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Pigments composition in monovarietal virgin olive oils from various sicilian olive varieties

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Abstract

This paper presents the first investigation of the chlorophyll and carotenoid pigments composition in sicilian monovarietal virgin olive oils from the three (*Cerasuola*, *Nocellara*, *Biancolilla*) main olive varieties cultivated in Sicily (Italy). In all, 19 compounds were identified and quantified in 24 olive oil samples. The application of reversed-phase liquid chromatography with photodiode array detection using a C-30 column in the simultaneous qualitative–quantitative analysis of virgin olive oils pigments, has been shown. The qualitative pigment pattern was similar among the varieties investigated, whereas quantitative differences were found among the different cultivars, which can all be considered as having an high pigment content. Pheophytin *a*, was the major component (19.36–25.04 ppm), followed by β-carotene (8.06–16.27 ppm). Pheophytin *a′* (2.92–4.17 ppm), lutein (2.28–4.49 ppm) and neoxanthin (1.54–2.11 ppm) were also well represented. The presence of carotenoid esters was also detected. The neoxanthin and β-carotene contents were higher compared to reports present in the literature for other olive oil varieties. This may be due to genetic factors and/or geographical differences. The ratio between the two isochromic pigment fractions, namely the chlorophyll and the carotenoid fractions, was around one in all varieties, showing that they were in balance. The lutein/β-carotene ratio was less than one in all cases. These parameters, along with other analytical parameters, could be used as indicators of typicality in olive oils. The presence of a specific pigment profile in olive oils could in fact be used to guarantee the genuineness of the product, since the quality control of food requires a precise knowledge of the pigments composition of the original products.

Keywords: Chlorophyll; Pheophytin; Carotenoid; Sicilian virgin olive oil

1. Introduction

Chlorophylls and carotenoids are very common pigments, which give colour to vegetables and several fruits, where they play key roles in photosynthesis. Neither chlorophylls nor carotenoids can be synthesized by animal tissues, though animal cells can chemically modify them for assimilation. Thus, these molecules must be obtained from food. Several reports have demonstrated that plant pigments play important roles in health (Franceschi et al., 1994; Mayne et al., 1996); in fact, the potential health benefit of a diet rich in carotenoids have been indicated in recent studies reporting their role as antioxidant, and as agents preventing cardiovascular diseases and degenerative eyes pathologies (Kritchevsky, 1999; Landrum & Bone, 2001); apart from the provitamin A value of the carotenoids with a β-ionone ring, numerous studies have also shown the anticancer activity of β-carotene and other carotenoids (Van Poppel & Goldbohm, 1995). Their presence in olive oil depends on olive fruits (*Olea europea*, L.) genetic factors (olive variety), the stage of fruits ripeness, environmental conditions, the extraction process and storage conditions. The extraction process causes losses of oil pigments, mainly chlorophylls, owing to structural transformation of pigments caused by liberation of acids, namely the transformation of chlorophylls into pheophytins by the removal of the Mg^{2+} ion. Chlorophyll pigments are responsible for the greenish hues of virgin olive oil. Among chlorophylls, pheophytin *a*, is found in major

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amounts in olive oils. The major ''yellow'' pigments of virgin olive oils are lutein and β-carotene. The study of chlorophyll and carotenoid pigments composition in edible products derived from olive is relatively recent. In comparison with classical C-18 stationary phase, the use of the much more hydrophobic C-30 phase has shown a better resolving power for compounds with a clear hydrophobic character, such as carotenoids and chlorophylls; in fact, the C-30 stationary phase provides sufficient phase thickness to enhance interaction with long chained molecules. Chlorophyll and carotenoids play an important role in the oxidative activity of processed foodstuff, due to their antioxidant nature in the dark and pro-oxidant activity in the light (Fakourelis, Lee, & Minn, 1987). Carotenoids, together with polyphenols and tocopherols provide oxidative stability to olive oils and have a synergistic antioxidant and anticarcinogenic action at physiological concentration. Sicily is the third main olive oil producing region in Italy, with a production of around 50,000 tons a year. The aim of this study was to evaluate the pigments in the three most important (*Cerasuola, Nocellara, Biancolilla*) single-variety virgin olive oils produced in Sicily and assess their levels. To our knowledge, no such data were available in the literature. Moreover, the presence of a specific pigment profile could became a requirement of virgin olive oil, and the ratio between pigments could be used to guarantee the authenticity of the product (Gandul-Rojas, Cepero, & Minguez-Mosquera, 2000).

2. Materials and methods

2.1. Chemicals

HPLC-grade solvents, organic solvents and reagents were purchased from Sigma–Aldrich (Milan, Italy). Chlorophyll *a* and *b* standards were supplied by Sigma. Pheophytins *a* and *b* were obtained by acidification with hydrochloric acid from the respective solutions of chlorophylls (Sievers & Hynninem, 1977). Chlorophyll *a* and *b* C-10 epimers were obtained according to the method of Watanabe et al. (1984). Pyropheophytins were obtained by heating in pyridine as described by Schwartz, Woo, and von Elbe (1981). β-Carotene, lutein and β-cryptoxanthin were purchased from Extrasynthese (Genay, France). Violaxanthin and neoxanthin were obtained after extraction with cold acetone and purification by OCC from curly lattuce, as described by Kimura and Rodriguez-Amaya (2002); in order to avoid contamination among the bands during OCC, only the main portion of each band of carotenoids was collected and the standards purity evaluated by HPLC was around 95% for neoxanthin and 96% for violaxanthin. Standards were stored under nitrogen in the dark at −18 °C.

2.2. Materials and pigments extraction

Twenty-four samples of three (*Cerasuola, Nocellara, Biancolilla*) sicilian monovarietal virgin olive oils were obtained from selected mills from the main producing areas of Sicily (Italy) during the 2004–2005 season. Olive oils were extracted from green fruits harvested at the same time using the centrifugal or *continuous* system technology. Fresh oil samples were stored at −20 °C in the dark before analysis in triplicate. Analyses were carried out within 3 months from olive oils production. Each sample was extracted by liquid-phase distribution (LPD) between *N,N*-dimethyl-formamide (DMF) and hexane, according to a method described by Minguez-Mosquera, Gandul-Rojas, and Gallardo-Guerrero (1992). The samples of virgin olive oils (25 g) were dissolved directly in DMF (150 ml) and treated with five successive 50 ml portions of hexane in a decanting funnel. Chlorophylls, chlorophyllic derivatives and xanthophylls were retained in the DMF phase. The hexane phase contained lipids and carotenes. The DMF phase was treated with a 2% Na_2SO_4 solution at 0 °C and transferred to 100 ml of a mixture of hexane/ethyl ether (1:1; v/v). The aqueous phase was discarded, eliminating polyphenols and other water-soluble compounds. The organic phase was evaporated to dryness in a rotovapor at 30 °C. The dry residue was dissolved in an appropriate volume of methanol, and analysed by HPLC. The study of recovery for lutein and pheophytin *a*, by the LPD extraction procedure from olive oil using the standard technique of enrichment, was of 97% for lutein and 95% for pheophytin *a*. The five hexane phases were combined, concentrated, filtrated, and made up to a known volume of hexane, to directly spectrophotometrically measure the β-carotene concentration, using a previously prepared calibration curve, as this phase only contains this pigment.

2.3. Pigments analyses

Chlorophylls, chlorophyllic derivatives and xanthophylls present in the olive oil extracts were analysed using an HPLC-PDA Shimadzu system equipped with two LC-10AD-Vp pumps, a SCL-10A-Vp system controller, a SPD-M10Avp photodiode array detector and a Rheodyne injector with a 5 μL loop. For HPLC separation a YMC 30 analytical column (YMC Europe, Schermbeck, Germany) with 5 μm C-30 reversed-phase material (250 × 4.6 mm i.d.), including a precolumn (YMC 30; S-5 μm, 10 × 4.0 mm i.d.) was used. The mobile phase consisted of a binary gradient of methanol/methyl-*tert*-butyl ether/water, MeOH/MTBE/H_2O (90:7:3; v/v/v) (A) and MeOH/MTBE/H_2O (7:90:3; v/v/v) (B), starting with 5% B, followed by a linear gradient to 35% B in 30 min, to 40% B at 40 min, and to 95% B at 50 min, then re-equilibrating the column to initial B conc., at a flow rate of 0.8 mL/min; chromatograms were recorded at five different wavelengths, λ_1 410 nm (Pheo *a*), λ_2 430 nm (Chl *a*), λ_3 444 nm (Lutein), λ_4 465 nm (Chl *b*), λ_5 665 nm, and UV–vis spectra were recorded in the range of 325–750 nm. Pigments in the samples were identified by comparison with standards and from their spectral characteristics, both absorption maxima and peak ratios. For chlorophylls and derivatives, the characteristic peak ratio

is that between the absorbance of the Soret band I and the absorption maximum III; for carotenoids, the height of the longest wavelenght absorption band III is expressed as a percentage of that of the middle absorption band II, the base line in each case being the minimum between the two maxima (100III/II) (Davies, 1975). HPLC quantification was carried out using the external standard method; standard curves were calculated by linear regression analyses, based on the available standards calibration curves, and the mean value of three determinations is reported. β-Carotene was quantified using a Shimadzu UV-2410PC UV–vis spectrophotometer and an appropriate previously prepared calibration curve and the mean value of three determinations is reported.

3. Results and discussion

This paper presents the first qualitative–quantitative investigation of the chlorophyll and carotenoid pigments composition in sicilian monovarietal virgin olive oils from the three (*Cerasuola*, *Nocellara*, *Biancolilla*) main olive varieties cultivated in Sicily (Italy). In all, 19 different compounds were identified and quantified in olive oil samples, including *cis*-isomers and esters. The application of reversed phase liquid chromatography with photodiode array detection using a C-30 column in the simultaneous chemical characterization of chlorophyll and carotenoid pigments present in virgin olive oils was shown also for the first time. The qualitative study of pigments in the 24 virgin olive oil samples demonstrated a common pattern, that is, not dependent on the three olive variety (*Cerasuola, Nocellara, Biancolilla*) considered in this work. This is consistent with qualitative reports on the pigments composition in olive oils from various spanish olive varieties (Gandul-Rojas & Minguez-Mosquera, 1996). A typical chromatogram of pigments extract from sicilian virgin olive oil is shown in Fig. 1. The pigments considered have a UV–vis spectra characteristic, therefore the use of the photodiode array detector (PDA) for the study of this pigments has been very extensive. Moreover, carotenoids esterification does not change the shape of the respective absorption spectrum. Table 1 shows the chromatographic and spectroscopic characteristics of the various pigments separated, including retention factor, absorption maxima measured by PDA, and peak ratios. The chromatographic and spectroscopic characteristic of these pigments after alkaline hydrolysis confirmed the tentative identification of the esters of neoxanthin, lutein and β-cryptoxanthin; olives are noncarotenogenic fruits and the presence of esterified xanthophylls in olive oil was previously only reported by Gandul-Rojas and Minguez-Mosquera (1996), in olive oil from the *Arbequina* variety. Virgin olive oils contained pigments from the fruit, plus derivatives associated with the extraction process, like pheophytins (*a* and *b*), and the 5,8-furanoid isomer luteoxanthin of the original xanthophyll violaxanthin bearing the 5,6-epoxide group, probably formed because of the acidity of the extraction medium. The industrial treatments can also lead to the formation of *cis*-isomers, which do not have the same vitamin activity as the all-*trans* isomers. This is important for the accurate determination of the dietary intake of these micronutrients and the development of comprehensive food tables. Table 2 shows the quantitative composition of chlorophylls and carotenoids of the oils investigated. CV% was under 9% in all HPLC measurements and under 1% in spectrophotometric measurements.

The three olive oil varieties studied in this work can be considered as having an high pigments content, with a green hue as the prevailing color. Pheophytin a was the major component (19.36–25.04 ppm; 36–49% total pigments), followed by β-carotene (8.06–16.27 ppm; 16–30%). Pheophytin *a′* (2.92–4.17 ppm; 6–8%), lutein (2.28–4.49 ppm; 5–8%) and neoxanthin (1.54–2.11 ppm; 3–4%) were also well represented. Oils from the *Nocellara* variety showed the highest pheophytin a (25.04 ppm) and the lowest β-carotene (8.06 ppm) contents. The highest β-carotene

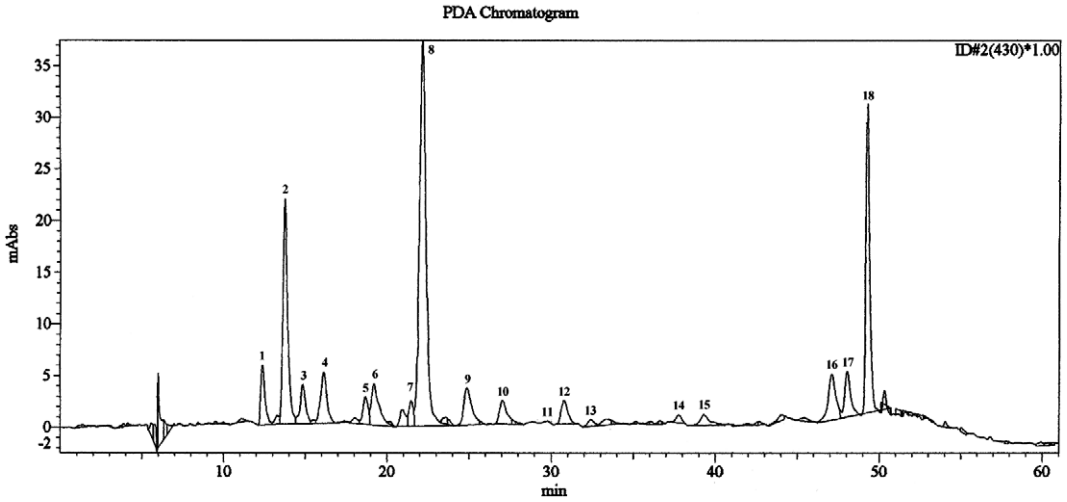

Fig. 1. Typical HPLC profile on a C-30 column at 430 nm of pigments separated in sicilian virgin olive oil. Peak numbers as in Table 1.

Table 1
Chromatographic and spectroscopic characteristics of pigments separated in sicilian virgin olive oil by HPLC

Peak number	Pigment	k[a]	Spectral data in HPLC eluent				
			Position of peak (nm)[b]			Peak height relationship[c]	
			I	II	III	100III/II	I/III
1	Neoxanthin isomer	2.3	415	438	467	88	–
2	Neoxanthin	2.7	415	438	467	88	–
3	Violaxanthin	3.1	413	434	464	93	–
4	Luteoxanthin	3.4	398	420	447	100	–
5	Antheraxanthin	4.1	(421)	444	472	45	–
6	*cis*-Violaxanthin isomer	4.2	413	434	464	93	–
7	Chlorophyll *b*	4.8	465	602	650	–	3.3
8	Lutein	5.1	423	444	472	63	–
9	β-Cryptoxanthin	5.8	(431)	450	477	28	–
10	*cis*-Lutein	6.3	416	438	466	38	–
11	Chlorophyll *a*	6.7	430	620	666	–	1.1
12	Neoxanthin ester	7.3	415	438	467	88	–
13	Lutein ester	7.8	423	444	472	63	–
14	β-Cryptoxanthin ester	9.2	(431)	450	477	28	–
15	Pyropheophytin *a*	9.7	406	506	666	–	2.2
16	Pheophytin *b*	11.8	434	601	652	–	4.1
17	Pheophytin *a*′	12.1	406	506	665	–	2.1
18	Pheophytin *a*	12.4	406	506	665	–	2.1

[a] Retention factor $(k) = t_r - t_m/t_m$, where t_r is the retention time of the pigment peak and t_m is the retention time of an unretained component.
[b] The values in parentheses indicate inflection points.
[c] Peak ratio I/III for chlorophylls and 100III/II for carotenoids.

Table 2
Concentrations (ppm) of individual carotenoids and chlorophylls and ratios between pigments fractions from monovariety sicilian virgin olive oils

Pigment	*Cerasuola* (ppm)	*Nocellara* (ppm)	*Biancolilla* (ppm)
Neoxanthin isomer	0.79	0.45	0.31
Neoxanthin	2.03	2.11	1.54
Violaxanthin	0.51	0.77	0.26
Luteoxanthin	0.74	0.8	0.51
Antheraxanthin	0.49	0.64	0.47
cis-Violaxanthin isomer	0.22	0.6	0.31
Chlorophyll *b*	1.55	1.16	1.03
Lutein	4.49	3.28	2.28
β-Cryptoxanthin	0.62	0.45	0.38
cis-Lutein	0.45	0.32	0.24
Chlorophyll *a*	0.12	0	0.2
Neoxanthin ester	0.45	0.34	0.28
Lutein ester	0.22	0.32	0.18
β-Cryptoxanthin ester	0.16	0.18	0.1
Pyropheophytin *a*	1.09	1.35	1.27
Pheophytin *b*	0.15	0.25	0.17
Pheophytin *a*′	3.47	4.17	2.92
Pheophytin *a*	19.72	25.04	19.36
β-Carotene	16.27	8.06	12.8
Total chlorophylls	26.1	31.97	24.95
Total carotenoids	27.44	18.32	19.66
Ratios			
Lutein/β-carotene	0.27	0.4	0.17
Chlorophylls/carotenoids	0.95	1.75	1.26

Means (ppm) value of eight samples, each sample in triplicate.
CV% under 9% in all HPLC measurements.
CV% under 1% in spectrophotometric measurements.

(16.27 ppm) and lutein (4.49 ppm) amounts were found in oils from the *Cerasuola* variety. The neoxanthin and β-carotene contents were higher compared to reports present in the literature for other olive oil varieties from Spain (Gandul-Rojas & Minguez-Mosquera, 1996) and from Greece (Psomiadou & Tsimidou, 2001). This may be attributed

to varietal differences with characteristic biosynthetic or catabolic pathways and/or to geographical differences. It is generally known that (Roca & Minguez-Mosquera, 2001) pigments concentration in olive fruits decreases during fruits ripening when the synthesis of anthocyanins intensifies, therefore maximum pigments content are found in olive oils extracted from green fruits; moreover, higher pigments content are found in oils produced by the *continuous* system technology, compared to oils produced by the traditional pressure system. The ratio lutein/β-carotene, which could be useful to differentiate oils from a single cultivar, was less than one in all the varieties analysed, and was similar to oils from Greek cultivars and different from Spanish cultivars were the ratio lutein/β-carotene was reported to range between 1.3 and 5.1. Independently of pigment content, the ratio between the chlorophyll and carotenoid fractions remained constant, with a value close to unity, in agreement with the report by Gandul-Rojas and Minguez-Mosquera (1996). Recently, Roca, Gandul-Rojas, Gallardo-Guerrero, and Minguez-Mosquera (2003) have shown that the oil authenticity parameters, as defined for example by the ratio of chlorophylls/carotenoids remained stable throughout a one year of oils storage at 15 °C in the dark. The presence of a specific pigment profile in olive oils could be used to guarantee the genuineness and typicality of the product, since the quality control of foods requires a precise knowledge of the pigment composition of the original products.

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