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Pigment profile and colour of monovarietal virgin olive oils from *Arbequina* cultivar obtained during two consecutive crop seasons

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ABSTRACT

Monovarietal virgin olive oils are labelled with the olive varieties giving them their distinctive character. There are numerous studies focussed on the characterisation and quantification of the minor fractions of virgin olive oils that have generated databases on varietal olive oils. However, few studies have focussed on the components of the pigment fraction of virgin olive oils. The aim of this work was to quantify the components of the chlorophyll and carotenoid fractions of the monovarietal virgin olive oils from the *Arbequina* cultivar, growing in the Spanish area of Catalonia, during two consecutive crop seasons. Additionally the pigment changes occurring during 24 months of oil storage were evaluated. The results of this study showed minor qualitative differences between monovarietal virgin olive oils from two consecutive seasons. The quantitative differences could be attributed to the harvest period in each season rather than to the year's weather conditions. Storage of the monovarietal virgin olive oils probably caused an important loss of the chlorophyll fraction, mainly chlorophyll *a*, during the first 6 months of storage. On the other hand, the carotenoid fraction was very stable and the retention of provitamin A was close to 80%, even after 24 months of storage.

1. Introduction

Olive oil production, in typical producer countries, has taken an unexpected new direction. During recent years, some Spanish olive-growing areas, such as Catalonia, have not opted for the massive cultivation of the olive tree, but rather have chosen a different model. The cultivation has been improved by renewing old trees, reducing the association with other crops, selecting the olive varieties suited to local agroclimates and planting new single-variety orchards. As a consequence, monovarietal virgin olive oils are labelled not only with the producers' name and usually the place of origin, but also with the olive varieties used to give them their distinctive character. In the olive-growing area of *Les Garrigues* (Catalonia, Spain), the native olive tree cultivar is the *Arbequina*, and the low production in this area allows the olives to be hand-picked, and the oil extraction takes place immediately after harvesting the fruit. As a consequence, the monovarietal virgin olive oils present distinct characteristics, directly related to the olive cultivar and the natural environment in which it grows.

Colour is one of the major attributes that affects consumer perception of quality of the virgin olive oil. During oil extraction, mass partitioning phenomena occur that determine the pigment distribution of the olive paste between the solid (pomace) and the liquid phases (oil and wastewater). The lipophilic nature of chloroplast pigments determines their affinity for the oily phase, and the more hydrophilic nature of anthocyanins determines their retention in the pomace and the wastewater (Ranalli, Malfatti, Lucera, Contento, & Sotiriuo, 2005). So, the chloroplast pigments (chlorophyll and carotenoids) are mainly responsible for the colour of virgin olive oil, ranging from yellow–green to greenish gold (Ayuso, Haro, & Escolar, 2004; Escolar, Haro, & Ayuso, 2007).

In addition to the colour, chlorophylls and carotenoids play an important role in the oxidative stability due to their antioxidant nature in the dark and pro-oxidant activity in the light.

Environmental factors, depicted by the year's climatology, may be more important than genetic factors for the chemical components that determine the monovarietal virgin olive oil quality (Beltrán, Aguilera, Del Rio, Sánchez, & Martinez, 2005; Romero, Tovar, Ramo, & Motilva, 2003; Salvador, Aranda, & Fregapane, 2001a; Salvador, Aranda, Gómez-Alonso, & Fregapane, 2001b). In addition to the environmental and varietal factors, storage and conditions, during the commercialisation of virgin olive oil, determine its pigment content. Olive oil generally has a relatively long shelf-life, during which only minor changes of its sensory characteristics occur. During storage, the plant material containing chlorophyll undergoes changes or loss of colour as a consequence of the pigment's chemical properties, the enzymes present and the physical conditions (e.g., temperature, light and oxygen). Chlorophyll degradation during storage is complex. The main difficulty in

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understanding the steps of chlorophyll degradation is that it occurs very rapidly and yields colourless and low-molecular-weight compounds as end-products (Gallardo-Guerrero, Gándul-Rojas, Roca, & Mínguez-Mosquera, 2005). The overlapping of the products with degradation products of other substances adds to the difficulty.

Consequently, the characterisation of the monovarietal virgin olive oils cannot be carried out with only a few chemical compounds or a simple statistical analysis. The factors affecting the product are represented in their proportions in the overall production, so the correct characterisation of these olive oils requires sampling (Aparicio & Luna, 2002). Major components, such as fatty acids, can only provide basic information (Alonso & Aparicio, 1993), and minor compounds can provide more useful information. These minor components could be the volatile and non-volatile compounds, including polyphenols, sterols, triterpenic alcohols and pigments. With the exception of the pigments, there are numerous studies focussed on the characterisation and quantification of these minor fractions of virgin olive oils that have generated databases on varietal olive oils.

However, few studies have focussed on the components of the pigment fraction of virgin olive oils. The first study focussing on the characterization of the pigment fraction of nine-single Spanish virgin olive oils was done by Gandul-Rojas and Mínguez-Mosquera (1996). Additionally, more recent studies have focussed on the pigment composition of monovarietal virgin olive oils from various Italian (Cerretani, Motilva, Romero, Bendini, & Lercker, in press; Giuffrida, Salvo, Salvo, La Pera, & Dugo, 2007; Ranalli et al., 2005) and Greek olive varieties (Psomiadou & Tsimidou, 2001). Thus, it is important to increase studies of the characterization of the pigment fraction of virgin olive oils produced from different olive cultivars growing in different countries and in different olive-growing areas to increase the databases on varietal virgin olive oils.

The aim of this work was to quantify the components of the chlorophyll and carotenoid fractions of the monovarietal virgin olive oils from the *Arbequina* cultivar growing in the Spanish area of Catalonia, during two consecutive crop seasons. Additionally the pigment changes occurring during 24 months of oil storage were evaluated.

2. Materials and methods

2.1. Materials

Thirty virgin olive oil samples from various olive oil mills, from all over the region of "Les Garrigues" (Catalonia, Spain), were obtained in two successive crop seasons, corresponding to 2002/03 (12 samples, period November 1 to December 15) and 2003/04 (18 samples, period November 1 to January 15). In this region, autumn frosts are very common, and the harvesting period covers the 3 months from November to January. The oils were taken directly from the production line under a protocol established by the Regulator Organism of the Protected Designation of Origin "Les Garrigues".

Daily weather data (temperature and rainfall) were obtained during two experimental years (2002–2003) from the meteorological station situated at La Granadella in the geographical centre of the "Les Garrigues" region. The maximum and minimum temperatures and the amount of precipitation for each day are shown in Fig. 1.

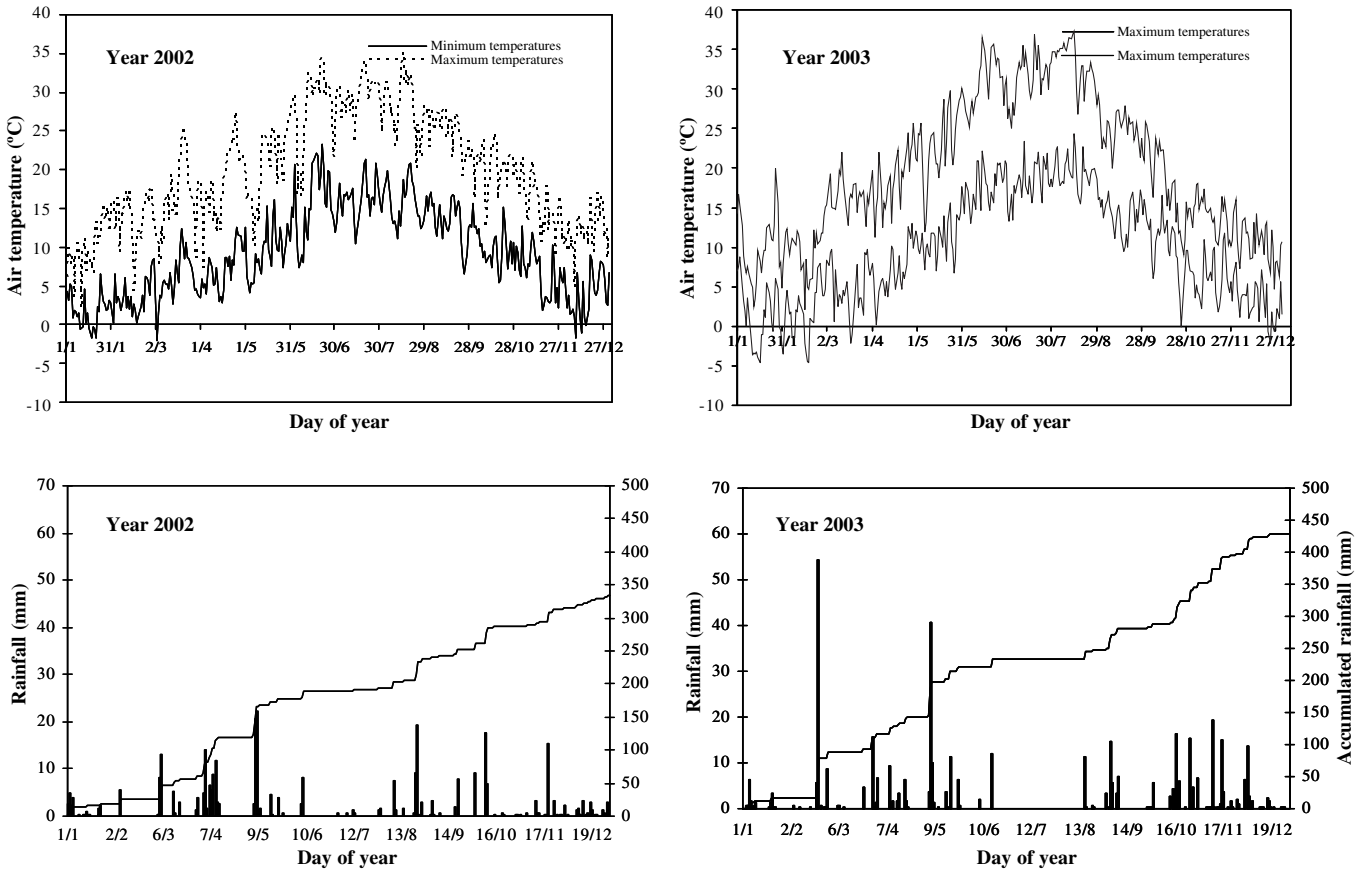

Fig. 1. Annual patterns of air temperature, daily and accumulated rainfall (mm) for 2002 and 2003.

The effect of storage was studied in oils from the 2002/03 crop season. After their first analysis (time = 0), they were kept in amber glass bottles (100 ml) closed with nitrogen atmosphere in the dark at 4 °C for 24 months. The pigment profile was analysed at 0, 6, 12 and 24 months.

2.2. Pigment extraction

Pigments were extracted from the virgin olive oil, following established procedures (Mínguez-Mosquera, Gandul-Rojas, & Gallardo-Guerrero, 1992). The method was based on a selective separation of components with *N*,*N*-dimetylformamide (DMF) and hexane. The hexane phase carried over lipids and the carotene fraction while the DMF phase retained chlorophylls, chlorophyllic derivatives and xanthophylls.

The sample of virgin olive oil (15 g) was dissolved in 150 ml of DMF saturated with $MgCO_3$ and treated five times, successively, with 50 ml of hexane. Two phases, hexane and DMF, were treated separately, as described by Criado, Motilva, Goñi, and Romero (2007). In a first step the *N*,*N*-DMF phase was treated with a 2% sodium sulphate solution at approximately 0 °C. Then, 70 ml of hexane and 70 ml of ethyl ether were added, shaken and kept until the phases had separated. The ether phase with the chloroplast pigments solution was washed three times with an aqueous solution of Na_2SO_4 (2%) at 0 °C. The ether was evaporated to dryness and dissolved in 1 ml of acetone.

The hexane phases were mixed with ether and saponified with 100 ml of 15.6% KOH in methanol, and strongly shaken to hydrolyze the lipids and purify any possible carotenoids. After one hour, distilled water was added and left until the phases had separated. The ether phase with the carotene fraction was washed three times with water to neutrality and another three times with an aqueous solution of Na_2SO_4. It was concentrated to dryness by rotary evaporation at reduced pressure and dissolved in 1 ml of acetone.

The chlorophyll and carotene extracts were stored in the dark in a freezer at −40 °C while awaiting the HPLC analysis. All extractions were performed in triplicate under a green light to prevent pigment alteration.

2.3. Pigment standards

Chlorophyll *a* (No. C-6144 from algae), chlorophyll *b* (No. C-5878 from spinach) and β-carotene (No. C-4582) were supplied by Sigma (St. Louis, MO, USA). Because of the absence of commercial pheophytin *a* and *b* standards, both were prepared from the respective solutions of chlorophylls by shaking the ether solution with 2–3 drops of 13% HCl until the green chlorophyll colour changed to the greyish pheophytin colour (Holden, 1976; Sievers & Hynninen, 1977).

The pheophorbide *a* standard was prepared by enzymatic de-esterification of pheophytin *a* (Hynninen, 1973). Chlorophyllides *a* and *b* were obtained from the respective solutions of chlorophylls by enzymatic de-esterification, using the method described by Jones, Butler, Gibbs, and White (1972). The enzymatic extract of chlorophyllase was obtained from *Ailanthus altissima* leaves according to the method of Terpstra and Lambers (1983).

Lutein, violaxanthin and neoxanthin standards were obtained from a pigment extract of fresh spinach, using the method described by Mínguez-Mosquera et al. (1992) and separated by TLC on silica gel GF254 (0.2 mm) on 20 × 20 cm plates, using petroleum ether (65–95 °C)/acetone/diethylamine (10:4:1, v/v/v). The chromatograms were developed for a distance of 16 cm, after which the lutein, violaxanthin and neoxanthin bands were scraped off individually and each was poured into a glass funnel attached to a small side-arm filtration flask. A total of 30 spots were collected separately for each band and each carotene was eluted with acetone and the absorbance was measured at 446 nm for lutein, 440 nm for violaxanthin and neoxanthin, using a spectrophotometer. The concentrations were calculated using the following formula (Davies, 1976):

$$\text{Concentration (g/ml)} = \frac{E}{E^{1\%}_{1\text{ cm}} \times 100}$$

where E is the absorbance and is the extinction coefficient in acetone (lutein and violaxanthin 2340, and neoxanthin 2050).

Antheraxanthin and β-cryptoxanthin standards were prepared from carotenoid saponified extract of red pepper, obtained according to the method described by Mínguez-Mosquera and Hornero-Méndez (1993). The antheraxanthin and β-cryptoxanthin were isolated by HPLC using a Spherisorb ODS-2 column (5 μm, 25 cm × 4.6 mm i.d., Teknokroma, Barcelona, Spain) connected to a fraction collector. The HPLC system was the same as that described below. The chromatographic conditions were: flow rate 1.5 ml/min^{-1} and injection volume 100 μl. The gradient was: 75% acetone and 25% water maintained for 5 min; after that the gradient changed to 95% acetone and 5% water and was maintained for 20 min. The chromatographic peaks corresponding to antheraxanthin and β-cryptoxanthin were collected, evaporated to dryness and dissolved in acetone with the absorbance measured at 446 and 452 nm, respectively. The concentrations were calculated with the formula shown above and extinction coefficients of 2349 and 2369, as reported by Mínguez-Mosquera (1997).

2.4. HPLC analysis of pigments

The HPLC system was made up of a Waters 717 plus Autosampler, a Waters 600 pump, a Waters column heater module and a Waters 996 photodiode array detector managed by Empower software (Waters Inc., Milford, MA).

The column was a stainless steel Spherisorb ODS-2 column (5 μm, 25 cm × 4.6 mm i.d., Teknokroma, Barcelona, Spain) equipped with a Spherisorb S5 ODS-2 (5 μm, 1 cm × 4.6 mm i.d., Teknokroma, Barcelona, Spain) pre-column. HPLC analysis was performed following the procedure of Mínguez-Mosquera et al. (1992). Separation was performed using an elution gradient (flow rate = 2 ml min^{-1}) with the mobile phases (A) water/ion pair reagent/methanol (1:1:8 v/v/v) and (B) methanol/acetone (1:1). The ion pair reagent was 0.05 M tetrabutylammonium acetate and 1 M ammonium acetate in water. The gradient elution was: 0–8 min, 25–75% phase B; 8–10 min, 75% B isocratic; 10–18 min, 75–90% B; 18–23 min, 90–100% B; 23–30 min, 100–25% (initial conditions). Detection was performed simultaneously at 410 nm to measure pheophytin *a* and pheophorbide *a*, 430 nm for chlorophyll *a*, 435 nm for pheophytin *b* and chlorophyllide *a*, 440 nm for neoxanthin, violaxanthin, luteoxanthin and esterified xanthophylls (neoxanthin and violaxanthin), 445 nm for antheraxanthin and mutatoxanthin, 446 nm for lutein and *cis*-α-carotene, 452 nm for β-cryptoxanthin, 454 nm for all-*trans*-β-carotene, 466 nm for chlorophyll *b* and 468 nm to measure chlorophyllide *b*. All pigments were identified by their spectral absorption maxima, peak ratios and cochromatography with commercial products or, when unavailable, with pigments obtained and purified in the laboratory.

External standard calibration was used for quantification. Individual pigments were quantified by a four-point regression curve on the basis of each standard obtained from commercial suppliers or by the different methods described below. The quantification of luteoxanthin (an intermediate compound of the acid transformation of violaxanthin), mutatoxanthin (a compound formed from the acid degradation of antheraxanthin) and α-carotene was carried out using the regression curves of violaxanthin, antheraxanthin and β-carotene, respectively. Individual pigments were expressed as mg kg^{-1} of olive oil.

The provitamin A value was obtained from the carotene contents with β-ionone rings and the results were expressed as μg of retinol kg^{-1} of oil, taking into account that μg retinol = 0.167 × μg β-carotene + 0.083 × (μg α-carotene + μg γ-carotene + μg β-cryptoxanthin) (Bauernfeind, Brucbacher, Kläui, & Marusich, 1971).

2.5. Oil colour

A spectrophotometer (Color-Eye 3000, Macbeth) with the Optiview 1.1 computer software was used to assess the oil colour. The oil colour was measured without dilution in a 1 cm transmission optical cell made of clear optical glass. Tristimulus values were calculated from the transmittances and the CIELAB system was applied, standard C illuminant with 2° for the foveal angle. The oil colour was expressed as chromatic ordinates L^*, a^* and b^* (Escolar, Haro, Saucedo, Ayuso, Jiménez, & Alvarez, 1994).

2.6. Statistical analysis

The statistical procedure was carried out with the 8.0 version SAS System package (SAS Institute Inc., Cary, NC, USA) to evaluate the effect of storage on the pigment contents.

3. Results and discussion

3.1. Chromatographic and spectral data of chlorophylls and carotenoids of virgin olive oil from Arbequina cultivar

The isolated pigments were characterized by their absorption spectra and by the ratio absorption maximum. Table 1 shows the chromatographic characteristics and the spectral absorption maxima of the isolated pigments (commercial and prepared standards) analysed by the chromatographic conditions described in Section 2 (λ nm, in-line). The solvents influenced the position of the absorption maxima, thus the solvents used to evaluate the spectral characteristics of the standards corresponded with the gradient solvent in the HPLC analysis of olive oil pigment extracts. The identification of chlorophylls and chlorophyll derivatives was based on the relationship between the Soret band (I) (the maximum absorption in the blue area) and the maximum absorption in the red area (III).

Carotenoids can specifically absorb light in the ultraviolet and visible regions of the spectrum. Each carotenoid is characterized by an electronic absorption spectrum. Accordingly, absorption spectroscopy is an important technique in carotenoid analysis. The position of the absorption maxima, usually three, is a function of the number of conjugated double bonds. All the compounds have a typical three-peaked carotenoid absorption spectrum with well-defined maxima and minima (Goodwin & Britton, 1988). The identification of carotenoids was based on the percentage of the III/II ratio (% III/II) that corresponds to the absorption in the red area (III) and the absorption at intermediate band (II) that usually corresponds with the maxima absorption (Davies, 1976).

Retention time alone is not a satisfactory criterion for identifying components in a chromatogram, i.e. comparing them with the retention times of standards. In this study, a combination of retention time and absorption spectrum was used to identify the components of the chlorophyllic and carotenoid fractions from the virgin olive oils. Fig. 2 shows the HPLC chromatogram of the chlorophylls, chlorophyll derivatives and carotenoids. A total of 16 peaks were resolved. Although each pigment was quantified at its maximum absorption, the chromatogram corresponding to Fig. 2A was obtained at 410 nm, used to measure the chlorophyll derivatives, pheophorbide *a* and pheophytin *a* (peaks 1 and 14, respectively). The pigment profile of the virgin olive oil comprises chlorophyll *a*, chlorophyll *b*, and derivative pigments associated with the acidic medium of the oil extraction process: pheophytin *a*, pheophytin *b*; and the carotenoids: lutein, β-carotene and the epoxide xanthophylls, neoxanthin, violaxanthin and antheraxanthin. The carotenoids, luteoxanthin and mutatoxanthin (peaks 4 and 6, respectively), and furanoid xanthophylls formed by acid transformation of violaxanthin and antheraxanthin, respectively, were quantified in minor concentrations and their chromatographic peaks were detected in the tail of their precursors (peaks 3 and 5). Besides these, the characteristic pigments of the *Arbequina* variety were detected: monoesterified violaxanthin and esterified neoxanthin (mono- and di-esterified xanthophylls), and pheophorbide *a*. Finally, the interest in the saponification of the carotenoid extract was for the detection and quantification of α-carotene (Fig. 2B, peak 15), present in minor concentrations in oils from the *Arbequina* variety.

3.2. The pigment contents of monovarietal virgin olive oils from two consecutive crop seasons

The chlorophyll and carotenoid contents of monovarietal virgin olive oils from the *Arbequina* variety are shown in Table 2. The only qualitative differences detected between seasons were of trace

Table 1
Spectral data of chlorophylls and carotenoids determined "in-line" with the gradient and mobile phases described in Section 2

Peak	Retention time (min)	Pigment	Absorption maximum λ (nm, in-line)[a]			Maximum ratio	
			I	II	III	I/III	% III/II
1	6.90	Pheophorbide *a*	409	508	666	2.22	
2	7.20	Neoxanthin	414	438	466		90
3	8.02	Violaxanthin	417	440	471		96
4	8.50	Luteoxanthin	400	423	450		105
5	9.50	Antheraxanthin	(425)	446	474		84
6	10.20	Mutatoxanthin	(408)	429	454		39
7	10.90	Lutein	424	446	474		91
8	14.02	Chlorophyll *b*	466	602	650	3.3	
9	14.85	β-Cryptoxanthin	(431)	452	479		25
10	17.20	Chlorophyll *a*	432	619	666	1.1	
11	19.35	Violaxanthin monoesterified	417	440	471		96
12	20.40	Pheophytin *b*	435	601	654	3.5	
13	21.50	Neoxanthin esterified	414	438	466		90
14	22.50	Pheophytin *a*	409	505	666	2.5	
15	19.15	*cis*-α-Carotene	(430)	448	478		60
16	20.05	All-*trans*-β-carotene	(432)	454	481		89

[a] A gradient mobile phases: (A) water/ion pair reagent/methanol (1:1:8 v/v/v) and (B) methanol/acetone described in Section 2.

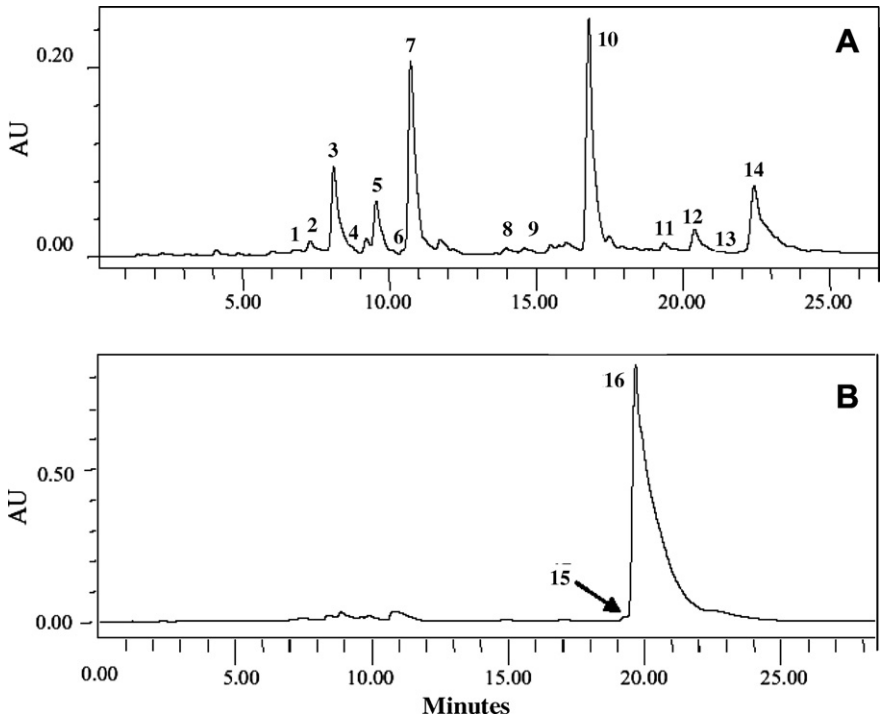

Fig. 2. (A) HPLC chromatogram at $\lambda = 410$ nm of chlorophylls, chlorophyll derivatives, xanthophylls and esterified xanthophylls. (B) HPLC chromatogram at $\lambda = 454$ nm of saponified extract of carotenoids. See Table 1 to identify the peaks.

Table 2
Pigment composition, chlorophylls and carotenoids, and chromatic ordinates of monovarietal virgin olive oils from the *Arbequina* cultivar from two consecutive crop seasons

Pigments (mg kg^{-1})	Crop season[d]				
	2002/2003		2003/2004		
	Mean	Range	Mean	Range	
Chlorophyll *a*	1.39	2.31–0.565	2.47	5.40–0.346	**
Chlorophyll *b*	0.196	0.369–0.094	0.534	1.11–0.062	**
Pheophytin *a*	0.549	0.987–0.182	1.62	2.71–0.170	**
Pheophytin *b*	ND	ND	ND	ND	
Pheophorbide *a*	0.148	0.258–0.017	0.102	0.292–0.0031	*
Total chlorophylls	2.28	3.92–0.858	4.73	9.22–0.870	**
Lutein	1.95	3.17–1.242	2.62	5.26–1.08	*
All *trans*-β-Carotene	1.11	1.33–0.890	1.28	2.13–0.753	*
Neoxanthin	0.140	0.269–0.060	0.149	0.326–0.021	NS
Violaxanthin	0.300	0.507–0.163	0.443	0.855–0.145	*
Antheraxanthin	0.326	0.400–0.218	0.304	0.467–0.126	NS
Luteoxanthin	tr	tr	0.014	0.034–0.0021	
Mutatoxanthin	tr	tr	tr	tr	
β-Cryptoxanthin	tr	tr	0.072	0.115–0.033	
X. esterified[a]	0.067	0.177–0.024	0.262	0.372–0.105	**
cis-α-Carotene	tr	tr	0.0065	0.018–tr	
Total carotenoids	3.89	5.85–2.60	5.15	9.58–2.27	**
Total pigments	6.17	9.77–3.46	9.88	18.80–3.14	**
Chlor/Car[b]	0.586	0.670–0.330	0.918	0.962–0.383	*
Lutein/β-carotene	1.76	2.38–1.40	2.05	2.47–1.43	*
Provitamin A[c]	185	223–148		364–129	
Chromatic ordinates					
L^*	87.0	91.4–81.7	88.1	92.6–86.9	NS
a^*	−2.98	−4.39 to −0.782	−4.14	−6.19 to −1.83	**
b^*	75.9	88.8–67.6	92.4	113.9–69.0	*
a/*b*	−0.039	−0.049 to −0.012	−0.044	−0.054 to −0.027	

NS, not significant ($p > 0.05$); * ($p < 0.05$); ** ($p < 0.01$).
ND, Not detected; tr, trace amounts.
[a] Xanthophylls esterified.
[b] Chlorophyll/carotenoid ratio.
[c] Expressed as μg retinol kg^{-1} oil.
[d] Significance level between seasons.

amounts of β-cryptoxanthin, the acidic derivative luteoxanthin and the *cis*-α-carotene in oils from the 2002/03 crop season. Quantitative differences were observed between seasons, mainly in concentration of chlorophylls *a* and *b*, and carotenoids (lutein and all-trans-β-carotene). These differences were probably a consequence of the wide range in the concentration of some pigments, mainly in oils from the 2003/2004 crop season. Thus, the concentration of total chlorophylls in oils from this season ranged from 9.22 to 0.870 mg kg^{-1} and total carotenoids from 9.58 to 2.27 mg kg^{-1}. The highest pigment concentration was from oils from the first days of the harvest period, obtained from olives with ripeness stage of small reddish spots, and the lowest pigment concentration corresponded to oils from the last days of the harvest period extracted from olives with the ripeness stage of black colour. By contrast, a minor range was observed in the pigment concentration of oils from the 2002/2003 season. This crop season was characterized by a short harvest period and low oil production in the study area. Thus, during this crop season, it was not possible to obtain green oils with the high pigment content characteristics of the first harvest period. This atypical olive harvest might have been a consequence of the low temperatures in the olive-growing area during the second part of December, 2001, which caused important damage to the olive trees (Morelló, Motilva, Ramo, & Romero, 2003). The trees were covered by snow for more than ten days, and the air temperature was below −5 °C for 110 consecutive hours, reaching minimal temperatures around −13 °C. As a result of these extreme conditions, an important reduction in the olive yield per tree was observed in the next harvest period (November, 2002–January, 2003) together with a faster ripening of the olive fruit. As a consequence, it was not possible to obtain highly pigmented oils, typical of the first harvest period. By contrast, the weather in 2003 (Fig. 1) was characterized by rainfall mainly in the spring (coinciding with the vegetative period of the olive tree) and autumn, which probably delayed olive ripening and allowed more highly pigmented oils to be obtained during the first harvest period.

According to this, the pigment content of oils from the 2003/2004 crop season should be more appropriate for the characterization of monovarietal virgin olive oils from the *Arbequina* cultivar growing in the study area, independently of the fact that no differences were observed in pigment profile between the two seasons studied and only some quantitative differences. In fact, from a commercial point of view, two types of oils were distinguished. Oils from the earliest harvest are greenish in colour, fruity and have a bitter almond-like taste, while those from the latest harvest are yellow in colour, more fluid and have a sweet taste.

From a nutritional point of view, the more highly pigmented oils from the 2003/2004 season showed a higher content of provitamin A (364 mg kg^{-1}). It is important to emphasize that the partial esterification of carotenoids in oils from the *Arbequina* cultivar has two important consequences. On the one hand, esterification increases the oxidative stability of the carotenoids, with the oil color thus being more stable (Gross, 1991). On the other hand, the esterification of carotenoid improves the bioavailability of these micronutrients (Van het Hof, West, Weststrate, & Hautvast, 2000).

For colour characterisation, coordinates based on the Hunter solid are widely used: L^* (lightness), a^*, and b, where + a represents red; $-a$, green; $+b$, yellow; and $-b$ blue. The a/b ratio is usually computed. It is negative for green plant material, approximately 0 for yellow material, such as virgin olive oil, and positive for orange or red material. The a/b ratio of the oils of this study was maintained at negative values near to 0 corresponding to yellow material and minor differences in this ratio were observed between seasons. The more important differences between seasons were observed in the b^* chromatic ordinate, corresponding to the yellow zone, with minor differences in the coordinate a^*, corresponding to the green zone (Table 2). This trend was similar for the chlorophyll and carotenoid pigment contents and the chlorophyll/carotenoid ratio was higher in oils from the 2003/2004 season. However, no significant differences were observed in the luminosity values (L^*). Usually this coordinate increases with the reduction in the pigment content of the oils, as the pigments would capture part of the light, instead of transmitting it. According to the results, the colour of the oils was more affected by harvest period than by season.

3.3. The effect of storage on the pigment content of monovarietal virgin olive oils

Chlorophyll degradation during storage is complex. The main difficulty in understanding the steps of chlorophyll degradation is that they yield different end-products, some of which are colourless. The overlapping of these products with degradation products adds to the difficulty. The oil pigment content, mainly the chlorophyll fraction concentration, decreased gradually with the storage time (Table 3), even with the most appropriate storage conditions (in absence of light and air). However, the transformations of the components of the pigment and carotenoid fractions during storage were different. The concentration of chlorophyll *a*, the most important pigment, decreased significantly during the first 6 months of storage and it was completely degraded between 6 and 12 months of storage, illustrating its greater instability compared with chlorophyll *b* (Rüdiger & Schoch, 1988). In parallel, the pheophytin *a* concentration increased significantly at 6 months of storage. Similarly, the concentration of chlorophyll *b* decreased significantly at 6 months of storage, but its transformation into pheophytin *b* was not as evident as that of chlorophyll *a*. Even at 24 months of storage, chlorophyll *b* was quantified. Pheophytins are the magnesium-free derivatives of the chlorophylls formed by the acidic medium during the virgin olive oil extraction process. The increase in the pheophytins *a* and *b* during oil storage is probably the result of a nonenzymatic process, which involves the formation and subsequent accumulation of free radicals, these being the main factors involved in the destruction of pigments. Pheophorbide formation is evidence of high chlorophyllase activity in olive paste from the *Arbequina* cultivar during the malaxation operation of the oil extraction process (Criado, Motilva, Ramo, & Romero, 2006; Roca & Mínguez-Mosquera, 2003). However, this enzymatic activity does not seem to occur during oil storage and the pheophorbide *a* concentration decreased with oil storage.

Table 3
Evolution during storage of the pigment content, chlorophylls and carotenoids, and chromatic ordinates of monovarietal virgin olive oils from the *Arbequina* cultivar

Pigments (mg kg^{-1})	Time of storage (months)[D]			
	0	6	12	24
Chlorophyll *a*	1.44[a]	0.137[b]	ND	ND
Chlorophyll *b*	0.207[a]	0.130[b]	0.103[b]	0.036[c]
Pheophytin *a*	0.585[a]	1.37[bc]	1.30[c]	1.01[d]
Pheophytin *b*	ND	ND	0.009[a]	0.049[b]
Pheophorbide *a*	0.149[a]	0.104[b]	0.099[c]	0.033[d]
Total chlorophylls	2.38[a]	1.75[b]	1.51[b]	1.13[c]
Lutein	1.97[a]	1.90[a]	1.82[a]	1.41[b]
All-*trans*-β-Carotene	1.11[a]	0.907[b]	0.848[b]	0.845[b]
Neoxanthin	0.147[a]	0.107[b]	0.094[b]	0.079[b]
Violaxanthin	0.320[a]	0.255[b]	0.107[c]	0.087[c]
Antheraxanthin	0.323[a]	0.206[b]	0.101[c]	0.081[c]
Luteoxanthin	ND	ND	0.062[a]	0.011[b]
Mutatoxanthin	ND	ND	0.050[a]	0.013[b]
β-Cryptoxanthin	ND	0.038[a]	0.031[b]	0.032[b]
X. esterified[A]	0.067[a]	0.055[a]	0.052[a]	0.017[b]
cis-α-Carotene	tr	tr	ND	ND
Total carotenoids	3.94[a]	3.47[b]	3.17[b]	2.57[c]
Total pigments	6.32[a]	5.22[b]	4.68[c]	3.70[d]
Chlor/Car[B]	0.603[a]	0.503[b]	0.477[b]	0.440[b]
Lutein/β-carotene	1.77[ab]	2.10[bc]	2.15[c]	1.66[a]
Provitamin A[C]	186[a]	155[b]	144[b]	144[b]
Chromatic ordinates				
L^*	86.3[a]	90.0[b]	93.3[c]	90.1[b]
a^*	−2.97[a]	−3.46[a]	−4.40[b]	−3.37[a]
b^*	81.4[a]	80.5[a]	78.9[a]	77.8[a]
a/b	−0.036[a]	−0.043[a]	−0.056[b]	−0.043[a]

ND: Not detected. tr: trace amounts.
[A] Xanthophylls esterified.
[B] Chlorophyll/carotenoid ratio.
[C] Expressed as μg retinol kg^{-1} oil.
[D] Different letters in the same row indicate a significant difference at $p < 0.05$.

In relation to the carotenoid fraction, all pigments were detected and quantified after two years of storage (24 months) with the exception of α-carotene. β-Carotene disappeared more rapidly, and was followed by the disappearance of violaxanthin and lutein. The order of carotenoid disappearance was the same as that reported many years ago in autumn leaves by Goodwin (1958) and by others, in studies on spinach leaf (Takagi, 1979; Takagi & Matsugami, 1977). The presence of oxygen in the headspace is a crucial factor in the β-carotene degradation. Even a low concentration leads to a significant loss of pigment (Gross, 1991). In spite of the inert atmosphere in the bottles during oil storage, the minor amount of oxygen dissolved in the oil probably favoured the reduction in β-carotene as observed during the first months of storage. From six months to the end of the study, no significant changes were observed in the β-carotene concentration. In parallel with this, the presence of free radicals might also accelerate the degradation rate of carotenoids. It was understood that the oxidation of carotenes and xanthophylls depended on the simultaneous oxidation of unsaturated fats. These unsaturated fats were probably oxidized in the first steps of the oil storage by lipoxygenase, and the oxidation product in turn oxidized the carotenoids.

It is important to emphasize the formation, during storage, of the acidic derivatives that were not detected in the olive oils at

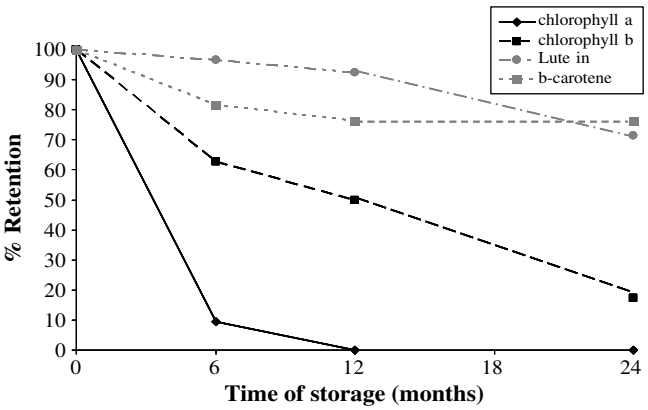

Fig. 3. Percentage of retention of chlorophyll *a*, chlorophyll *b*, lutein and β-carotene of the virgin olive oil during storage period (100% retention corresponds to the concentration of each pigment at time = 0).

time 0 of storage (Table 3), like those observed with the acidic transformation of the chlorophylls *a* and *b* to their respective pheophytins. Thus, luteoxanthin, an intermediate product of the acid transformation of violaxanthin, and mutatoxanthin, a product resulting from the acid transformation of antheraxanthin, were quantified after 12 months of storage.

As a consequence of the different kinetic degradations of the chlorophyll and carotenoid fractions, the ratio between the two pigment fractions (Chlor/Car) decreased slightly during the first 6 months of storage and, after this period, the reduction was not statistically significant (Table 3). The kinetics of the degradation of the more important pigments during oil storage was expressed in terms of the percentage of retention (Fig. 3). The most important losses were observed after 12 months of storage. Chlorophyll *a* was not quantified and 60% of the initial chlorophyll *b* content was retained, which confirms the greater instability of chlorophyll *a*. The main components of the carotenoid fraction, lutein and β-carotene, showed a great stability with oil storage. The retention curves showed two regions corresponding to an induction period (the initiation of free radicals takes place), more extensive in the case of lutein, and a main oxidative period, typical of an autocatalytic reaction. Thus, after 12 months of storage, the percentages of retention for lutein and β-carotene were 90 and 80%, respectively. Even after 24 months of oil storage, both carotenes showed retention of near to 80%, thus confirming the great stability of this fraction when the virgin olive oil is stored under optimal conditions. As a consequence of this great stability, the provitamin A content was well preserved.

Along with the decrease of the oil pigment concentration, the chromatic ordinate L^* increased and the intensity of the oil colour decreased. The ordinate a^* was at negative values, close to 0, throughout the storage period, and no statistical differences were observed with exception of the oils after 12 months of storage. Similarly, the storage time seemed to have no important effect on the ordinate b^* value (yellow zone) which decreased slightly but not statistically significantly, as was observed in a previous study by the authors (Morelló, Motilva, Tovar, & Romero, 2004). The pheophytination of chlorophylls *a* and *b*, forming chlorophyll derivatives, probably involved a transformation and not a loss of pigments. This could explain the minor changes observed in colour with oil storage time.

4. Conclusions

The results of this study showed minor qualitative differences between monovarietal virgin olive oils from two consecutive seasons. The quantitative differences could be attributed to the harvest period in each season rather than the year's weather conditions. The storage of the monovarietal virgin olive oils involved an important loss of the chlorophyll fraction, mainly chlorophyll *a*, during the first 6 months of storage. On the other hand, the carotenoid fraction was very stable and the retention of provitamin A was close to 80%, even after 24 months of storage. The production of monovarietal virgin olive oil in the olive-growing area of this study allows commercialization no more than one year after extraction, enabling the organoleptic characteristics (colour) and nutritional properties, based on the provitamin A content to, be preserved.

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