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Synchronous Fluorescence Spectroscopy of Edible Vegetable Oils. Quantification of Tocopherols

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The study demonstrates the application of front face and right angle synchronous fluorescence spectroscopy for the characterization of edible oils. The method enables monitoring of tocopherols, pheophytins, and other fluorescent components in edible oils. Principal component analysis of synchronous fluorescence spectra revealed sample clustering according to the type of oil. Partial least-squares regression was utilized to develop calibration models between fluorescence spectra and total tocopherol content determined by HPLC. The regression models showed a good ability to predict tocopherol content. The best fitting results were obtained for 1% v/v diluted oils and for bulk samples using the entire spectrum, yielding the regression coefficient, *r*, of 0.991, and root mean square error of cross-validation of ∼8%. The results presented confirm the capabilities of the fluorescence techniques as a tool for the analysis of edible oils.

KEYWORDS: Tocopherols; pheophytins; vegetable oils; synchronous fluorescence spectroscopy

INTRODUCTION

Fluorescence spectroscopy has a growing importance in food analysis. The main advantage of fluorescence spectroscopy is its high sensitivity and selectivity as compared to other spectroscopic methods. The analytic potential of fluorescence is considerably improved by the application of multidimensional measurement techniques, such as total luminescence and synchronous scanning fluorescence (*1*). The application of multivariate methods for the analysis of fluorescence data further increases its feasibility, especially in studies of the more complex systems (*2*). Fluorescence techniques have been successfully used in the analysis of various food products. In particular, fluorescence spectroscopy was used for monitoring colored precursors in sugar industry processes (*3*−*6*) and oxidation in fish and meat (*7*, *8*), flour (*9*, *10*), dairy products (*11*, *12*), and beers (*13*).

Several papers have discussed the potential of fluorescence in the analysis of edible oils. Fluorescence spectroscopy was used to characterize various types of oil (*14*). It was shown that despite general similarity in the fluorescence characteristic, various kinds of oils exhibit unique emission properties, which may serve as fingerprints for their identification and authentication. Various chemometric approaches have been used for discrimination between different kinds of edible oils and differently processed olive oils (*15*, *16*). Fluorescence spectroscopy has been successfully used for monitoring changes occurring in oil during frying (*17*) and bleaching process (*18*) as well as for quantitative analysis of chlorophylls and pheophytins in olive oils (*19*). The published results and an increasing number of papers devoted to this subject during recent years indicate the wide field of possible applications of fluorescence techniques in edible oils analysis. On the other hand, they indicate that there is a need for in-depth studies and validation before full advantage may be taken of these techniques.

In most of the studies, right angle geometry was used to measure either diluted or bulk oil samples. In such arrangement, the measured fluorescence spectra depend on the actual oil concentration and the solvent used. Recently, Zandomeneghi et al. (*20*) compared the native fluorescence of virgin olive oils using right angle and front face fluorescence techniques. The front face technique eliminates or reduces the artifacts of the right angle geometry, occurring in strongly absorbing samples, for example, primary and secondary inner filter effects. On the other hand, the spectra of individual components may be disturbed by molecular interactions, which occur in concentrated samples. All of these effects result in differences between spectra measured for diluted and bulk oil samples. Our own previous study also showed that fluorescence characteristics of oils change considerably depending on the sample concentration (*21*). As we compared bulk and strongly diluted samples, in which inner filter effects were negligible, we ascribed the observed differences to the molecular interactions such as energy transfer and

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quenching occurring in bulk oils. Despite the difficulties in interpreting the fluorescence spectra of individual components, the front face technique applied for bulk samples has important advantages. Namely, the front face fluorescence measurements do not require any sample preparation and may be conducted directly on oil samples, which can be particularly attractive in the routine analytical applications. Moreover, they allow components with low fluorescence intensities, which could not be detected in diluted samples, to be identified.

The aim of our investigation was to compare the two instrumental arrangements, front face and right angle geometry, with the objective of testing their performance in the characterization and discrimination between various edible oils. Moreover, we studied the possibility of quantitative assessment of total tocopherol content using synchronous fluorescence spectra of both diluted and undiluted oils. Exploratory analysis of the synchronous fluorescence spectra was performed by means of principal component analysis (PCA). Additionally, partial least-squares regression (PLSR) was used to develop calibration models between the fluorescence spectra and the total tocopherol content independently determined by the HPLC method.

EXPERIMENTAL PROCEDURES

Materials. The studies were performed on commercially available edible oils acquired in a local supermarket. Three to four bottles each of olive, grapeseed, rapeseed, soybean, sunflower, peanut, and corn oils were analyzed. The replicate bottles contained the same brand of oil. α-Tocopherol (97%) and *n*-hexane (HPLC grade) were purchased from Aldrich (Steinheim, Germany), γ- and δ-tocopherols were from Sigma (St. Louis, MO) and were used as received.

Fluorescence Measurements. Fluorescence spectra were obtained on a Fluorolog 3-11 Spex-Jobin Yvon spectrofluorometer, with a xenon lamp source used for excitation. Excitation and emission slit width was 2 nm. The acquisition interval and the integration time were maintained at 1 nm and 0.1 s, respectively. A reference photodiode detector at the excitation monochromator stage compensated for the source intensity fluctuations. Individual spectra were corrected for the wavelength response of the system. Right angle geometry was used for the oil samples diluted in *n*-hexane (0.2 and 1% v/v) in a 10 mm fused-quartz cuvette. The front face option was used for bulk oil samples.

Three-dimensional spectra were obtained by measuring the emission spectra in the range from 290 to 700 nm repeatedly, at excitation wavelengths from 250 to 450 nm, spaced by 5 nm intervals in the excitation domain. Fully corrected spectra were then concatenated into an excitation−emission matrix.

The synchronous scan fluorescence spectra were collected by simultaneously scanning the excitation and emission monochromators in the 250−700 nm range, with a constant wavelength difference, $\Delta\lambda$, of 10 nm between them. Fluorescence intensities were plotted as a function of the excitation wavelength. The oil from each bottle was sampled and measured in triplicate.

Reference Analysis. The analysis of tocopherol content was performed by using a HPLC method described in detail in ref *22*. Briefly, the analysis was carried out at room temperature on a Waters 600 high-performance liquid chromatograph equipped with a Symmetry C_{18} column (150 mm × 3.9 mm, 5 μm) fitted with μBondapak C_{18} cartridge guard column (all from Waters, Milford, MA). A mobile phase of 50% acetonitrile and 50% methanol was used with the flow rate of 1 mL/min. Samples of oils were weighed (0.0400−0.1200 g) and dissolved in 1 mL of 2-propanol. Vortex-mixed samples were directly injected onto the HPLC column without any additional sample treatment. The injection volume was 20 μL; a rheodyne type injector was used. The eluate was detected using a Waters 474 scanning fluorescence detector set for the emission at 325 nm and for excitation at 295 nm. The emission slit width was 10 nm, fluorometer gain 100, and attenuation 1. Tocopherols were identified by comparing their retention times with those of the corresponding standards and by spiking the samples with an appropriate standard. Additionally, a Waters 996 photodiode array detector was used to identify the compounds by their absorption spectra.

Fluorescence Data Analysis. PCA and PLSR were the two chemometric tools used in the multivariate evaluation of the fluorescence data; both of these techniques are based on linear decomposition of data.

PCA is a bilinear modeling method, which gives an interpretable overview of the main information contained in a multidimensional data set (*23*). The information included in the original variables is projected onto a smaller number of variables called principal components (PCs). PCs are linear functions of the original variables. They are estimated to contain, in decreasing order, the main structured information in the data. A PC is the same as a score vector and is also called a latent variable.

In the matrix representation, a model with a given number of components is represented by the equation

$$X = TP^T + E = \text{structure} + \text{noise}$$

where X is a data matrix, representing a number of samples and variables, T is the scores matrix, P is the loadings matrix, and E is the error matrix. The combination of scores and loadings is the structured part of the data. The remaining contribution is called error or residual and represents the fraction of variation that cannot be interpreted by the model.

By plotting the principal components, one can view the relationships between different variables and detect and interpret sample patterns, groupings, similarities, or differences. The scores plots visualize the relationship between the oil samples for each of the PCs, whereas the loadings plots are used for the interpretation of the corresponding spectral variations.

All of the spectra recorded were used for the PCA, including replicates, analyzed as separate samples. Data pretreatment consisted in mean centering the spectra to eliminate the common spectral information. We considered an optimal number of components the lowest number that allowed describing no less than 95% of total variance.

PLSR is a method for relating the variations in one or several response variables (Y variables) to the variations of several predictors (X variables), with explanatory or predictive purposes (*24*). PLS models both the X and Y matrices simultaneously to find such latent variables in X that will best predict the latent variables in Y. These PLS components are similar to principal components. Interpretation of the relationship between X data and Y data is simplified as this relationship in concentrated on the smallest possible number of components. By plotting the first PLS components one can view main associations between the X variables and Y variables and also interrelationships within X data and within Y data.

PLS was used for testing the correlation between the synchronous fluorescence spectra (X) and the total tocopherol content (Y) in the oils samples measured by a HPLC method. An average of three recorded spectra per sample, for 25 bottles of oil, was used in the analysis. Data pretreatment involved mean centering.

Full cross-validation was applied for all regression models. Cross-validation is a strategy for validating calibration models based on systematically leaving out groups of samples in the modeling and testing the left-out samples in a model based on the remaining samples.

The regression models were evaluated using the correlation coefficient (r) and the validation parameter, root mean square error of cross-validation (RMSECV), as a term indicating the prediction error of the model. The RMSECV is defined by the equation

$$\text{RMSEC} = \sqrt{\frac{\sum_{i=1}^{N} (y_i^{\text{pred}} - y_i^{\text{ref}})^2}{N}}$$

where, y_i^{pred} is the predicted concentration value for a sample in the cross-validation procedure, y_i^{ref} is the reference value, and N is the number of samples.

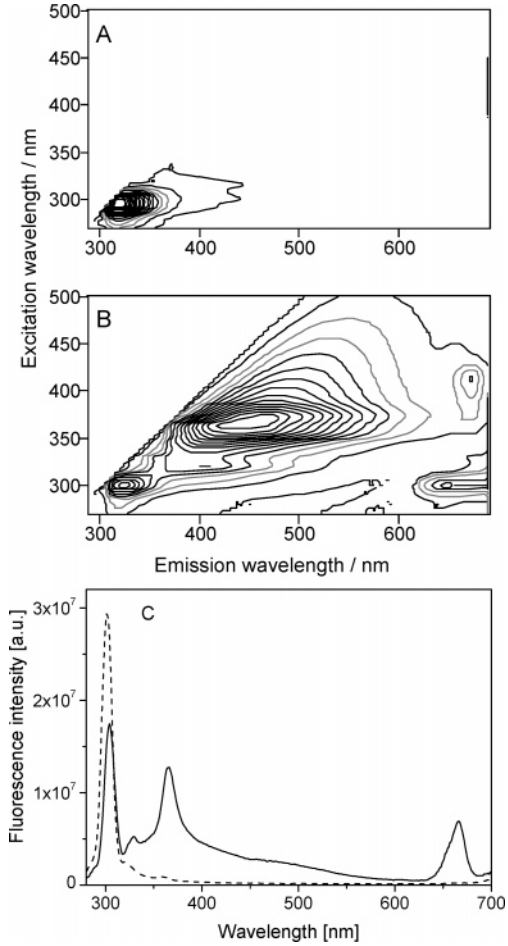

Figure 1. Total luminescence spectra of the corn oil: (**A**) sample diluted in *n*-hexane, 1% v/v; (**B**) bulk oil. (**C**) Synchronous fluorescence spectra of oil diluted in *n*-hexane, 1% v/v (dashed line) and bulk (solid line).

The data analysis by PCA and PLS was performed using Unscrambler 9.0 software (CAMO, Oslo, Norway).

RESULTS AND DISCUSSION

Fluorescence Characteristics of the Oils. Total luminescence spectra for corn oil diluted in *n*-hexane (1% v/v) and bulk sample are shown in **Figure 1**. The spectra show common features characteristic for all of the oils studied.

The assignment of the emission bands to specific chemical components, based on comparison of three-dimensional and single excitation and emission spectra with those of the respective reference compounds, was described in detail previously (*21*, *25*). The relatively intense band, observed in diluted oil samples, with excitation in the range of ~270−310 nm and emission in the range of ~300−350 nm has been ascribed to tocopherols. A less intense fluorescence band at ~300 nm in excitation and ~400 nm in emission is present in refined oils and was tentatively ascribed to the oxidation products of oils. A similar band had been noted by us previously in a different set of edible oils (*21*, *25*) and was observed also by other authors (*26*). Synchronous fluorescence spectra of oils (**Figure 1C**) reveal bands similar to those observed in the total luminescence spectra. The spectra of diluted oils measured at $\Delta\lambda = 10$ nm show the presence of one intense band with its maximum at 303 nm, ascribed to tocopherols, and a shoulder at longer wavelengths, which indicates the presence of other fluorescent components.

It was recently suggested that fluorescence observed in extra virgin olive oils in the 300−350 nm range may originate from phenolic compounds, such as gallic acid, vanillic acid, and hydrocaffeic acid (*20*). However, the oils presently studied were mainly commercially available refined vegetable oils. The content of polyphenols is expected to be much lower in such oils than in freshly pressed extra virgin olive oil. For example, the total phenol content in olive oils was determined in the range of 55−126 mg of caffeic acid equivalents/kg, whereas for sunflower and corn refined oils it varied in the 0.5−3.2 mg/kg range (*27*). Therefore, we believe that the main components responsible for the short-wavelength emission in refined oils are tocopherols; this assumption will be positively verified in the following sections.

As is evident from **Figure 1**, the spectra of diluted oils contain primarily the information related to the emission ascribed to tocopherols. We have recently shown that using synchronous fluorescence spectra of 1% v/v oils, recorded at various excitation−emission wavelengths offset ($\Delta\lambda = 10$, 30, 60, 80 nm), it is possible to correctly classify the oils by means of the *k*-nearest-neighbors method (*25*).

Concentration of the oil samples has a pronounced effect on the fluorescence spectra observed. Higher oil concentrations correspond to stronger tocopherol emission. However, in more concentrated samples, the intensity of tocopherol emission decreases again, and the spectra reveal the presence of other fluorescent species, which were not observed in diluted samples. Total luminescence spectra recorded for bulk oils using front face geometry differ considerably from those measured for diluted oils (**Figure 1**). The intensity of the short-wavelength emission ascribed to tocopherols is considerably reduced. Concurrently, an intense structured emission band is observed in the intermediate-wavelength region. Additionally, for bulk oils, the long-wavelength band appears, at 350−420 nm in excitation and at 660−700 nm in emission, characteristic for the fluorescence of pigments of the chlorophyll group (*19*), which includes chlorophylls *a* and *b* and pheophytins *a* and *b*. The pigments of the chlorophyll group mainly occur in crude oils, obtained directly by the extraction from the oilseed, and are removed during processing (*28*). The spectra presented show that some traces of chlorophyll pigments remain even in refined oils.

Figure 2 shows synchronous fluorescence spectra of bulk oil samples. Synchronous fluorescence spectra of bulk oils show the presence of three main emission regions, namely, the band at 303 nm, ascribed to tocopherols, another at 666 nm, ascribed to chlorophylls, and a broad emission band in the intermediate region, exhibiting several maxima, which may indicate several distinct emissive species in this region (see **Figures 1** and **2**).

The fluorescence characteristics change with increasing oil concentration; thus, the spectra of bulk oils contain the most comprehensive information about the fluorophores present in oils. In addition to the tocopherol and chlorophyll emissions common to all of the oils studied, the spectra reveal a broad emission band in the intermediate wavelength region. This emission should be the most relevant for the discrimination of oils because it shows the largest variability depending on the kind of oil.

Exploratory Analysis of the Oil Fluorescence. PCA models were developed using synchronous fluorescence spectra of diluted and bulk oils. PCA modeling for oils diluted in *n*-hexane at concentration of 0.2 and 1.0% v/v was performed for synchronous fluorescence spectra measured at $\Delta\lambda = 10$ nm with right angle geometry. All of the oils were included in the analysis. The resulting scores and loadings graphs are shown in **Figure 3**.

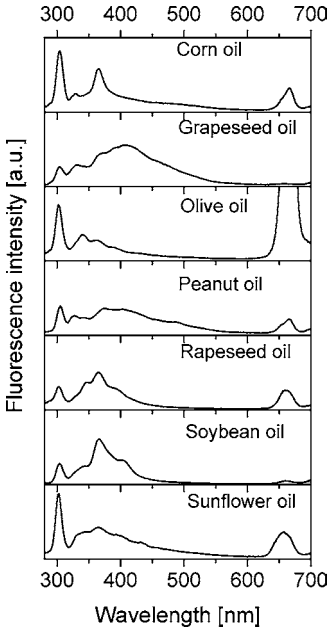

Figure 2. Synchronous fluorescence spectra of bulk oils measured at $\Delta\lambda = 10$ nm, with a common fluorescence intensity scale used for all samples.

The PCA for the lowest oil concentration reveals the sample distribution according to the PC1 value, which describes 95% of the total variance. This component is related to the tocopherol emission, as is evident from the loadings plots. Some clustering reflecting kinds of oils is observed; however, these clusters are strongly overlapped. The observed sample distribution in the score plot, along the PC1 axis, seems to agree with the total tocopherol content of oils determined by the HPLC method (**Table 1**).

The sample clustering according to the kind of oil is greatly improved at 1% v/v concentration, mainly due to variations in PC2, which corresponds to the fluorescent band at 320−400 nm and describes 10% of the total variance. PC1 (87% of total variance) still describes the tocopherol emission and is related to the tocopherol concentration.

PCA for bulk oils was performed on the synchronous fluorescence spectra measured at $\Delta\lambda = 10$ nm using front face geometry. Note that the olive oil samples were excluded from the final PCA model. As noted in the synchronous fluorescence spectra, the olive oil differs from all of the others by a very high intensity of the chlorophyll band. Therefore, with the olive oil included in the analysis, the PCA model described primarily the differences between it and other samples due to the chlorophyll band. In contrast to the models obtained for diluted samples, four PCs are needed to describe >95% of total variance. PC1 describes 50% of the total variance and is related mainly to the emission at 300−350 nm and above 400 nm. PC2 describes 29% of the total variance and is related to the 300, 320−380, and 620−700 nm emission bands. The first two components are the most effective in achieving sample clustering according to the oil type, whereas additional components do not improve clustering in a noticeable manner.

The present results allow us to conclude that the spectral range at ~300 nm has the highest loadings (and the most important variables for the sample classification) in diluted oils and correlates with the tocopherol content. Additional fluorophores become important and improve oil discrimination in more concentrated and bulk oil samples.

Total Tocopherol Content. Linear regression analysis between fluorescence intensity at 301 nm and the total tocopherol concentration was performed to test the quantitative relationship between fluorescence ascribed to tocopherols and real vitamin E content in oils. The regression yielded the correlation coefficients of 0.953 and 0.945, respectively, for 0.2 and 1% v/v oil solutions. These high correlations indicate that the fluorescence band with the maximum at 301 nm in synchronous spectra can be ascribed mainly to tocopherol emission, confirming our previous assumption that tocopherols are the main components responsible for this emission in refined oil. No significant correlation was found between the fluorescence intensity at 301 nm and the total tocopherol concentration in bulk oil samples. Thus, the univariate regression gives relatively good results for diluted samples, whereas for bulk oils, in which a strong influence of the matrix constituents on tocopherol emission can occur, the univariate analysis fails. We expected that a model based on multivariate input data would be more robust against possible matrix interferences and differences in background fluorescence; therefore, PLS analysis was applied.

The PLS modeling was performed for synchronous fluorescence spectra measured at different instrument arrangements and sample dilutions. The synchronous fluorescence spectra at $\Delta\lambda = 10$ nm were used as the *x* variable, whereas the concentration of tocopherols determined by the HPLC method was used as the *y* variable. The calibration results are summarized in **Table 2** and **Figure 4**. **Figure 4** shows a typical plot of the total tocopherol content predicted by the PLS model based on the synchronous fluorescence spectra versus the concentration obtained using the reference analysis. The linearity of the plot indicates good performance of the model in predicting the concentration of tocopherols.

It is well-known that the spectral range used in the analysis is the critical parameter in the application of the PLS algorithm. Models that include unnecessary spectral information tend to overfit the data. On the other hand, information related to an analyte may be ignored if an insufficient spectral region is chosen for the analysis. The optimal spectral range should include information related to the concentration of the constituents of interest and other interfering constituents, eliminating any regions including noise or artifacts. Therefore, different spectral ranges were selected to include various fluorescence bands: tocopherols only, tocopherols and minor fluorescent components, and the full spectral range. In total, nine PLS models were investigated, including all possible combinations of three different oil dilutions with three different spectral ranges.

Overall, all of the PLS models underscore a strong correlation between the fluorescence and the total tocopherol concentration. The final PLS models for 0.2% v/v oils, built with three latent variables, give relatively high correlation coefficients; however, the respective RMSECV values are relatively high. The best fitting parameters were obtained using the 290−460 nm spectral range. More variables, four or five, were required to build reliable PLS models for 1% v/v samples. Here, the best fitting results were obtained with the full-range spectrum. Narrowing of the analyzed spectral range makes the fitting parameters worse; however, they remain generally better than those of the most diluted samples. The PLS analysis of spectra of bulk oils, using both the full and the 290−580 nm range, gives results comparable to those for 1% v/v samples. However, for the 290−320 nm region, the correlation coefficient was rather low,

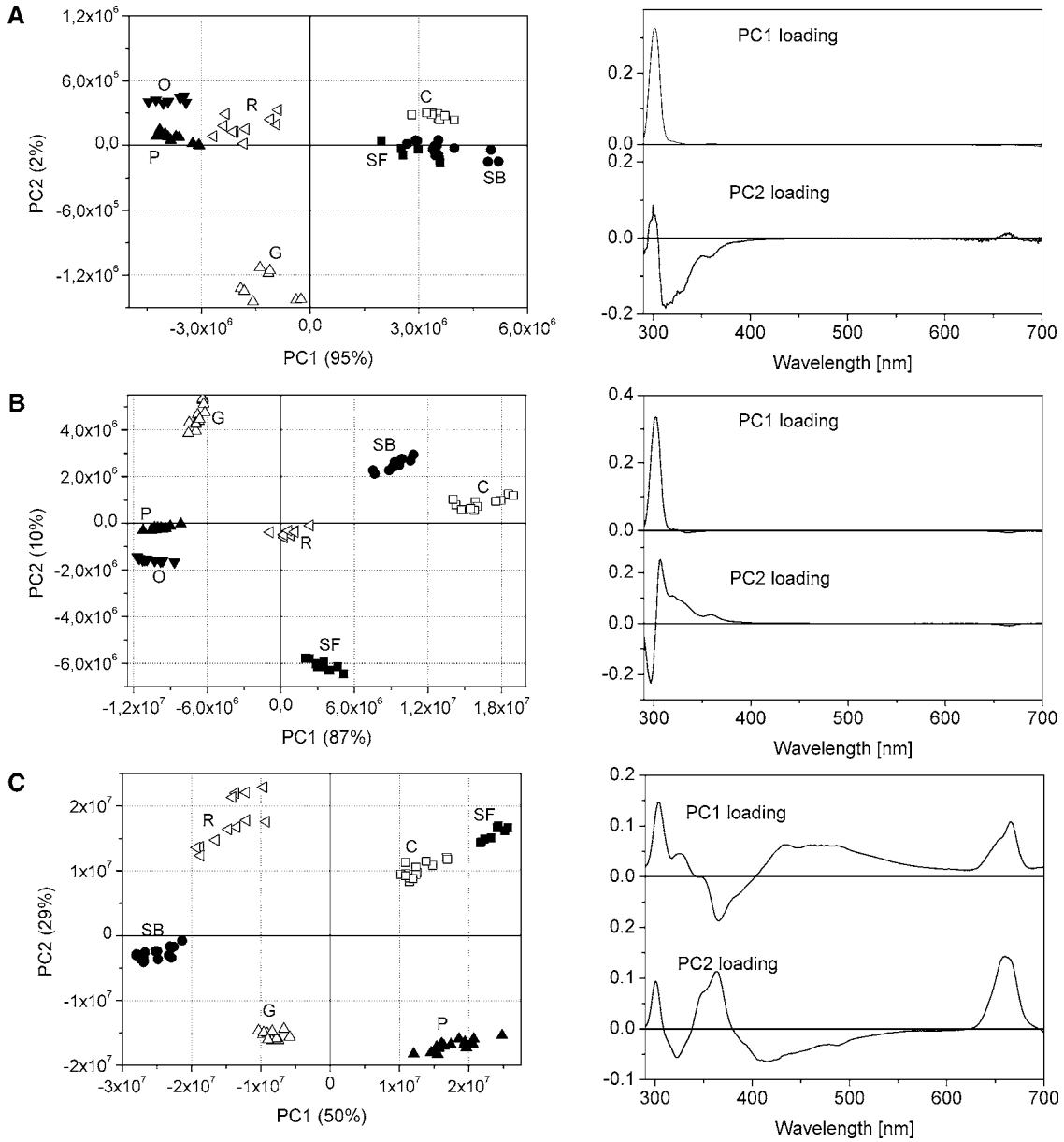

Figure 3. PCA models of the synchronous fluorescence spectra, at $\Delta\lambda = 10$ nm, scores plot for PC1 versus PC2 components, and loadings plot for PC1 and PC2 componentsL (**A**) 0.2% v/v oils in *n*-hexane, right angle geometry; (**B**) 1% v/v oils in *n*-hexane, right angle geometry; (**C**) bulk oils, front face geometry. Oils: C, corn; G, grapeseed; O, olive; P, peanut; R, rapeseed; SB, soybean; SF, sunflower.

Table 1. Average Total Tocopherols Content in Various Oils Determined by Means of HPLC

oil	total tocopherol[a] (mg/dm^3)
corn	775 (25)
peanut	205 (2)
grapeseed	112 (0.2)
rapeseed	421 (33)
sunflower	671 (13)
soybean	759 (15)
olive	158 (3)

[a] Mean values of total tocopherol content determined for three or four separate bottles of each kind of oil. Standard deviations are given in parentheses.

Table 2. Results of PLS Regression of Tocopherol Content and Synchronous Fluorescence Spectra at $\Delta\lambda = 10$ nm

PLS model	spectral range (nm)	no. of samples	latent variables	*r*	RMSECV (mg/dm^3)
diluted oils	290–700	22	3	0.970	62
0.2% v/v	290–460	22	3	0.974	57
	290–320	22	3	0.973	59
diluted oils	290–700	25	5	0.991	36
1% v/v	290–460	25	4	0.988	40
	290–320	25	5	0.983	49
bulk oils	290–700	24	7	0.991	37
	290–580	24	6	0.985	46
	290–320	24	9	0.918	106

accompanied by a high RMSECV value. The PLS models for bulk samples required even more latent variables, from six to nine.

Summarizing the results of the PLS calibration, we conclude that more latent variables are required at higher sample concentrations to appropriately reproduce the correlation between the spectra and the tocopherol concentration. This may result from the increasing influence of the more concentrated matrix on the observed tocopherol fluorescence and also from the internal filter effects. Improvement of the fitting parameters, when wider spectral regions were analyzed, also shows that other

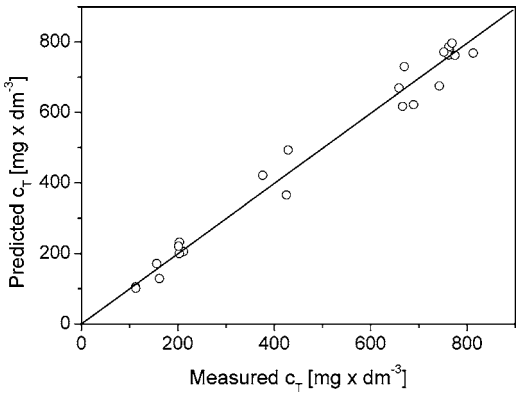

Figure 4. Plot of the total tocopherol content predicted by the PLS model based on the synchronous fluorescence spectra versus the concentration obtained from the reference HPLC analysis (PLS regression for front face synchronous fluorescence spectra of bulk oils, with the entire spectra used in the calculations).

fluorescent constituents should be included in the model if the tocopherol content were to be predicted correctly. This becomes especially important for the more concentrated samples. The inferior model performance for the most diluted samples may indicate that although the matrix influence is reduced, some additional spectral information important for tocopherol quantification may be missed at such high dilutions.

The RMSECV error for the best model has the value of 36 mg/dm^3, corresponding to 8% of the average tocopherol content of 443 mg/dm^3 in the set of oils studied. This relatively high value could result from our attempt to model a diversity of oils, seven various kinds, with tocopherol contents ranging from 158 to 775 mg/dm^3, and diverse matrix constituents. We believe the predicting ability of the model can be improved with an oil kind specific calibration. Such a calibration should also reduce the number of latent variables.

In summary, the potential of the synchronous fluorescence spectroscopy for qualitative and quantitative analysis of edible oils has been demonstrated. The results showed that the discrimination of oils based on their fluorescence can be performed either directly on bulk oils or after dilution. However, the analysis of bulk samples seems to be more reliable as fluorescence spectra of such samples contain more information, which is lost upon dilution. Excellent oil discrimination was achieved with two primary components in PCA of fluorescence spectra of 1% v/v diluted oils. The PLSR models show good correlation between the synchronous fluorescence spectra and the total tocopherol content as determined by the HPLC method. The best fitting results were achieved for 1% v/v diluted oils and for bulk samples using the entire spectrum.

The results of the present study proved that rapid fluorescence measurements can be conducted directly on the oil samples and used for oil discrimination and for quantitative determination of vitamin E after an appropriate calibration. Further studies are needed to resolve various issues that are important for practical application of the fluorescence techniques, among which are the method verification for specific kinds of oil and its applicability for the quantification of other fluorescent oil constituents, such as phenolic compounds. They are important for practical and common applications of different fluorescence techniques in the quality assurance of oils.

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