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Simultaneous Fluorometric Determination of Chlorophylls *a* and *b* and Pheophytins *a* and *b* in Olive Oil by Partial Least-Squares Calibration

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The resolution of quaternary mixtures of chlorophylls *a* and *b* and pheophytins *a* and *b* has been accomplished by partial least-squares (PLS) multivariate calibration, applied to the fluorescence signals of these pigments. The total luminescence information of the compounds has been used to optimize the spectral data set to perform the calibration. After preliminary studies, a method is described in acetone media, to avoid emulsions with the olive oil samples. Different scanning paths have been selected for each method. For the simultaneous determination of the pigments in olive oil samples, a comparative study of the results found by using excitation, emission, and synchronous spectral data, as analytical signal, was performed. The excitation spectra were selected as the better analytical signals for the determination of the pigments in olive oil samples. The optimum wavelength range to record the excitation spectra ($\lambda_{em} = 662$ nm) was selected to minimize the contribution of pheophytin *a* and to maximize the contribution of the other pigments, which are the minor constituents in olive oil. Determination of these pigments in olive oil samples was effected from the excitation spectra of dissolutions o suitable aliquots in acetone. Recovery values from olive oil, spiked with chlorophylls *a* and *b* and pheophytins *a* and *b,* were in the ranges of 70−112, 71−111, 76−105, and 82−109%, respectively.

KEYWORDS: Chlorophylls; pheophytins; fluorescence; partial least-squares; olive oil

INTRODUCTION

Virgin olive oil has a color ranging from green-yellow to gold, depending on the variety and degree of the fruit's ripeness. The composition of the total content of pigments, naturally present in the oil, is an important quality parameter because it is correlated to color, which is a basic attribute for evaluating olive oil quality. Pigments are also involved in mechanisms of autoxidation and photooxidation. Two classes of natural pigments occur in olive oil: (1) chlorophylls and pheophytins and (2) carotenoids. The color of olive oil is dependent on the pigments in the fruit: green olives give a green oil because of the high chlorophyll and pheophytin content, and ripe olives give a yellow oil because of the carotenoid (yellow red) pigments. Chlorophyll *a* (chl *a*) may be present in a virgin olive oil just after production, but its pheophytinization occurs rapidly and pheophytin *a* is the main pigment. Chlorophyll *a* and pheophytin *b* (phe *b*) were detected only in oils from green fruits, whereas chl *b* was not found. Psomiadou and Tsimidou (*1*) analyzed 52 samples of virgin olive oil and found that in >70% of the samples the main pigment was phe *a* (>10 mg kg^{-1}).

Determination of these pigments in olive oil usually involves HPLC methods with UV detection. Rahmani and Csallany (*2*) proposed the determination of chlorophyll pigments in vegetable oils by isocratic HPLC. Mínguez-Mosquera et al. (*3*) proposed the separation of 17 pigments with a gradient elution and at 430 and 410 nm, using a programmable photodiode array detector. By applying this method, the change in the natural ratio between chlorophylls and carotenoids in olive fruit during processing for virgin olive oil has been analyzed (*4*). Simultaneous HPLC determination of tocopherols, carotenoids, and chlorophylls, at 290 nm, with gradient elution and with hexane/propan-2-ol as mobile phase, has been studied for monitoring their effect on virgin olive oil oxidation (*5*).

Two papers, based on multivariate methods and applied to the determination of chlorophylls and pheopigments in phytoplankton, have been published. In the first, parallel factor analysis (PARAFAC) was applied to fluorescence excitation−emission matrices of chlorophylls and pheopigments (*6*). The same authors (*7*), in another paper, present the validation of a previously described multivariate partial least-squares (PLS) method (*8*), based on spectrophotometric measurements, for determining these pigments.

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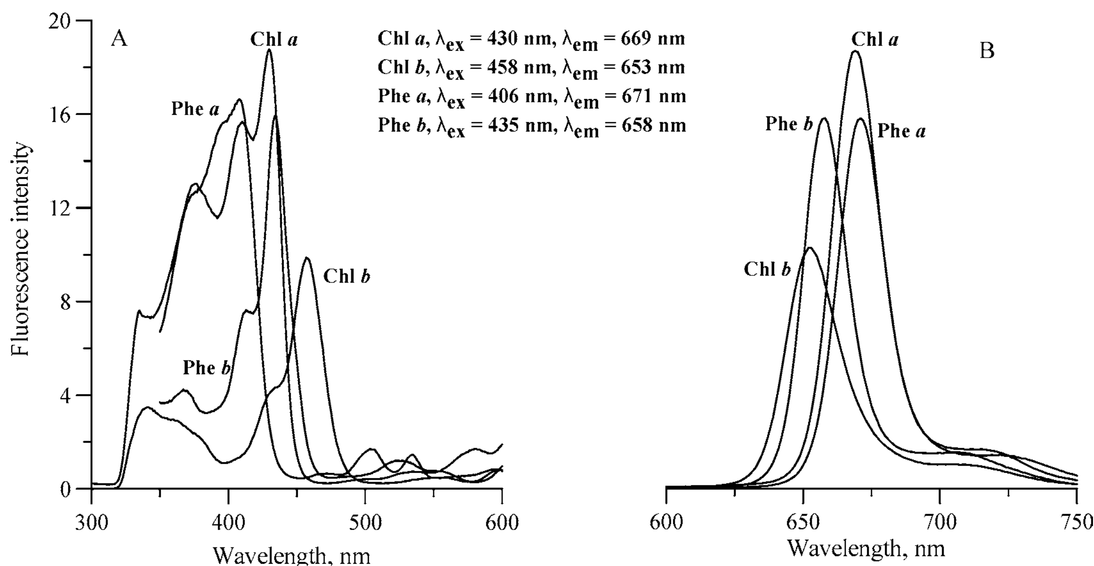

Figure 1. Excitation (**A**) and emission spectra (**B**) of pigments in acetone/water (9:1) medium.

The enhancement of the fluorescence of chl *a* and *b* in a nonionic micellar medium has been used for the establishment a method for the determination of these pigments (*9*).

We have not found methods for the simultaneous determination of chlorophylls and pheopigments in olive oil based on spectrofluorometric analysis.

In this paper the fluorometric behavior of chlorophylls *a* and *b* and pheophytins *a* and *b* has been studied. The description of the results obtained in the resolution of quaternary mixtures, using multivariate calibration techniques, specifically the partial least-squares type 1 (PLS-1) method, is divided into two parts concerning the study of quaternary mixtures in acetone/water media and the modifications introduced for the determination of the pigments in olive oil. To perform the determination, a previous optimization of the scanning paths in acetone/water media and in acetone media, for the determination of the pigments in olive oils, is carried out using the three-dimensional representation of the excitation−emission data matrices as contour plots. Finally, we compare three procedures for the analysis of the pigments in olive oil and, from the results, we propose a method for the simultaneous determination of the four pigments in olive oils without pretreatment of the samples.

EXPERIMENTAL PROCEDURES

Apparatus. Fluorescence measurements were made on an SLM Aminco-Bowman, series 2, spectrofluorometer, equipped with a 150 W continuous xenon lamp, interfaced by a GPIB card and driver with a Pentium PC microcomputer. Data acquisition and data analysis were performed by the use of AB2 software, version 5.00, running under Windows 98. The excitation and emission slits were 4 nm for both. The scan rate of the monochromators was maintained at 10 nm s^{-1} in the recording of conventional spectra and at 30 nm s^{-1} when three-dimensional excitation−emission spectra were recorded. All measurements were performed in 10 mm quartz cells, at 20 °C, by use of a thermostatic cell holder and a Selecta model Frigiterm 382 thermostatic bath.

Software. The GRAMS-386 Level I version 2.0 software package, with the PLS plus version 2.1 application software (Galactic Industries, Salem, NH) (*10*), was used for statistical treatment of the data, and the application of the PLS analysis ws based on multivariate calibration methods. The digitalized spectra, acquired with the series 2 luminescence instrument, were converted to ASCII XY format with the converter included in the AB2 software and imported to the GRAMS 386 program through the included ASCII XY converter. A homemade converter program, running in BASIC, was used to transform the bidimensional files, in ASCII XY format, to the software package SURFER (Golden Software, Golden, CO, 1994) (*11*) for Windows to obtain the three-dimensional excitation−emission matrices presented as contour plots. The contour plots (two dimensions: excitation and emission wavelengths) are generated by linking points of equal fluorescence intensity to form the contour map.

Reagents. Preparation of Stock Solutions. Stock solutions of chl *a* (Fluka, Riedel-de Haën, Germany) and chl *b* (Aldrich Chemical Co.) were prepared by dissolving the contents of ampules containing 1 mg, of each chlorophyll, in 25 mL of acetone. The acetone was of pro-analyse grade (Merck, Darmstadt, Germany). Pheophytin stock solutions were prepared according to a previously described procedure (*6*). Ten milliliters of the chl *a* or chl *b* stock solution was taken and acidified with 10 drops of 1 M HCl. Then, the solution was extracted with 15 mL of diethyl ether by vigorous shaking during a few minutes. The aqueous phase was discarded, and the organic phase was washed with water five times. Ten milliliters of acetone was added, and the diethyl ether was evaporated under nitrogen. The resulting solutions had a final concentration of 40 μg mL^{-1} of phe *a* or phe *b* in acetone. Solutions of the four pigments of lower concentration were prepared by appropriate dilution of these.

Sep-Pak Plus cartridges from Waters (Milford, MA) were used for the solid-phase extraction (SPE) procedures. All other chemicals utilized were of analytical reagent grade or better.

Procedure for the Analysis of Chl *a*, Chl *b*, Phe *a*, and Phe *b* in Olive Oil. Suitable amounts of the olive oil (between 0.05 and 1 g of olive oil depending on the stage of fruit ripeness) were accurately weighed and transferred to 10 mL calibrated flasks, diluting to volume with acetone. The excitation (λ_{em} = 662 nm) spectra of the solutions were obtained, and the optimized PLS-1 method was applied to analyze these spectra and determine the concentrations of the pigments in the olive oil samples. To perform the PLS-1 calibration, a set of samples with final concentration ranges varying from 140 to 560 ng mL^{-1} for phe *a*, from 10 to 40 ng mL^{-1} for chl *a* and phe *b*, and from 20 to 80 ng mL^{-1} for chl *b* was used as calibration matrix.

RESULTS AND DISCUSSION

Fluorometric Studies of the Pigments. The native fluorescence of these compounds is well-known. Initially, we selected a 9:1 acetone/water medium to carry out preliminary studies on the influence of pH, because these studies have been already made for chlorophyls in this medium (*9*). The emission spectra are very similar for the four components, and their maxima are very close, being 669 nm for chl *a*, 653 nm for chl *b*, 671 nm for phe *a*, and 658 nm for phe *b*. The greater differences correspond to the excitation spectra (**Figure 1**). Phe *a* presents

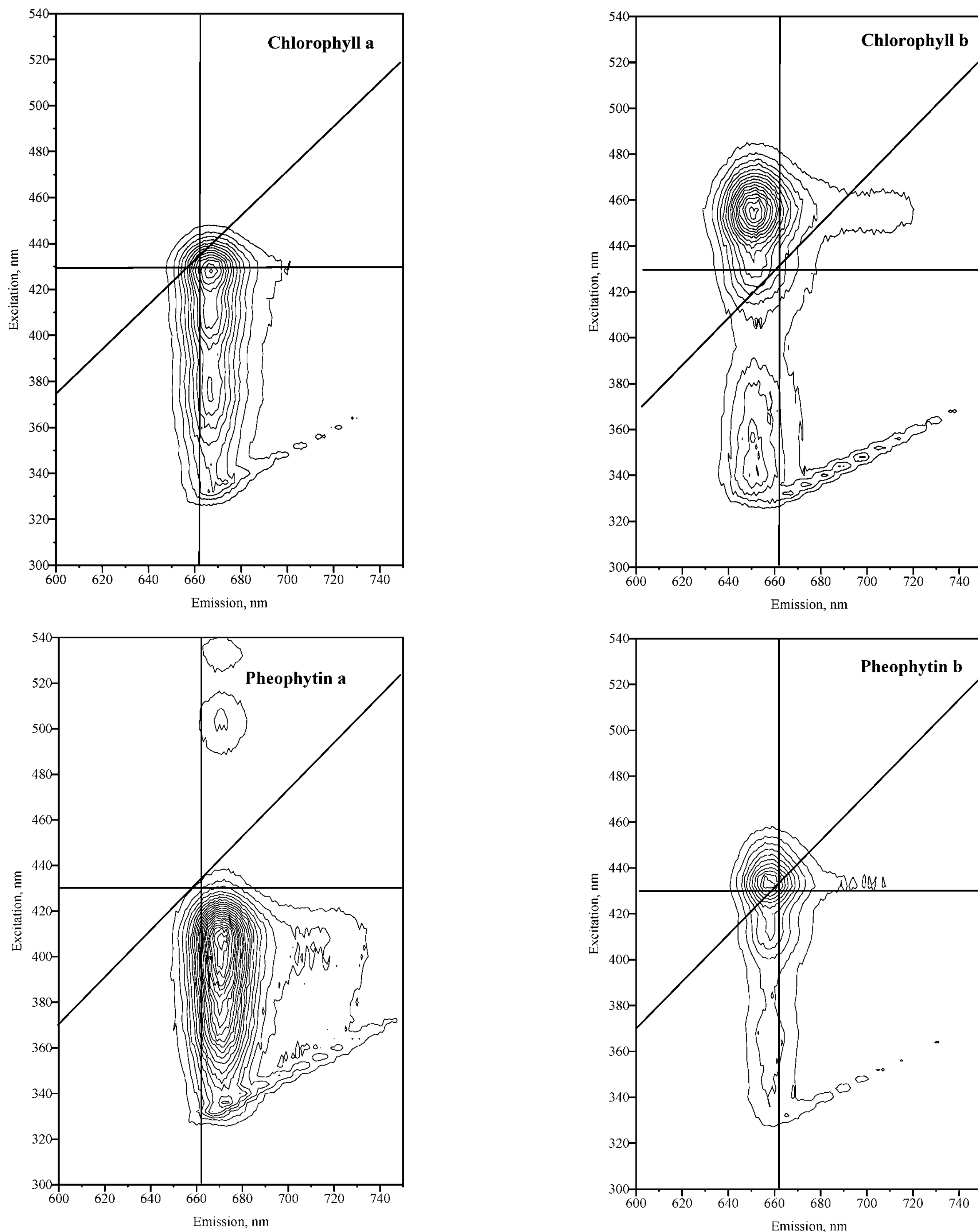

Figure 2. Contour plots of the total fluorescence spectra of chl *a*, chl *b*, phe *a*, and phe *b* in acetone media. The selected paths for scanning the excitation (λ_{em} = 662 nm), emission (λ_{ex} = 430 nm), and synchronous ($\Delta\lambda$ = 230 nm) spectra are shown by the solid lines slicing the data matrices.

the higher difference between emission and excitation maxima (Stokes shift of 271 nm) and phe *b* the minor (Stokes shift of 223 nm).

Santana et al. (*9*) studied the influence of pH for chl *a* and *b* and observed that the relative fluorescence intensity remains constant from pH 2.5 to 12 for chl *a* and from 3 to 12 for chl

Table 1. Statistical Parameters of the Calibration Graph at the Wavelengths Selected for the Determination of the Four Pigments in Olive Oil

	chl *a*	chl *b*	phe *a*	phe *b*
emission (λ_{ex} = 430 nm)				
slope (*m*)	2.78×10^{-1}	5.42×10^{-2}	3.06×10^{-2}	1.66×10^{-1}
intercept (*b*)	-1.08×10^{-1}	1.45	4.53×10^{-1}	2.33
SD[a] of slope (S_m)	1.28×10^{-3}	3.65×10^{-4}	3.40×10^{-4}	1.78×10^{-3}
SD of intercept (S_b)	2.10×10^{-1}	2.14×10^{-1}	1.33×10^{-1}	4.39×10^{-1}
regression SD ($S_{y/x}$)	3.85×10^{-1}	4.71×10^{-1}	2.38×10^{-1}	8.90×10^{-1}
R^2	0.9999	0.9995	0.999	0.9991
LOD[b]	2.26	11.88	13.08	7.92
analytical sensitivity ($S_{y/x}/m$),[c] ng mL^{-1}	1.38	8.69	7.78	5.36
excitation (λ_{em} = 662 nm)				
slope (*m*)	2.36×10^{-1}	6.37×10^{-1}	1.12×10^{-1}	1.50×10^{-1}
intercept (*b*)	-2.90×10^{-1}	3.12	2.76	1.96
SD of slope (S_m)	2.30×10^{-3}	5.99×10^{-4}	1.37×10^{-3}	1.70×10^{-3}
SD of intercept (S_b)	3.76×10^{-1}	3.52×10^{-1}	5.40×10^{-1}	4.20×10^{-1}
regression SD ($S_{y/x}$)	6.89×10^{-1}	7.73×10^{-1}	1.10×10^{-1}	8.51×10^{-1}
R^2	0.9994	0.999	0.9988	0.999
LOD	4.77	16.62	14.46	8.38
analytical sensitivity ($S_{y/x}/m$), ng mL^{-1}	2.91	1.21	0.98	5.67
synchronous ($\Delta\lambda$ = 230 nm)				
slope (*m*)	2.18×10^{-1}	4.45×10^{-2}	1.29×10^{-2}	1.57×10^{-1}
intercept (*b*)	-2.96	1.08	1.24×10^{-1}	2.27
SD of slope (S_m)	3.84×10^{-3}	3.45×10^{-4}	1.57×10^{-4}	1.75×10^{-3}
SD of intercept (S_b)	6.28×10^{-1}	2.0×10^{-1}	6.17×10^{-2}	4.31×10^{-1}
regression SD ($S_{y/x}$)	1.15	4.46×10^{-1}	1.10×10^{-1}	8.74×10^{-1}
R^2	0.998	0.9993	0.9988	0.999
LOD	8.61	13.69	14.3	8.23
analytical sensitivity ($S_{y/x}/m$), ng mL^{-1}	5.27	10.02	8.52	5.56

[a] Standard deviation. [b] Detection limit according to the method of Clayton et al. (*14*). [c] Reference *15*.

b. Our studies confirm these results. With respect to the pheophytins, the relative fluorescence intensity remains constant from pH 2.5 to 8 and in acidic media, the fluorescence signal decreases with decreasing pH, and in basic media the fluorescence signal decreases with increasing pH. Changes in the spectra have not been observed.

The fluorescence signal, obtained for each of these pigments at its maximum $\lambda_{exc}/\lambda_{em}$ pair, varies linearly with the concentration, in the range assayed (50−500 ng mL^{-1}).

Simultaneous Determination of Chl *a*, Chl *b*, Phe *a*, and Phe *b* by PLS-1: Optimization of the Scanning Path in the Excitation−Emission Matrices and Analysis of the Pigments in Olive Oil. The analysis of mixtures of these pigments by conventional spectrofluorometry is not feasible, because the excitation and emission spectra are overlapped (**Figure 1**). Because of this, the simultaneous determination of the four pigments was approached by the PLS-1 multivariate method. A 28-sample calibration matrix was taken, resulting in a central composite design with four variables (one for each component) at three concentration levels. The final concentrations of chl *a* and phe *b* were varied between 0 and 500 ng mL^{-1} and those of chl *b* and phe *a* between 0 and 570 ng mL^{-1}. All samples were prepared in a 10 mL volumetric flask and in acetone/water (9:1). The excitation spectra were recorded at λ_{em} = 661 nm and between 300 and 600 nm, and the emission spectra were recorded at λ_{ex} = 427 nm and between 600 and 750 nm.

Once optimized, the models show very good values of the statistical parameters: root-mean-square difference (RMSD), which is an indication of the average error in the analysis by cross-validation for each component in the calibration matrix

$$\mathrm{RMSD} = [1/N\sum_{i=1}^{N}(\hat{x}_i - x_i)^2]^{0.5}$$

where x_i is the true concentration of the analyte in the sample, $\hat{x}_i$ represents the estimated concentration of the analyte in sample *i*, and *N* is the total number of the samples used in the prediction set; and the square of the correlation coefficient (R^2), which is an indication of the quality of fit when plots of actual versus predicted concentration were constructed.

The PLS-1 method was then applied to the two spectral data sets of a series of problem mixtures, and satisfactory results were obtained for the recovery values in all of the samples analyzed (recovery values between 96 and 108% in all cases); also, they are similar with both scanning paths, the excitation spectra and the emission spectra. Therefore, for acetone/water samples, both data sets can be used with good results, and we have proven that the PLS multivariate method can be a feasible way of analyzing this mixture. This procedure could be interesting for some samples of aqueous matrix, but in the case of olive oil samples an acetone/water medium gives rise to emulsions, and therefore we decided to use pure acetone to prepare the samples. The results obtained in this medium are described next.

We must take into account that the concentration range of phe *a* in olive oil is higher than those of the other three pigments. Phe *a* is the predominant pigment in olive oil, and its concentration makes up ∼70−80% of the total; if oils are extracted from black olives, phe *a* is practically the only pigment of the class present (*1*, *12*). These differences in the concentrations made nonviable the prediction with the previously optimized method because the concentrations of the reagents in the calibration set were similar. Taking into account these considerations, a new calibration set was designed and the scanning paths have been selected to minimize the fluorescence signal from phe *a* and to maximize the fluorescence signals of the other three pigments.

The overlap between spectra can be best examined by collecting a total luminescence spectrum of each of the compounds, in the form of an emission−excitation matrix (EEM). The EEMs were collected by scanning the emission spectrum, between 600 and 750 nm, at increments of the excitation wavelength of 4 nm, between 300 and 600 nm.

To obtain the contour plots in the excitation−emission plane, the binary spectral data obtained with the SLM Aminco Bowman series 2 spectrofluorometer were converted to ASCII

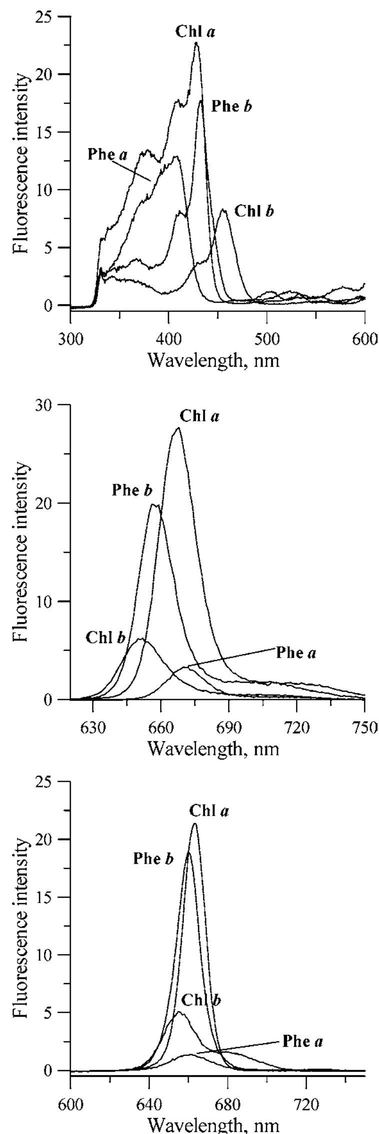

Figure 3. Excitation (λ_{em} = 662 nm), emission (λ_{ex} = 430 nm), and synchronous spectra ($\Delta\lambda$ = 230 nm), selected for the determination of chl *a*, chl *b*, phe *a*, and phe *b* in olive oil by PLS-1. [chl *a*] = 100 ng mL^{-1}, [chl *b*] = 100 ng mL^{-1}, [phe *a*] = 100 ng mL^{-1}, [phe *b*] = 100 ng mL^{-1}.

XY format with the ASCII converter included in the AB2 software. A program was written in BASIC to produce the contour plots by connecting points of equal fluorescence intensity by contour lines. The equifluorescence intensity points were calculated by linear interpolation between neighboring points in the EEM to find the $\lambda_{ex}/\lambda_{em}$ pairs corresponding to the contour plot. **Figure 2** shows the contour plots corresponding to chl *a*, chl *b*, phe *a*, and phe *b*, respectively.

The scanning paths, represented as solid lines, were selected as compromise values, essentially to pass as close as possible to the excitation and emission maxima of chl *a*, chl *b*, and phe *b*. In addition, and considering the complexity of the matrix of the olive oil, three different data sets (the excitation spectra, the emission spectra, and the synchronous spectra) were evaluated to perform the determination. The excitation spectra were recorded at a constantly maintained emission wavelength of 662 nm and the emission spectra at an excitation wavelength of 430 nm; the synchronous paths were scanned with a difference between the emission and excitation wavelengths of 230 nm. The corresponding spectra are represented in **Figure 3**.

Table 2. Composition of the Different Mixtures Used in the Calibration Set for the Determination of the Four Pigments in Olive Oil

sample	chl *a*, ng mL^{-1}	chl *b*, ng mL^{-1}	phe *a*, ng mL^{-1}	phe *b*, ng mL^{-1}
1	10	50	350	10
2	40	80	560	10
3	40	80	140	10
4	40	20	140	10
5	10	20	140	40
6	25	50	350	25
7	40	80	560	25
8	10	20	140	10
9	25	80	560	40
10	40	20	140	40
11	40	80	560	40
12	25	80	560	25
13	25	20	140	25
14	25	20	140	40
15	40	20	140	25
16	10	80	560	40
17	10	80	560	25
18	40	80	560	10
19	10	80	560	10
20	10	20	140	25
21	40	50	350	10
22	10	20	140	40
23	10	80	560	40
24	40	50	350	40
25	10	80	560	10
26	40	80	560	40
27	40	20	140	40
28	10	20	350	40
29	10	0	560	10
30	0	80	560	25
31	10	80	560	0
32	25	50	0	0
33	40	0	140	0
34	0	50	350	0
35	40	0	0	10
36	0	80	0	40
37	0	0	350	25
38	10	0	0	0
39	0	50	0	0
40	0	0	350	0
41	0	0	0	25
42	0	0	0	0

Table 3. Statistical Parameters of the PLS-1 Method Using the Three Spectral Data Sets for the Determination of Chlorophylls *a* and *b* and Pheophytins *a* and *b* in Olive Oil

	excitation		emission		synchronous	
component	RMSD	R^2	RMSD	R^2	RMSD	R^2
chl *a*	0.857 (5)[a]	0.997	1.303 (4)	0.992	1.782 (4)	0.987
chl *b*	1.073 (5)	0.998	1.533 (4)	0.997	1.941 (8)	0.996
phe *a*	7.309 (5)	0.998	9.881 (4)	0.997	8.985 (7)	0.998
phe *b*	0.921 (5)	0.996	0.891 (4)	0.996	2.037 (4)	0.982

[a] Values in parentheses correspond to the number of factors used in the prediction stage.

Before proceeding to the design of the calibration matrix, we constructed the calibration graphs at these wavelengths previously selected because, as we show in **Figure 2**, the conditions are adverse for chl *b* and very adverse for phe *a*. The calibration graphs were obtained by preparing samples in triplicate with increasing concentrations of each pigment, and the study was realized between 20 and 240 ng mL^{-1} for chl *a*, between 100 and 900 ng mL^{-1} for chl *b*, between 100 and 600 ng mL^{-1} for phe *a*, and between 50 and 400 ng mL^{-1} for phe *b*. The results obtained are summarized in **Table 1**. The values of the determination coefficient, R^2, are very good for all pigments.

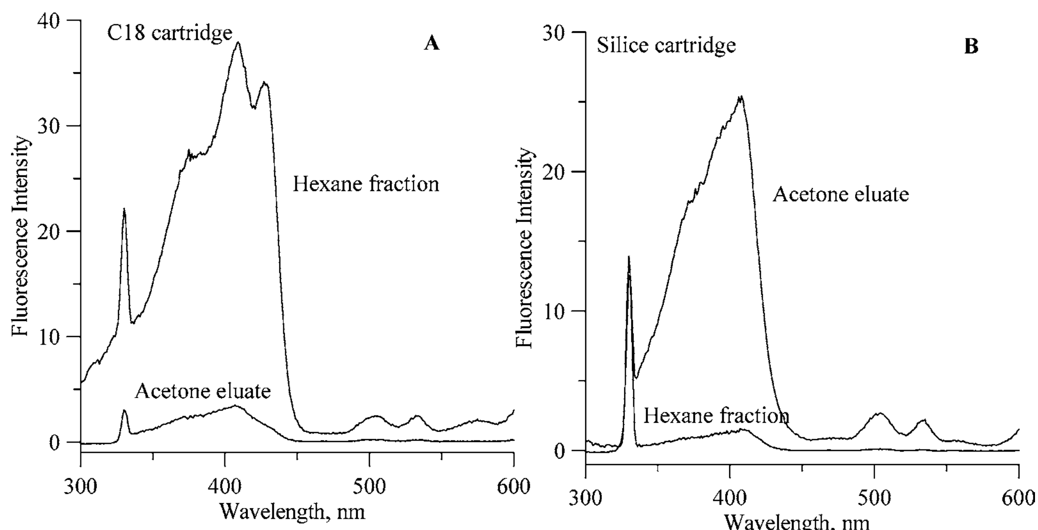

Figure 4. Excitation spectra of the different fractions collected in the SPE procedures, with C18 cartridges (**A**) and with silica cartridges (**B**).

Table 4. Recovery of Chlorophylls *a* and *b* and Pheophytins *a* and *b*, Using Solid-Phase Extraction for the Preparation of the Olive Oil Samples

	added, mg kg^{-1}	% recovery	
		Sep-Pak, C18[a]	Sep-Pak, silica
chl *a*	4	57.6	76.9
	2	50	82.9
chl *b*	4	50.6	74.9
	2	77	76.1
phe *a*	2.4	139.5	103.8
	4.2	84.1	82.9
phe *b*	4	113.9	95.5
	2	110.2	89.2

[a] These recoveries correspond to the hexane fractions and were predicted with a PLS-1 method previously optimized with a calibration set of samples prepared in hexane.

Calibration Matrix and Selection of the Spectral Zones for the Analysis. To establish a quantitative model for the system and taking account the concentration of the pigments in olive oil, the calibration set was prepared in accordance with a Doehlert optimal design (*13*) with four variables and three levels for each one. This calibration set is formed with 29 quaternary samples, but we completed it with binary and ternary mixtures and, finally, 42 calibration samples were used. The concentration of each pigment was varied between 0 and 560 ng mL^{-1} for phe *a*, between 0 and 40 ng mL^{-1} for chl *a* and phe *b*, and between 0 and 80 ng mL^{-1} for chl *b* (**Table 2**). The excitation spectra (λ_{em} = 662 nm) were registered between 300 and 600 nm, the emission spectra (λ_{ex} = 430 nm) between 600 and 750 nm, and synchronous spectra ($\Delta\lambda$ = 230 nm) between 300 and 600 nm.

The spectral regions between 330 and 600 nm (271 experimental points) for the excitation spectra, between 600 and 750 nm (151 experimental points) for the emission spectra, and between 370/600 and 520/750 nm (151 experimental points) for synchronous spectra were selected for the analysis because these are the zones with the maximum spectral information.

To determine the correct number of loading vectors to be used for the modeling of the data, the cross-validation for all samples was performed. In **Table 3** the optimum number of factors, RMSD, and R^2 values are summarized. As can be observed, the lowest number of factors for all compounds is obtained with the emission spectra data set and the R^2 values are similar for the three spectral data sets.

Application of the Optimized PLS-1 Method to Olive Oil. Five monovarietal virgin olive oil samples obtained from olives at different stages of fruit ripeness were analyzed with the

Table 5. Recoveries of Chlorophylls *a* and *b* and Pheophytins *a* and *b* in Oil Samples from Olives in Different Ripening Stages, Using the Excitation Spectral Data Set

	olive oils from black fruits[a]							olive oil from semiblack olives[b]			olive oil from green fruits[c]		
		olive oil 1		olive oil 2		olive oil 3							
	added, mg kg^{-1}	found, mg kg^{-1}	% rec ± RSD	found, mg kg^{-1}	% rec ± RSD	found, mg kg^{-1}	% rec ± RSD	added, mg kg^{-1}	found, mg kg^{-1}	% rec ± RSD	added, mg kg^{-1}	found, mg kg^{-1}	% rec ± RSD
chl *a*		0.01										0.36	
	0.25	0.211	84.5 ± 4.3	0.175	70.0 ± 7.8	0.253	101.4 ± 0.8	0.5	0.336	66.4 ± 2.5	5	5.64	112.0 ± 5.2
	0.5	0.411	82.2 ± 1.7	0.403	80.6 ± 3.3	0.417	83.5 ± 2.0	1	0.756	75.5 ± 3.4	10	11.01	106.5 ± 4.3
chl *b*		0.01				0.018						0.216	
	0.25	0.216	86.3 ± 2.1	0.165	72.1 ± 3.2	0.233	93.2 ± 1.8	0.5	0.418	83.7 ± 0.8	5	5.56	111.0 ± 3.3
	0.5	0.436	87.3 ± 0.9	0.355	71.0 ± 3.2	0.485	97.0 ± 1.8	1	0.87	87.0 ± 0.8	10	10.86	108 ± 3.5
phe *a*		0.875		2.534		0.1063			4.5			53.86	
	1.5	1.183	78.9 ± 2.2	1.292	86.2 ± 2.0	1.167	77.9 ± 3.2	3	3.044	101.5 ± 3.4	30	152.01	101.5 ± 2.1
	3.19	2.365	77.1 ± 0.9	2.552	80.3 ± 2.2	2.732	75.9 ± 1.5	6.4	6.17	85.7 ± 1.4	63.8	66.7	105.0 ± 1.8
phe *b*		0.01		0.38					0.222			2.94	
	0.25	0.249	99.9 ± 2.1	0.205	82.4 ± 1.8	0.259	103.8 ± 2.3	0.5	0.428	85.8 ± 0.8	5	5.4	108.3 ± 2.0
	0.5	0.418	105.0 ± 2.3	0.445	89.1 ± 1.7	0.516	103.2 ± 2.6	1	0.821	82.1 ± 1.1	10	10.92	109.0 ± 1.6

proposed methods and, previously, to select the best method for the preparation of the samples, several procedures were studied. Two of them used SPE with cartridges with different sorbents, and another one is simply the dissolution of the sample with acetone. These methods are described below.

SPE with Sep-Pak Plus C18 (360 mg) Cartridges. The cartridges were conditioned with 8 mL of methanol and with 8 mL of hexane and, afterward, a suitable aliquot of olive oil (between 0.05 and 1 g of olive oil depending on the stage of fruit ripeness), dissolved in 4 mL of hexane and spiked with different amounts of each pigments, was injected and finally eluted with 10 mL of acetone. These two different fractions were re-collected, one in hexane and the other in acetone. In **Figure 4A** the excitation spectra of these fractions are represented. It can be seen that in these conditions the analytes were not retained and all of them eluted in the hexane phase. To predict the concentrations of the pigments in this fraction, a new matrix of calibration was built, but the samples of the calibration set were prepared in hexane. The percentages of recovery found are shown in **Table 4**.

SPE with Sep-Pak Plus Silica (690 mg) Cartridges. The sample (between 0.05 and 1 g of olive oil depending on the stage of fruit ripeness) of olive oil, spiked with different amounts of each pigment, was dissolved in 4 mL of hexane and passed through the cartridge. It was washed with 5 mL of hexane until lipids had been eliminated, and the pigments were eluted with 10 mL of acetone. The first drops of this fraction contain hexane and were discarded. The fluorescence spectra of the hexane and acetone fractions were registered (**Figure 4B**), and the proposed PLS-1 method was then applied as described under procedure. The percentage recoveries found are shown in **Table 4**. We concluded that, in this case, phe *a* was largely retained but a small fraction (∼5%) eluted in the hexane phase. Chlorophylls *a* and *b* and phe *b* were retained, and the recoveries in the acetonic solutions were acceptable.

Dissolution of the Olive Oil with Acetone. The samples (between 0.05 and 1 g of olive oil depending on the stage of fruit ripeness), spiked with different amounts of each pigment, were diluted to 10 mL with acetone. The excitation, emission, and synchronous spectra were registered in the conditions selected, and the proposed PLS-1 methods were applied. The recovery values ranged from 41 to118% for the emission spectral data set and from 60 to 160% for the synchronous spectral data set. The best recoveries were obtained when the excitation spectral data set was used, and in **Table 5** the results obtained with these spectra are summarized.

The phe *a* content found here agrees well with the bibliographic data, and it is always the principal pigment present in olive oil. From our results, this content, in olive oils from black fruits, ranges between 0.2 and 2.5 mg kg^{-1}, and it is higher in oil from semiblack olives, ∼4.5 mg kg^{-1}. Phe *b* content varies between 0 and 0.4 mg kg^{-1} in oil from black olives, and its concentration increases with the decrease in fruit ripeness. In oil from green olives a content of 3 mg kg^{-1} has been found. The major concentrations of the pigments have been found in oil from green olives, ∼54 mg kg^{-1} of phe *a*, and in this case the presence of chl *a* is notable, ∼0.4 mg kg^{-1}.

Conclusions. The simultaneous fluorometric determination of mixtures of chlorophylls *a* and *b* and pheophytins *a* and *b,* using PLS multivariate calibration techniques, is demonstrated to be feasible. The observation of the total fluorescence information, contained in the three-dimensional excitation−emission matrices, allows us the selection of the best scanning paths to perform the determination of each of the components of the mixture. With regard to the determination of these pigments in olive oil, we have proven that the previous isolation of them, by SPE, is not necessary, and the simple dissolution of a suitable aliquot of the olive oil in acetone is enough. When the results obtained by the three data sets examined (excitation, emission, and synchronous spectra of these solutions) were compared, better results for the quantification of all components have been obtained with excitation signals. β-Carotene and lutein are not fluorescent and, therefore, this procedure allows the determination of chlorophylls and pheophytins without interference from the other pigments present in olive oil.

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