c B	12.82 ± 0.1 d A	1.84 ± 0.01 d B	2.38 ± 0.16 a A	2.32 ± 0.01 b A	0.15 ± 0.01 c B	0.20 ± 0.02 b A	0.22 ± 0.06 b A
Oregano-flavored olive oil	0.45 ± 0.08 a,b A	0.45 ± 0.00 b A	0.46 ± 0.04 b A	13.8 ± 1.4 b B	13.9 ± 0.2 b B	14.8 ± 0.1 c A	1.91 ± 0.02 c B	1.92 ± 0.08 c,d B	2.22 ± 0.09 c A	0.21 ± 0.01 b B	0.16 ± 0.03 c C	0.24 ± 0.02 b A
Hot pepper-flavored oil	0.31 ± 0.04 c B	0.34 ± 0.01 c A	0.34 ± 0.01 c A	14.8 ± 1.3 a B	15.8 ± 0.7 a A,B	16.2 ± 0.1 a A	1.98 ± 0.02 b B	1.99 ± 0.00 c B	2.03 ± 0.03 d A	0.17 ± 0.02 c B	0.19 ± 0.04 b A,B	0.23 ± 0.07 b A
Rosemary-flavored olive oil	0.39 ± 0.01 b B	0.51 ± 0.01 a A	0.53 ± 0.04 a A	13.7 ± 1.5 b A,B	14.0 ± 0.1 b B	15.3 ± 0.1 b A	2.14 ± 0.04 a B	2.16 ± 0.054 b B	2.71 ± 0.21 a A	0.35 ± 0.01 a A	0.36 ± 0.03 a A	0.35 ± 0.05 a A

In columns, different lowercase letters indicate significant differences ($P < 0.05$) among flavored and unflavored oils

In lines, different capital letters indicate significant differences ($P < 0.05$) during the storage of each oil

growing conditions and/or individual characteristics), and the parameters fixed for the oil extraction process.

After 6 months of storage (Table 1), only the mean K_{270} value of the rosemary-flavored oils was higher than the limit established for an extra-virgin olive oil.

After 9 months of storage (Table 1), the unflavored oils and those flavored with garlic maintained their chemical parameters within the limits fixed for extra-virgin olive oils. The other oils showed value of K_{270} higher than 0.22.

Evolution of the Phenolic Content

The total phenolic contents of the oils are reported in Fig. 1. At production, the highest phenolic concentration was detected in the extract of the unflavored oils (181.3 ± 7.2 mg gallic acid equivalents/kg of oil), followed, in a decreasing order, by the oils flavored with lemons, hot pepper, oregano, rosemary, and garlic (30.6 ± 1.4 mg/kg). The huge differences existing among the phenolic contents of unflavored and flavored oils could be explained on the basis of interactions taking place between the olives and the flavoring agents during the extraction phase being responsible for the formation of bonds between phenolics and components of spices, herbs and fruits. For example, it is well known the reaction of addition of thiols to polyphenolic compounds catalyzed by polyphenol oxidases or peroxidases, which is on the basis of the removal of allium-specific volatile sulfur compounds from foods containing garlic [29]. Other authors [30] studied binding among polyphenolics and polysaccharides of the plant cell walls and found that the molecular size of polyphenols and their conformational flexibility are important to the binding, which, instead, would seem independent of pH. They also noticed that small changes in the structure of either the polyphenol or the polysaccharide resulted in marked changes in their affinity for each other. Furthermore, it is well known the interaction polyphenolics–proteins as well as the formation of soluble and insoluble complexes between them [31]. The higher phenolic contents detected on unflavored oils were not in agreement with the results obtained by Damechki et al. [12] though these differences should be explained on the basis of both the different starting oil and the specific flavoring method applied. In fact, they used an olive oil instead of an extra-virgin one (thus starting from a lower phenolic content) and produced rosemary and oregano flavored oils by infusion at 40 °C, for a time ranging from 24 to 72 h, allowing a longer time of contact between oil and flavoring agents and thus a transfer of potent antioxidants from the herbs to the oil matrix. Instead, in the present work, the flavoring agents were added directly to the olives. This means that the time of contact was shorter, the temperature was lower and, furthermore, the phenolic compounds were subjected to interactions with some components of spices, herbs and fruits in different amounts depending on the nature of the flavoring agents and were also partitioned between the aqueous (with whom they have a higher affinity) and the oily fractions.

A decrease in the phenolic content was noted during storage, especially after 9 months of storage. The phenolic loss was of about 81% for the unflavored oils and those flavored with garlic and lemon; 72% for the hot pepper-flavored oils; 66% for the rosemary-flavored oils; and 53% for the oils flavored with oregano. After 9 months, the phenolic contents were, in a decreasing order: unflavored (35.0 ± 3.7 mg/kg), oregano, hot pepper, lemon, rosemary and garlic (6.3 ± 0.4 mg/kg) flavored oils.

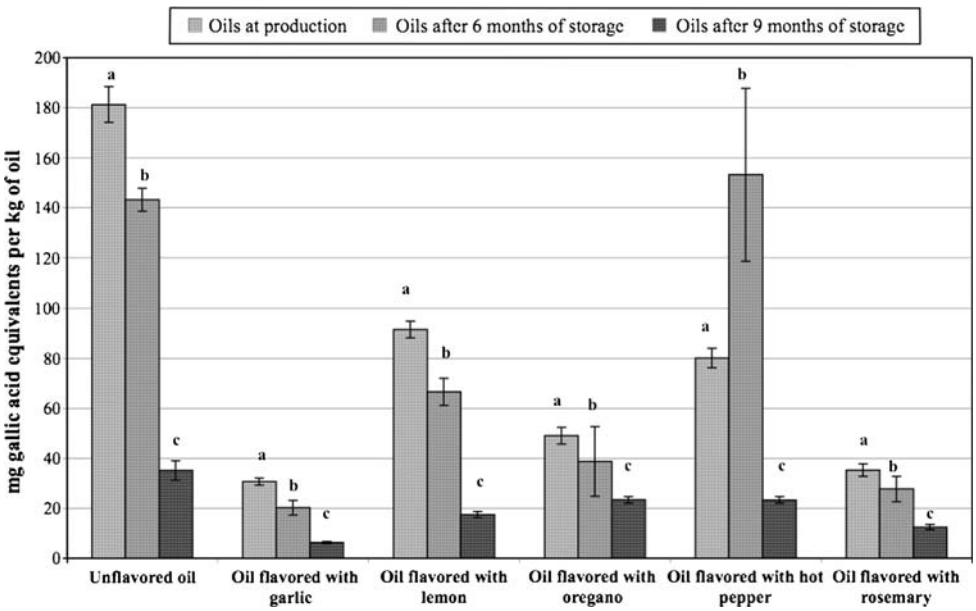

Fig. 1 Total phenolic content of unflavored and flavored oils at production, 6 and 9 months of storage. Different letters indicate significant differences ($P < 0.05$)

As showed in Fig. 1, the phenolic content of the oils flavored with hot pepper would seem to increase from production to 6 months of storage and then to decrease. Taking into account the well-known low selectivity of the Folin–Ciocalteau method, the anomalous behavior of total phenols in oils flavored with hot pepper could be due to interferences from ascorbic acid, capsaicin, reducing sugars, and other reducing compounds present in the spice composition and thus in the oils. The high standard deviation would seem to strengthen this hypothesis.

Phenolic Profiles of Oils

Figure 2 shows an example of an HPLC phenolic profile relative to the unflavored oil at production. The phenolic composition of unflavored and flavored oils at 0, 6, and

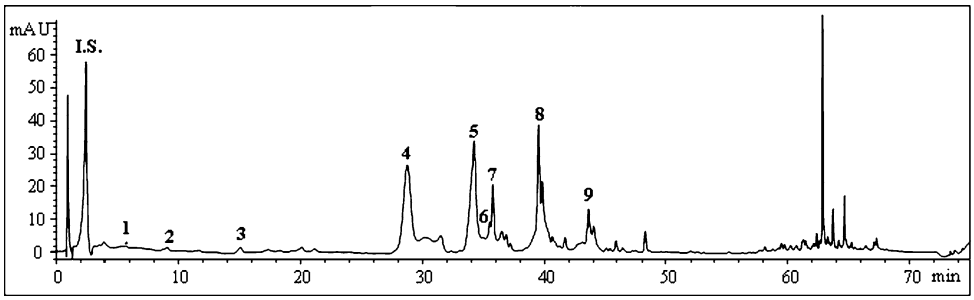

Fig. 2 Phenolic profile of unflavored oil recorded at 280 nm. I.S. internal standard, gallic acid *1* 3,4-DHPEA (3,4-(dihydroxyphenyl) ethanol or hydroxytyrosol,); *2* *p*-HPEA (*p*-(hydroxyphenyl)ethanol or tyrosol,); *3* Vanillin; *4* 3,4-DHPEA-EDA (dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol); *5* *p*-HPEA-EDA (dialdehydic form of the decarboxymethyl elenolic acid linked to tyrosol); *6* 1-acetoxypinoresinol; *7* pinoresinol; *8* 3,4-DHPEA-EA (3,4-dihydroxyphenylethanol-elenolic acid, an isomer of oleuropein aglycone); *9* *p*-HPEA-EA (*p*-4-hydroxyphenylethanol-elenolic acid, an isomer of ligstroside aglycone)

Table 2 Phenolic composition of unflavored and flavored olive oils during storage

Flavoring agents	3,4-DHPEA	*p*-HPEA	Vanillin	3,4-DHPEA-EDA	*p*-HPEA-EDA	1-Acetoxypinoresinol	Pinoresinol	3,4-DHPEA-EA	*p*-HPEA-EA
Oils at production									
Unflavored	0.16 ± 0.05	0.25 ± 0.02	0.37 ± 0.01	13.03 ± 0.41	13.13 ± 0.13	1.93 ± 0.02	2.96 ± 0.21	10.23 ± 3.16	2.50 ± 0.17
Garlic	0.95 ± 0.01	–	0.40 ± 0.01	0.80 ± 0.01	5.02 ± 0.01	0.31 ± 0.01	0.91 ± 0.01	1.06 ± 0.01	1.37 ± 0.01
Lemon	–	–	0.36 ± 0.07	9.22 ± 4.22	9.40 ± 4.43	–	3.97 ± 1.85	6.76 ± 3.64	2.37 ± 1.05
Oregano	–	–	0.15 ± 0.04	0.84 ± 0.07	1.03 ± 0.02	–	4.13 ± 0.17	1.32 ± 0.01	4.69 ± 0.06
Hot Pepper	–	0.28 ± 0.00	0.45 ± 0.10	–	10.63 ± 0.13	0.59 ± 0.02	1.89 ± 0.09	1.70 ± 0.37	3.51 ± 0.33
Rosemary	–	–	0.25 ± 0.00	0.64 ± 0.07	4.31 ± 0.16	0.28 ± 0.02	0.25 ± 0.02	0.70 ± 0.01	2.02 ± 0.02
Oils after 6 months									
Unflavored	0.90 ± 0.02	0.59 ± 0.04	0.46 ± 0.01	12.66 ± 0.78	12.55 ± 0.05	0.97 ± 0.24	1.93 ± 0.10	9.56 ± 0.14	2.88 ± 0.01
Garlic	0.75 ± 0.01	0.20 ± 0.04	0.43 ± 0.02	0.76 ± 0.04	5.29 ± 0.19	0.29 ± 0.02	0.31 ± 0.00	0.81 ± 0.11	1.36 ± 0.14
Lemon	0.47 ± 0.12	0.35 ± 0.04	0.54 ± 0.02	6.54 ± 0.13	7.28 ± 0.14	1.09 ± 0.08	1.08 ± 0.08	5.43 ± 0.04	1.56 ± 0.15
Oregano	–	0.18 ± 0.01	0.27 ± 0.03	0.92 ± 0.09	4.03 ± 0.30	0.20 ± 0.00	0.16 ± 0.06	1.09 ± 0.05	4.01 ± 0.15
Hot Pepper	0.23 ± 0.02	1.62 ± 0.00	0.34 ± 0.00	0.99 ± 0.38	6.95 ± 0.09	0.55 ± 0.03	0.49 ± 0.03	0.76 ± 0.00	1.92 ± 0.01
Rosemary	0.11 ± 0.05	0.13 ± 0.02	0.17 ± 0.00	0.40 ± 0.04	2.90 ± 0.17	0.20 ± 0.02	0.20 ± 0.03	0.48 ± 0.01	1.62 ± 0.16
Oils after 9 months									
Unflavored	1.30 ± 0.03	1.36 ± 0.13	0.10 ± 0.02	0.21 ± 0.06	0.90 ± 0.15	0.11 ± 0.01	0.14 ± 0.02	0.63 ± 0.06	0.23 ± 0.04
Garlic	–	–	0.21 ± 0.02	0.06 ± 0.08	0.70 ± 0.02	–	–	0.11 ± 0.01	0.20 ± 0.03
Lemon	0.13 ± 0.08	0.13 ± 0.02	0.10 ± 0.03	0.25 ± 0.01	0.43 ± 0.02	0.13 ± 0.06	0.10 ± 0.04	0.43 ± 0.02	0.21 ± 0.02
Oregano	0.11 ± 0.04	0.37 ± 0.03	0.27 ± 0.00	0.53 ± 0.03	2.94 ± 0.16	0.41 ± 0.04	0.33 ± 0.05	0.85 ± 0.00	3.39 ± 0.20
Hot Pepper	0.16 ± 0.00	1.38 ± 0.12	0.23 ± 0.07	0.20 ± 0.05	2.15 ± 0.04	0.36 ± 0.01	0.15 ± 0.02	0.30 ± 0.03	0.45 ± 0.07
Rosemary	–	0.13 ± 0.00	0.08 ± 0.00	0.23 ± 0.02	0.88 ± 0.09	0.38 ± 0.01	0.31 ± 0.01	0.30 ± 0.03	0.59 ± 0.08

Results are expressed as mg/kg oil

9 months is reported in Table 2. Chromatograms of the flavored oils (not shown) were quite different with respect to that of the unflavored oils due to the absence of some peaks and the presence, especially in the case of the oils flavored with oregano and lemon, of unidentified peaks eluted during the final part of the chromatographic run and probably deriving from the flavoring agents.

At production, the most abundant compounds present in the unflavored and lemon-flavored oils were 3,4-DHPEA-EDA and *p*-HPEA-EDA. *p*-HPEA-EDA was the most abundant compound in the oils flavored with hot pepper and rosemary. 3,4-DHPEA was absent from all the flavored oils with the exception of those flavored with garlic whereas *p*-HPEA showed a detectable concentration only in the unflavored and hot pepper-flavored oils.

After 6 months, 3,4-DHPEA-EDA and *p*-HPEA-EDA were still the main phenolic compounds of the unflavored and lemon-flavored oils but decreases of about 3–7% (unflavored) and 20–30% (lemon-flavored) in comparison to the initial concentration were observed. This difference appears to be well correlated with the faster increase of the K_{232} measurement detected in the lemon-flavored oils during the first 6 months of storage (Table 1).

At the end of the storage period, increases in 3,4-DHPEA and *p*-HPEA were measured in almost all the oils due to the degradation of 3,4-DHPEA-EDA and *p*-HPEA-EA (oleuropein and ligstroside aglycones). The concentrations of compounds such as 3,4-DHPEA-EDA and *p*-HPEA-EDA decreased strongly, showing the highest losses in the unflavored oils (98 and 93%, respectively) and in the lemon-flavored oils (97 and 95%, respectively).

Antioxidant Activity of the Oil Phenolic Extracts Measured According to the β-Carotene Bleaching Assay

The results detected at production and after 6 and 9 months of storage are shown in Fig. 3. At production, the percentages of the antioxidant activity coefficient were in this order: rosemary-flavored oils (79.5 ± 3.1) > unflavored and hot pepper-flavored oils > lemon and oregano-flavored oils > garlic-flavored oils (24.4 ± 5.8). During storage, the antioxidant activity significantly decreased and, after 9 months, the antioxidant activity of the rosemary-flavored oils was still the highest (51.3 ± 4.2) followed by those flavored with hot pepper (21.9 ± 7.9), lemon (17.3 ± 1.1), oregano (15.4 ± 2.4), the unflavored oil (13.9 ± 1.4), and the garlic-flavored ones (8.5 ± 0.7).

The normal decreasing trend of the antioxidant activity (measured according to the β-carotene bleaching assay) of the phenolic extracts of hot pepper-flavored oils strengthens the aforementioned hypothesis of the interference of reducing compounds on the phenolic content analysis.

Though the low phenolic content and antioxidant activity value (measured according to the β-carotene bleaching assay) of garlic flavored oils, they showed the lowest values of primary and secondary oxidation. This could be due to a non-phenolic compound named allicin that was found to be able to scavenge hydroxyl radicals (·OH) [32]. The existence of a linear correlation between total phenolic content and antioxidant activity measured by the β-carotene bleaching assay was checked and the resulting *R* value (0.386) demonstrated a low contribution

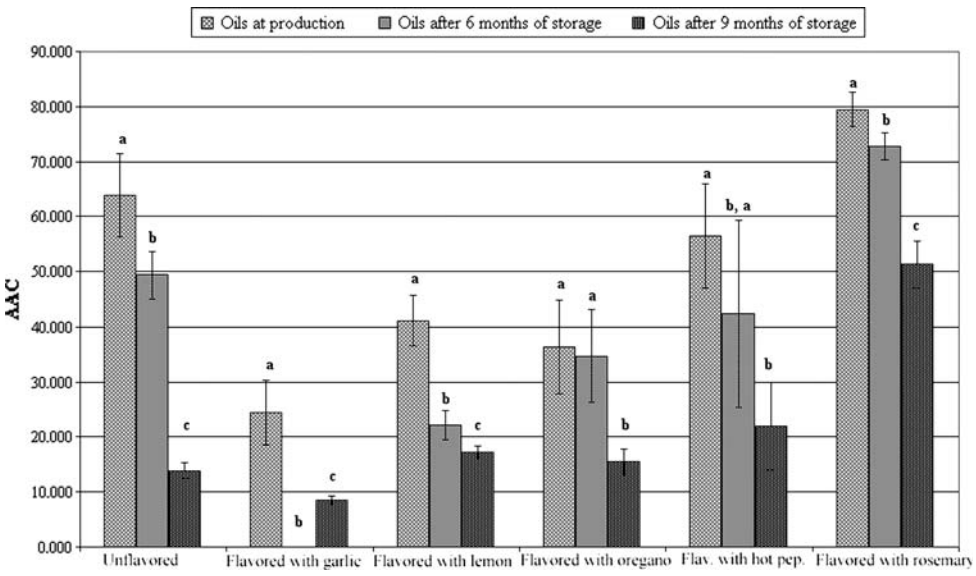

Fig. 3 Antioxidant activity (AAC), measured according to the β-carotene bleaching assay, of unflavored and flavored oils at production, 6 and 9 months of storage. Different letters indicate significant differences ($P < 0.05$)

of phenolics to the ability of the flavored oils in preventing the oxidation of a lipidic substrate. Different findings have been highlighted by previous researches performed on unflavored olive oils [33, 34]. Probably, the ability of the flavored oils in preventing the oxidation of a lipidic substrate is mainly due to compounds different from phenolics. Furthermore, the antioxidant activity depends not only on the phenolic concentration, but also on polarity (lipid oxidation in an oil-in-water emulsion increases with decreasing antioxidant polarity) and the specific chemical structure of each phenolic compound (degree of hydroxylation and extent of conjugation) and some works in the literature report examples of hierarchies for antioxidant activity and reduction potential of phenols [35]. This matter could also explain the contrast between the high antioxidant activity and the low total phenolic content of rosemary-flavored oils. In fact, according to the literature [36], the high antioxidant activity and capacity of rosemary extracts are due to the presence of high concentrations of carnosic acid, carnosol and a number of unidentified phenolics among which synergism occurs. Carnosic acid and carnosol are less reactive compounds due to their low polarity and low number (two) of hydroxyl groups but, as it is well known, the less reactive phenols regenerate the more active ones so increasing the antioxidant power. Furthermore, due to their low polarity, carnosic acid and carnosol are located at the water/oil interface where they inactivate free radicals, avoiding hydroperoxide breakdown and so preventing further degradation to more active oxidizing forms. In fact, as previously described for rosemary-flavored oils, though the indices of primary oxidation (P.V. and K_{232}) increased after 6 months of storage, K_{270} which measures the secondary oxidation (hydroperoxide degradation) remained unchanged.

Conclusions

During 9 months of storage, only the unflavored oils and those flavored with garlic showed all the chemical parameters within the limits fixed for extra-virgin olive oils. The phenolic content and profiles of the considered flavored oils depended to a great extent on the flavoring substance added. During the whole storage time, the highest phenolic content was measured in the unflavored oils whereas the lowest content was detected in the extract of the oils flavored with garlic. After 9 months of storage, the phenolic loss ranged from about 81% in the unflavored oils to about 53% of the oils flavored with oregano. Noticeable differences were found among the phenolic profiles of unflavored and flavored oils. During storage, increases in 3,4-DHPEA and *p*-HPEA were measured in almost all the oils due to the occurring of oxidation reactions. According to the β-carotene bleaching assay, the rosemary-flavored oils showed the highest value of antioxidant activity during the whole storage period whereas the lowest antioxidant activity coefficients were shown by the oils flavored with garlic. The loss of antioxidant activity ranged from 79% in the unflavored oils to 36% in the oils flavored with rosemary. The correlation coefficient between phenolic content and antioxidant activity of the flavored oils was very low indicating that (a) the antioxidant activity could depend on the specific chemical structure of each phenolic compound and, thus, on the concentration of each of them (b) the prevention of lipid oxidation could be due mainly to compounds different from phenolics.

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