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The freezing process helps to preserve the quality of extra virgin olive oil over time: A case study up to 18 months

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ABSTRACT

The effects of a long storage period on some Tuscan EVOOs filtered and frozen at −23 °C in comparison with the same specimens maintained at room temperature in the dark, were evaluated by monitoring the evolution of their phenolic composition and aromatic profile.The oils were analyzed immediately after opening and after 10 days up to 18 months after oil bottling. A non parametric statistical analysis and principal components analysis (PCA) coupled to linear discriminant analysis (LDA) was applied for assessing the differences among the trials.

Increments in tyrosol, hydroxytyrosol and % of hydrolysis were observed for EVOOs stored at room temperatures starting from 3-months storage, and increased thereafter. The frozen EVOOs were statistically undistinguishable over time, differently from the correspondent ones at room temperature. All the frozen EVOOs showed negligible differences in aromatic profile until 12 month of storage. Some compounds potentially related to off-odor sensations were significantly increased in the unfrozen specimens only at 18 months.

1. Introduction

Extra Virgin Olive Oil (EVOO) is obtained from freshly pressed fruits of *Olea europaea* and can be considered like "a natural juice". The typical taste and flavor of EVOO results from molecules either originally present in cell oleosomes or formed during the oil making process. EVOO is well known as one of the few oils with high resistance to oxidative deterioration. As assessed, the length of the lag-phase before the production of peroxides during the oil autoxidation is strongly related to the fatty acid composition, but in the EVOOs the minor components, including phenols, play an important protective role. It is now recognized that EVOO is the only oil for human consumption which naturally contains appreciable amounts of minor polar phenolic compounds, largely responsible for oil stability with respect to auto and thermo-oxidation.

This complex fraction is constituted by simple phenols, such as tyrosol and hydroxytyrosol, cinnamic acids, flavonoids in trace amounts, as well as secoiridoidic derivatives of oleuropein and ligstroside, (Mateos et al., 2001; Montedoro, Servili, Baldioli, & Miniati, 1992; Romani et al., 2001). The presence of lignans, (+)-1-acetoxypinoresinol and (+)-pinoresinol, has been also highlighted within the phenolic fraction of Spanish (Brenez, Garcia, Garcia, Rios, & Garrido, 1999; Gomez-Alonso, Salvador, & Fregatane, 2002; Mateos et al., 2001) and of Italian EVOOs (Oliveras-Lopez et al., 2007; Servili et al., 2007).

The phenolic content in EVOOs is, in turn, strongly affected by several factors, such as the cultivar and the ripening stage (Kalua, Allen, Bedgood, Bishop, & Prenzler, 2005), the milling process (Amirante et al., 2002; Salvador, Arand, Gomez-Alonso, & Fregapane, 2003; Servili et al., 2004), the presence of CO_2 during malaxation (Parenti, Spugnoli, Masella, Calamai, & Pantani, 2006), the olive stoning before milling (Mulinacci et al., 2005; Servili et al., 2007). To date, the phenolic compounds are recognized of paramount importance for the EVOOs quality and conservation because of their antioxidant properties (Bendini et al., 2007; Servili et al., 2004). Several scientific evidences pointed out a beneficial effect of EVOOs rich in phenolic constituents (Beauchamp et al., 2005; Bendini et al., 2007) particularly on the cardiovascular system (Covas, 2007) with an higher activity for those molecules containing O-diphenolic group, i.e. hydroxytyrosol and oleuropein derivatives (Visioli, Poli, & Galli, 2002).

EVOO is generally consumed within 1 year from its production, but the current legislation (RIS-2/8-IV/98 COI/T.15/NC n 2/Rev. 8, del 25/

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11/98), allow the commercialization within 18 months since the bottling date. It is well known that the overall quality and the phenolic content of EVOOs, even when properly packaged, decrease during storage at room temperature (Gomez-Alonso, Mancebo-Campos, Desamparados Salvador, & Fregapane, 2007; Kalua, Bedgood, Bishop, & Prenzler, 2006; Tsimidou, Georgiu, Koidis, & Boskou, 2005). It was also demonstrated that the degradation rate of the phenolic compounds during aging is strongly related to their initial concentration. Two EVOOs, analyzed up to 18 months of conservation at room temperature, showed the highest decrease in secoiridoids (close to 50%) in the sample with the lowest initial amount, while in the richest samples the decrease was below 20% (Romani et al., 2007).

Even at low temperature, e.g. at 1 °C for 12 months without headspace, a decreasing of the phenolic content was pointed out (Kalua et al., 2006). Recently it has been demonstrated that olive oil control materials stored at 0–8 °C, showed unchanged peroxide value, acidity, waxes and delta-K until 24 months (Bosque-Sendra et al., 2011). Nevertheless the authors did not report data related to the volatile constituents and the phenolic compounds.

To the best of the authors' knowledge, no article has been published to date on evaluating the changes of a long-term freezing, up to 18 months, on the EVOOs.

The aim of this study was to evaluate the effects of storage period at −23 °C on two groups of EVOOs up to 9 and 18 months, respectively, mainly by monitoring their phenolic composition and aromatic profile in comparison with the same oils maintained at room temperature. All the selected samples were filtered, and maintained in dark (at least for the second group of oils) or frozen for comparison and analyzed periodically. With the aim of simulating a domestic use, for each sampling time the analyses were done on the specimens immediately after thawing and 10 days after bottles opening. The phenolic content was determined by an HPLC/DAD/MS method, the aromatic fraction was determined by SPME-GC–MS and all the data were statistically evaluated.

2. Material and methods

2.1. Pilot experiment

A preliminary experiment was set up selecting 6 clear Tuscan EVOOs (named 1–6), all collected in 2006 and filtered directly by the producers (Table 1). The original bottles of all specimens (750 mL) were frozen directly on 30 January 2007 or stored in the dark at room temperature for comparison. An aliquot of each sample was analyzed after 9 months from the date of freezing and compared with the corresponding oil at room temperature.

Table 1
Analytical parameters evaluated for frozen (F) and unfrozen (U) samples after 9 months of storage (pilot experiment).

Oils	Peroxide value (meq O_2 kg^{-1})	% of 3,4-DHPE-DEA on Total Phenols	% of Hydrolysis	% of Hydroxytyrosol on 3,4 DHPE-DEA
1-U	14.54 ± 0.6	39.4 ± 2.0	3.7 ± 0.1	4.5 ± 0.1
1-F	5.93 ± 0.3	30.7 ± 1.7	3.7 ± 0.2	4.3 ± 0.2
2-U	21.21 ± 0.7	28.4 ± 0.8	3.8 ± 0.1	7.8 ± 0.2
2-F	6.95 ± 0.2	23.9 ± 0.8	1.8 ± 0.1	2.4 ± 0.1
3-U	11.52 ± 0.6	32.7 ± 1.1	3.3 ± 0.2	7.5 ± 0.3
3-F	5.08 ± 0.2	28.7 ± 1.4	1.4 ± 0.1	1.6 ± 0.0
4-U	13.5 ± 0.4	9.4 ± 0.4	1.6 ± 0.0	14.7 ± 0.7
4-F	8.67 ± 0.4	6.3 ± 0.3	0.6 ± 0.0	4.6 ± 0.1
5-U	15.9 ± 0.7	38 ± 1.0	5.9 ± 0.3	10.6 ± 0.6
5-F	7.55 ± 0.4	35.9 ± 1.0	5.6 ± 0.3	9.2 ± 0.3
6-U	17.54 ± 0.8	18.6 ± 0.5	6.0 ± 0.2	19.9 ± 0.9
6-F	8.67 ± 0.4	16.3 ± 0.6	4.7 ± 0.1	8.0 ± 0.4

2.2. Final experiment

Following the indications obtained after the pilot experiment a larger experiment was carried out the following year. Three limpid Tuscan EVOOs (named CA, SA, SD throughout the paper), collected in 2007 and filtered directly by the producers were selected for this experiment. These oils were bottled and frozen at −23 °C on 28 January 2008.

For each EVOO 24 identical brown glass 50 mL bottles (conversely to the pilot study where large different 750 mL bottles were used) were filled to the neck, to a minimum headspace volume (close to 2 mL). For each EVOO the sample lot was divided in two equal subsets one of which was stored in a freezer at −23 °C, and the other at room temperature in the dark. At time intervals of 0, 3, 6, 12, and 18 months two bottles for each sample, either stored at room temperature or frozen, were randomly selected from each subset, one of which was immediately analyzed and the other was opened, half of the oil was discarded and the remaining part was analyzed after 10 days from opening (at room temperature) to simulate the decay occurring in house held bottles during home consumption. At each sampling time the phenolic composition together with the % of hydrolysis defined as (Tyrosol + Hydroxytyrosol)/total phenols) × 100, was determined by HPLC/DAD. Moreover, the acidity (expressed as % oleic acid), the peroxide value (expressed in meq O_2 kg^{-1} oil by the CDR instrumentation — see next paragraph) and volatile compound profile (by head-space solid phase microextraction-gas chromatography–mass spectrometry, HS-SPME-GC–MS) were also determined.

2.3. Reference compounds

The following commercial compounds were used: tyrosol (Tyr), oleuropein, luteolin, and apigenin, were purchased from Extrasynthèse (Genay, France). The analytical standards and internal standards for the quantification of the volatile fraction were purchased by Sigma-Aldrich.

2.4. Extraction and quantification of the Minor Polar Compounds (MPC)

Twenty milliliters of each oil sample were extracted with 60 mL of ethanol/acidic water (70:30 v/v) as previously described (Oliveras-Lopez et al., 2007). Briefly, the raw alcoholic extract of each sample was brought to dryness under reduced pressure, redissolved in 2 mL of the extraction solvent, and analyzed by HPLC/DAD/MS.

The quantitative evaluation of the MPC was performed by HPLC/DAD through the use of authentic standards, tyrosol, oleuropein, luteolin 7-*O*-glucoside and apigenin aglycone, which were used to prepare five-point regression lines ($R^2 \geq 0.999$). Tyrosol, hydroxytyrosol, and lignans concentrations were calculated at 280 nm using tyrosol as the reference. The flavonoids were evaluated at 350 nm and using luteolin 7-O-glucoside as external standard. The secoiridoids were calculated at 280 nm using oleuropein as the reference; elenolic acid and elenolic acid derivatives were evaluated at 240 nm using oleuropein as the reference at the same wavelength. For some of these compounds a correction of the molecular weight was applied as previously reported (Mulinacci et al., 2005). All the samples were analyzed in triplicate and the standard deviation was <5% for peroxide and % of hydrolysis; <6% for the others parameters.

2.5. HPLC/DAD/MS analysis

All reagents used were of analytical grade. Phenolic compounds were identified by comparison with pure standards, UV spectra, relative t_R and mass spectra in ESI.

Analysis of the MPC extract was performed on an Agilent 1100 Liquid Chromatograph equipped with a 1100 autosampler, column heater module, binary pump and DAD; the MS detector was a HP 1100 MSD API-electrospray, all from Agilent Tech. (Palo Alto, AC,

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USA). The Luna RP18 (Phenomenex-Torrance CA) column, 250 × 4.6 mm (5 μm), was used to analyze all the phenolic compounds, with the exception of deacetoxyoleuropein aglycon (3,4 DHPE-EDA), and elenolic acid (EA). These latter compounds were separated by the use of a LiChrosorb RP18 (Merk Hibar) column, 250 × 4.6 mm (5 μm), according to the method previously described (Mulinacci et al., 2005). For both columns the eluents were H_2O at pH 3.2 by formic acid (A) and acetonitrile (B). The analyses carried out on the Luna column were executed applying a multistep linear solvent gradient as follow: from A 100% to A 85% in 5 min; 10 min to A 70% then a plateau of 5 min to A 65% in 5 min and a plateau of the same time; 7 min to A 55% and a plateau for 5 min to B 100% within 5 min, and a final plateau of 3 min. Total time of analysis 50 min, equilibration time 20 min, oven temperature 26 °C; flow rate 0.8 mL min^{-1}.

The operative conditions of the MS detector were: capillary voltage from 3000 to 3500 V, working in negative ionization, with a variable fragmentor potential in the range of 80–150 V.

2.6. Volatile profile by HS-SPME-GC–MS analysis

The volatile compound profile was analyzed by HS-SPME–GC–MS technique. An Agilent 7890 GC-Chromatograph equipped with a 5975A MSD with EI ionization was employed. A 3 g aliquot of each EVOO sample was weighed in 10 ml screw cap head space vials, supplemented with 20 μl of an internal standard mixture (ISTD mix) and immediately sealed. A Triphase Carboxen/PDMS/DVB 65 μm SPME fiber, (Supelco, USA) was exposed in the head space (HS) of the vials at 60 °C for 30 min sampling after a five-minutes equilibration time. A Gerstel MPS2 XL autosampler equipped with magnetic transportation adapter and thermostated agitated tray was used for ensuring consistent SPME extraction conditions.

The chromatographic settings were as follows: injector in split less mode set at 260 °C, J&W innovax column (30 m, 0.25 mm i.d., 0.5 μm df); oven temperature program: initial temperature 40 °C for 1 min, then 2 °C min^{-1} until 80 °C, then 5 °C min^{-1} until 150 °C, then 10 °C min^{-1} until 220 °C, then 30 °C min^{-1} until 260 °C, hold time 5 min.

MS detector was operated in scan mode in the m/z range 29–330, at three scans s^{-1}. Since the response of SPME fibers varies depending upon wearing and sample complexity, the addition of suitable internal standards (ISTDs) to the samples prior to analysis was necessary. An ISTD mix were selected on the basis of chemical similarity to the compounds present in the EVOOs, and contained 2-methyl undecanal, ethyl acetate-d_8, ethyl hexanoate-d_{11}, acetic acid-d_3, hexanoic acid-d_{11}, 5-methylhexanol. These compounds were dissolved into isooctane, stored as solutions at −80 °C, and added to either samples or calibration standards before the analysis, then vortexed for 1 min for homogenization.

Calibration lines (1st order) were constructed for each analyte in ranges including the concentration present in EVOO samples, and referred to the most suitable ISTD. Deodorized corn oil was used as a matrix for constructing the calibration lines.

2.7. Acidity and peroxide value

The acidity and the peroxides values (PV) of the oils were evaluated by the Oxitester ® instrument (CDR SrL, Florence-Italy). The applied peroxide test is in agreement with the Reg. CEE N. 2568/91 and the data are expressed as mEq O_2 kg^{-1}. To measure at the end point the obtained Fe3+ ions as a red complex at 505 nm, 5 μL of EVOO were used.

The acidity values were determined on 2.5 μL aliquots of EVOO added to an alcoholic solution of KOH and phenolphthalein derivative. The free fatty acids react with a chromogenic reagent, the measure is carried out at 630 nm, the acidity is expressed as % of oleic acid. This method was in agreement with the reference method ISO 660.

2.8. Statistical analyses

Statistical analyses were performed with the SYSTAT 12.0 software (Systat Software Inc., Richmond, California, USA). For the data of phenolic compounds non parametric Friedman and Kruskal–Wallis tests were used for sample comparison at each sampling time. In addition, principal component analysis, PCA (for constructing latent variables) and linear discriminant analysis, LDA on the constructed latent variables were applied for sample supervised classification. A variable number of principal components was selected for LDA i.e. the number of PCs before the first flex in the scree plot (see also supporting information, Fig. A1).

3. Results and discussion

The physical state changing of the EVOO resulting from temperature variation could affect its resistance to oxidation processes. In this context the little amount of emulsified water, typical of veiled oils, can play a crucial role. In these oils, the phenolic constituents remains partially solubilized in the lipid phase, while another part is only suspended in the oil and can precipitate during freezing–thawing steps. This phenomenon can reduce the oxidative resistance particularly of those unfiltered EVOOs mainly related to a non homogeneous distribution of its antioxidant compounds within the sample (Cerretani, Bendini, Biguzzi, Lercker, & Toschi, 2005; Lerker, Frega, Bocci, & Servidio, 1994). Recent reviews focused on the filtration systems applied to EVOOs (Fregapane, Lavelli, León, Kapuralin, & Salvador, 2006; Lozano-Sánchez, Cerretani, Bendini, Segura-Carretero, & Fernández-Gutiérrez, 2010), highlighted that, albeit this process removes part of the phenolic compounds located within the water droplets in the oil, at the same time, it reduces the hydrolysis of the secoiridoids during the storage. In light of the above mentioned aspects and in order to avoid inconsistencies or in homogeneity during defrosting induced by the residual water, only EVOOs directly filtered by the producers were selected for this research in which a preliminary experiment was done before to develop a successive more controlled study.

The analysis of the volatile compounds was done only on the samples of the final experiment.

3.1. Pilot experiment

Six Tuscan filtered oils were selected to investigate the effect of the freezing on EVOO quality, in a pilot experiment where all the specimens were maintained over time, in their original commercial bottles, in dark.

The main results obtained by the comparison of frozen (and unfrozen oils after 9 months) highlighted that the peroxides remained below 9 meq O_2 kg^{-1} in all the frozen oils, while up to 12.5–21.2 meq O_2 kg^{-1} were recorded in the correspondent samples at room temperature (Table 1).

As expected, the acidity values were not affected by freezing and no changes occurred for the classes of lignans and flavonoids (data not shown). Nevertheless, increments of tyrosol, hydroxytyrosol and elenolic acid, ranging between 30% to over 64%, were pointed out in the specimens at room temperature after 9 months (data not shown). These molecules, mainly produced from the hydrolysis of the secoiridoids over time, are responsible of the higher values observed for the % of hydrolysis ((Tyrosol + Hydroxytyrosol)/total phenols) ×100) in unfrozen oils (Table 1).

Unexpectedly, the 3,4-DHPE-DEA, one of the main constituent in fresh EVOOs of higher quality, increased in the EVOOs at room temperature with respect to the corresponding frozen ones. At the same time the % of Hydroxytyrosol/3,4-DPE-EA and the % of hydrolysis increased in all the unfrozen oils, with the only exception of oil 1, confirmed an higher hydrolysis of the secoiridoids at room temperature associated to an accelerated aging process. Consequently a better quality of the

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corresponding frozen oils was highlighted. To confirm these preliminary results, a final experiment was designed applying more controlled experimental conditions (the same dark bottles, with the same headspace volume and caps for all the oils) and longer storage time.

3.2. Final experiment

The three EVOOs selected for this experiment were initially characterized for their total phenolic content with 217,3 mg L^{-1} for CA, 349,7 mg L^{-1} for ST and 407 mg L^{-1} for SD. The peroxides were below 15 meq O_2 kg^{-1} (14.2 meq O_2 kg^{-1} for CA; 10.5 meq O_2 kg^{-1} for ST and 11.3 meq O_2 kg^{-1} for SD) and acidity below 0.35% (0.34 for CA; 0.2 for SD and 0.22 for ST).

The LDA score plot at time 0 on PCA latent variables was constructed with the data of phenolic composition, % of hydrolysis, peroxide and acidity values of the three EVOOs. The PC1 and the PC2 have been able to explain more than 75% of the total variance (see Fig. A1 in additional file) and the LDA score plot of Fig. 1 showed an evident and significant clustering by sample type.

Therefore, the samples were considered enough representative of typical Tuscan EVOOs and suitable to carry out the study over time.

Differently from the pilot experiment, also the volatile constituents were determined for each sampling time. A pairwise comparison of the EVOOs under trial for phenolic and volatile components, immediately after opening and 10 days after bottle opening was done.

For each of the selected oils at time 0 and 10 days after opening, no significant differences after Kruskal–Wallis non parametric test were pointed out for tyrosol, hydroxytyrosol, % of hydrolysis, and elenolic acid at all sampling times. Consequently, for these parameters these samples were considered as replicates for the further statistical elaborations. Conversely, statistically significant differences were found between 0 and 10 days after opening for several secoiridoidic compounds and peroxide values and therefore, these parameters were analyzed separately for the above mentioned variables. In the successive paragraphs are shown and discussed the main differences highlighted over time for the volatile and phenolic fraction.

3.2.1. Phenolic fraction

In regards to the phenolic compounds and in agreement with the findings of the pilot experiment, significant differences already at 3 months (with only the exception of CA oil), and more pronounced starting from 6 months up to 18 months were highlighted for the oils at room temperature (Fig. 2). The hydroxytyrosol amounts were from 1.8 to 5.2 mg L^{-1} for CA, from 2.9 to 11.2 mg L^{-1} for SD and from 3.3 to 8.1 mg L^{-1} for ST. An analogous behaviour was observed also for the Tyr in CA and SD samples, with the exception of ST oil at room temperature where no increment was observed. The concentration of the elenolic acid increased in the unfrozen oil in a similar way. As expected by the previous data, the % of hydrolysis increased over time in all the unfrozen oils showing a similar trend (from 3.53 to 7.62% for CA, from 1.81 to 5.82 for SD and from 2.77 to 5.84 for ST). On the opposite, these parameters remained almost unaltered, for the correspondent frozen samples while the lignans, expressed as sum of acetoxypinoresinol and pinoresinol, remained unaltered over time in both the frozen and unfrozen oils (Fig. 2).

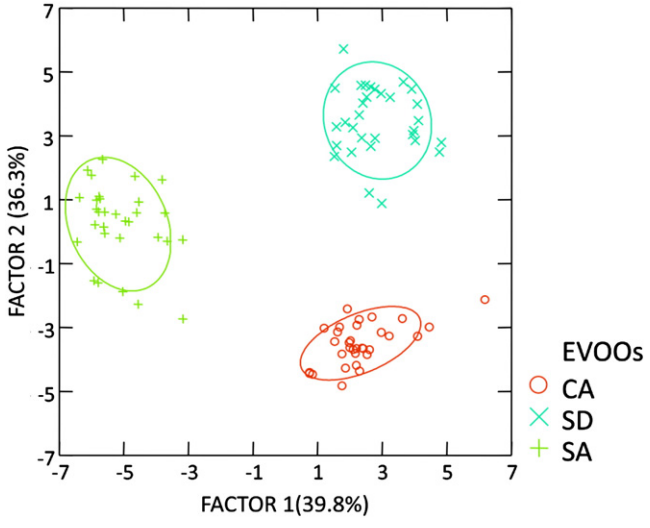

Fig. 1. Score plots from PCA analysis (on all data except the volatile components) of the EVOOs at time 0 (final experiment).

Even if the hydroxytyrosol is widely known as a potent bioactive compound for human health and several food supplements containing high content of this phenol are nowadays on the market, paradoxically low concentrations of this molecule are desirable in the EVOOs from a quality standpoint. In fact, it is widely recognized that the simple phenols, tyrosol and hydroxytyrosol, increase over time due to hydrolytic processes of the secoiridoidic derivatives representing their linked forms. It has been verified that % of hydrolysis, defined as [(Tyrosol + Hydroxytyrosol)/total phenols] × 100, typically increases in aged EVOOs therefore the quality of the product must be also correlated to lower values of this parameter (Brenes, Garcia, Garcia, & Garrido, 2001). The frozen EVOOs analyzed in both the pilot and final experiment, showed significantly lower values for this parameter when compared with those measured for the corresponding samples at room temperature (Table 1 and Fig. 2).

Unexpected results, even if in agreement with the findings of the pilot experiment (Fig. 3), were the higher amounts of 3,4-DHPE-EDA, oleochantal and oleuropein aglycone observed in the EVOOs at room temperature (Fig. 4). This class of molecules is constituted by different isomeric and isobaric forms particularly related to mono and dialdehydic derivatives and to open and cyclic forms (Servili et al., 2004) all characterized by a low stability. The co-presence of numerous and very similar isomers is a determinant factor for explaining the not complete chromatographic resolution of these components observed in several published methods even if different methodological approaches have been suggested to overcame this problem. To date, all the proposed HPLC methods are not able to completely distinguish the various secoiridoidic isomers and also the coupling with the mass detector is not resolving due to the numerous isobaric forms. In the oil these molecules become to successive transformations, i.e. hydrolysis of the secoiridoidic compounds, leading also to an increase of the tyrosol and hydroxytyrosol amounts during the oil aging. Furthermore, it is well known to the researchers working on this matter that the unavailability of commercial standards for these phenolic compounds influences negatively their quantitative estimation.

Both in the pilot and in the final experiment apparent higher secoiridoidic amounts in the unfrozen oils with respect to the corresponding frozen samples were observed (Figs. 3 and 4).

It can be hypothesized that the equilibrium among these secoiridoids within the oil is temperature dependent and the relative amount of the isobaric forms are different in the frozen and unfrozen oil. This different equilibrium can affect the measured absorbance values at 280 nm, the wavelength widely used in all the proposed HPLC/DAD methods for their determination. Furthermore, no data are available, on the molar extinction coefficients at 280 nm for the isobaric derivatives (open and close forms) of oleuropein aglycone and its deacetoxy isobaric forms. The same is true also for the ligstroside aglycone and its derivatives such as oleochantal. In addition, the Extract Ion technique at m/z 539 Th, allowed to exclude the presence of oleuropein in all the oils of pilot and final experiments, ruling out the possibility that the observed increments of 3,4DHPE-EDA or of oleuropein aglycone in the frozen oils can be related to the hydrolysis of the original glycoside.

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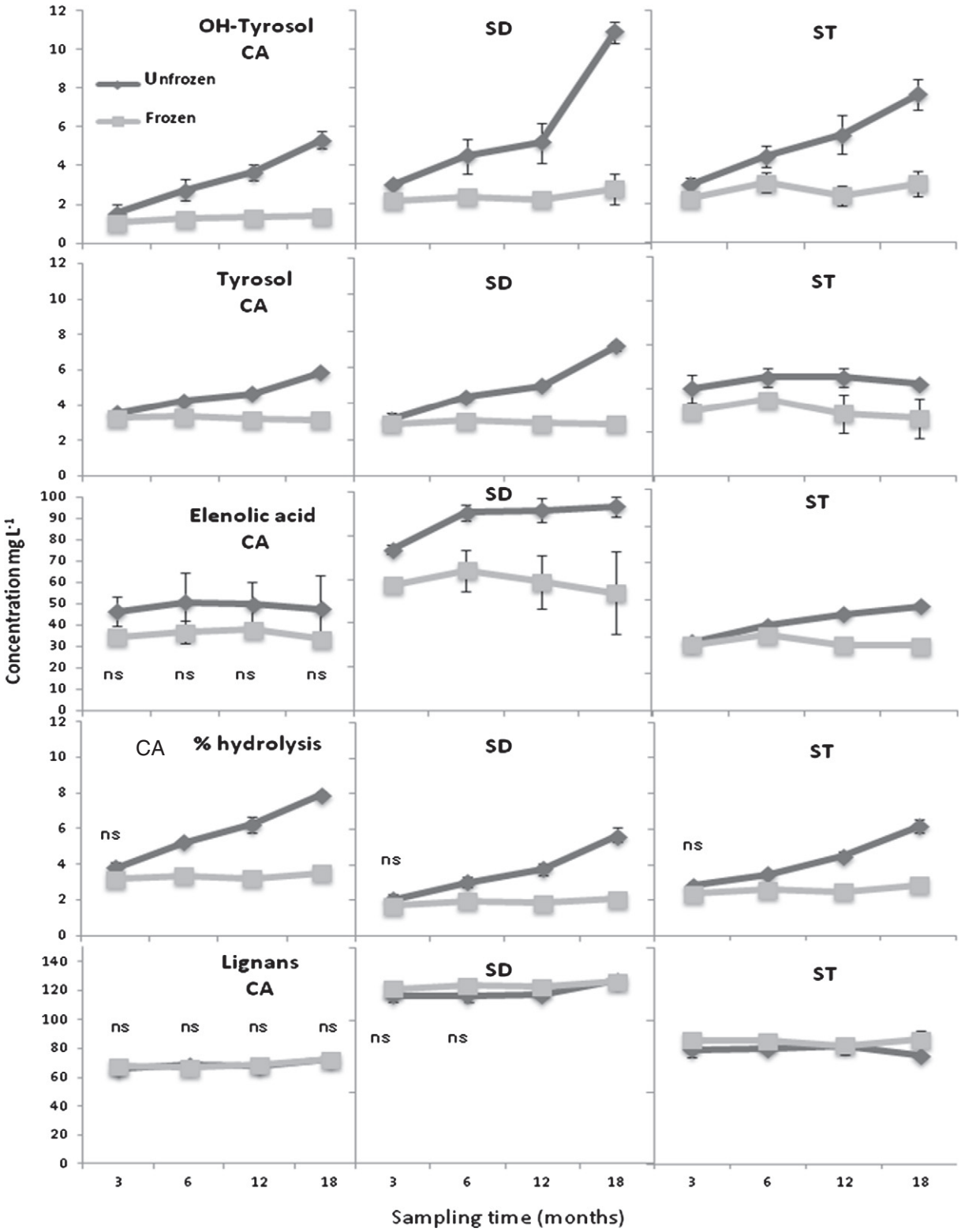

Fig. 2. Distribution over time (3–18 months) of tyrosol, hydroxytyrosol, EA, lignans and % of hydrolysis for the three EVOOs analyzed immediately upon opening. Bars represent means ± S.E. of measurements; bars not visible indicate S.E. smaller than the symbol. When not indicated the differences between frozen and unfrozen samples at the respective times were significant (Mann–Whitney U test, $p < 0.05$).

The PCA analysis indicated that almost 80% of the total variance is explained by the first 3 PC (see additional files — Fig. A_1 and A_2). In this case, by the time that each principal component is orthogonal to the others in the direction of maximal covariance, several principal components are required to account at least 80% variance of the dataset. Therefore the scores of these PCs were used for LDA Analysis. The factor loadings on these latent variables indicated that the secoiridoidic compounds were the most important in clustering (high values on PC1) while the simple phenols and % of hydrolysis is important in PC2. These results essentially confirm what evidenced

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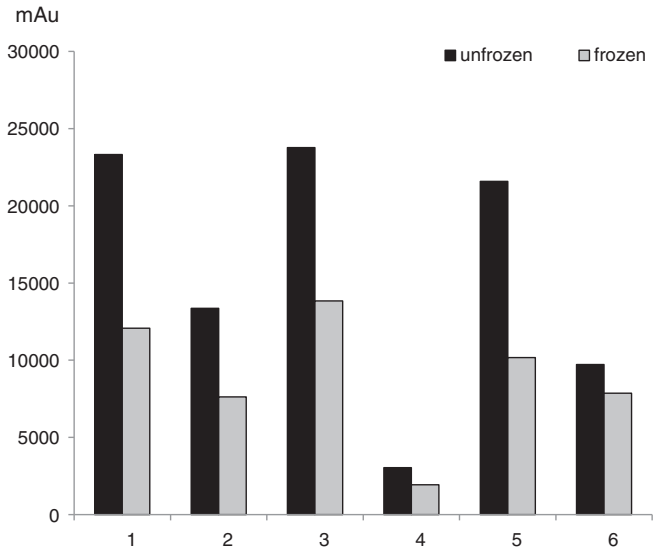

Fig. 3. Pilot experiment: a pairwise comparison of 3,4DHPE-EDA after 9 months in frozen and unfrozen oils. Data are expressed as area values in mAu of absorbance and, the numbers 1–6 are related to the EVOOs (see also Table 1).

by the pair wise comparison of Figs. 2 and 4, while the analytical parameters not related to the phenolic fraction (e.g. acidity and peroxide value) seem to play a minor role in clustering.

The PCA-LDA approach to the entire chemical analytical dataset evidenced a clustering starting from 3 months storage in the EVOOs maintained at room temperature while no cluster separation was observed for the corresponding frozen ones (see additional files — Fig. A2 and A3). The leave-one-out (Jackknifed) classification performed upon dataset re-sampling (see additional files — Table A2) indicated that a correct reattribution of the samples at their time cluster was obtained for the frozen samples only at 18 months storage, while for the EVOOs stored at room temperature as many as 88% of the samples were correctly reattributed already after 3-month storage. These evidences indicate that a delayed ageing occurred in the frozen EVOOs.

3.2.2. EVOOs 10 days after thawing and bottle opening

Main objective was to verify if the EVOOs after thawing underwent to a more rapid degradation compared with the unfrozen oils. This part of the study was done simulating a domestic use of an EVOO after thawing and 10 days have been considered as the required average time to completely consume one small oil bottle.

For almost all the frozen EVOOs at 18 months, increments of 3,4-DHPE-EDA, oleuropein aglycone and oleochantal were highlighted 10 days after opening (Fig. 4). The observed increments of those secoiridoids usually abundant in the fresh oils were an unusual result and it can be defined as an apparent increment. Analogously to what discussed for the data shown Figs. 3 and 4 on frozen and unfrozen oils immediately after bottle opening, also in this case different ratios among the co-eluting isobaric forms of the secoiridoid compounds can be hypothesize with a consequent variations of absorbance values at 280 nm. Moreover it is well known that the presence of higher amount of oxygen can accelerate the oxidation of the phenolic compounds in the EVOO and consequently it is impossible to obtain a real increment in the concentration of the secoiridoids under these experimental condition. On the other hand, the statistically significant differences between 0 and 10 days after opening, observed for the peroxide values both in frozen and unfrozen oils confirms as expected, an increment of the primary products of autoxidation being well known that higher amount of oxygen in the headspace of the bottle accelerate this process.

However, the differences among the oils stored at room temperature and those frozen, are still largely significant for tyrosol, hydroxytyrosol and % of hydrolysis, in agreement with previously observed findings (Fig. 2), indicating a far superior quality of EVOOs stored at −23 °C. In addition, it must be noted, that PCA-LDA approach showed that the little differences between bottles immediately opened and analyzed after 10 days, were significant only for the frozen samples.

3.2.3. Volatile fraction

It is well known that the volatile fraction influences the organoleptic quality of the extra virgin olive oil and some of these components, can be related to positive and negative sensations (Angerosa et al., 2004; Flamini, Coini, & Morelli, 2003), and therefore the analysis of the volatile fraction was carried out on the oils of the final experiment.

The complete list of all the identified compounds for frozen/unfrozen EVOOs is reported in Table 2. High quality olive oils have a profile of volatile compounds – mainly constituted of aldehydes, esters, alcohols and ketones – that generates a balanced flavor of green and fruity sensory characteristics (Aparicio & Morales, 1998). Some of these molecules confer positive odor connotations and are typical of high quality EVOOS while others (e.g. E,E 2,4-heptadienal, nonanal, E 2-decenal, E 2-undecenal) are potentially associated with off odor sensations and organoleptic defects when present in elevated concentrations. A recent review of the most compounds commonly found in rancid olive oil along with their olfactory sensations and thresholds are published by Morales, Luna, and Aparicio (2005).

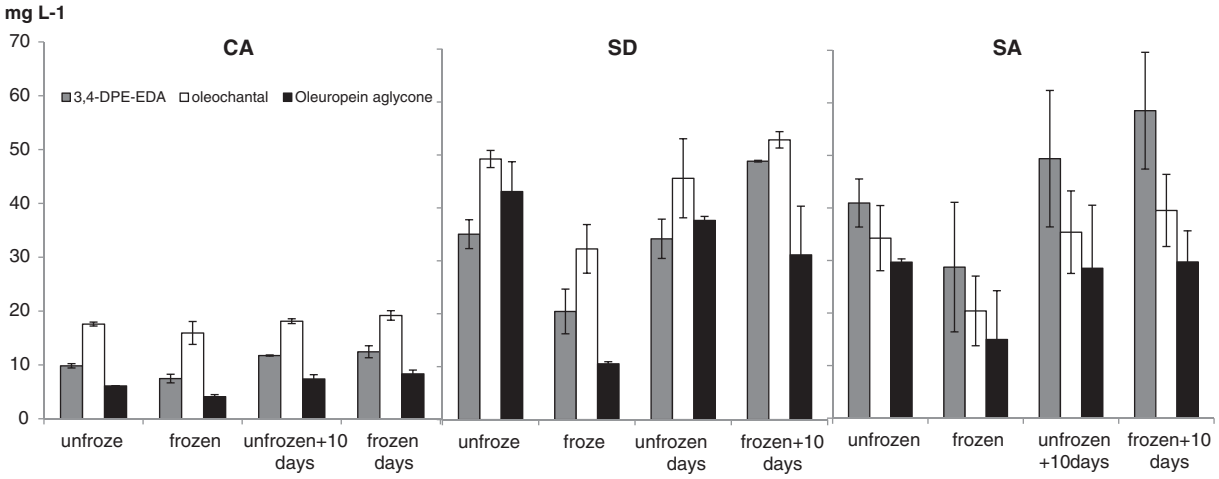

Fig. 4. Final experiment at 18 months, samples analyzed immediately and 10 days after opening bottle; data are expressed as mg L^{-1}. When not indicated the differences between frozen and unfrozen samples at the respective times were significant (Mann–Whitney U test, $p < 0.05$).

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Table 2
Final experiment: factor loadings after PCA analysis of all the volatile components for frozen and unfrozen EVOOs at 12 and 18 month.

Compounds	PC1	PC2	PC3	PC4	PC5
2,4-Heptadienal (E,E)	−0.12759	−0.41239	0.072861	−0.82154	0.066242
Nonanal	−0.13271	0.283757	0.146371	−0.00722	0.665172
2-Decenal	0.020518	−0.05594	0.095591	−0.89855	−0.31109
2-Undecenal	−0.21411	−0.49488	−0.45575	−0.4965	−0.23434
Ethyl-acetate	0.86171	−0.2933	0.23833	0.08234	0.151821
Methyl-salicylate	0.112524	0.643299	−0.33894	−0.4659	0.316008
Acetic-acid	−0.28584	0.243872	0.801949	0.335534	0.164897
2-Hexenal (E)	−0.31744	0.594496	0.110656	0.340058	0.394336
Hexanal	0.27451	−0.4735	0.678066	−0.17021	0.021693
1-Penten-3-One	0.801299	0.502145	0.105979	0.15322	0.200665
Pentanale	0.089268	0.210887	0.166977	0.278356	0.748683
2-pentenal (E)	0.65905	0.443559	0.498214	0.290994	0.052312
Butanal–2-methyl + 3-methyl	0.574801	0.131283	0.590239	0.368925	−0.04196
Ethanol	0.093985	0.93985	−0.0784	0.089042	0.178259
1-Penten-3-ol	0.964206	−0.01763	0.139488	0.023289	0.020083
Hexanol	0.052951	0.218781	0.383163	−0.14943	0.078637
3-hexen-1-ol (Z)	0.678484	−0.26971	0.338152	0.049163	−0.23303
2-hexen-1-ol (E)	0.012209	0.301536	0.631604	0.612206	0.22192
Benzaldheyde	0.229056	0.76453	0.450307	0.23885	0.271927
3-hexen-1-yl acetate (Z)	0.931244	0.015315	−0.00726	−0.12822	−0.04647
Phenylethyl alcohol	0.238781	0.769942	0.451301	0.103229	−0.15845
1-decanol	−0.20772	0.324878	−0.69974	−0.18996	0.058316
2,6-octadiene-2,6-dimethyl	0.373506	−0.19124	0.524579	0.497868	0.421474
3-ethyl-1-5-octadiene	0.558732	0.056564	0.589447	−0.12216	0.190726
α-farnesene	−0.17803	0.878853	0.045978	0.198471	0.301401
Benzyl alcohol	−0.205	0.728849	−0.05419	0.053381	0.033744
Butanoic acid	0.428376	−0.07552	0.61121	−0.00802	0.554047
Hexanoic acid	0.223832	0.126952	0.821778	−0.24744	0.245352
Octanoic acid	0.415758	0.095471	0.626857	0.194186	0.077114
O-Cymene	0.428781	0.554614	0.122955	0.290094	0.577708

Conversely to the phenolic fraction, the differences observed between each couple of frozen/unfrozen oils were negligible up to 6 months (data not shown) and the variations recorded in the GC profile at 12 months, by the PCA-LDA approach, were not significant (p-value 0.14 after Wilks'-lambda test). Five latent variables constructed by PCA were used for LDA analysis of volatile compounds which explained over 75% of the dataset variance (Table 2 and Fig. 5). In addition, the leave-one-out (Jackknifed) classification performed upon dataset re-sampling showed a poor reclassification. Conversely, at 18 months the differences were significant (p-value 0.03156 after Wilks'-lambda test) and all the frozen EVOOs were differently classified from the respective ones stored at room temperature with a 100% reattribution.

The volatile profile of bottles opened and analyzed after 10 days were significantly different from those immediately opened both for frozen and for room temperature-stored samples (data not reported).

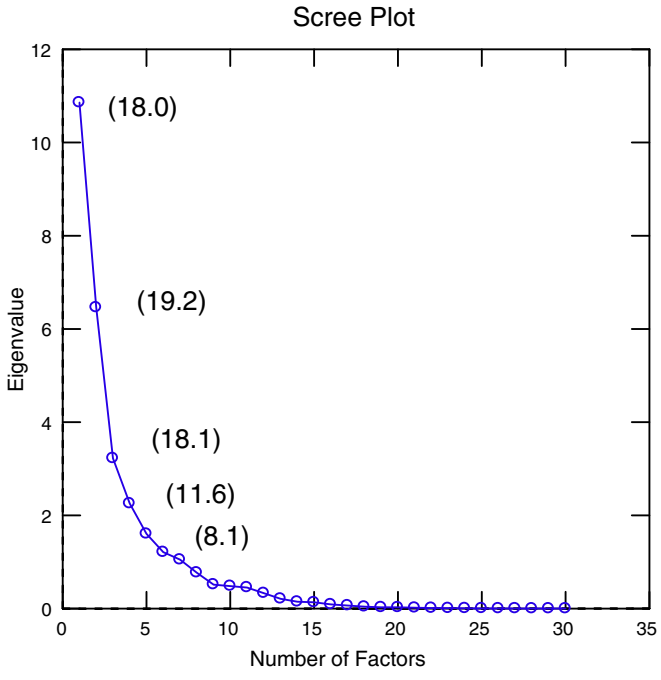

Fig. 5. Volatile components at 12 and 18 months: fraction of total variance with the % of explained variance reported in brackets.

4. Conclusions

The results of this study indicate that EVOOs maintained a superior quality profile upon freezing up to 18 months in comparison with those stored at room temperature both for phenolic and volatile compounds. The quality decay in sealed and light protected bottles is mostly evident in the phenolic components where differences are evidenced starting from 3 months storage, while for the volatile components are significant only after 12–18 months. Unexpectedly, increments of some secoiridoids were measured in the unfrozen oils over time. These results can be explained taking into account that the chemical equilibrium among different isobaric forms of the main secoiridoidic compounds is temperature dependent and this affect the absorbance values used for the quantitative study. The quality decay, 10 days after bottle opening, occurs in EVOOs at room temperature as well as in frozen ones for volatile profile, while it is more evident in frozen EVOOs for the phenolic fraction a large gain in these compounds is still guaranteed by the previous conservation at −23 °C indicating a far superior quality of frozen EVOOs.

These results could help in maintaining over time better nutraceutical properties of EVOOs in comparison with the conventional storage at room temperature and the cryopreservation can help to store for long time selected EVOOs of higher quality. The inserting of $EVOO_S$ in the cold chain could also improve the organoleptic properties of those oils intended for export that often are not optimal.

Moreover, it must also be underlined that the oil freezing could also be useful to ensure a stability over time of EVOOs used for

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pharmacological–nutritional intervention studies, that often involve a daily oil intake in a wide number of humans for long time.

To confirm these results further investigations involving a wide number of $EVOO_S$ are desirable.

Abbreviation

3,4-DHPE-EDA	3,4 dihydroxyphenylethanol- Elenolic acid Dialdheyde
EVOOs	extra virgin olive oils
MPC	Minor Polar Compounds

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2013.03.052.

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