SPECIAL ISSUE PAPER

Raquel Mateos · José A. García-Mesa

Rapid and quantitative extraction method for the determination of chlorophylls and carotenoids in olive oil by high-performance liquid chromatography

Abstract Colour is an organoleptic characteristic of virgin olive oil and an important attribute that affects the consumer perception of quality. Chlorophylls and carotenoids are the main pigments responsible for the colour of virgin olive oil. A simple analytical method for the quantitative determination of chlorophylls and carotenoids in virgin olive oils has been developed. The pigments were isolated from small samples of oil (1.0 g) by solid-phase extraction using diol-phase cartridges (diol-SPE), and the extract was analysed by reverse-phase HPLC with diode-array UV detection. Chromatographic peak resolution, reproducibility (coefficient of variation (C.V.) <4.5%) and recovery (>98.4%) for each component were satisfactory. A comparative study of the proposed method was performed versus classical liquid-liquid extraction (LLE) with N,N'-dimethylformamide and solid-phase extraction using a C18 column (C18-SPE). While 96.4% of the pigments were recovered by LLE, only 51.3% were isolated by C18-SPE in comparison to diol-SPE. Likewise, a higher alteration of pigment composition was observed when such LLE and C18-SPE procedures were used. In this sense, a higher ratio of pheophytin in comparison to that isolated by the diol-SPE procedure was achieved with both extraction procedures, indicating a greater extent of the pheophytinization reaction. Therefore, quantification of pigments from virgin olive oil by diol-SPE followed by RP-HPLC was found to be rapid, simple, required only a small amount of sample, consumed only small amounts of organic solvents, and provided high recoveries, accuracy and precision.

Keywords Carotenoids · Chlorophylls · Virgin olive oil · HPLC · Solid-phase extraction

R. Mateos · J. A. García-Mesa (✉)
C.I.F.A. Venta del Llano, IFAPA,
Ctra. Bailén-Motril Km 18.5,
23620 Mengíbar, Spain
e-mail: josea.garcia.mesa.ext@juntadeandalucia.es
Tel.: +34-953-370150
Fax: +34-953-374017

Introduction

Virgin olive oil obtained from olive fruit (*Olea europaea*) presents colours ranging from greenish-yellow to gold, depending on the fruit's ripeness. Colour is an organoleptic parameter of virgin olive oil and an important attribute that affects the consumer's perception of quality. Chlorophylls and carotenoids are the main pigments responsible for the colour of virgin olive oil. During the storage period, chlorophyll undergoes specific changes that imply an alteration of the pigment profile commonly associated with recently extracted virgin olive oil [1]. There is no loss of pigments; rather, qualitatively there is a general shift in the chlorophyll pheophytinization reaction initiated during the extraction process, as a consequence of the released acidity, implying that pheophytin *a* and lutein are the major components of the chlorophyll and carotenoid fractions, respectively, in oils stored for a long time before marketing.

Differences have been observed in chlorophyll and carotenoid composition, depending on the fruit variety, the growing region, the degree of fruit ripeness, the extraction process, and the storage conditions of the oil [2, 3]. In addition, chlorophylls and carotenoids play an important role in stability, related to their antioxidant nature in the dark and pro-oxidant activity in the light, which depends on their concentration in the fruit [4–10].

The levels of these compounds have been determined traditionally with spectrophotometric methods by measuring the total content in chlorophylls and carotenoids, since the absorption spectra of virgin olive oils present typical bands for chlorophylls and carotenoids in the visible part of the spectrum [3]. However, to better characterize the compounds belonging to the pigment fraction of virgin olive oil, the isolated substances were analysed by HPLC. In this sense, the pigment fraction of olive oils is currently isolated by liquid-liquid extraction based on a selective separation of pigments with N,N'-dimethylformamide (DMF) and hexane [11]. Although this extraction procedure is the most widely used, this procedure is very laborious and the composition of the pigment fraction can be slightly affected. Consequently, attempts have been

made to isolate the pigment fraction by solid-phase extraction (SPE) using C_{18} cartridges [11], resulting in low recovery of chlorophyll fractions due to their partial coextraction with lipid compounds. The analysis of the pigment fraction has also been performed by reversed-phase high-performance liquid chromatography (RP-HPLC) with a UV-vis spectrophotometer [11–13] or in series with a fluorescence spectrometer [14].

On the other hand, major carotenoids and chlorophylls in conjunction with tocopherol have also been analysed by the direct injection of an oily solution onto an HPLC with a photodiode detector [15], electrochemical detector [16], or UV-Vis spectrophotometer in series with a fluorescence detector [17]. These methodologies avoid the isolation step of pigments from virgin olive oil but are less sensitive at detecting minor components such as xanthophylls, quantifying only the main components of the pigment fraction.

In this work, a simple, rapid and precise analytical method was developed for the qualitative and quantitative determination of chlorophylls and carotenoids in virgin olive oils. The pigment fraction was isolated from oil samples by solid-phase extraction using diol-phase cartridges, and the extract was analysed by reverse-phase HPLC and a UV-visible detector. The results are compared with those obtained by extraction with N,N'-dimethylformamide and by solid-phase extraction using a C_{18} column.

Materials and methods

Virgin olive oil samples

Several virgin olive oils (VOO) of the '*Picual*' variety were extracted, using a laboratory-scale Abencor Oil mill (Abengoa, Spain), comprising a hammer mill, a thermo-beater and a vertical centrifuge. Olive paste was kneaded at 28 °C for 30 min [18] and the oil was collected by centrifugation of the paste. After being decanted for 30 min, the oil samples were filtered and then stored at −4 °C until analysed.

Analytical materials, reagents and standards

All reagents were of analytical or chromatographic grade.

Sodium carbonate anhydrous, sodium sulphate anhydrous, potassium hydroxide 85 %, *n*-hexane, N,N'-dimethylformamide and methanol were acquired from Panreac (Barcelona, Spain). Anhydrous diethyl ether and acetone, triethylamine and ammonium acetate were purchased from Sigma Chemical Co. (Madrid, Spain). Acetone and methanol were of HPLC grade (Romil Ltd., Cambridge, UK). SPE cartridges packed with diol-phase (500 mg/3 mL) were from Supelco (Bellefonte, PA, USA), and SPE-cartridges packed with C_{18}-phase (1 g/6 mL) were from J.T. Baker (Phillipsburg, NJ, USA).

The standards used to quantify the pigment were as follows: chlorophyll *a*, and chlorophyll *b* standards were supplied by Sigma (St. Louis, MO, USA). Pheophytins *a* and *b* were obtained from the respective solution of chlorophyll by acidification with hydrochloric acid until the green chlorophyll colour changed to the greyish pheophytin colour [19]. Lutein and β-cryptoxanthin were purchased from Extrasynthese (Genay, France). Antheraxanthin, violaxanthin and neoxanthin were obtained from a pigment extract of fresh spinach and separated by TLC on silica gel GF_{254} (0.2 mm) on 20×20 cm plates using petroleum ether (65–95 °C)/acetone/diethylamine (10:4:1) [11].

Analytical procedures

Liquid extraction of pigments with N,N'-dimethylformamide

The method used to obtain the pigment extract is based on the selective separation of pigments using N,N'-dimethylformamide (DMF) and hexane. Lipids and the carotene fraction appeared in the hexane phase, while the DMF phase retained chlorophylls and xanthophylls [11]. The sample of virgin olive oil (7.5 g) was dissolved in 75 mL of DMF saturated with Na_2CO_3 and treated with five successive 50 mL portions of hexane.

The DMF phase was transferred to a 1,000 mL separatory funnel containing 200 mL of 2 % sodium sulfate solution at approximately 0 °C. A 70 mL portion of a mixture of hexane/ethyl ether (1:1, v/v) was added, and the mixture was shaken and then held until the phases had separated. The aqueous phase was discarded, eliminating water-soluble compounds. The organic phase that contained the pigment solution was dried three times with an aqueous solution of Na_2SO_4 (2 %) at 0 °C and evaporated to dryness in a rotary evaporator. The dry residue was dissolved in an appropriate volume of acetone, which was used for the HPLC pigment analysis. To obtain the overall spectrum corresponding to the pigment solution, the volume of the organic phase was adjusted to get a final concentration of 10% (w/v).

Hexane fractions were purified according to the protocol reported by Criado et al. [20]. In this sense, the five hexane phases were combined in a separatory funnel containing 100 mL of ether, saponified with 100 mL of 15.6% KOH in methanol, and vigorously shaken to hydrolyse the lipids and purify the possible carotenoids. Then distilled water was added and, after being shaken for 1 min, the ether phase with the β-carotene was separated and washed three times with an aqueous solution of Na_2SO_4. Finally, to quantify the β-carotene present in the hexane fraction, a suitable amount of hexane was added to yield a 10% (w/v) solution (with respect to oil). The β-carotene concentration was measured directly in this solution using the extinction coefficient $E_{454}^{1\%} = 2592$ [21].

Solid-phase extraction (SPE) of pigments using a C_{18} cartridge

Solid-phase extraction (SPE) using a C_{18} cartridge was carried out according to the method of Mínguez-Mosquera et al., [11]. The column was conditioned with 6 mL of methanol and then with 6 mL of hexane. A sample of virgin olive oil (1.0±0.001 g) was weighed and dissolved in 4 mL of hexane. The oil solution was applied to the column and the fraction of hexane was recollected in a 10 mL flask. Then another 3 mL of hexane was added to wash away any lipid fraction that the column might retain, and again this hexane fraction was recollected in the preceding volumetric flask. The hexane phase contained the β-carotene fraction and some of the pheophytin a, which were measured directly in this solution (after volume adjustment to 10 mL) using their respective extinction coefficients in hexane: $E_{454}^{1\%} = 2592$ (β-carotene) and $E_{660}^{1\%} = 613$ (pheophytin a) [22]. Finally, the pigments were eluted with 5 mL of acetone, and the solvent was removed in a rotary evaporator at room temperature in vacuo until dryness. The residue was reconstituted in 0.3 mL of acetone. An aliquot (20 μL) of the final solution was injected into the HPLC system.

Solid-phase extraction (SPE) of pigments using a diol cartridge

A diol-bonded phase cartridge was placed in a vacuum-elution apparatus, and conditioned by the consecutive passing of 6 mL of methanol and 6 mL of hexane. The vacuum was then released to prevent drying of the column. A sample of virgin olive oil (1.0±0.001 g) was weighed and dissolved in 4 mL of hexane. The oil solution was applied to the column, and the solvent was collected in a volumetric flack. The column was washed with 5 mL of hexane, which were recombined with the preceding fraction of hexane. The hexane phase retained the β-carotene fraction that was measured after volume adjustment to 10 mL, using the coefficient of extinction $E_{454}^{1\%} = 2592$ [21].

Finally, the column was eluted with 3 mL of acetone, and the solvent was evaporated in a rotary evaporator at room temperature in a vacuum until dryness. The residue was redissolved in 0.3 mL of acetone. An aliquot (20 μL) of the final solution was injected into the HPLC system.

Spectral evaluation of isolated fractions from previous extraction procedures

In order to compare the performances of the different procedures, the spectra of all of the isolated fractions were studied. The hexane and acetone fractions corresponding to 1 g of oil were diluted to 10 mL and their spectra were recorded over the 350 to 775 nm range.

High-performance liquid chromatography (HPLC) procedure

HPLC analyses were performed on an Agilent 1,100 liquid chromatographic system equipped with a diode-array UV/vis detector and rheodyne injection valve (20 μL loop). A 250 mm x 4.6 mm i.d., 5 μm particle size Inertsil ODS 2 column (Sugelabor) was used. Agilent Chemstation software system controlled the equipment and carried out the data processing. Elution was performed at a flow rate of 1.0 mL/min at room temperature, using as the mobile phase a mixture (8:2 v/v) of methanol/water containing 0.025 % ammonium acetate and 0.05 % triethylamine as phase A and methanol/acetone (1:1 v/v) as phase B. The pigments were eluted according to the solvent gradient summarized in Table 1. Detection was performed simultaneously at 410 nm to measure pheophitin a, 430 nm to measure chlorophyll a, 435 nm to measure pheophitin b, 446 nm to measure lutein and the other xanthophylls present in the sample, and at 466 nm to measure chlorophyll b. HPLC quantification was carried out using the external standard method. Standard curves were calculated by linear regression analysis, plotting HPLC peak absorbance area vs concentration of the standard. The results were given as milligrams per kilogram of virgin olive oil.

Results and discussion

SPE isolation of the pigment fraction

The evaluation of the pigment fraction of virgin olive oil has previously involved the liquid-liquid extraction of pigments with N-N'-dimethylformamide (DMF). However, this procedure requires several extraction and cleanup steps to eliminate undesired compounds. It is a laborious method, where pigment alteration may occur. Consequently, attempts to isolate the pigments by solid-phase extraction (SPE) using C_{18} cartridges (C18-SPE) [11] have been proposed. However, chlorophylls cannot be quantitatively analysed due to their partial coextraction with lipid matrix.

Nevertheless, solid-phase extraction (SPE) has been classified as an effective technique for isolating virgin olive oil components due to its advantages compared to

Table 1 Gradient scheme used for the HPLC separation of the olive oil pigments

Time (min)	Mobile phase	
	A (%)	B (%)
0	75	25
10	50	50
12.5	50	50
14.0	20	80
16.0	20	80
21.0	0	100
35.0	0	100
40.0	75	25

traditional liquid-liquid extraction (LLE) methods, such as higher selectivity, smaller sample and solvent requirements, easier handling, absence of emulsion and less time required.

Several sorbents for the SPE isolation of minor compounds in olive oil have been proposed. Silica gel, a classical stationary phase, was assayed for pigment isolation. However, major reactivity of this phase was observed when the pigments were eluted. Following a similar procedure to that described for the diol phase in the Experimental section, the silica gel showed permanent browning after pigment elution with acetone. This fact was interpreted as a degradation and/or irreversible retention in the column, which was confirmed as follows: both the hexane and the acetone fractions were mixed and evaporated to dryness and reconstituted in hexane. The spectrum of the whole extract was similar in profile but lower in absorbance intensity than that corresponding to the initial oil, corroborating the ineffectiveness of this phase at isolating pigments (data not shown). Moreover, this phenomenon hindered the reuse of the column for later analysis.

Another sorbent used to isolate minor compounds in olive oil, the amine phase, was rejected owing to its high reactivity, as demonstrated by the drastic changes in the composition of the phenolic compounds isolated from olive oil [23].

The diol phase has been successfully used to isolate polyphenols, with better performance than the C_{18} phase [23, 24]. Therefore, the isolation of the pigment fraction from virgin olive oil by SPE is proposed here based on a diol-phase cartridge (diol-SPE).

A green VOO was used to adjust the solvents and their rates for cartridge conditioning, lipid elution and optimal analyte recovery. The passage of the hexane solution of the olive oil through a diol-cartridge retained the pigment fractions on the solid phase. Hexane washing eliminated the lipid matrix. Later, the pigments were eluted with acetone and the final solution was evaporated, yielding a greenish-yellow solid residue. Finally, the pigment residue was dissolved in 0.3 mL of acetone for HPLC analysis.

All of the variables were optimised as a compromise between the least amount of solvent needed to completely remove the lipid matrix and the maximum pigment recovery. The conditions evaluated for the amount of oil, hexane, and acetone ranged from 0.5 to 3 g; 1 to 6 mL and 1 to 6 mL, respectively, and the eluates obtained in each trial were studied by evaluating the spectra recorded over the wavelength range of 350 to 775 nm. The optimal conditions needed to reach the best recovery of the pigment fraction and thus the maximum absorbance of acetone fraction in the range 350 to 700 nm were 1 g of oil sample, 5 mL of hexane to elute free-pigmented oil, and 3 mL of acetone to elute the pigment fraction.

Figure 1 shows a comparison of the visible spectra of the hexane fractions (using hexane as blank) after three different extraction procedures (LLE, C_{18}-SPE and diol-SPE) under the above described conditions. As reflected in Fig. 1, all of the curves showed a broad absorption zone between 375 and 525 nm, with three well-defined maxima at 410, 450, and 470 nm. This zone corresponds to blue absorption that transmits the complementary yellow colour. Also, an appreciable peak appears at 670 nm, corresponding to the C_{18}-SPE eluate, but no absorbance was observed in the eluates from LLE and diol-SPE. This zone corresponds to red absorption that transmits the complementary green colour characteristic of chlorophyll pigments. Evaluations of the spectra lead to the conclusion that the C_{18} phase is not able to retain the chlorophyll pigments efficiently, because the presence of an appreciable absorption peak at around 670 nm indicated the partial coextraction of chlorophyll pigments with the lipid matrix when the column was washed with hexane. In this sense, the poor retention capacity of the C_{18} column hampered the quantitative analysis of chlorophyll compounds by C_{18}-SPE. This drawback is described in the original paper [11], where the authors determine the pheophytin a in both the hexane and the acetone fractions.

Moreover, spectrophotometric measurements at 454 nm and 660 nm have been used to quantify β-carotene and pheophytin a, respectively, which elute in the hexane fraction [11]. However, the chlorophyll pigments coeluted in the hexane fraction show a slight absorption at 454 nm, implying an overestimation of the amount of β-carotene when the measurement was made at this wavelength. On the other hand, other chlorophyll components present in the oil (such as pheophytin b and chlorophyll a and b) show an intense absorbance at 660 nm, resulting in a overestimation of pheophytin a.

The spectrum of the hexane fraction obtained with the diol column (Fig. 1) showed the characteristic profile of β-carotene, implying the absence of the rest of the carotenoids (xanthophylls and lutein) and the chlorophyll pigment in this fraction as well as good retention capacity in the diol column. Finally, the LLE hexane fraction showed a profile similar to that corresponding to the diol spectrum but at a lower intensity. This indicated a lower recovery for the LLE procedure, owing to the losses in the consecutive extraction steps inherent to this isolation procedure.
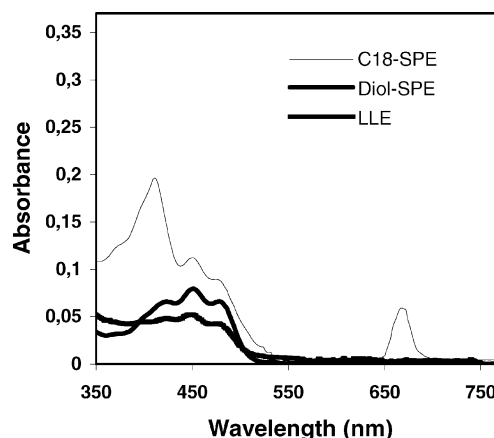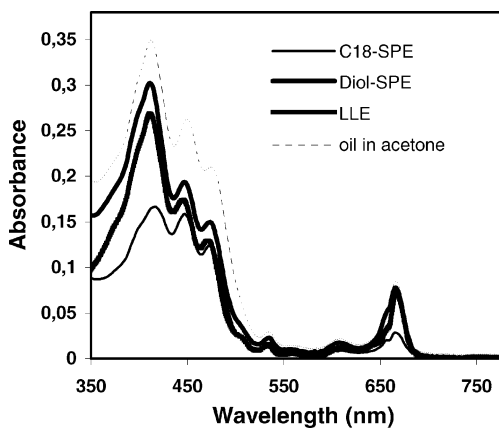

Fig. 1 Comparisons of absorption spectra of hexane fractions from virgin olive oil obtained by liquid-liquid extraction with N,N'-dimethylformamide (LLE) and solid-phase extraction using the C18 column (C18-SPE) and the diol column (diol-SPE)

Fig. 2 Comparisons between the absorption spectra from virgin olive oil dissolved in acetone and acetone fractions obtained by liquid-liquid extraction with *N,N′*-dimethylformamide (LLE) and solid-phase extraction using a C18 column (C18-SPE) and a diol column (diol-SPE)

the spectra from the acetone eluates obtained from LLE, C$_{18}$-SPE and diol-SPE. Again, all of the curves show a broad absorption zone between 375 and 500 nm, with three well-defined peaks at 414, 450 and 476 nm corresponding to xanthophylls and lutein next to three peaks at 534, 614 and 670 nm characteristic of chlorophyll pigments (pheophytins and chlorophylls).

When the β-carotene eluted with hexane fraction (Fig. 1) was taken into account, and the spectra from the acetone extracts were compared with the spectrum that results when oil is dissolved in acetone (Fig. 2), the percentage recoveries for the xanthophylls and lutein were found to be 82, 95 and 99% with C$_{18}$-SPE, LLE and diol-SPE, respectively, whereas for chlorophyll compounds the recoveries were 40, 93 and 98% for C$_{18}$-SPE, LLE and diol-SPE, respectively. According to these results, diol-SPE showed the best performance for pigment isolation from virgin olive oil.

In order to evaluate the potential analyte alteration during the isolation process with diol-SPE, a free-pigmented lipid matrix was spiked with the pigment fraction obtained from olive oil using the procedure detailed in the Experimental section. The rest of the acetone was evaporated under vacuum, and the resulting spiked sample

Simultaneously, the spectrophotometric evaluation of acetone eluates from the three specified extraction procedures was monitored. Figure 2 shows the spectra corresponding to oil dissolved in acetone in comparison to

Fig. 3a–c HPLC chromatogram of the pigment from 'Picual' variety virgin olive oil. Peaks measured at 410 nm: 1, neoxanthin; 1′, neoxanthin isomer; 2, violaxanthin; 3, luteoxanthin; 4, antheraxanthin; 4′, antheroxanthin isomer; 5, mutatoxanthin; 6, lutein; 6′ and 6″, lutein isomers; 7, chlorophyll *b*; 8, β-cryptoxanthin; 9, chlorophyll *a*; 9′, chlorophyll *a′*; 10, pheophytin *b*; 10′, pheophytin *b′*; 11, pheophytin *a*; 11′, pheophytin *a′*. Isolation of pigments by solid-phase extraction using a diol column (**a**); by liquid-liquid extraction with *N,N′*-dimethylformamide (**b**) and by solid-phase extraction using C18 column (**c**)
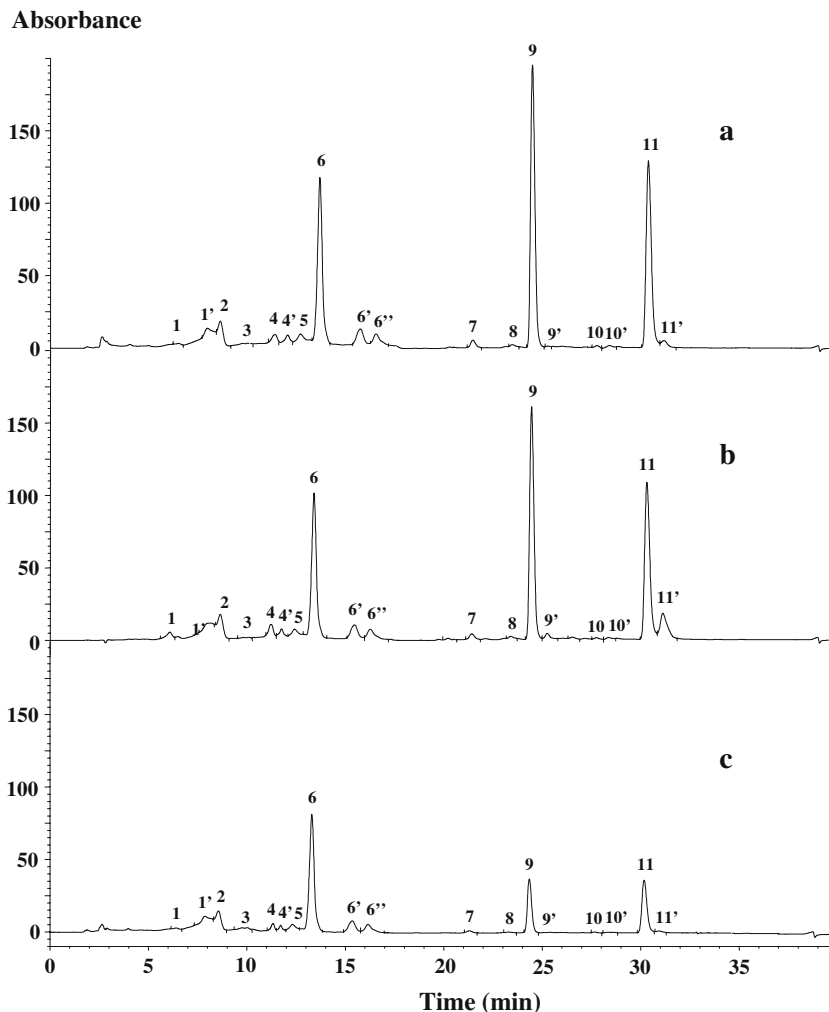
Table 2 Pigment contents (means±standard deviation, $n=4$) isolated from virgin olive oil by different procedures: liquid-liquid extraction with N,N'-dimethylformamide (LLE), solid-phase extraction using a C_{18} (C_{18}-SPE) and a diol column (diol-SPE)

Peak	Compound	Diol-SPE (ppm)	LLE (ppm)	C18-SPE (ppm)
1	Neoxanthin	0.036±0.002	0.04±0.01	0.025±0.002
1′	Neoxanthin isomer	0.76±0.04	0.79±0.04	0.8±0.1
2	Violaxanthin	0.578±0.007	0.57±0.01	0.50±0.04
3	Luteoxanthin	0.12±0.04	0.03±0.04	0.08±0.02
4	Antheroxanthin	0.24±0.01	0.25±0.02	0.17±0.01
4′	Antheroxanthin isomer	0.192±0.002	0.16±0.01	0.164±0.002
5	Mutatoxanthin	0.202±0.002	0.184±0.002	0.157±0.004
6	Lutein	5.4±0.3	5.3±0.3	4.7±0.3
6′	Lutein isomer	0.43±0.02	0.53±0.01	0.32±0.04
6″	Lutein isomer	0.401±0.007	0.36±0.02	0.23±0.02
7	Chlorophyll b	0.40±0.02	0.32±0.04	0.13±0.02
8	β-cryptoxanthin	0.058±0.002	0.042±0.003	0.030±0.002
9	Chlorophyll a	5.8±0.2	5.3±0.3	1.4±0.2
9′	Chlorophyll a'	0.007±0.003	0.14±0.02	0±0
10	Pheophytin b	0.10±0.01	0.07±0.01	0.041±0.006
10′	Pheophytin b'	0.173±0.008	0.11±0.02	0±0
11	Pheophytin a	5.18±0.02	4.43±0.03	1.6±0.2
11′	Pheophytin a'	0.22±0.01	0.94±0.09	0.07±0.01
TOTAL		20.3±0.9	19.6±0.9	10.4±0.9
Carotenoids		8.4±0.5	8.3±0.5	7.2±0.5
Chlorophylls		11.9±0.5	11.3±0.4	3.2±0.4

was again submitted to the diol-SPE isolation procedure. The HPLC analysis of both the initial and the re-extracted pigment fraction yielded the same HPLC profile (Fig. 3a). This indicated negligible alteration during the extraction steps on the diol-phase. Up to fifteen extractions were made in the same column, without altering the components and with no loss of performance of the diol phase.

RP-HPLC analysis

A pool of pigment fraction obtained from a very green olive oil was used to optimize the solvent composition and elution gradient. Peak resolution was assessed by changing the flow (1.0–1.5 mL/min) and the elution gradients of the solvents in different trials. The best chromatographic

Table 3 Carotenoid and chlorophyll contents (means±standard deviation, $n=4$) isolated from virgin olive oil by different procedures: liquid-liquid extraction with N,N'-dimethylformamide (LLE), solid-phase extraction using a C_{18} (C_{18}-SPE) and diol column (diol-SPE), and percentage composition with respect to the total of each fraction[a]

		Diol-SPE (ppm)	%	LLE (ppm)	%	C18-SPE (ppm)	%
Carotenoid fraction	Minor car[b]	2.19±0.03	26.16	2.1±0.2	25.11	1.9±0.1	26.87
	Lut	6.2±0.4	73.84	6.2±0.3	74.89	5.25±0.4	73.13
	Total	8.4±0.5		8.3±0.5		7.2±0.5	
Chlorophyll fraction	Chl[a]	5.8±0.5	48.94	5.4±0.2	47.92	1.4±0.2	42.76
	Phy[a]	5.40±0.03	45.39	5.4±0.1	47.66	1.7±0.2	51.87
	Chl[b]	0.41±0.05	3.40	0.32±0.04	2.81	0.13±0.02	4.10
	Phy[b]	0.27±0.02	2.27	0.18±0.03	1.61	0.04±0.01	1.27
	T Chl	6.2±0.5	52.35	5.7±0.3	50.73	1.5±0.2	46.86
	T Phy	5.67±0.01	47.65	5.6±0.1	49.26	1.7±0.2	53.14
	Total	11.9±0.5		11.3±0.4		3.2±0.4	

[a] Abbreviations: Chl, chlorophyll; Phy, pheophytin; Lut, lutein; Minor car, minor carotenoids; T Chl, total chlorophyll; T Phy, total pheophytin
[b] Values are the sum of neoxanthin, violaxanthin, antheroxanthin, mutatoxanthin, β-cryptoxanthin

separation resulted from a flow of 1.0 mL/min and an elution gradient (described in detail in the Experimental section) using a mixture (8:2, v/v/v) of methanol/water containing 0.05% triethylamine and 0.025% ammonium acetate as phase A and acetone/methanol (1:1, v/v) as phase B, in an ODS2 reverse-phase column. A typical chromatogram of pigment extract from virgin olive oil obtained by SPE using a diol column is depicted in Fig. 3a, which shows a satisfactory chromatographic peak resolution, free from interfering peaks.

Identification of pigments

The pigments were identified by their chromatographic behaviour on analytical RP-HPLC and the spectroscopic characteristics (λ_{max} and peak ratios) of each pigment published by other authors [11, 25]. All of the pigments are listed in Table 2 in the order of chromatographic elution on analytical RP-HPLC. The pigment constituents of virgin olive oil consisted of two types of compounds: (a) xanthophyll group (oxygenated carotenoids) and (b) chlorophyll group. A total of 18 pigments were identified and quantified in virgin olive oil (Fig. 3). Of these, 11 were xanthophylls (neoxanthin, violaxanthin, luteoxanthin, antheroxanthin, mutatoxanthin, lutein, β-cryptoxanthin, and their isomers) while seven were chlorophyll pigments (chlorophyll *b*, chlorophyll *a*, pheophytin *b*, pheophytin *a*, and their isomers).

Comparisons of the HPLC chromatograms of pigments obtained by the three extraction procedures yielded HPLC profiles containing the same chromatographic peaks but in different proportions (Fig. 3a–c). Figure 3a and b show similar qualitative chromatographic profiles but with quantitative differences, where the intensities of the three main peaks (lutein, chlorophyll *a*, and pheophytin *a*) from extract made with diol-SPE (Fig. 3a) were higher than those from the extract obtained by LLE (Fig. 3b). Also, the higher intensities of chlorophyll *a'* and pheophytin *a'* (Fig. 1b) indicate a slight alteration of the pigment compound due to the higher manipulation involved in the LLE procedure in comparison to the diol-SPE procedure.

However, drastic differences arose when Fig. 1c, corresponding to C_{18}-SPE, was compared to HPLC chromatograms of pigments extracted by diol-SPE (Fig. 1a) and LLE (Fig. 1b). While lutein and the preceding peaks belonging to the xanthophyll group quantitatively exhibited similar behaviour irrespective of the extraction procedure used, the chlorophyll compounds showed much lower concentrations based on the C_{18}-SPE procedure. This fact agrees with the spectra in Figs. 1 and 2, which indicate that chlorophyll compounds coelute with matrix lipid in the cleanup step, showing the inadequacy of this extraction procedure at quantifying pigments from virgin olive oil.

The concentrations of individual chlorophylls and carotenoids obtained by the three extraction procedures are summarized in Table 2. Again, lower recoveries, 96.4% and 51.3%, were obtained for pigments isolated by LLE and C_{18}-SPE, respectively, in comparison to diol-SPE.

Table 3 indicates the percentages of chlorophyll and carotenoid compounds in relation to the total composition of each fraction. The carotenoid fraction showed more stable behaviour, both qualitatively, by the absence of chemical transformation between them and no formation of artefacts, as well as quantitatively, due to the excellent recovery obtained using LLE (98.7%) and the slightly lower recovery obtained using C_{18}-SPE (85.6%). However, the chlorophyll fraction behaved differently. It is well-known that a proportion of the native chlorophyll is transformed into pheophytins when the central Mg^{2+} ion of the porphyrin ring is substituted by H^+, during the extraction process. In this sense, the largest percentage of the pheophytin was obtained using the C_{18}-SPE (53.1%) followed by LLE (49.3%) and diol-SPE (47.6%), indicating the progress of the pheophytinization reaction with LLE and to an even greater extent when the C18 column was used. Moreover, quantitatively, more differences were observed in comparison to the carotenoid fraction. While 94.7% of the chlorophyll pigments were recovered by LLE, only 27.2% were isolated by C_{18}-SPE.

Finally, the free-pigmented lipid matrix was obtained from virgin olive oil (VOO) by purification through the diol column. Thus, recovery studies of the olive oil pigments were performed in order to compare the extraction/purification procedures. Extracts of pigments obtained by diol-SPE were isolated and spiked in free-pigmented lipid matrix. The results from four determinations in spiked lipid-matrix olive oil indicated good reproducibility (CV, *n*=4) of the complete method. The recoveries relative to each component of the pigment extract were excellent (Table 4).

Table 4 Reproducibility (coefficient of variation (C.V.)) and recovery via HPLC of free-pigmented lipid matrix spiked with pigment extract and submitted to an overall extraction procedure using a diol column (*n*=4)

Peak	Compound	C.V. (%)	Recovery (%)
1	Neoxanthin	2.1	99.12
1′	Neoxanthin isomer	3.2	99.23
2	Violaxanthin	2.9	98.52
3	Luteoxanthin	1.8	99.72
4	Antheroxanthin	4.5	99.37
4′	Antheroxanthin isomer	1.4	99.52
5	Mutatoxanthin	3.3	98.98
6	Lutein	1.4	99.27
6′	Lutein isomer	1.6	99.74
6″	Lutein isomer	2.6	99.71
7	Chlorophyll *b*	1.5	99.91
8	β-cryptoxanthin	2.1	99.22
9	Chlorophyll *a*	3.2	99.57
9′	Chlorophyll *a'*	2.1	99.42
10	Pheophytin *b*	2.4	98.68
10′	Pheophytin *b'*	3.1	99.37
11	Pheophytin *a*	4.1	98.51
11′	Pheophytin *a'*	4.5	98.43

In summary, the isolation procedure described in the present paper, based on the solid-phase extraction of pigments from virgin olive oil using a diol column, provided excellent results. The method proposed is highly selective and requires small quantities of sample and solvent volumes, and shows satisfactory precision and recoveries.

References

1. Roca M, Gandul-Rojas B, Gallardo-Guerrero L, Mínguez-Mosquera MI (2003) J Am Oil Chem Soc 80:1237–1240
2. Serani A, Piacenti D (1992) J Am Oil Chem Soc 69:469–470
3. Mínguez-Mosquera MI, Rejano-Navarro L, Gandul-Rojas B, Sánchez-Gómez AH, Garrido-Fernández J (1991) J Am Oil Chem Soc 68:332–336
4. Usuki R, Endo Y, Kaneda T (1984) J Am Oil Chem Soc 48(4):991–994
5. Usuki R, Suzuki T, Endo Y, Kaneda T (1984) J Am Oil Chem Soc 61(4):785–788
6. Endo Y, Usuki R, Kaneda T (1985) J Am Oil Chem Soc 62(9):1375–1378
7. Endo Y, Usuki R, Kaneda T (1985) J Am Oil Chem Soc 62(9):1387–1390
8. Rahmani M, Csallany AS (1998) J Am Oil Chem Soc 75:837–843
9. Psomiadou E, Tsimidou M (2002) J Agric Food Chem 50:716–721
10. Psomiadou E, Tsimidou M (2002) J Agric Food Chem 50:722–727
11. Minguez-Mosquera MI, Gandul-Rojas B, Gallardo-Guerrero ML (1992) J Agric Food Chem 40:60–63
12. Su Q, Rowley KG, Itsiopoulos C, O'Dea K (2002) Eur J Clin Nut 56:1149–1154
13. Luaces P, Pérez AG, García JM, Sanz C (2005) Food Chem 90:169–174
14. Cicheli A, Pertesana GP (2004) J Chromatogr A 1046:141–146
15. Psomiadou E, Tsimidou M (1998) J Agric Food Chem 46:5132–5138
16. Puspitasari-Nienaber NL, Ferruzzi MG, Schwartz J (2002) J Am Oil Chem Soc 79:633–640
17. Seppanen CM, Rahmani M, Csallany AS (2003) J Food Sci 68(5):1644–1647
18. Martínez JM, Muñoz E, Alba J, Lanzón A (1975) Grasas y Aceites 26:379–385
19. Sievers G, Hynninen PH (1977) J Chromatogr 134:359–364
20. Criado MN, Ramón-Morelló J, Motilva MJ, Romero MP (2004) J Am Oil Chem Soc 81:633–640
21. Foppen FH (1971) Chromatogr Rev 14:133–298
22. Minguez-Mosquera MI, Garrido-Fernández J, Gandul-Rojas B (1990) J Agric Food Chem 38:1662–1666
23. Mateos R, Espartero JL, Trujillo M, Ríos JJ, León-Camacho M, Alcudia F, Cert A (2001) J Agric Food Chem 49:2185–2192
24. Gutiérrez F, Albi MA, Palma R, Ríos JJ, Olías JM (1989) J Food Sci 54:68–70
25. Edelenbos M, Christensen LP, Grevsen K (2001) J Agric Food Chem 49:4768–4774