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Origin of French Virgin Olive Oil Registered Designation of Origins Predicted by Chemometric Analysis of Synchronous Excitation−Emission Fluorescence Spectra

NATHALIE DUPUY,*,† YVELINE LE DRÉAU,† DENIS OLLIVIER,§ JACQUES ARTAUD,‡ CHRISTIAN PINATEL,|| AND JACKY KISTER†

Laboratoire GOAE, Université Paul Cézanne, UMR CNRS 6171, case 561, 13397 Marseille Cedex 20, France; Laboratoire de Marseille, Direction Générale de la Concurrence, de la Consommation et de la Répression des Fraudes, 146 traverse Charles-Susini, 13388 Marseille Cedex 13, France; Laboratoire de Chimie Analytique de l'Environnement, Université Paul Cézanne, UMR CNRS 6171, IFR PMSE 112, B.P. 80, Aix-Marseille III, France; and Association Française Interprofessionnelle de l'Olive, (AFIDOL), Maison des Agriculteurs, 22 avenue Henri-Pontier, 13626 Aix-en-Provence Cedex, France

The authentication of virgin olive oil samples requires usually the use of sophisticated and very expensive analytical techniques, so there is a need for fast and inexpensive analytical techniques for use in a quality control methodology. Virgin olive oils present an intense fluorescence spectra. Synchronous excitation−emission fluorescence spectroscopy (SEEFS) was assessed for origin determination of virgin olive oil samples from five French registered designation of origins (RDOs) (Nyons, Vallée des Baux, Aix-en-Provence, Haute-Provence, and Nice). The spectra present bands between 600 and 700 nm in emission due to chlorophylls *a* and *b* and pheophytins *a* and *b*. The bands between 275 and 400 nm in emission were attributed to α-, β-, and γ-tocopherols and to phenolic compounds, which characterize the virgin olive oils compared to other edible oils. The chemometric treatment (PLS1) of synchronous excitation−emission fluorescence spectra allows one to determine the origin of the oils from five French RDOs (Baux, Aix, Haute-Provence, Nice, and Nyons). Results were quite satisfactory, despite the similarity between two denominations of origin (Baux and Aix) that are composed by some common cultivars (Aglandau and Salonenque). The interpretation of the regression coefficients shows that RDOs are correlated to chlorophylls, pheophytins, tocopherols, and phenols compounds, which are different for each origin. SEEFS is part of a global analytic methodology that associates spectroscopic and chromatographic techniques. This approach can be used for traceability and vindicates the RDOs.

KEYWORDS: Virgin olive oil; synchronous excitation−emission fluorescence; PLS; quality control; origin determination; traceability

INTRODUCTION

The Mediterranean basin is the most important olive oil producing area in the world. Oils come from very many olive varieties, which are generally related to a soil that confers specific sensory and chemical properties. These characteristics are developed by attribution by national or European organizations of mark of quality as the registered designation of origin (RDO) in France or the protected designation of origin (PDO) in Europe. In France, seven RDOs were recently created from two. The RDOs are regulated by specific articles and conditions that rule all of the official channels from growth to fabrication. Because of their high cost, notably with the RDO label, virgin olive oils could be the subject of fraudulent practices, which consist of mixing with various amounts of seed oils, low-cost olive oils, or olive pomace oils. Nowadays, one of the major problems in the agricultural−food industry is to set objective tools to determine the origin of primary materials as well as finished products so that we can follow the products from the producer to the consumer.

The search for the origin and the authenticity of olive oils has been the object of numerous studies in the past few years using the extremely varied physical−chemical determinations that are associated with a chemometric treatment. The studies could be classified in two main categories. In the first one, the samples are chemically treated to determine the composition in different constituents: fatty acids, triacylglycerols (*1*, *2*), sterols (*3*), aroma (*4*), etc. The second one is based on spectroscopic studies on samples without preliminary treatment: ^{1}H

* Corresponding author (e-mail nathalie.dupuy@univ.u-3mrs.fr).
† Laboratoire GOAE.
§ Laboratoire de Marseille, Direction Générale de la Concurrence, de la Consommation et de la Répression des Fraudes.
‡ Laboratoire de Chimie Analytique de l'Environnement.
|| Association Française Interprofessionnelle de l'Olive (AFIDOL).

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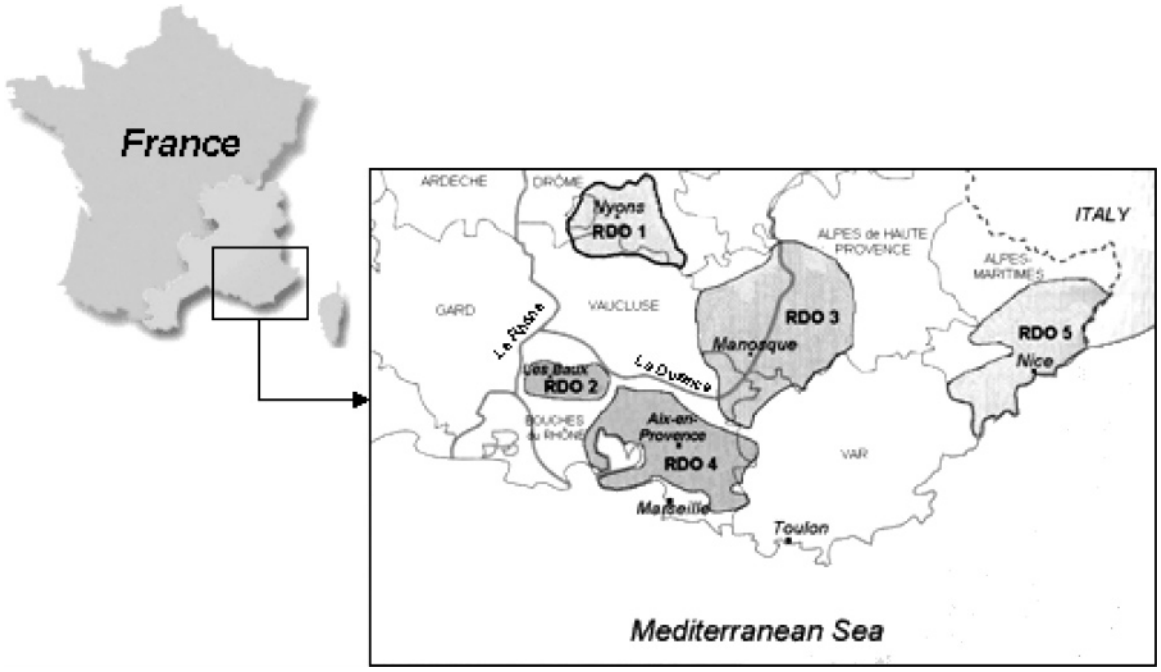

Figure 1. Geographical areas of the five French RDOs: RDO 1, Nyons; RDO 2, Vallée des Baux de Provence; RDO 3, Haute-Provence; RDO 4, Aix-en-Provence; RDO 5, Nice.

Table 1. Cultivars of Five French RDOs

	Aix	Baux	Haute-Provence	Nice	Nyons
primary cultivars	Aglandau Cayanne Salonenque	Aglandau[a] Grossane Salonenque Verdale BdR[b]	Aglandau	Cailletier	Tanche
secondary cultivars	Bouteillan Grossane Picholine Verdale BdR[b]	Picholine	Bouteillan Picholine Tanche	no	no
local and old cultivars	Ribiére Sabine Saurine Sigoise Triparde	yes	Boube, Colombale Estoublaisse Filayre Grapié	Araban Blanquetier Blavet Nostral Ribeyrou	no

[a] Synonym of Bégurette. [b] Verdale des Bouches du Rhône.

and ^{13}C NMR (*5*, *6*), near-infrared spectroscopy (NIR) (*7*, *8*), and Fourier transform infrared spectroscopy (FTIR) (*9*).

Few papers are reported in the literature for vegetable oils analysis by fluorescence spectroscopy. Fluorescence spectroscopy was used to analyze minor components (chlorophylls, tocopherols, phenols, riboflavin, etc.) present in olive oils (*10*−*12*). Fluorescence spectra were successfully used to characterize and discriminate edible oils (*11*, *13*, *14*). Moreover, this technique, generally associated with chemometrics treatment, was used to distinguish between commercial samples of virgin olive oils, pure olive oils, and olive pomace oils (*11*, *15*, *16*).

The aim of this study was to develop by synchronous excitation−emission fluorescence spectroscopy (SEEFS) a direct and rapid test method to discriminate olive oils according to their RDOs. Samples were discrimined by applying statistical methods to excitation−emission fluorescence spectra of oils.

MATERIALS AND METHODS

Reagents and Standards. Methanol and hexane (spectrosol grade) were purchased from SDS (Peypin, France). Tyrosol and caffeic acid were purchased from Extrasynthèse (Genay, France); *p*-coumaric acid was from Sigma (Saint Quentin Fallavier, France), and α-tocopherol was from Merck (Darmstadt, Germany).

Oil Samples. Industrial virgin olive oil samples (n = 133) were obtained from the French Inter-Professional Olive Oil Association (AFIDOL), Aix-en-Provence, France. Samples were obtained from two successive crops (2002/2003 and 2003/2004). They came from five French RDOs including four that received a European protected designation of origin (PDO) (**Figure 1**). Eight foreign commercial virgin olive oils (2004) were obtained from the Direction Générale de la Concurrence de la Consommation et de la Répression des Fraudes (DGCCRF).

Table 1 shows the different constituted cultivars of RDOs. The RDOs are made up of primary and secondary cultivars, as well as local and old varieties. Nyons (n = 26), Haute-Provence (n = 26), and Nice (n = 23) are made up of one unique principal cultivar. Aix-en-Provence (Aix) (n = 28) and Vallée des Baux de Provence (Baux) (n = 30) have up to three or four principal cultivars, of which at least two present, but their proportions were not specified.

Instrumentation. Synchronous excitation−emission fluorescence spectra were recorded with a Perkin-Elmer LS-50B spectrometer interfaced to a personal computer. The source was a xenon flash lamp, power equivalent to 20 kW for 8 μs duration. Virgin olive oil samples were filled into a 10 mm fused quartz cell. All of the spectra were

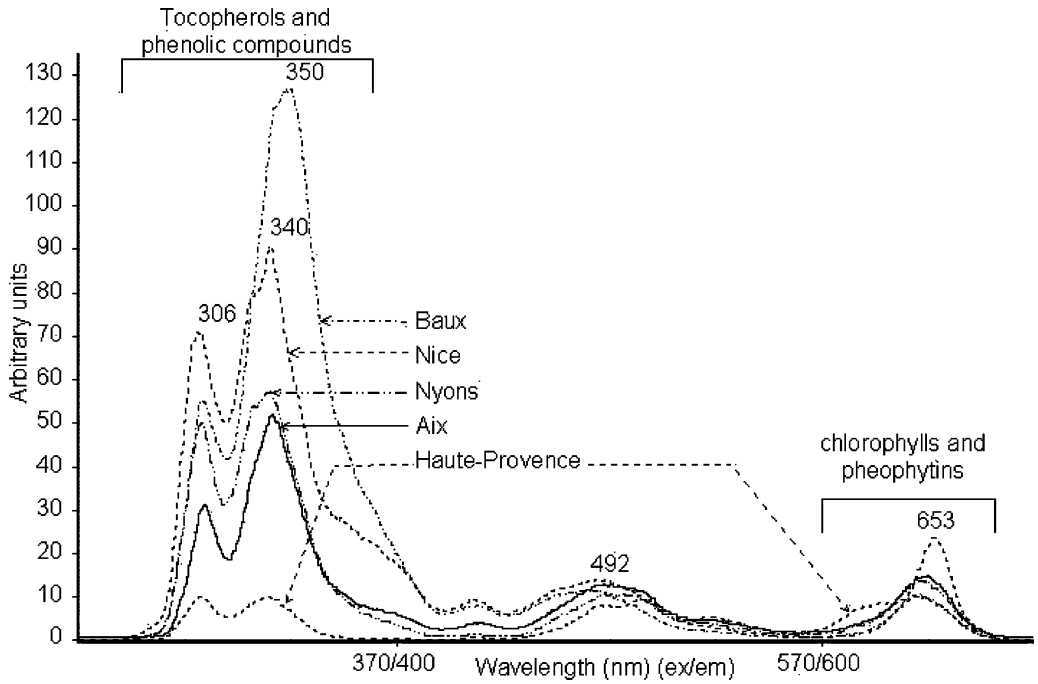

Figure 2. Synchronous excitation–emission fluorescence spectra ($\delta\lambda = 30$ nm) of each mean virgin olive oil RDO.

computed at 1 nm resolution between 250 and 700 nm. The synchronous fluorescence spectra were collected by synchronously scanning the excitation and emission monochromator in the 250−700 nm range with constant wavelength difference $\delta\lambda = 30$ nm between them. The step size and band-pass of the monochromator were set to 5 and 4 nm, respectively.

Fluorescence Measurements. Virgin olive oil samples were analyzed without any prior treatment, and the spectral data were used for the chemometric analysis.

Pure phenolic compounds (~2 mg) were dissolved in methanol (2 mL) and then extracted by hexane (2 mL). The hexanic phase was analyzed by fluorescence spectroscopy after concentration adjustment. The obtained spectra were compared with a virgin olive oil at 1% (v/v, hexane) spectrum.

Principal Component Analysis (PCA). PCA is a method for extraction of the systematic variations in one data set (*17*). This method can be used for classification as well as for description and interpretation. PCA is oriented toward modeling the variance/covariance structure of the data matrix into a model that represents the significant variations and considers the noise as an error. The components are found during the calibration, one by one. Each principal component, called a vector of loading, represents the main systematic variation in the data set, which can be modeled after the extraction of the previous ones. Common characteristics of all spectra are modeled in one or several principal components for which the scores are not significantly different according to the species. On the contrary, the information that differentiates the species contributes to the principal component that has significant scores (*18*). PCA is a tool for unsupervised learning, for example, extracting regularities directly from the input data without referring to classes known in advance.

Partial Least-Squares Regression (PLS). This supervised analysis was based on the relationship between the signal intensity and the origin of the sample (*19*). Interference and overlapping of the spectral information may be overcome by using powerful multicomponent analysis such as PLS regression (*20*, *21*), which allows a sophisticated statistical approach using the full or partial spectral region rather than unique and isolated analytical bands. The algorithm is based on the ability to mathematically correlate spectral data to a property matrix of interest while simultaneously accounting for all other significant spectral factors that perturb the spectrum (*22*). It is thus a multivariate regression method that uses the full spectral region selected and is based on the use of latent variables. Samples of known origins are used as calibration samples, and then the origins of unknown samples are directly calculated using the resulting equation under the same conditions. In our case, we are looking for only chemical modifications and not real concentrations using the same data processing. PLS1 deals with only one variable response at a time, and PLS2 handles several responses simultaneously and is used when variables are collinears. We used the PLS1 algorithm with mean centered variables and not PLS2 because the geographical origins were not correlated with each other.

The root mean square error of prediction (RMSEP) gives an estimation of the prediction performance during the step of validation of the calibration eq 1

$$\mathrm{RMSEP} = \sqrt{\left(\frac{\sum_{i=1}^{M}(C_i - C'_i)}{M}\right)} \qquad (1)$$

where C_i is the known value, C'_i is the value calculated by the calibration equation, and M is the number of samples in the prediction set. For some applications, the spectral data were first derived with the algorithm developed by Savitzky and Golay (*23*) to remove unwanted spectral variations as offsets, and a smoothing with two polynomial orders was performed.

For the codification of olive origin, the five origins corresponding to RDOs are arbitrarily classified in the order Aix, Baux, Haute-Provence, Nice, and Nyons, so for each sample the origin may be represented by a five-dimensional output vector with 1 at the position corresponding to geographic origin and 0 at the other positions. For instance, sample 1, which is of Aix origin, will be codified by the vector 1,0,0,0,0.

Deviation Standard. The repeatability of the prediction is defined by the relative standard deviation (RSD).

Software. The chemometric applications were performed by UNSCRAMBLER software version 6 from CAMO (Computer Aided Modelling, Trondheim, Norway).

RESULTS AND DISCUSSION

Figure 2 shows the mean SEEF spectra of each virgin olive oil RDO. All of the spectra exhibit a similar profile with variable

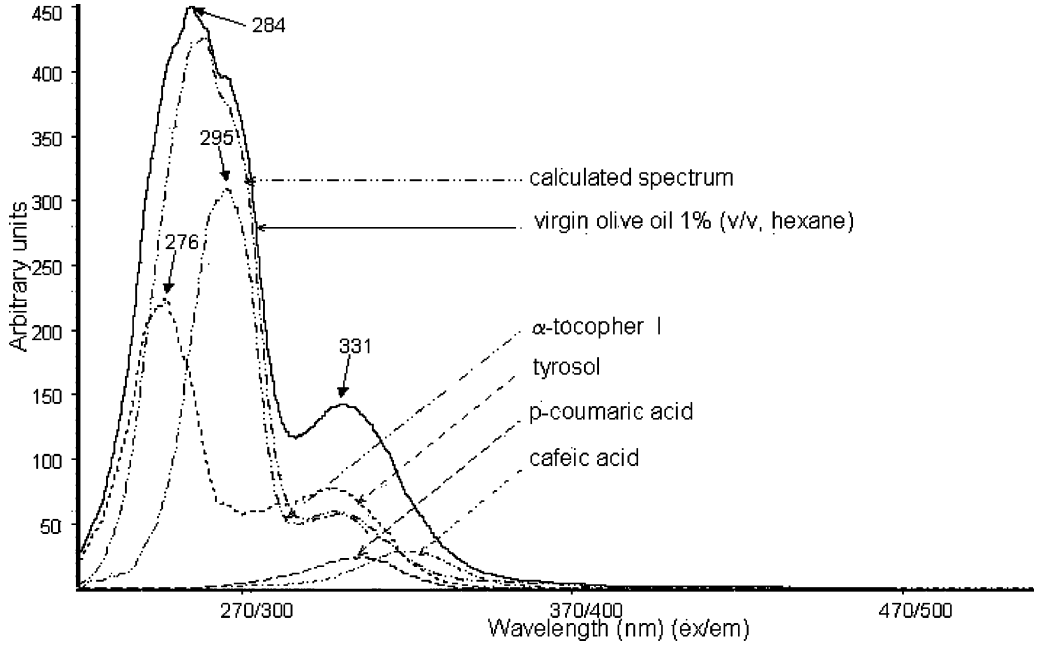

Figure 3. Synchronous excitation–emission fluorescence spectra of some phenolic compounds, α-tocopherol, virgin olive oil in hexane, and calculated spectrum.

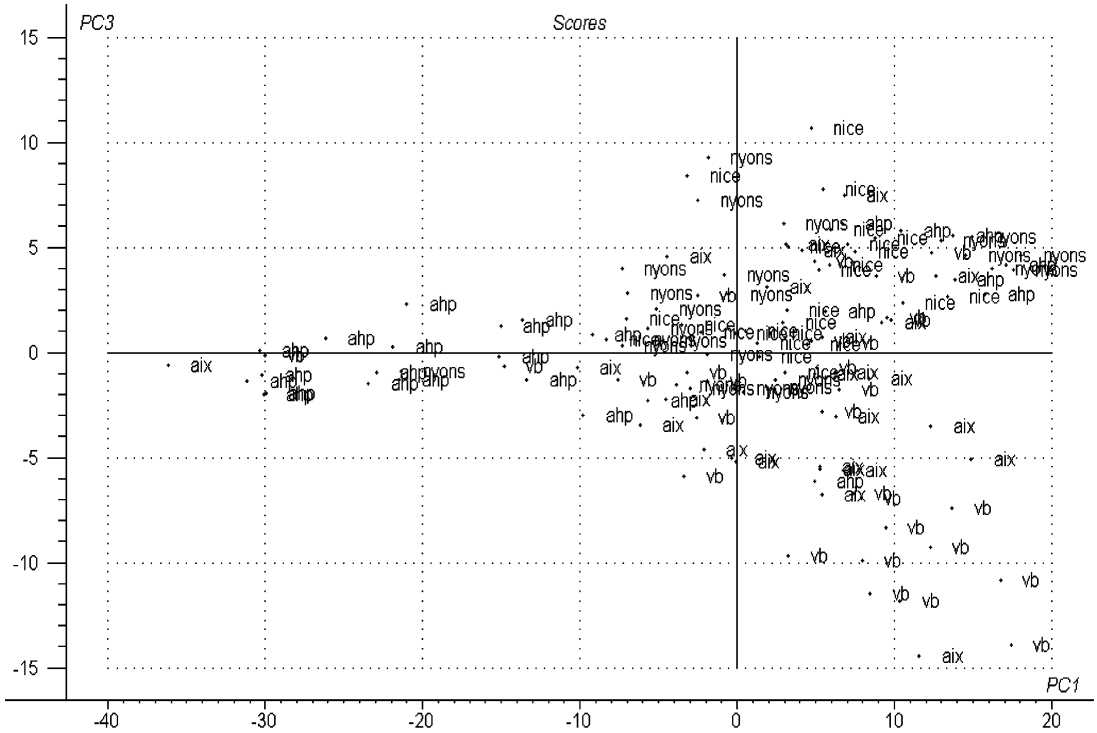

Figure 4. PCA on the 250–700 nm range with first derivatized and normalized spectra from all 133 oil samples (vb, Baux; ahp, Haute Provence).

intensities and wavelength maxima. Bands between 600 and 700 nm in emission have been attributed to chlorophylls *a* and *b* and to pheophytins *a* and *b* (*11*, *13*−*15*, *24*, *25*).

Some small bands are present between 400 and 600 nm in emission. Some identification attempts were made, but attributions remain uncertain. According to Kyriadikis et al. (*11*), they could be due to oxidation products of vitamin E.

Between 250 and 400 nm, spectra exhibit a medium or intense fluorescence signal with two maxima, one at ∼300 nm and the second at ∼340−350 nm. Sikorska et al. (*14*) showed that α-tocopherol in hexane presented, in synchronous fluorescence, a peak near 300 nm [the wavelength varies according to the difference ($\delta\lambda$) between the excitation and emission wavelengths]. The SEEF spectrum of α-tocopherol in **Figure 3** confirms this attribution. Moreover, it is well-known that virgin olive oils contain tocopherols (α, β, γ), of which α-tocopherol is the most concentrated one. Their analysis is carried out by HPLC with a fluorimetric detection (λ_{ex} = 290 nm, λ_{em} = 330 nm) (*26*).

Virgin olive oils are distinguished from other edible oils by the presence of minor compounds belonging to phenols, acid phenols, secoiridoids (oleuropeine, ligstroside aglycons, and derived) (*27, 28*) and lignan families (*29*−*31*). Some of these compounds are powerful antioxidants, which confer to the oil its stability toward oxidation. The phenolic compounds can be detected by fluorescence after separation by HPLC (λ_{ex} = 264

Table 2. PLS Regression Results

sample	Aix	Baux	Haute-Provence	Nice	Nyons
RMSEP	0.24	0.29	0.32	0.22	0.27
factor number	13	9	12	7	13
preprocessing	normalization	normalization	normalization	normalization	normalization

Table 3. Predicted RDOs with PLS Regression (Samples < 0 Are Denoted 0; Samples > 1 Are Denoted 1)

	Aix		Baux		Haute-Provence		Nice		Nyons	
sample	PO[a]	GO[b]	PO	GO	PO	GO	PO	GO	PO	GO
3	0.205	0	0.683	1	0	0	0.164	0	0.151	0
4	0.789	1	0.211	0	0.219	0	0	0	0.039	0
5	0.745	1	0.325	0	0	0	0.164	0	0.026	0
6	0.626	1	0.275	0	0.083	0	0.094	0	0	0
8	0	0	1	1	0	0	0	0	0.175	0
9	0.858	1	0.314	0	0	0	0.066	0	0	0
12	0.662	1	0.247	0	0.317	0	0	0	0.022	0
29	0	0	0.261	0	0.908	1	0	0	0	0
48	0	0	0.203	0	0.785	1	0.010	0	0.164	0
49	0.237	0	0.133	0	0.778	1	0	0	0	0
51	0.340	0	0.117	0	0.652	1	0	0	0.072	0
63	0.324	0	0.943	1	0	0	0	0	0.438	0
64	0.316	0	0.605	1	0	0	0.334	0	0	0
65	0.245	0	0.631	1	0	0	0.198	0	0	0
66	0.044	0	0	0	0.209	0	0.057	0	0.842	1
67	0.094	0	0.017	0	0.155	0	0.138	0	0.722	1
68	0.075	0	0.373	0	0	0	0.314	0	0.700	1
69	0.066	0	0	0	0.225	0	0	0	0.651	1
70	0.061	0	0.121	0	0.142	0	0	0	0.831	1
74	0.129	0	0.142	0	0.156	0	0	0	0.753	1
79	0	0	0.301	0	0.041	0	0.937	1	0	0
80	0.167	0	0	0	0.090	0	0.672	1	0.225	0
82	0.020	0	0.202	0	0	0	0.759	1	0.075	0
83	0	0	0	0	0.216	0	0.653	1	0.240	0
91	0.324	0	0	0	0.088	0	0.925	1	0	0
106	0.693	1	0.379	0	0.033	0	0	0	0	0
92	0.332	0	0	0	0	0	0.873	1	0	0
16	**0.720**	**0**	**0**	**1**	**0.025**	**0**	**0**	**0**	**0.220**	**0**
28	**0.646**	**0**	**0.274**	**0**	**0**	**1**	**0**	**0**	**0.293**	**0**

[a] Predicted origin. [b] GO: 1, geographic origin; 0 no geographic origin.

nm, $\lambda_{em} = 354$ nm; and $\lambda_{ex} = 310$ nm, $\lambda_{em} = 430$ nm) (*32*) or ($\lambda_{ex} = 280$ nm, $\lambda_{em} = 320$ nm) (*33*).

Figure 3 shows SEEF spectra of three phenolic compounds present in the virgin olive oils. Each spectrum presents a fluorescence band in the spectral region (275−350 nm). We can see in **Figure 3** the mathematically calculated emission spectrum from the three phenolic compounds and α-tocopherol spectra. The comparison with the virgin olive oil spectrum shows a great similarity of profiles. This allows one to conclude that the fluorescence emission between 275 and 400 nm is not only due to the whole of tocopherols but also to other fluorescent compounds including the phenolic compounds present in the virgin olive oils. The 400−600 nm emission spectral range could be due to fatty oxidation products (*11*), vitamin B_2, or some of the chemically correlated molecules and carotenoids (*12*).

Registered Designation of Origin. First, PCA was used to study the spectral data structure. The best result was obtained for the 250−700 nm range with first derivatized (5 points, polynomial order = 2) and normalized spectra. The number of significant components was 5 (98% of explained variance); cross-validation with a random cancellation matrix with 20 cancellation groups was applied. The best classification was done in the space of the first and third principal components (**Figure 4**), and we could differentiate two groups (Haute-Provence and Baux) but with high overlaps. As this classification was unsatisfactory, we chose to discriminate the sample with PLS discriminant analysis. As a matter of fact, soft independent modeling of class analogy (SIMCA) and principal component regression−discriminant analysis (PCR-DA) were based on the principal component coefficient, so as PCA does not lead to one group for each origin, it was better to use another chemometric treatment. In PLS-DA analysis, we assume that a sample has to be a member of one of the classes included in the analysis. The membership or not of one class was resolved by a binary discriminant variable coded 1 or 0. This variable was the *Y* variable in the model. The optimal number of factors in the regression was determined by full cross-validation.

Table 4. Repeatability of the Origin Prediction Calculated on 10 Spectra of the Same Virgin Olive Oil

sample	Aix	Nice	Haute-Provence	Nyons	Baux
1	–0.006	–0.070	0.172	0.842	0.163
2	0.014	–0.049	0.171	0.722	0.162
3	–0.003	–0.057	0.170	0.876	0.157
4	0.024	–0.065	0.160	0.833	0.146
5	0.006	–0.073	0.166	0.810	0.148
6	0.052	–0.120	0.197	0.775	0.185
7	0.071	–0.126	0.199	0.763	0.195
8	0.072	–0.131	0.194	0.737	0.193
9	0.045	–0.121	0.207	0.706	0.187
10	0.052	–0.113	0.218	0.744	0.162
RSD[a]	0.029	0.032	0.020	0.057	0.018

[a] Relative standard deviation.

Table 5. Prediction of the Origin on Eight Foreign Virgin Olive Oils (Samples < 0 Are Denoted 0; Samples > 1 Are Denoted 1)

sample	Baux	Aix	Haute-Provence	Nice	Nyons
127	0.299	0.309	0.000	0.117	0.168
128	0.125	0.000	0.503	0.000	0.338
129	0.375	0.232	0.106	0.256	0.000
130	0.190	0.074	0.249	0.000	0.399
131	0.425	0.019	0.219	0.000	0.000
133	0.161	0.000	0.549	0.000	0.000
134	0.336	0.285	0.360	0.000	0.000
135	0.476	0.555	0.198	0.000	0.000

To predict the RDO of different oils, the PLS regression on the SEEF spectra was used; 104 samples were used in the calibration set, and 29 samples were used in the prediction set. In the calibration, we used a cross-validation to optimize the number of factors used in the regression (**Table 2**). In **Table 2**, the RMSEP was done on the real predicted values. The results obtained for the test set and the five variables are given in **Table 3**. Considering the difficulty in calibrating and predicting the origin with variable concentrations in each cultivar, it was necessary to discriminate the results between the initial value of 0 or 1. In **Table 3**, the values are adjusted at 0 for the negative ones and at 1 for the values superior to one. The predicted origins never give 0 or 1 results, but this result was justified by the different amounts of fluorescent compounds into the samples. As a matter of fact, there is a natural variation of the concentrations in fluorescent compounds, which can also be function of the olive treatment to obtain oil.

If all of the values between 0 and 0.400 are considered to indicate a non-RDO sample and the values between 0.600

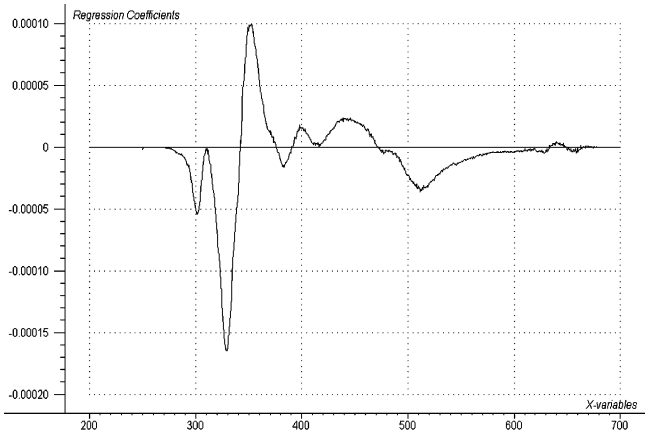

Figure 5. First regression vector of Aix RDO ($X = 90\%$, $Y = 7\%$).

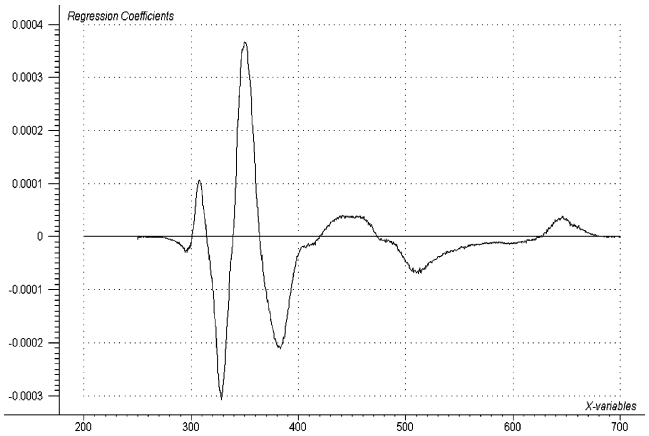

Figure 6. First regression vector of Baux RDO ($X = 82\%$, $Y = 15\%$).

and 1 a RDO sample, all of the origin samples were correctly predicted, except the two samples in bold in **Table 3**. These two samples were predicted as Aix RDO instead of Baux RDO (0.720) and Haute-Provence RDO (0.646), respectively. For these samples, the fatty acid and triacylglycerol compositions determined by gas and liquid chromatographies confirm the fluorescence results (*2*, *34*). Therefore, they are not in accordance with the other samples from the Baux and Haute-Provence RDOs. The geographical proximity (**Figure 1**) and the varietal composition (**Table 1**) may explain this bad prediction because the Aix, Baux, and Haute-Provence RDOs are constituted of the Aglandau variety as one of the primary cultivars, without a fixed precise amount of it. Therefore, it was not aberrant for some oils to obtain bad classification.

To evaluate the repeatability of the model we predict the origin for a new sample provided by the Nyons RDO. The spectra were recorded 10 times, for 10 replicates. As seen in **Table 4**, the repeatability was good with a relative standard deviation of <6%. In **Table 4**, the results are not discriminated to 0 for Aix and Nice in order to obtain results comparable to the ones of other geographic origins.

Finally, the designation of origin was tested on eight foreign oils in order to evaluate the risk of assigning these samples as belonging to one of the five RDOs. The results are presented in **Table 5**. If all of the values between 0 and 0.400 are considered to indicate a non-RDO sample and the values between 0.600 and 1 to indicate a RDO sample, four samples were suspicious, with values between 0.400 and 0.600, but any one was classified as RDO. For the suspicious samples, the fatty acid

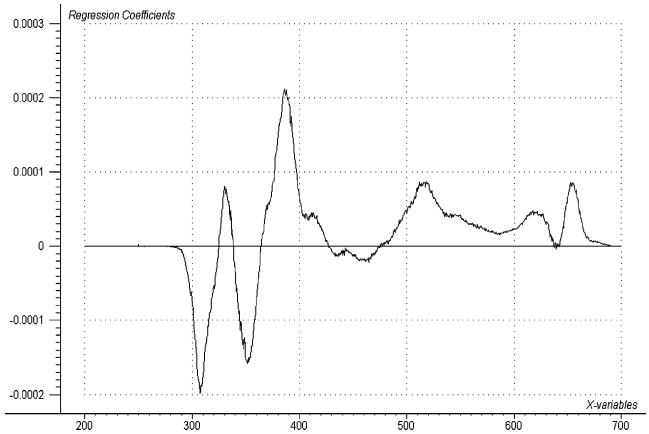

Figure 7. First regression vector of Haute-Provence RDO ($X = 85\%$, $Y = 10\%$).

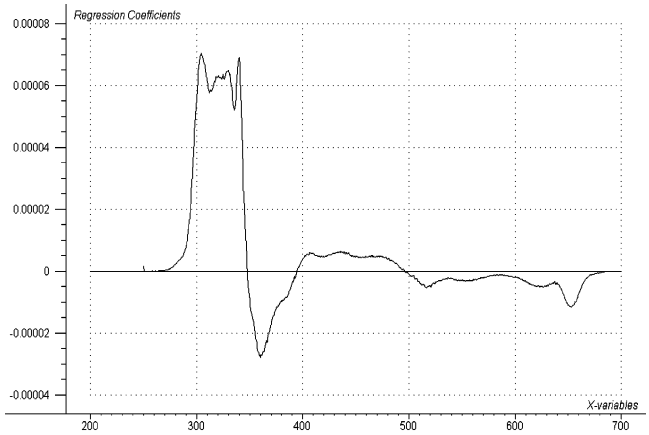

Figure 8. First regression vector of Nice RDO ($X = 71\%$, $Y = 10\%$).

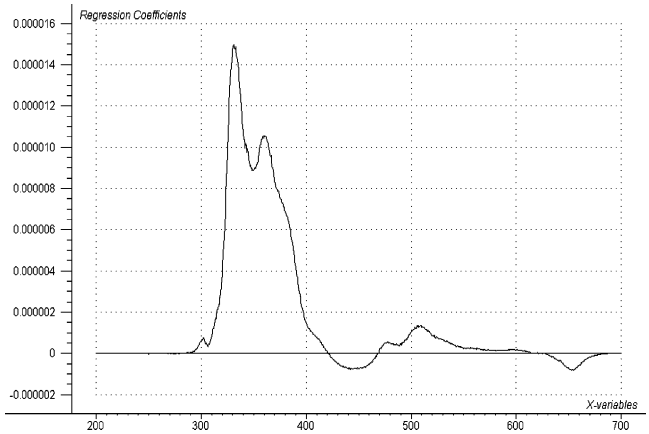

Figure 9. First regression vector of Nyons RDO ($X = 87\%$, $Y = 23\%$).

and triacylglycerol compositions determined by gas and liquid chromatographies allow the samples to be identified as foreign ones.

As it was possible to classify samples as a function of their geographical origin, it is interesting to understand how this classification was done on the basis of the fluorescence data. It is well-known that the first coefficient of regression was a good approximation of the pure compound spectrum (*21*) in the case of PLS regression between spectra and concentration data of this compound. Therefore, the first regression vectors obtained for all of the origins were an approximation of the original feature of each French RDO. **Figures 5**−**9** show the first vector regression obtained for each regression. The comparison of the

Table 6. Maxima and Minima Reported to the First Regression Vector of Each RDO

RDO	λ (nm) positive part	λ (nm) negative part	% of explained variance spectral data	% of explained variance origin
Aix	**353**, **397**, 447	302, 329, 648	90	7
Haute-Provence	330, **387**, 520, 653	307, 351	85	10
Baux	308, **350**, 650	328, **380**, 514	82	15
Nice	301, 320, **340**	358, 652	71	10
Nyons	**329**, 361, 511	442, 653	87	23

regression vectors shows that they were very different. **Table 6** presents the maxima and the minima of each regression vector. These maxima and minima are very different for each RDO. The Haute-Provence and VB origin present positive correlation, respectively, to the 653 and 650 nm bands and so to the chlorophyll and pheophytin contents that were usually very characteristic ofo these RDOs. The spectral region between 300 and 450 nm seems to be essential for the four other RDOs, and each RDO was correlated with a very precise zone (**Table 6**) different from the other RDOs. Therefore, it is probable that tocopherol and phenolic compounds were highly characteristics of the precise geographic origin. In each fluorescence spectrum of olive oil, a fingerprint of the origin could be found. This approach can be used for traceability and vindicates the RDOs. SEEFS is a rapid method for the determination of the origin of olive oil taking place in a global analytic methodology that associated spectroscopic and chromatographic techniques.

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