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Classification of Pumpkin Seed Oils According to Their Species and Genetic Variety by Attenuated Total Reflection Fourier-Transform Infrared Spectroscopy

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ABSTRACT: Attenuated total reflection Fourier-transform infrared spectroscopy (ATR-FTIR), followed by multivariate treatment of the spectral data, was used to classify seed oils of the genus *Cucurbita* (pumpkins) according to their species as *C. maxima*, *C. pepo*, and *C. moschata*. Also, *C. moschata* seed oils were classified according to their genetic variety as RG, Inivit C-88, and Inivit C-2000. Up to 23 wavelength regions were selected on the spectra, each region corresponding to a peak or shoulder. The normalized absorbance peak areas within these regions were used as predictors. Using linear discriminant analysis (LDA), an excellent resolution among all categories concerning both *Cucurbita* species and *C. moschata* varieties was achieved. The proposed method was straightforward and quick and can be easily implemented. Quality control of pumpkin seed oils is important because *Cucurbita* species and genetic variety are both related to the pharmaceutical properties of the oils.

KEYWORDS: Fourier-transform infrared spectroscopy, genetic variety prediction, linear discriminant analysis, pumpkin seed oil, prediction of botanical origin

INTRODUCTION

A wide variety of *Cucurbita* species are grown all around the world.[1] Among these, the most cultivated and studied species are *C. maxima*, *C. pepo*, and *C. moschata*. In addition, up to 18 varieties of *C. moschata* are currently registered by the official list of commercial varieties.[1] Most of these varieties are tolerant to hot and humid weather, and for this reason they are widely grown in tropical countries. The seven *C. moschata* varieties most cultivated in Cuba are RG, Inivit C-88, Inivit C-2000, Cuba-Cueto 85-79, Santa Monica, Mancha, and Fifi.[1]

Cucurbita seed oils are of interest from the point of view of gastronomical, nutritional, and pharmaceutical applications. Thus, the seed oils of *C. maxima*, *C. pepo*, and *C. moschata* are used as antihelmintics to treat tapeworm infestation and as mild anti-inflammatory agents to relieve disorders of the prostate gland and urinary bladder caused by hyperplasia.[2,3] For many years, particularly in Europe, pumpkin seed oils, especially from *C. pepo*, have been used as popular remedies to alleviate the effects of benign prostatic hyperplasia.[4] These medical effects have been attributed to pumpkin oil's high content of linoleic and oleic acids and to the presence of several phenolic compounds. The oil contains ca. 70% unsaturated fatty acids, as well as hydrocarbons, terpenoids, carotenoids, tocopherols, and phytosterols as minor components. Among the fatty acids, the most abundant are palmitic, stearic, oleic, and linoleic.[2,3]

In a global market with different regional legal regulations, the assurance of the quality of food products, including traceability according to botanical and geographical origins and to industrial processing, has become a field of increasing importance. Then, to protect consumers increasingly concerned about food origin, legislation is being progressively developed in many countries.[5]

Edible oil authentication may refer to either the botanical or geographical origin or to the industrial processing of the oil. Pumpkin seed oils of different species and varieties differing in their organoleptic, antioxidant, and pharmaceutical properties are found at high prices within the organic farming and gastronomical commercial circuits. Therefore, authentication methods capable of establishing the origin and other quality parameters of pumpkin seed oils are required.

To establish the authenticity of edible oils, a number of chromatographic,[6−8] atomic (ICP-AES),[9,10] and molecular spectroscopic methods, the latter including fluorescence,[11] FTIR,[12−20] FT-Raman,[12,13] NMR,[21] and mass spectrometry (MS)[22−24] methods, in most cases followed by multivariate statistical analysis of data, have been described. For this purpose, the contents of fatty acids,[18,25] tocopherols,[11] volatile compounds,[26] amino acids,[22] and sterols,[24,27] among other oil components, have been used. The triglyceride composition, established by HPLC-APCI-MS followed by linear discriminant analysis (LDA) of the spectral data, has been also used to distinguish oils from different species, including pumpkin seed oil.[6] Also, ICP-MS has been used to establish the geographical origin of pumpkin seed oils from the Styrian variety of *C. pepo*.[9]

FTIR is a rapid, nondestructive powerful analytical tool, very suitable for the study of edible oils and fats, requiring minimum sample preparation. Furthermore, attenuated total reflectance (ATR) is a very useful tool to obtain FTIR spectra of fats and oils. ATR-FTIR data have been used to distinguish butters from

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Table 1. FTIR Spectral Regions Selected as Predictor Candidates for LDA and Assignments to Vibrational Transitions According to References 31 and 32

identification no.	range, cm^{-1}	functional group	nominal frequency	vibration mode
1	3016−2952	=C—H (cis)	3006	stretching
2	2952−2900	−C—H (CH_2)	2924	stretching (asym)
3	2900−2866	−C—H (CH_2)		stretching
4	2866−2778	−C—H (CH_2)	2853	stretching (sym)
5	1796−1675	−C=O (ester)	1746	stretching
		−C=O (acid)	1711	stretching
6	1539−1429	−C—H (CH_2)	1465	bending (scissoring)
		−C—H (CH_3)	1450	bending (asym)
7	1418−1361	=C—H	1400	bending
		−C—H (CH_3)	1377	bending (sym)
8	1344−1300	not assigned	1319	bending
9	1300−1257	=C—H (cis)	1294	bending
10	1257−1218	−C—O—CH_2−	1238	stretching
				bending
11	1218−1147	−C—O—CH_2−	1163	stretching
			1163	bending
12	1147−1128	−C—O	1138	stretching
13	1128−1108	−C—O	1118	stretching
14	1108−1069	−C—O	1097	stretching
15	1069−1043	−C—O		stretching
16	1043−1007	−C—O	1033	stretching
17	1007−930	−HC=CH− (trans)	968	bending (out of plane)
18	930−887	−HC=CH− (cis)?	914	bending (out of plane)
19	887−804	=CH_2	850	wagging
20	801−751	−C—H		bending (out of plane)
21	751−710	−$(CH_2)_n$	723	rocking
		−HC=CH− (cis)		bending (out of plane)
22	710−665	C....C	685	bending (out of plane)
23	665−639	O—H	650	bending (out of plane)

vegetable margarines by principal component analysis[14] and to classify vegetable oils according to their botanical origin[19] and extra virgin olive oils according to their genetic variety,[20] using in both cases LDA models.

In this work, ATR-FTIR, followed by LDA of the spectral data, was used to classify pumpkin seed oils from three different species (*C. maxima*, *C. pepo*, and *C. moschata*). Moreover, oils from *C. moschata* species were also classified according to their genetic variety. For these purposes, FTIR spectra were divided into 23 wavelength regions, using normalized absorbance peak areas as predictor variables.

MATERIALS AND METHODS

Oil Samples. A total of 30 pumpkin seed oils from three different species (6 samples of *C. maxima*, 6 of *C. pepo*, and 18 of *C. moschata*) were collected; *C. maxima* and *C. pepo* oil samples were purchased in the European market, whereas *C. moschata* samples, of three different genetic varieties (6 of RG, 6 of Inivit C-88, and 6 of Inivit C-2000), were obtained by pressing pumpkin seeds collected at different geographical origins at Cuba. For this purpose, a hydraulic press was used. In all cases, the species and the genetic variety of the samples were certified by the suppliers. All samples were stored in amber glass bottles at −20 °C prior to analysis.

ATR-FTIR Spectra. ATR-FTIR spectra were obtained with a Jasco 4100 type A spectrophotometer (Jasco, Easton, MD) provided with a deuterated L-alanine-doped triglycine sulfate detector (DLATGS) with Peltier temperature regulation. The ATR accessory (ATR-PRO410-S, Jasco) was equipped with a single-reflection ZnSe crystal. All analyses were carried out at room temperature. Spectra were recorded in the absorbance mode from 3200 to 600 cm^{-1}, with 2 cm^{-1} resolution, by accumulating 15 scans. Data handling was performed with the Spectra Manager v. 2.07.00 software (Jasco).

For each sample, ca. 50 μL was deposited on the ZnSe crystal surface. The spectrum was collected against a background obtained with the empty ATR cell. Three spectra were recorded for each sample. At the beginning of the working session and between runs, the ATR crystal was repeatedly cleaned with a cellulose tissue soaked in *n*-hexane, rinsed with acetone, and dried.

Data Treatment and Statistical Analysis. The ATR-FTIR spectra were divided into the 23 wavelength regions described in Table 1. At the sight of the spectra of oils of the different species and varieties, wavelength regions, all of them corresponding to a peak or a shoulder, were selected. In this way, each region conveyed absorbance associated with structural or functional group information. Peaks or shoulders were not necessarily specific, because they could be due to contributions of more than a single transition, arising from a given compound or from several compounds, including lipids or minor components of the samples (see Table 1). The 23 selected areas were measured in all of the spectra. To minimize variability associated with sources of variance affecting the intensities of all peaks, such as radiation source intensity, normalized rather than absolute area was used. For this

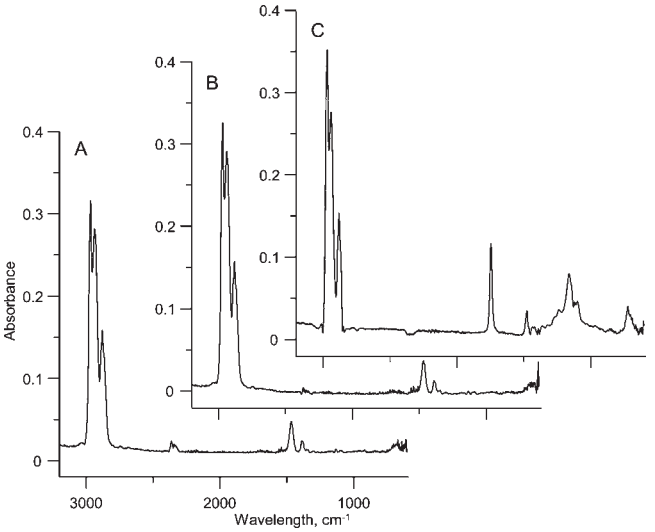

Figure 1. ATR-FTIR spectra of pumpkin seed oil samples of *C. maxima* (A), *C. pepo* (B), and *C. moschata* (Inivit C-88 variety) (C).

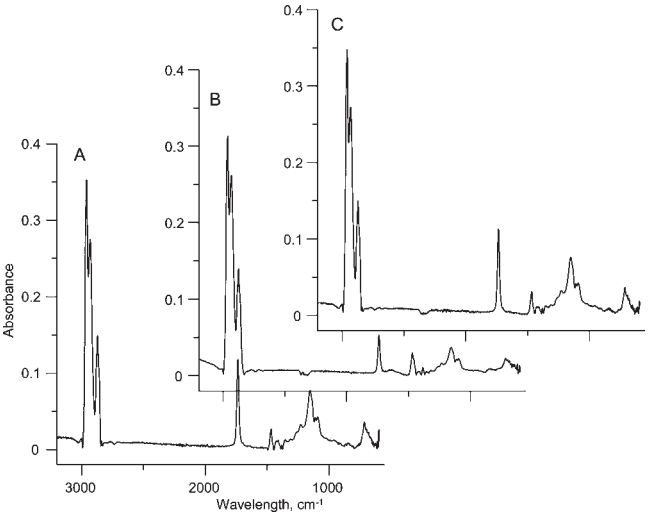

Figure 2. ATR-FTIR spectra of *C. moschata* pumpkin seed oil samples of the RG (A), Inivit C-88 (B), and Inivit C-2000 (C) genetic varieties.

purpose, the area of each spectral region was divided by each of the areas of the other 22 regions; in this way, and because each pair of areas should be considered only once, $(23 \times 22)/2 = 253$ normalized predictors were obtained. These predictors were used to construct LDA models. For this purpose, the SPSS software package (v. 12.0.1, SPSS Inc., Chicago, IL) was used. LDA, a supervised classificatory technique, is widely recognized as an excellent tool to obtain vectors showing the maximal resolution between a set of previously defined categories. The LDA discriminating vectors are frequently obtained by minimizing a parameter called Wilks' lambda (λ_W).[28] This parameter is calculated as the sum of squares of the distances between points belonging to the same category, divided by the total sum of squares. Values of λ_W approaching 0 are obtained with well-resolved categories, whereas overlapped categories made λ_W approach 1. Up to $N - 1$ discriminant vectors are constructed by an LDA, with N being the lowest value for either the number of predictors or the number of categories.

Selection of predictors to be included in the LDA models was performed by using the SPSS stepwise algorithm. According to this algorithm, a predictor is selected when the reduction of λ_W produced after its inclusion in the model exceeds the entrance threshold of a test of comparison of variances or F test (F_{in}). However, the entrance of a new predictor modifies the significance of those predictors that are already present in the model. For this reason, after the inclusion of a new predictor, a rejection threshold, F_{out}, is used to decide if one of the other predictors should be removed from the model. The process terminates when there are no predictors entering or being eliminated from the model. The significances of F_{in} and F_{out} were set to 0.01 and 0.10, respectively.

RESULTS AND DISCUSSION

ATR-FTIR Analysis. ATR-FTIR spectra of the 30 pumpkin seed oil samples studied in this work were collected. Spectra of three samples, corresponding to *C. maxima*, *C. pepo*, and *C. moschata* seed oils, are shown in Figure 1. As observed in this figure, the spectrum of *C. moschata* seed oil exhibited important differences with respect to the oils of the other two species, the spectra of *C. pepo* and *C. maxima* being rather similar to each other. As shown in the literature,[29,30] different pumpkin cultivars may show significant differences in the seed oil composition. These differences are mainly due to the profiles of linoleic, oleic, and stearic acids and also to variations in the profiles of tocopherols. However, independently from the chemical origin of the spectral differences, a common practice in multivariate classification techniques is to collect as many variables as possible, looking for variables carrying discriminant information. Then, these variables are used as "blind" candidates to be included in the model as predictor variables. It should be also noted that the spectral differences observed could be due to genetic differences, growing conditions differences, or the different processing systems used to obtain the oils, because *C. pepo* and *C. maxima* oil samples were commercially available, whereas *C. moschata* oils were produced by pressing at laboratory scale. The spectra of three *C. moschata* samples, corresponding to the three genetic varieties RG, Inivit C-88, and Inivit C-2000, are compared in Figure 2. These spectra were quite similar to each other; however, as shown below, the spectra contained the necessary information to allow prediction of both the species and variety of the oil samples by using multivariate statistical techniques.

Classification of Pumpkin Seed Oils According to Their Species. A first LDA model was constructed to classify the oil samples according to the species as *C. maxima*, *C. pepo*, and *C. moschata*. For this purpose, a matrix containing 30 objects, which corresponded to the means of the 3 spectra recorded for each sample, and the 253 predictors obtained after application of the normalization procedure indicated above, was constructed. The objects were then divided into training and evaluation sets. Hence, 22 objects were included in the training set (4 of *C. maxima*, 4 of *C. pepo*, and 14 of *C. moschata*), whereas the 8 remaining objects (2 of *C. maxima*, 2 of *C. pepo*, and 4 of *C. moschata*) were reserved as evaluation set. A response column, containing the three categories (*C. maxima*, *C. pepo*, and *C. moschata*), was also added to these two matrices.

The variables selected by the SPSS stepwise algorithm and the corresponding standardized coefficients of the model are given in Table 2. According to this table, the main wavelength regions selected by the algorithm to construct the LDA model corresponded to −C—H (CH_2, asym- and sym-stretching), −C═O (ester, stretching), −C—O (stretching), and ═C—H (cis, bending). A score plot on the plane of the two discriminant functions is shown in Figure 3. In agreement with the very low λ_W of 0.024, a large resolution between the three pairs of categories

Table 2. Predictors Selected and Corresponding Standardized Coefficients of the Discriminant Functions f_1 and f_2 of the LDA Model Constructed To Predict the Species of Pumpkin Seed Oils

predictor[a]	f_1	f_2
1/3	3.43	−14.94
1/14	−0.89	4.37
1/18	−0.37	−0.45
1/23	2.90	0.23
2/3	10.53	2.02
2/4	−14.74	15.92
2/11	1.33	−0.36
3/4	−0.03	−1.73
5/9	9.24	0.31
5/14	−5.18	0.01
7/9	−0.15	1.64
8/9	3.47	−3.79
10/14	21.99	−13.23
12/14	−4.03	12.57
14/15	3.36	2.65
16/19	−0.77	−0.24

[a] Area ratios of the wavelength regions identified according to Table 1.

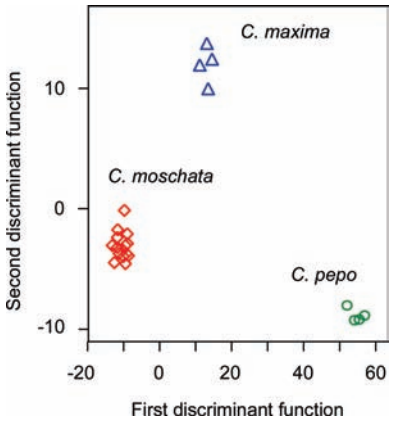

Figure 3. Score plot on the plane of the two LDA discriminant functions of the model constructed to classify pumpkin seed oils according to their species.

was achieved. Furthermore, despite the similarity of their spectra, *C. pepo* and *C. moschata* seed oils were very well resolved along the first discriminant function, whereas resolution between them and *C. maxima* oil was mainly achieved along the second discriminant function. Also, when leave-one-out validation was applied to the training set, all of the samples were correctly classified. With regard to the evaluation set, all of the objects were also correctly assigned with an assignment probability of >95%.

Classification of *C. moschata* Seed Oils According to Their Genetic Variety. Another LDA model was constructed to classify pumpkin seed oils from *C. moschata* species according to their genetic variety. In this case, the 18 available objects (6 samples × 3 genetic varieties) were divided in two sets: 12 objects were used as training set (4 samples × 3 genetic varieties), whereas the remaining 6 objects were reserved to constitute the evaluation set. A response variable containing the three categories (RG, Inivit C-2000, and Inivit C-88) was also added

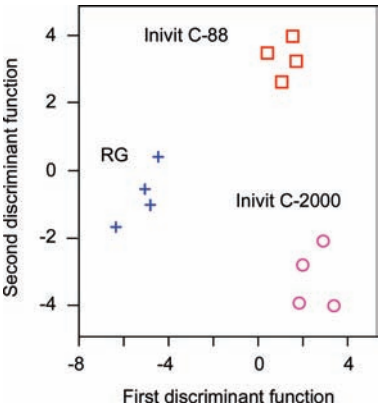

Figure 4. Score plot on the plane of the two LDA discriminant functions of the model constructed to classify *C. moschata* pumpkin seed oils according to their genetic variety.

Table 3. Predictors Selected and Corresponding Standardized Coefficients of the Discriminant Functions f_1 and f_2 of the LDA Model Constructed To Predict the Genetic Variety of *C. moschata* Pumpkin Seed Oils

predictor[a]	f_1	f_2
2/4	0.31	1.04
3/12	1.75	0.09
3/15	−0.84	0.53
3/23	−3.70	−3.18
4/16	2.99	2.37
7/14	3.21	0.51
16/18	0.83	1.66
16/19	0.85	−1.84
16/23	−3.56	1.87
17/18	−0.53	0.78
19/23	8.01	1.79

[a] Area ratios of the wavelength regions identified according to Table 1.

to the matrices. A score plot showing the projection of the oil samples on the plane of the two discriminant functions is shown in Figure 4 ($\lambda_W = 0.106$). A large resolution between the RG and the Inivit C-88/Inivit C-2000 pair was achieved along the first discriminant function, whereas the Inivit C-88/Inivit C-2000 pair was clearly resolved along the second discriminant function. The variables selected by the SPSS stepwise algorithm and the corresponding standardized coefficients of the model are given in Table 3. According to this table, the main wavelength regions selected by the algorithm to construct the LDA model corresponded to −C—H (CH_2, sym-stretching), ═C—H (bending), −C—H (CH_3, sym-bending), −C—O (stretching), ═CH_2 (wagging), and O−H (bending out of plane). When leave-one-out validation was applied to the training set, all of the points were correctly classified. With regard to the evaluation set, all of the objects were also correctly assigned with an assignment probability of >95%. Then, the quick classification of pumpkin seed oils according to their species, as well as the classification of *C. moschata* seed oils according to their genetic variety, using ATR-FTIR followed by LDA of the spectral data, was demonstrated. Furthermore, an excellent resolution among the seed oil species was achieved despite having included oils of three different varieties of *C. moschata* seeds in the training and

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evaluation sets; therefore, the capability of the proposed method to predict both species and variety by the sequential application of two LDA models has been demonstrated.

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