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High-performance liquid chromatographic analysis of chlorophylls, pheophytins and carotenoids in virgin olive oils: chemometric approach to variety classification☆

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Abstract

This work evaluate the possibility to get from the quali-quantitative determination of the pigments contained in monovarietal olive oils (chlorophylls, pheophytins and carotenoids) and from the multivariate statistical analysis of these measures, parameters able to distinguish within the cultivars. The chemometric variables used have concurred to obtain preliminary interesting results. Liquid-phase distribution and solid-phase extraction/purification procedures has been compared: recoveries for both are resulted higher than 94% for all the pigment classes and the R.S.D. values were below 10%. HPLC analysis, allowing the simultaneous pigment determination, and fluorescence detection, allowing a better green pigments measure (detection limits from 5 to 80 ppb), are revealed a fundamental solution.
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1. Introduction

The chemical composition of the olive oils (*Olea europaea*, L.) varies widely depending on fruit variety, degree of fruit ripeness, environmental conditions, growing region and techniques of processing and storage [1,2]. These factors influence oil colour, which is one of the basic quality characteristics of virgin olive oils. The green-yellowish colour is due to various pigments, i.e. chlorophylls, pheophytins and carotenoids. Such natural pigments can also affect considerably the preservation of the product as prooxidant, in synergy with metals eventually present. In particular the chlorophylls and the pheophytins in presence of the light act as catalysts in the formation of singlet state oxygen [3] and therefore they promote the first phases of the autoxidation process. Moreover, some researches underline the delaying role of the carotenoids in the photooxidation process [4,5]. The analysis of the chlorophylls and pheophytins has been recently considered to identify the technological treatments, like deodorization, used in a fraudulent way in the commerce of mixed olive oils [6].

The level of these compounds has been traditionally determined with spectrophotometrical methods by the measure of the total content in chlorophylls and carotenoids with values ranging, as regards the chlorophylls, from 1 to 10 ppm, and for the carotenoids from fews up to 100 ppm; it is well known in fact the absorption curve of the virgin olive oils in the visible spectrum, characterized by typical bands for the chlorophylls and the carotenoids.

Has seemed interesting to us to analyse in detail such compounds using, after a preliminary pigments separation obtained with solid-phase extraction (SPE) and liquid-phase

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distribution (LPD) procedures, the HPLC analysis coupled with contemporaneous UV–vis and fluorescence detection because this method allows to measure simultaneously chlorophylls and carotenoids and to obtain less discordant data than the obtainable ones with spectrophotometrical methods [7–11].

In this work the analysis of olive oil samples of different variety and geographic origin obtained with various extraction and conservation technologies and characterized by different maturity degrees has been made in order to evaluate and measure how these variables affect the content in pigments: in particular has been adopted a chemometric approach to identify into the pigments patterns of every monovarietal sample some parameters able to distinguish olive cultivars and genuineness of olive oils.

2. Experimental

2.1. Equipment

HPLC pigments separation was performed with a Perkin-Elmer liquid chromatographic system (Norwalk, CT, USA) equipped with a Perkin-Elmer LC 250 binary pump and a Rheodyne Model 7125 injector whit a 20 μl fixed loop (Cotati, CA, USA). Pigments detection was performed with two detector systems, a Perkin-Elmer LS 30 fluorescence spectrometer and a Perkin-Elmer LC 95 UV–vis spectrophotometer. The used column was a C_{18} reversed-phase Waters Spherisorb ODS-2 (5 μm particle size, 250 mm × 4.6 mm internal diameter) protected by a guard-cartridge system packed with the same material. A Büchi R 3000 rotavapor was also used for sample preparation.

2.2. Reagents and standards

HPLC-grade solvents (methanol, acetone) and analytical grade DMF (*N*,*N*-dimethylformamide), *n*-hexane and diethyl ether were purchased from Sigma (St. Louis, MO, USA). Ultra-pure water generated by the Milli-Q system (Millipore, Bedford, MA, USA) was used.

Chlorophylls *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 [12,13]. Carotenoids (lutein, violaxanthin and neoxanthin) standards were obtained after extraction with cold acetone and purification by OCC from curly lettuce [14].

2.3. Sampling and sample preparation

In the last 3 years have been sampled directly at the oil mill and analyzed not more than three months after the production 94 virgin olive oils of the harvests 2000, 2001, 2002; these samples were different for varietal and geographic origin, ripening degree and extraction technology. Among these 56 were monovarietal oils (Tortiglione, Dritta, Leccino, Gentile, Frantoio, Bianchera, Picholine, Peranzana, Coratina, Ogliarola, Buga, Carbonera cvs.), 12 obtained by the pressing of fruits deriving from different varieties (two or three in well-known ratios) and the remaining oils were mixtures in unknown proportions. The sampled olive oils have been analyzed by liquid chromatography techniques to determine the quali-quantitative profiles, measuring the chlorophylls *a* and *b* and the respective products of transformation, the pheophytins *a* and *b*; moreover have been measured the carotenoids lutein, violaxanthin and neoxanthin.

Has been use the analytical procedure setted by Minguez Mosquera [15]: this technique is characterized by a preliminary extraction of the pigments which must be studied in one of the following ways: LPD and SPE. LPD is realized using as solvents *n*-hexane and DMF; the hexanic fraction retains lipids and carotenes, DMF fraction chlorophylls, chlorophyllic derivatives like pheophytins and xanthophylls. The last fraction is therefore treated with a Na_2SO_4 2% solution and reextracted with *n*-hexane–diehyl ether (1:1); of the two phases so obtained, one organic and the other watery, the aqueous one is discarded removing poliphenols and other hydrophilic compounds, the organic phase dried and resuspended with acetone for the injection in HPLC. SPE is carried out using octadecyl disposable cartridges (C_{18}): the pigments contained in the oil sample (1 g), dissolved in *n*-hexane (4 mL), loaded on column and washed with the same solvent are eluted using 5 mL of acetone.

Recovery studies of the olive oil pigments have been made to compare the extraction/purification procedures. In general SPE extraction is slightly less effective than LPD: the recover capability values are instead comparable for both the methods. In Table 1, the data relative to the single pigments are shown.

2.4. HPLC analysis

We have used reversed-phase ion-pair chromatography to separate olive oil pigments; the eluents used were (A) water-ion-pair reagent (0.05 M tetrabutylammonium and 1 M ammonium acetate aqueous solution)–methanol (1:1:8, v/v) and (B) acetone–methanol 1:1 (v/v). The pigments were eluted at a rate of 1.5 mL/min following the scheme in Table 2. The clorophyllic pigments was detected fluorometrically (with a fluorescence spectrometer) using excitation and emission wavelenghts of 440 and 660 nm respectively. Carotenoids detection was obtained spectrophotometrically at 430 nm.

2.5. Statistical analysis

Multivariate analysis [factor analysis with principal component analysis (PCA) method and hierarchical clustering] was done by SPSS statistic software package.

Table 1
Study of recovery of virgin olive oil pigments by LPD and SPE

	Chlorophylls (mg/kg olive oil (ppm))		Pheophytin *a* (mg/kg olive oil (ppm))		Pheophytin *b* (mg/kg olive oil (ppm))		Lutein (mg/kg olive oil (ppm))	
	LPD	SPE	LPD	SPE	LPD	SPE	LPD	SPE
Virgin olive oil	0.50	0.42	9.12	8.45	0.40	0.25	4.71	4.55
Pigment addition	0.32	0.32	2.33	2.33	0.11	0.11	2.07	2.07
Enriched oil	0.80	0.75	11.40	10.83	0.54	0.34	6.46	6.54
Recovery (%)	*97.56*	*101.35*	*99.56*	*100.46*	*105.88*	*94.44*	*95.28*	*98.79*
R.S.D. (%, $n = 3$)	*2.60*	*4.70*	*1.22*	*2.38*	*7.53*	*7.78*	*1.12*	*1.94*

3. Results and discussion

From a comprehensive evaluation, between the olive oil pigments detected pheophytin *a* and lutein represent the most substantial fraction (more than 80% for all the samples) with values ranging between 2.06 and 37.06 ppm for the pheophytin *a* and between 3.96 and 14.78 ppm for lutein; the clorophyll *a* not always has turned out detectable, often revealed only in traces; easier the quantification of the other pigments (Table 3). The method chosen for the analysis and in particular the use of a fluorescence spectrometer as detector has turned out very useful. This allowed us to obtain a good detection of the signals concerning the pheophytins (*a*, *b* and relative epimers a' and b') and to have detection limits 10 times lower than obtainable ones with a detector UV–vis (Table 4).

The obtained data, mean and median values (Table 5), substantially agree with analogous measures reported in literature [16–19] as regards the absolute and relative amounts.

In particular with respect to the different processing technologies, variety and ripening degree we can observe that: (i) with the newer extraction technology (centrifugal or *continuous* system) the olive oils samples show a greater amount of pigments as regards the traditional (pressure system); (ii) variety lead to significant difference on the pigment composition of the end product; instead geographical origin affects mainly pigment amounts; (iii) the level of maturation of the fruits is closely correlated with the pigment amount: the collection of cherry olives for all the varieties guarantees a more elevated content in these substances than the productions obtained in complete or late maturation. Tables 6 and 7 summarize this results.

Table 2
Gradient scheme used for the HPLC separation of the olive oil pigments

Time (min)	Mobile phase		Elution curve[a]
	A (%)	B (%)	
0	75	25	
7	25	75	Linear, 1
10	25	75	
20	10	90	Convex, −5
24	0	100	Concave, +5
30	75	25	Concave, +5

[a] The numbers refer to the curve slope used by the methods of the Perkin-Elmer LC 250 binary pump.

Table 3
Typical pigment distribution

Olive oil pigment	Fraction (%)
Chlorophyll *b*	4
Chlorophyll *a*	1
Pheophytin *b*	4
Pheophytin *a*	48
Neoxanthin	4
Violaxanthin	4
Lutein	35

HPLC analysis proved to be useful for the study of the olive oil pigments in terms of separation of the various compound classes and quantitative determination of the single terms: the obtained data have point out the opportunity of a systematic study of all the fractions, for a more complete characterization of the olive oil productions. Furthermore this analysis can be applied to identify olive oil adulteration with natural or synthetic food colourings. Fig. 1 shows the comparison between three different samples: a virgin, an refined and a commercial adultered olive oil: the quali-quantitative pigment composition join the drastic decrease in pigments of the refined oil and the anomalous ratio between pheophytin *a* and pheophytin a' epimer peaks shows clearly the adulteration of the commercial as regards the virgin olive oil. In fact as regards the virgin olive oils in no case the peak concerning the a' epimer is superior than the basic epimer.

The second aim of this work was to evaluate the possibility to obtain varietal identification parameters leaving from the content in pigments of the various examined oils. To this purpose the data concerning monovarietal samples with comparable maturity degree have been analysed exploiting the tools supplied by the multivariate statistics: we have considered only four varieties in this study because only for this ones we had a sufficient number of useful samples.

Multivariate statistical analysis has been used to recognize which chemometric information coming from measured parameters of the olive oil is able to discriminate the olive cultivars. For every sample have been considered like descriptive variables the following ones: the content in single pigment (seven variable ones distinguished) and, more, new variables derived from the combination of the previous ones: for example new variables were the relationship in weight carotenes/green pigments or still the relationship

Table 4
Detection limits of the olive oil pigments (ppm)

Olive oil pigment	UV–vis detector (λ 430 nm)	Fluorescence spectrometer (λ_{exc} 440 nm, λ_{em} 660 nm[a])
Chlorophyll *b*		0.027
Chlorophyll *a*		0.005
Pheophytin *b*		0.009
Pheophytin *a*		0.080
Neoxanthin	0.013	
Violaxanthin	0.010	
Lutein	0.013	

[a] λ_{exc}, λ_{em} are respectively excitation and emission wavelengths.

Table 5
Virgin olive oil pigments: summarizing table

Olive oil pigment	Mean values (mg/kg olive oil (ppm))	Median values (mg/kg olive oil (ppm))	Range (mg/kg olive oil (ppm))
Chlorophyll *b*	0.92	0.41	0.00–5.19
Chlorophyll *a*	0.29	0.01	0.00–6.18
Pheophytin *b*	1.20	0.92	0.05–9.72
Pheophytin *a*	12.09	10.75	2.06–37.06
Neoxanthin	0.91	0.86	0.12–2.36
Violaxanthin	0.89	0.51	0.00–5.15
Lutein	7.82	6.82	3.96–14.78

Table 6
Comparison between olive oil extraction technologies: centrifugal or *continuous* system versus traditional or *pressure* system

Olive oil pigment	Traditional system[a]		Continuous system[a]		Continuous vs. traditional
	Mean	Median	Mean	Median	
Chlorophyll *b*	0.52	0.23	1.23	1.06	−57.44 %
Chlorophyll *a*	0.27	0.01	0.37	0.01	−28.05 %
Pheophytin *b*	1.17	0.98	1.44	1.10	−18.31 %
Pheophytin *a*	10.74	8.75	14.46	13.60	−25.72 %
Neoxanthin	0.24	0.25	1.08	0.95	−77.47 %
Violaxanthin	0.35	0.37	0.67	0.16	−48.20 %
Lutein	5.44	5.47	9.30	9.39	−41.54 %

[a] mg/kg olive oil (ppm).

Table 7
Correlation between the level of maturation of the fruits and the pigment amounts

Olive oil pigment	Green olives (mg/kg olive oil (ppm))	Cherry olives (mg/kg olive oil (ppm))	Black olives (mg/kg olive oil (ppm))
Chlorophylls	1.80	1.56	1.13
Pheophytins	17.74	16.74	12.25
Carotenoids	12.97	12.44	9.54
Total pigments	32.51	30.75	22.92

Table 8
List of variables describing olive oil samples used in multivariate statistical analysis

Abbreviation	Variable	Abbreviation	Variable
1. Chlb	Chlorophyll *b*	10. $\sum$Y	Sum of *yellow* pigments
2. Chla	Chlorophyll *a*	11. $\sum$Chls	Sum of chlorophylls
3. Pheob	Pheophytin *b*	12. $\sum$Pheos	Sum of pheophytins
4. Pheoa	Pheophytin *a*	13. Y/G	Ratio *yellow*/*green* pigments
5. Neoxanthin	Neoxanthin	14. Chls/Lut	Ratio chlorophylls/lutein
6. Violaxanthin	Violaxanthin	15. fPheos·fLut	$\sum$Pheos/$\sum$G·lutein/$\sum$G
7. Lutein	Lutein	16. fChls·fLut	$\sum$Chls/$\sum$G·lutein/$\sum$G
8. $\sum$	Sum of all the pigments	17. fChla	Chla/($\sum$Chls + $\sum$G-lutein)
9. ΣG	Sum of *green* pigments	18. fChlb	Chlb/($\sum$Chls + $\sum$G-lutein)

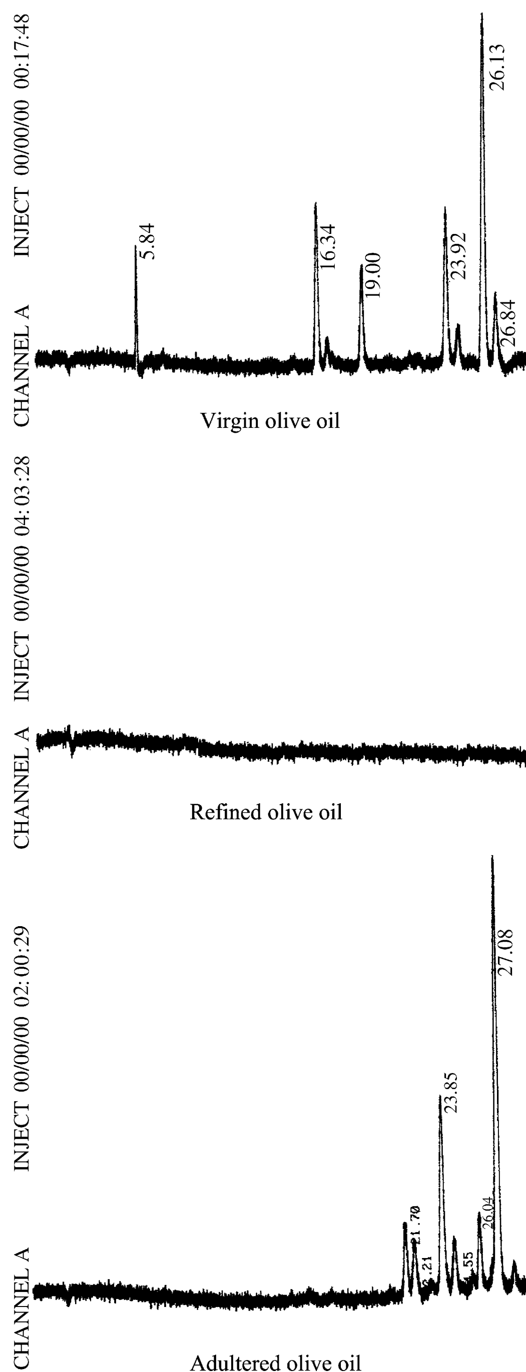

Fig. 1. Comparison between three different olive oil samples.

chlorophylls/lutein. A complete list of the variables considered is reported in Table 8. In order to identify between the variables taken in consideration the ones able to explain the variance shown by the olive oil samples, the first statistical method used has been the factor analysis, and the extraction method PCAi used; this allows to focalize our attention only on few mainly meaningful variables excluding the others.

The entire data matrix for each sample was subjected to PCA. This analysis is a well known technique which provides a significant insight into the structure of a data set. PCA generates a set of new orthogonal variables (axes), the principal components (PCs), linear combination of the original variables, so that the maximal amount of variance contained in the starting data set is concentrated in the first principal components. Therefore, PCA is suitable to reduce the dimensionality of large data matrices by eliminating the non-significant principal components and facilitating successive analyses on the reduced data. The data were auto-scaled before PC computation in order to asses the same weight to each variable. Analysing the covariance matrix, four principal component were needed to account for about 94% of the total variation (Table 9). The loadings associated to each variable on the first four principal component identify the variables that mostly define them (Table 10).

Table 9
Percentage variance contributions by the first 10 PCs

PCs	Variance (%)	Cumulative variance (%)
1	54.90	54.90
2	22.36	77.26
3	10.75	88.01
4	5.92	93.93
5	3.08	97.01
6	1.20	98.21
7	0.87	99.08
8	0.59	99.67
9	0.18	99.85
10	0.07	99.92

The projections of the loadings on the plane defined by the first two principal components are illustrated in Fig. 2. These projections allow us to visualize the position of the variables in the plane and the corresponding correlations. In fact, if two variables are distant (the angle between the respective vectors is for example 90°) they are less correlated because the correlation coefficient is the cosine of this angle (cos 90° = 0).

Table 10
Loadings of variables on the first four components

Variable	PC1	PC2	PC3	PC4
Chlb	0.953	−0.151	− 0.127	0.053
Chla	0.072	−0.915	0.253	−0.247
Pheob	0.753	0.514	0.335	−0.080
Pheoa	0.942	0.269	0.123	−0.141
Violaxanthin	0.341	−0.174	0.592	0.681
Lutein	0.890	0.116	0.321	−0.163
Neoxanthin	0.898	0.101	0.038	0.012
$\sum$	0.955	0.193	0.208	−0.086
$\sum$G	0.951	0.241	0.125	−0.126
$\sum$Y	0.903	0.071	0.381	0.011
$\sum$Pheo	0.932	0.293	0.143	−0.136
$\sum$Chl	0.924	−0.319	−0.073	0.004
Y/G	−0.842	−0.051	0.433	0.047
Chls/Lut	0.729	−0.477	−0.428	0.100
fPheo·fLut	−0.566	0.552	0.008	−0.577
fChls·fLut	0.536	−0.736	−0.368	0.105
fChla	0.047	−0.928	0.228	−0.232
fChlb	0.713	−0.085	−0.633	−0.180

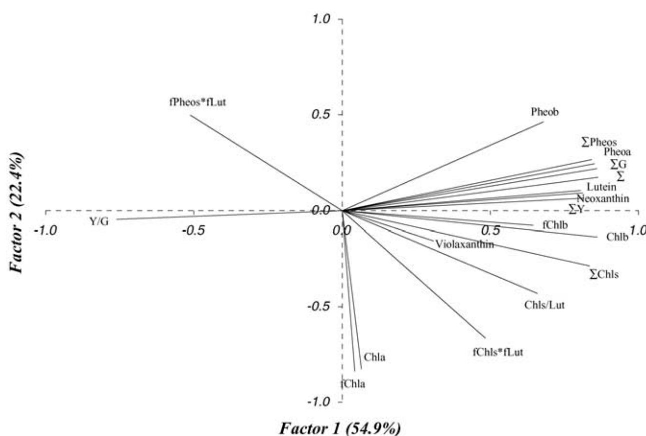

Fig. 2. Projections of loadings of the variables on the first two PCs.

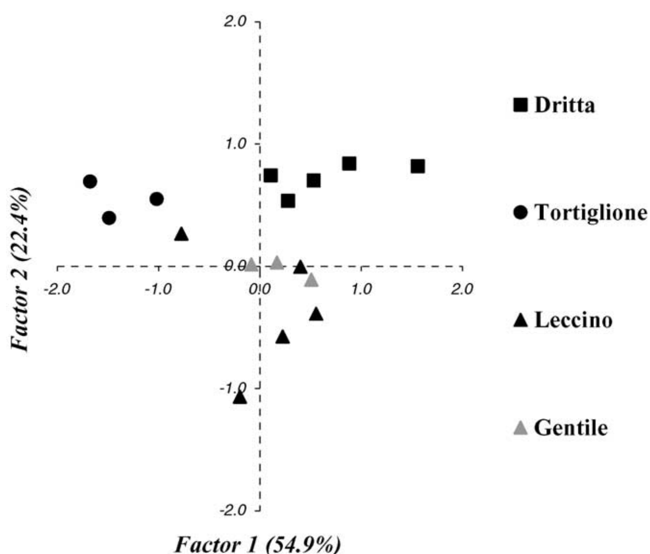

Fig. 3. Scores of samples on the first two PCs.

The scores of data plotted on the first two principal components shows clearly three grouping of olive varieties (Fig. 3). Comparing the Figs. 2 and 3 we can easily to recognize the variables characterizing the varieties considered.

4. Conclusion

The multivariate statistical approach applied on olive oil pigment data obtained using HPLC techniques allow to recognize, among many descriptive variables, the most significant ones, able to cluster olive oil sample and able to lead us to a first classification of olive variety.

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