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High performance size-exclusion chromatography analysis of polar compounds applied to refined, mild deodorized, extra virgin olive oils and their blends: An approach to their differentiation

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ABSTRACT

An investigation was carried out to evaluate the use of High Performance Size-Exclusion Chromatography (HPSEC) of polar compounds of refined, mild deodorized, extra virgin olive oils as well as of their blends, in attempting to reveal significant differences in the amounts of the substance classes constituting polar compounds among these oils. Two sets of blends were prepared by mixing an extra virgin olive oil with both refined and mild deodorized olive oils in increasing amounts. The obtained data highlighted that the triacylglycerol oligopolymers were absent or present in traces in the extra virgin olive oil, while their mean amount was equal to 0.04 g/100 g and 0.72 g/100 g in mild deodorized and refined olive oils, respectively. Oxidized triacylglycerols and diacylglycerols were more abundant in mild deodorized oil and refined oil than in extra virgin olive oil. The Factorial Discriminant Analysis of the data showed that the HPSEC analysis could reveal the presence of refined/mild deodorized oils in extra virgin olive oils. In particular, the classification functions obtained allowed designation of mixtures containing at least 30 g/100 g of mild deodorized oil and all those containing refined olive oil as deodorized oil, therefore as oils subjected to at least a mild refining treatment.

1. Introduction

Olive oil is one of the oldest known vegetable oils, widely consumed in the Mediterranean countries (Hrncirik & Fritsche, 2005). According to the trade standard of the International Olive Council for olive oils and olive-pomace oils and to the European Community (EC) Regulations (International Olive Council, 2009; Official Journal of European Community, 2001), extra virgin olive oil is the oil obtained from the fruit of the olive tree solely by mechanical or other physical means, under conditions that do not lead to alterations in the oil, and which has not undergone any treatment other than washing, decanting, centrifuging, or filtration.

The growing interest for extra virgin olive oil over the past few years can be attributed not only to its superior organoleptic characteristics (aroma and taste), but also to its potential health benefits and remarkable antioxidant properties (Jafari, Kadivar, & Keramat, 2009; Owen et al., 2000).

These properties confer to extra virgin olive oil a great value and cause its relatively high price on the market, thus encouraging fraudulent practices such as mixing cheaper oils, from other vegetable sources or lower quality olive oils, with extra virgin olive oil (Jafari et al., 2009).

The newest, most common adulterations of extra virgin olive oil are additions of both refined olive oil and mild deodorized olive oil. The latter is obtained from a virgin oil with an unpleasant flavor, subjected to a mild thermal deodorization (80–120 °C) for removing undesired substances that negatively influence its flavor. Subsequently this mild refined oil can be blended with extra virgin olive oil in variable amounts (Saba, Mazzini, Raffaelli, Mattei, & Salvadori, 2005).

In general these blends, and in particular the blend between extra virgin olive oil and mild thermal deodorized oil, do not produce easily detectable modifications of the chemical composition. Indeed, the parameters usually checked as quality indicators, such as triacylglycerols composition, sterols, and newly formed steroid hydrocarbons are not appreciably altered by the blending (Saba et al., 2005).

Different approaches to this issue have been used, on the basis of both common analytical techniques, such as gas chromatography

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(GC) and HPLC, and more advanced ones. Fragaki, Spyros, Siragakis, Salivaras, and Dais (2005) applied nuclear magnetic resonance (NMR) spectroscopy and multivariate statistical analysis to detect the adulteration of extra virgin olive oil with *lampante* and refined olive oil. Chiavaro et al. (2008) evaluated the potential application of differential scanning calorimetry (DSC) to discriminate among olive oils of different commercial categories (extra virgin olive oil, olive oil, refined olive oil, olive-pomace oil and refined olive-pomace oil). Jafari et al. (2009), instead, investigated the usefulness of DSC, NMR spectroscopy and GC analysis of fatty acid methyl esters in the detection of adulteration of olive oil with soybean, sunflower, and canola oils. In a study carried out by Oliveros, Concepcion, Pavon, and Luis (2002), electronic olfactometry was used for the detection of samples of virgin olive oil adulterated with sunflower oil and olive-pomace oil. Mildner-Szkudlarz and Jelen (2008) investigated the effectiveness of three rapid methods of volatile compounds analysis, with subsequent principal component analysis (PCA) treatment of data, in differentiating among virgin olive oil samples adulterated with hazelnut oil. Pérez-Camino, Cert, Romero-Segura, Cert-Trujillo, and Moreda (2008) determined the concentration of fatty acid alkyl esters for detecting the blends of extra virgin olive oil and mildly refined low quality olive oils. The identification of oils of different botanical origin (sunflower, corn, peanut, and coconut oils) in extra virgin olive oil has been also investigated by Priego Capote, Ruiz Jimenez, and Luque de Castro (2007), by means of chemometric treatment of chromatographic profiles. Tay, Singh, Krisnan, and Gore (2002), instead, have investigated the application of Fourier transform infrared spectroscopy to identify the adulteration of extra virgin olive oil with several seed oils.

High Performance Size-Exclusion Chromatography (HPSEC) of polar compounds (PC) – which allows the separation and the quantitation of triacylglycerol oligopolymers (TAGP), oxidized triacylglycerols (ox-TAG), and diacylglycerols (DAG) – could be useful to discriminate among extra virgin olive oils, refined and mild deodorized ones. TAGP, which develop during bleaching and especially deodorization in the refining process (Eder, 1982; Gomes & Caponio, 1998; Gomes, Caponio, & Delcuratolo, 2003), are a reliable index of secondary oxidative degradation of an oil, because they are stable and not influenced by processing conditions. On the contrary, ox-TAG, which comprise all form of triacylglycerols oxidation, could give information about the primary oxidation level of oil. Finally, DAG constitute a reliable parameter for a proper evaluation of the real level of hydrolytic degradation of refined oils.

The HPSEC analysis was already used to assess the actual level of oxidative and hydrolytic degradation of oils, either refined or subjected to treatments requiring high process temperatures (Dobarganes, Pérez-Camino, & Márquez-Ruiz, 1988; Gomes & Caponio, 1998; Hopia, 1993). Moreover, it was used also to monitor the changes occurring in oil during frying (Arroyo, Cuesta, Garrido-Polonio, López-Varela, & Sánchez-Muñiz, 1992; Garrido-Polonio, Sánchez-Muñiz, Arroyo, & Cuesta, 1994; López-Varela, Sánchez-Muñiz, Garrido-Polonio, Arroyo, & Cuesta, 1995) as well as to assess the quality of the lipid fraction of various foods (Caponio & Gomes, 2004; Caponio, Gomes, & Summo, 2003; Caponio, Gomes, Pasqualone, & Summo, 2007; Summo, Bilancia, & Caponio, 2008). It is worth noting that the products of triacylglycerol oxidation, at high amounts, could have harmful effects on the consumer's health (Billek, 2000; Saguy & Dana, 2003), as well as a pro-oxidative activity (Frankel, Neff, Selke, & Brooks, 1988; Gomes, Delcuratolo, & Paradiso, 2008; Mistry & Min, 1988), that could negatively influence the shelf-life of oils.

In this paper, HPSEC analysis of PC was applied in attempting to reveal differences in the classes of substances constituting them in refined, mild deodorized, extra virgin olive oils and their blends. Multivariate statistical analysis was also applied for a first approach to their differentiation.

2. Materials and methods

2.1. Sampling and blends preparation

Forty different extra virgin olive oils (EVO) produced between 2005 and 2009 crop seasons were provided by local cooperatives; twenty-seven refined olive oils (RO) were sampled directly from refining industries between 2005 and 2009; and six mild deodorized olive oil (DO) were provided by industrial plants between 2006 and 2009. The samples of both RO and DO were representative of large batches of oils. The small number of mild deodorized olive oils was due to the hard difficulties to find such oils.

Two sets of blends were prepared in the laboratory, by mixing an EVO with DO and RO at different increasing amounts: 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, and 75 g/100 g. All the oils used for the blends were produced in 2009.

2.2. Analytical determinations

Free fatty acids, peroxide value, UV spectrophotometric constants, and stigmastadienes were determined as prescribed by the official analytical methods of EC Regulations (Official Journal of European Community, 1991, 1995).

PC were separated from the oil samples by silica gel column chromatography, according to the AOAC method (Association of Official Analytical Chemists, 2003). After elution of the non-polar components with 150 mL of petroleum ether-diethyl ether (87:13, v/v), the PC were recovered with 150 mL of diethyl ether. The efficacy of separation was checked by thin layer chromatography as recommended by the same method. Then, the PC recovered in CH_2Cl_2 were analyzed by means of HPSEC analysis to determine the amounts of TAGP, ox-TAG, and DAG. The chromatographic system consisted of a Perkin-Elmer pump, series 200, a 7125S sample injector (rheodyne), a 50 μL injector loop, a PL-gel guard column (Perkin-Elmer, Beaconsfield, UK) of 5 cm length × 7.5 mm i.d., and a series of three PL-gel columns (Perkin-Elmer, Beaconsfield, UK) of 7.5 mm i.d. × 30 cm in length each. The columns were packed with highly crosslinked styrene divinylbenzene copolymers with a particle diameter of 5 μm and pore diameters of 500 Å, 500 Å and 100 Å, respectively. The detector was a differential refractometer (series 200A, Perkin-Elmer, Beaconsfield, UK). The elution solvent used was CH_2Cl_2 for HPLC at a flow rate of 1.0 mL/min. Peaks on the chromatograms were identified by polystyrene standards (Supelco, Milan, Italy) of known molecular weight (MW = 4000 and 2000 g/mol) as well as tristearin, distearin, and monostearin standards (Sigma-Aldrich, St. Louis, MO, USA). For each standard, the elution volume was measured under the same conditions as used in our

Table 1
Mean values and standard deviation of the polar compounds and of the main substance classes constituting them in extra vergin olive oil (EVO), mild deodorized olive oil (DO) and refined olive oil (RO) groups, as well as the results of the ANOVA and the post-hoc Tukey's HSD test.

Parameters	EVO ($n = 40$)		DO ($n = 6$)		RO ($n = 27$)		F valvue
	Mean	SD	Mean	SD	Mean	SD	
PC	2.30	0.61B	3.96	0.71A	4.71	1.10A	69.0779
TAGP	0.00	0.00B	0.04	0.02B	0.72	0.36A	90.6295
ox-TAG	0.42	0.11B	0.97	0.44A	0.86	0.20A	55.5134
DAG	1.37	0.29C	2.08	0.37B	2.79	0.64A	78.5855

PC, polar compounds (g/100 g); TAGP, triacylglycerol oligopolymers (g/100 g); ox-TAG, oxidized triacylglycerols (g/100 g); DAG, diacylglycerols (g/100 g). Different letters mean significant difference at $p \leq 0.001$.

Table 2
Analytical characteristics of the oils used for making the blends.

Parameters	EVO		DO		RO	
	Mean	SD	Mean	SD	Mean	SD
FFA	0.67	0.04A	0.54	0.02A	0.29	0.04B
PV	3.8	0.2B	18.0	0.2A	1.9	0.1C
K232	1.310	0.008C	2.445	0.023A	1.729	0.009B
K270	0.120	0.007C	0.167	0.006B	0.714	0.006A
PC	2.40	0.04C	4.38	0.14B	4.75	0.11A
TAGP	0.00	0.00C	0.05	0.01B	0.31	0.01A
ox-TAG	0.26	0.01C	1.36	0.03A	0.76	0.01B
DAG	1.20	0.02C	2.07	0.02B	3.03	0.01A
Stig	0.00	0.00B	0.10	0.01B	5.17	0.35A

EVO, extra virgin olive oil; DO, mild deodorized olive oil; RO, refined olive oil. FFA, free fatty acids (g/100 g); PV, Peroxide value (meqO2/kg); K_{232}, specific absorption at 232 nm; K_{270}, specific asborption at 270 nm; PC, polar compounds (g/100 g); TAGP, triacylglycerol oligopolymers (g/100 g); ox-TAG, oxidized triacylglycerols (g/100 g); DAG, diacylglycerols (g/100 g); Stig, stigmastadienes (mg/kg). Different letters mean significant difference at $p \leq 0.05$.

analysis. The log of MW as a function of elution volume was plotted, and the line of best fit was drawn by using the least square method. From the elution volume of each separated peak in a chromatogram, the corresponding MW could then be obtained using the calibration curve (Gomes, 1992). For quantitative determination of the peaks, known amounts of TAGP, ox-TAG, and DAG were obtained by preparative gel permeation chromatography of PC derived from a refined peanut oil and then used as standards in HPSEC method. The amount collected for each standard, corresponding to a given class of compounds, was used to prepare a stock solution in CH_2Cl_2 and solution containing different concentrations after successive dilutions. These solutions were analyzed by HPSEC following the analytical method we developed. The calibration curves were obtained by plotting the amounts of standards (μg) that had been injected into the HPSEC system loop, against the areas of the corresponding chromatogram peaks (Gomes & Caponio, 1999). In order to identify the free sterols and triterpene diols, the unsaponifiable matter of an olive oil was fractionated by thin layer chromatography. The bands corresponding to sterols and triterpene diols were recovered in CH_2Cl_2 and analyzed by HPSEC analysis: these substances were eluted together as one peak before the free fatty acids peak. Subsequently the GC analysis of these bands showed a typical gas chromatogram of sterols and triterpene diols of the olive oil (Gomes & Caponio, 1996). The free fatty acids were identified by standard of oleic acid but the quantitative determination was carried out by acid-basic titration (Official Journal of European Community, 1991). To evaluate the accuracy and the precision of the HPSEC method, ten independent replicate analyses of PC of the same oil were carried out. The percent coefficient of variation obtained from the replications ($n = 10$) was 1.4% for PC, 1.2% for TAGP, 1.3% for ox-TAG, and 1.6% for DAG. The limits of detection for each class of compounds constituting the PC were less than 0.01 g/100 g of fat.

All the analyses were carried out twice.

2.3. Statistical analysis

Linear regressions and one-way analysis of variance (ANOVA) were performed by XlStat software (Addinsoft, USA, 2008.1.01 version). The significance level was set at $p < 0.05$. Correlations and Factorial Discriminant Analysis (FDA) were performed by Statistica software (Statgraphics, USA). To perform FDA and build discriminant functions, a factorial design (to degree 3) for the independent variables and the best subsets model building were adopted. Equal a priori probabilities were set.

3. Results and discussion

Table 1 reports the mean values and standard deviation of the PC and the different substance classes constituting them detected in EVO, RO and DO groups, as well as the results of the ANOVA and the post-hoc Tukey's HSD test. PC showed higher levels in both DO and RO respect to EVO. As regards TAGP, they were absent or present in traces in EVO, but present in quite high amounts in RO (mean value equal to 0.72 g/100 g of fat). It is noteworthy that DO showed little but appreciable levels of oligopolymers (mean value equal to 0.04 g/100 g of fat). This could be of great interest, since TAGP are stable oxidation products, that do not undergo further degradation. Also ox-TAG showed differences amongst the different types of oils, being RO and DO significantly richer in these substances than EVO. A great variability for this parameter was observed for DO. Finally, DAG were found in different levels in the three classes of oils: in particular, RO had the highest amounts, while EVO showed the lowest ones. The higher amount of TAGP in RO compared to DO is attributable to the higher temperatures

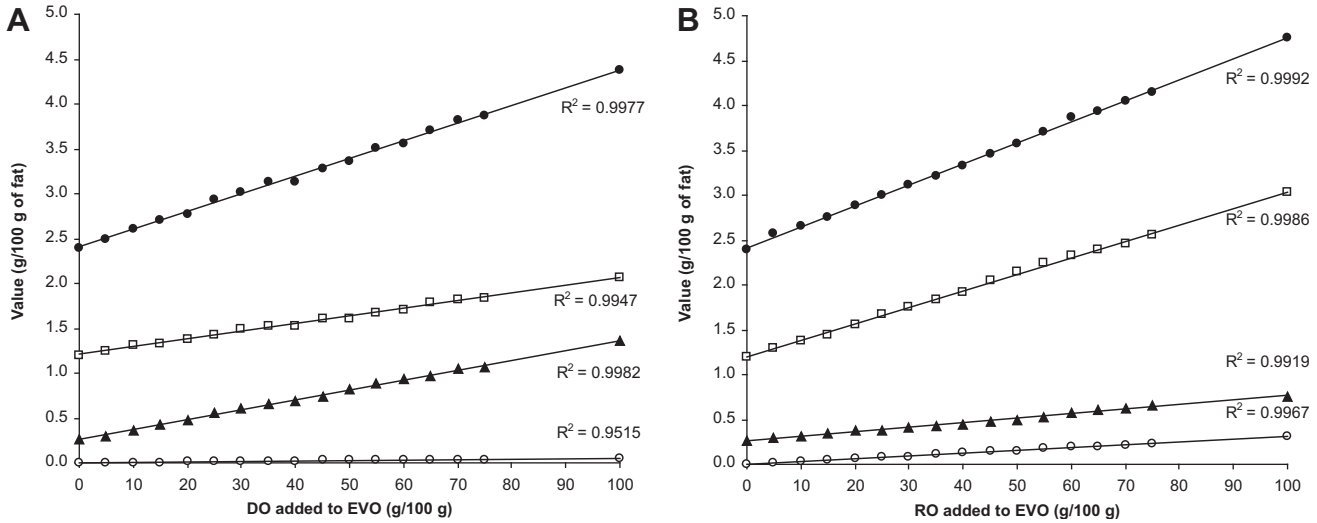

Fig. 1. Linear regressions of polar compounds (PC, ●), triacylglycerol oligopolymers (TAGP, ○), oxidized triacylglycerols (ox-TAG, ▲) and diacylglycerols (DAG, □) with the amount (g/100 g) of both mild deodorized olive oil (DO, A) and refined olive oil (RO, B) added to extra virgin olive oil (EVO).

achieved during the entire refining process, taking also in account that TAGP are the product of the polymerization reactions of ox-TAG (Gomes et al., 2003). A significant and negative correlation between these two parameters, in fact, was found in studies on the refining process of edible oils (Gomes & Caponio, 1996, 1998; Gomes et al., 2003). The higher amount of DAG detected in RO than in DO is an index of a more intense hydrolytic degradation in the first ones.

In Table 2 the analytical characteristics of the oils used for making the blends in the experimental work are reported. The parameters usually checked as quality indicators fell within the limits prescribed by EC regulations for EVO and RO (Official Journal of European Community, 2007). The values of the above cited parameters for DO could allow to classify it as an extra virgin olive oil, but since this oil was subjected to a mild deodorization, it should be classified as a refined olive oil (Official Journal of European Community, 2001). The values of free fatty acids and peroxide value, higher than 0.3 g/100 g and 5 meq O_2/kg respectively, confirmed that DO was not subjected to neutralization and bleaching, steps in which free fatty acids are removed and hydroperoxides partially degraded (Bernardini, 1983; Hui, 1996).

As regards stigmastadienes – which are formed by the acid catalyzed sterol dehydration reaction during bleaching process (Cert, Lanzon, Carelli, & Albi, 1994; Grob, Giuffre, Biedermann, & Bronz, 1994; Zschau, 2001) or during the deodorization process, promoted by high temperatures (Kim & Nawar, 1991) – their content in EVO was consistent with their commercial category. The low amount determined in DO, instead, was due to the fact that this oil was not subjected to bleaching but only to a mild deodorization. Moreover, it is already known that the formation of stigmastadienes can be avoided by conveniently modulating the processing parameters during refining (Paganuzzi, 1997), thus obtaining oils with a stigmastadienes content similar to that of a virgin olive oil.

Regarding PC, preliminarily separated from oils by silica gel column chromatography, the definitively lower values detected in EVO with respect to those determined in refined and deodorized oils, indicate a higher hydrolytic and oxidative degradation of the last ones.

In Fig. 1 the percent amounts of TAGP, ox-TAG and DAG determined for the single blends EVO + DO and EVO + RO, respectively, are reported. All parameters showed a very high linearity for both series of blends – as shown by R^2 as well as by very low values of mean squared error and standardized errors (data not shown) – throughout the whole range of the adopted blending proportions.

With the aim to assess the usefulness of the HPSEC indices in helping to discriminate extra virgin olive oils adulterated by the addition of refined or mild deodorized olive oils, factorial discriminant analysis (FDA) was performed. Classification functions were built using PC, TAGP, ox-TAG and DAG values of the set of known sample oils (40 EVO, 27 RO, and 6 DO) and combining them in a 3-degree factorial design, i.e. a polynomial model including the first-order terms for the predictors as well as the combinations of the predictors to obtain terms up to degree 3. The best subset approach allowed to compare simplified submodels obtained from the full model (i.e. polynomial models comprising a smaller number of terms selected among the terms of the full model) and select the best possible submodel for discriminating the samples. The selected model included *TAGP* and *PC* × *TAGP* × *ox-TAG* variables. Table 3 reports the standardized coefficients of the classification functions, used to obtain the coordinates of the class centroids and of the oil samples in order to subsequently classify them. In the lower part of the Table, the means of each class are also reported. The different classes, as can be observed, could be discriminated considering the balance between the overall degradation (both oxidative and hydrolytic) – expressed by the *PC* × *TAGP* × *ox-TAG* factor – and the level of the polymerization products, *TAGP*. In fact, both mild and traditional refining processes cause a shift of this balance in favour of the oligomeric compounds. Thus, TAGP in particular could offer a significant contribute in discriminating oils. The significance of TAGP in the classification functions confirms their potential contribute to detect the presence of low quality (such as refined or deodorized olive oils) olive oils blended with extra virgin olive oils: they are stable end-products, their formation is mainly due to processing and they are absent or present in trace levels in good quality extra virgin olive oils.

Table 3
Standardized coefficients and class means relative to the FDA.

	Standardized coefficients		Class means		
	TAGP	PC × TAGP × ox-TAG	EVO	DO	RO
Function 1	3.45219	−2.95060	−2.02193	−1.68447	3.369774
Function 2	−1.39546	2.27132	0.00193	−0.01370	0.000191

Table 4
Classification of the mixtures of extra vergin olive oil (EVO) with mild deodorized olive oil (DO) or with refined olive oil (RO) by factorial discriminant analysis.

DO added to EVO (g/100 g)	Classification	RO added to EVO (g/100 g)	Classification
5	EVO	5	DO
10	EVO	10	DO
15	EVO	15	DO
20	EVO	20	DO
25	EVO	25	DO
30	DO	30	DO
35	DO	35	DO
40	DO	40	DO
45	DO	45	DO
50	DO	50	DO
55	DO	55	DO
60	DO	60	DO
65	DO	65	DO
70	DO	70	DO
75	DO	75	DO

The classification functions obtained gave no misclassifications in the starting set of oil samples. When applied to the oil mixtures (Table 4), they classified mixtures containing up to 25 g/100 g of DO as EVO; all other mixtures (those containing more than 25 g/100 g of DO and all those containing RO) were classified as deodorized oil, therefore as oils subjected to at least a mild refining treatment.

4. Conclusions

The obtained data highlighted the lower levels of oxidative and hydrolytic degradation of extra virgin olive oils respect to other oils. The FDA of the data indicated that the HPSEC analysis could give a contribute in revealing the presence of refined or mild deodorized oils in extra virgin olive oils. These preliminary results encourage in applying HPSEC analysis to more complex experimental designs including several blends of oils.

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