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Differentiation Between Origins of Extra Virgin Olive Oils by GC–C-IRMS Using Principal Component Analysis, Linear Discriminant Analysis, and Hierarchical Cluster Analysis

Validation of international food products has become increasingly important due to the proliferation of food products shipped globally. This study classified extra virgin olive oils from Portugal and Turkey using gas chromatography–combustion-isotope ratio mass spectrometry (GC–C-IRMS) to evaluate the isotope ratios of the fatty acid methyl esters (FAMEs) extracted from the oils. Linear discriminant analysis (LDA) and hierarchical clustering analysis (HCA) were applied to the carbon isotopic results to determine the geographic origin of the oils. Results showed that the isotope ratios of the individual FAMEs were statistically different, and that modeling the individual FAMEs enabled the differentiation of the oils by geographic region.

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Rapid and accurate chemical methods of food quality assurance are important for validation and the integrity of the products sold to consumers (1–3), Food quality standards vary internationally, thus, products shipped across international borders are particularly in need of a standardized method of quality assurance (4,5). One method to determine the origin of food products is isotope ratio mass spectrometry (IRMS). Due to isotopic fractionation, plants from different geographic regions exhibit different metabolic pathways and rates, thus, they will accumulate different iso-

tope ratios of common elements (6). Plants have three different metabolic pathways to fix carbon during photosynthesis. We may distinguish between C3 plants, C4 plants, and CAM plants (crassulacean acid metabolism). Plants that survive solely on C3 fixation (C3 plants) tend to thrive in areas where both sunlight intensity and temperatures are moderate, carbon dioxide concentrations are around 200 ppm or higher, and ground water is plentiful. The C3 plants, which originated during Mesozoic and Paleozoic eras, predate the C4 plants and still represent approximately 95% of the Earth's plant biomass,

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including olive trees. C3 plants lose 97% of the water taken up through their roots to transpiration (7). The isotopic signature of C3 plants shows higher degree of ^{13}C depletion than the C4 plants (8). CAM plants, such as cacti, can grow in dry environments and can resist little and irregular rainfall. The isotope ratio of an olive is not only influenced by metabolic pathways, but also by regional factors such as water-use efficiency.

Extra Virgin Olive Oils

The word virgin implies that the oil was produced by the use of physical, and not chemical, means. Extra-virgin olive oil (EVOO) comes from cold pressing of the olives, contains no more than 0.8% acidity (IOOC Standard), and is judged to have a superior taste. The expectation of extra-virgin and virgin olive oil is that they do not contain refined oil (9).

Chemical Composition of Olive Oils

Olive oils contain about 98–99% fatty acids, which are bonded in mono-, di-, and triesters of glycerol. Natural oils contain triglycerides as the major component (for example, 98%); trace amounts of diglycerides; and phytonutrients such as vitamins and antioxidants at 1–2% concentrations.

The most dominant fatty acids in olive oil are palmitic (16:0), palmitoleic acid (16:1), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3). These structures are given in Figure 1.

Because the polarity of the carboxyl group causes tailing under gas chromatographic (GC) separations with polar stationary phases, the acids usually are transformed chemically to fatty acid methyl esters (FAMEs). Therefore, the polarity and tailing is reduced and the separation can be achieved in an appropriate time window.

GC–C-IRMS

The differences in carbon isotopic abundances result from the different growing locales and seasons. The analyzed samples representing the different olive oils cannot be distinguished to the point of geographical origin by GC analysis itself. According to the results, the FAME profiles of the two oils
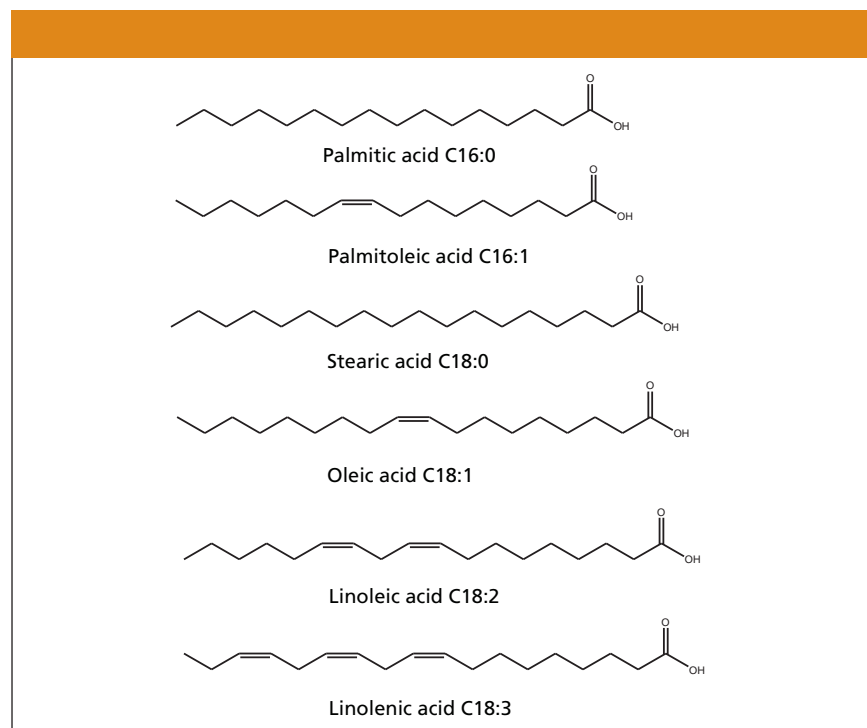

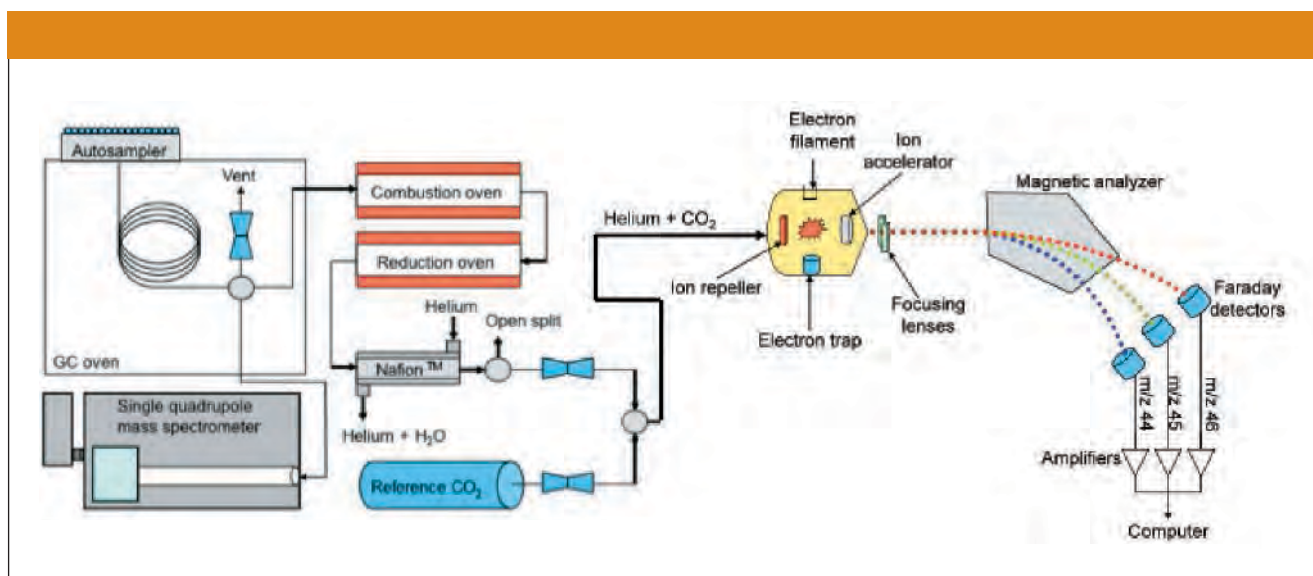**Figure 1:** Structures of the fatty acids detected in olive oil.

Figure 2: Schematic of the GC–C-IRMS system used in this work. Note the use of a single-quadrupole MS system to identify compounds based on their fragmentation patterns before combustion and isotope ratio analysis.

were not distinguishable by their chromatograms. Therefore, IRMS was used to distinguish between the olive oils by measuring isotope ratio differences that arise from different regional influences for each FAME (for example, different atmospheric carbon dioxide concentrations and water-use efficiency). The FAMEs from the oils were evaluated with respect to their isotope ratios, and then statistically compared using two-way analysis of variance (ANOVA).

The isotope ratio δ is usually measured relative to a standard reference material. For this study, values were normalized to the carbon from carbonate in the shells obtained from Vienna Pee Dee Belemnite (VPDB). The δ is calculated as

$$\delta = 1000(R_{Sample} - R_{Standard})/R_{Standard}$$

[1]

for which R_{Sample} is the abundance ratio of the heavier isotope to the lighter isotope (for example, $^{13}C/^{12}C$). The $R_{Standard}$ values usually are selected because they represent a stable material that is highly enriched in the heavier isotope. Most substances are depleted with respect to the heavy isotope in comparison to the standard, and therefore δ values less than zero are expected (10).

Experimental

Materials

Six bottles of commercial extra virgin olive oils (New Market, Athens, Ohio) were purchased: three bottles of "Azeite de Oliveira Virgem Extra" (origin: Portugal) and three bottles of "Sultan 100% Extra Virgin" (origin: Turkey). The color of the Portuguese oils varied noticeably. The first bottle (A1) was yellow-clear while the other bottles (A2 and A3) could be described as yellow-green. Oil from each bottle was analyzed as a separate sample. A Triglyceride C16-C22 Standard Mix (LA83308, Supelco, Bellefonte, Pennsylvania) was used to help identify the fatty acids.

Sample preparation

For the preparation of the FAMEs, the AOAC method (11) was modified as follows: 0.5 g of the oil sample was weighed into a 30-mL vial; 5 mL of 0.5 M methanolic sodium hydroxide solution was added for saponification and heated to 60–70 °C for 10 min. Then 4 mL of 14% BF_3 in methanol was carefully added to the vials (attention: strong bubbling occurs!) and heated at the same temperature for another 10 min.

The solutions were stirred periodically with a glass rod. After cooling to room temperature, the oily drops in the solution disappeared. The FAMEs were extracted three days later. The samples were stored in glass screw cap vials and secured with Parafilm.

For the extraction 10 mL of saturated sodium chloride solution and 5 mL of *n*-hexane (reagent grade) were added to the sample vials. The organic phase was separated from the aqueous and transferred to another 5-mL vial. A spatula of anhydrous sodium sulfate was added to the vial to remove any residual water. The organic phase was then decanted into a new 5-mL vial, sealed with a screw-cap, wrapped with Parafilm, and stored in a freezer.
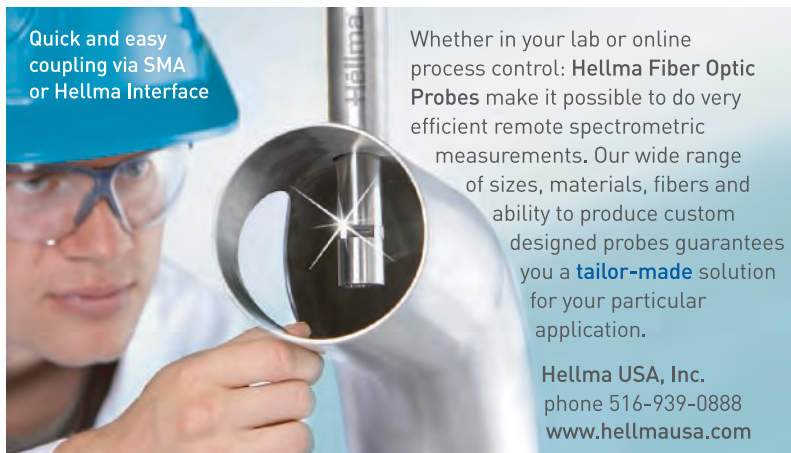
GC–C-IRMS

GC–MS analyses were performed using a GC system (Trace GC, Thermo Finnigan, Waltham, Massachusetts) with an IRMS detector (Delta plus Advantage, Thermo Finnigan, Waltham, Massachusetts). The GC effluent was split using a low-dead-volume X-connector so that approximately 10% of the effluent flowed to the single quadrupole mass spectrometer for structural elucidation and 90% to the IRMS system for isotopic analysis. The GC effluent was directed into a combustion oven to convert the organic materials to carbon dioxide before ionization. In GC–IRMS with combustion (GC–C-IRMS), ion chromatograms of three isotopic peaks are collected by three individual ion collectors with different sensitivities — that is, m/z 44 ($^{12}C^{16}O_2$), the isobaric ions m/z 45 ($^{13}C^{16}O_2$ and $^{12}C^{16}O^{17}O$), and m/z 46 ($^{12}C^{16}O^{18}O$). Samples were injected using an autosampler (AS3000, Thermo Finnigan, Waltham, Massachusetts). The data acquisition was accomplished with the standard software of the instrument Isodat 2.0 (Thermo Finnigan, Waltham, Massachusetts). Table I shows the parameters used in the GC analyses.

Different temperature programs were used to achieve the best separation results. While the chromatographic peaks of methyl palmitoleate and methyl palmitate could be measured with good resolution ($R_s = 1.9$) by applying temperature program 1 and splitless injection, methyl linoleate, methyl oleate, and methyl stearate could not be resolved at these conditions. To achieve separation for those substances, temperature program 2 and a split ratio of 1:10 was used.

Due to a high concentration of methyl oleate, the combustion chamber was flushed with He for 830 s after the injection while using temperature program 1 to reduce the concentration of CO_2 entering the reduction oven. The flush was turned off after 1820 s for the remaining duration of the run. When applying temperature program 2, the combustion chamber did not need to be flushed to limit the CO_2 from entering into the reduction oven because the injection was split with a 1:10 ratio.

Results

Quantitative determination of major components

The contents of the olive oils were quantified relatively using the peak areas. These results are reported in Table II.

Two-way analysis of variance

A total of four FAME Peaks were observed. Unfortunately, only three peaks gave reproducible isotope ratios. The three peaks correspond to the FAMEs methyl palmitoleate, methyl palmitate, and methyl oleate.

The first evaluation compared the isotope ratios of the three FAME peaks. If the peaks do not vary significantly they would not be useful for multivariate methods like PCA or LDA.

The other factor that was studied was whether the oils from two different origins had different or similar isotope ratios. Two-way ANOVA was applied to investigate the differences among the three FAME peaks and two geographical sources. The results were calculated by MS Excel and are presented in Table III.

The factors of peaks and origins were significantly different at a 95% level of

Table I: Parameters

GC column	DB-5 (30 m x 0.25 mm x 0.25 µm, J & W Scientific, Folsom California)
Carrier gas	Helium
Carrier gas flow rate	1 mL/min
Ion source	Electron energy 70 eV
Injection temperature	230 °C
Detector temperature	230 °C
Combustion oven temperature	940 °C
Reduction oven temperature	640 °C
Temperature program 1	Start: 100 °C, 10 °C/min to 240 °C, 5 °C/min to 300 °C, 5 min at 300 °C
Temperature program 2	Start: 70 °C, 2 min at 70 °C, 3 °C/min to 200 °C, 10 °C/min to 300 °C
Injection volume	1 µL
Split	Splitless for temperature program 1 1:10 for temperature program 2

Table II: Average relative quantities of olive oil components with 95% confidence intervals

		A – Portuguese [%]	B – Turkish [%]
Methyl palmitoleate	C16:1	0.5 ± 0.3	0.7 ± 0.2
Methyl palmitate	C16:0	15 ± 2	17 ± 3
Methyl linoleate	C18:2	2.1 ± 0.4	3.2 ± 0.4
Methyl oleate	C18:1	79 ± 5	75 ± 5
Methyl stearate	C18:0	2.0 ± 0.4	3.5 ± 0.3

Table III: Two-way ANOVA for peaks, geographic origin (oils), and interaction

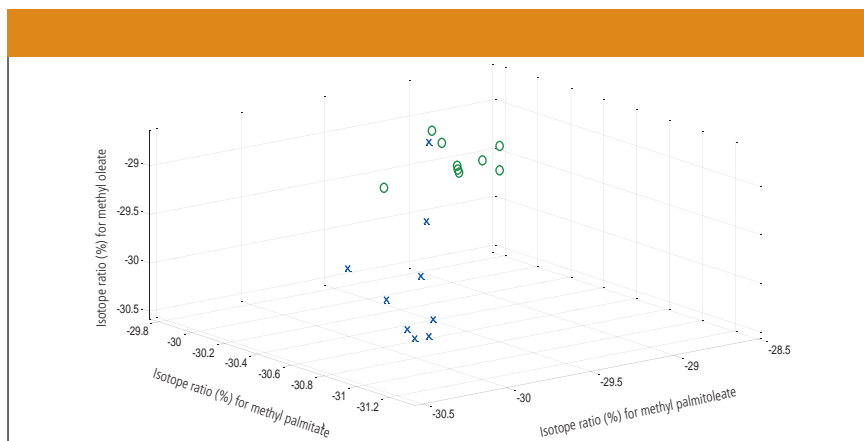| ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Peaks	12.6	2	6.29	18.4	2×10^{-7}	3.10
Oils	12.1	1	12.1	35.4	5×10^{-8}	3.95
Interaction	0.541	2	0.270	0.791	0.457	3.10
Within	30.8	90	0.342			
Total	56.0	95				

Figure 3: Samples plotted with respect to the isotope ratios of the three FAME peaks $^{13}C/^{12}C$ δ values (x = Portuguese, o = Turkish).

significance. Interaction between these two factors was insignificant.

Principal component analysis

Figure 3 gives the distribution of olive oil samples with respect to the isotope ratios of the three FAMES. One can see from this figure that by using only a single isotope ratio that the two geographic regions cannot be resolved. Figure 4 is a plot of the principal component scores. The scores can be understood as a linear combination of the pretreated isotope ratios, while the loadings express the relative weighting of the isotope ratio. Before PCA, the data were normalized to unit vector and mean-centered. The scores of the Portuguese oils formed a larger cluster than the Turkish oils. The Portuguese oils varied with respect to color as well, and their isotope ratios were less precise, as presented in Table IV.

Additionally, the Turkish oil isotope ratios clustered by bottle, whereas the Portuguese oils did not. ANOVA was used with one factor as the geographic region and the other factor was the three different GC peaks. In Table III, the results indicate that each GC peak and corresponding FAME gave a different isotope ratio. Interaction was not significant, so the isotope ratios for each peak and oil combination did not vary significantly.

Hierarchical cluster analysis

The measured isotope rations were now used to calculate the Euclidean distance matrix. A MATLAB script was written to draw a dendrogram using an average linkage algorithm. The dendrogram is given in Figure 5.

Good clustering is achieved from the isotope ratios among the two olive oils. The isotope ratios were not distinct for each bottle.

Linear discriminant analysis

Linear discriminant analysis calculates a linear function of the variables D, which maximizes the ratio of the between-group variance to the within-group variance. Geometrically, the discriminant is a line through the clusters of points, such that the projections of the points of the two groups are separated as much as possible (Figure 6) (13).

LDA (14) was used to establish collected classifier for the olive oils. The

Table IV: Average δ values of methyl ester peaks and 95% confidence intervals.			
	Methyl Palmitoleate	**Methyl Palmitate**	**Methyl Oleate**
Portuguese	-29.9 ± 0.6‰	-30.7 ± 0.4‰	-29.9 ± 1.0‰
Turkish	-28.9 ± 0.5‰	-29.9 ± 0.4‰	-29.4 ± 0.3‰
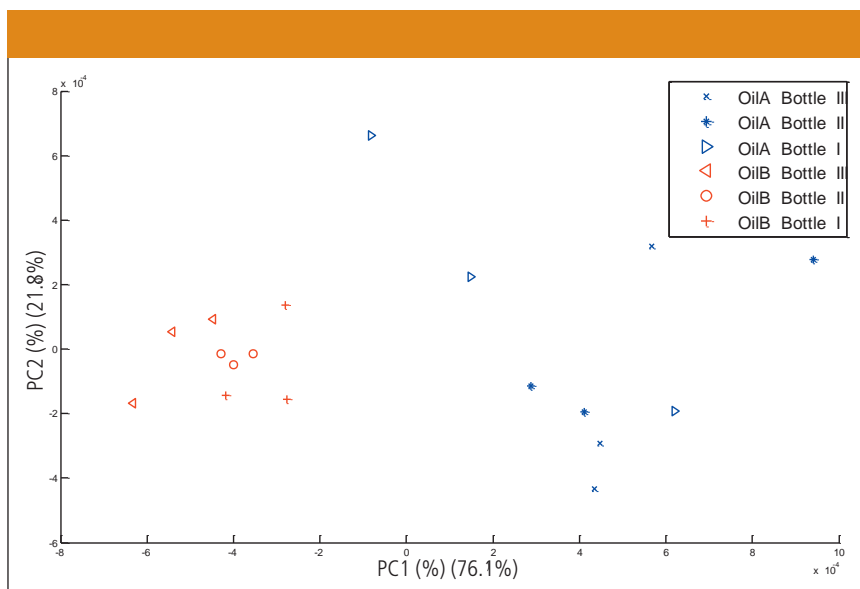
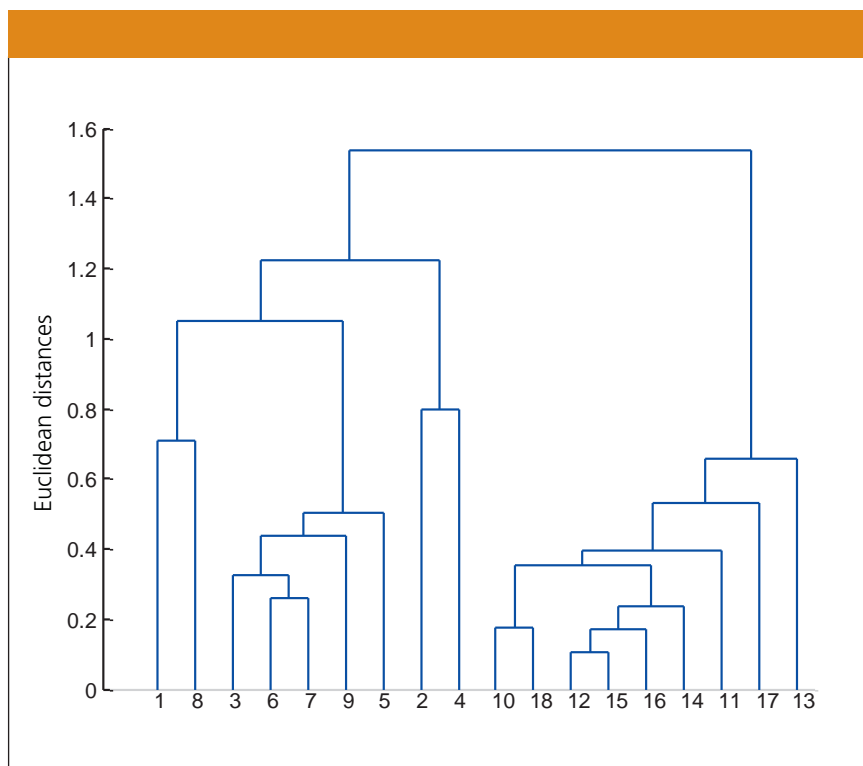**Figure 4:** Principal component scores of the normalized, mean-centered isotope ratios of the three FAME peaks.
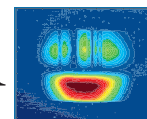
Figure 5: Hierarchical clustering using a dendrogram (Portuguese, 1–9; Turkish, 10–18). Bottles are consecutive groups of three.

Figure 6: Situation where principal component (PC) and linear discriminant function (DF) are (a) essentially the same and (b) orthogonal (13).

following model (15) was applied to discriminate between the two geographic origins of the oils:

$$D = w_0 + w_1x_1 + w_2x_2 + \ldots + w_nx_n$$

[2]

or using matrices

$$d_i = w_0 + w^Tx_i$$

[3]

$$w_0 = -0.5(x_1 - x_2)^TS^{-1}(x_1 - x_2)$$

[4]

$$w^T = (x_1 - x_2)^TS^{-1}$$

[5]

$$S = A^TA + B^TB/n_1 + n_2 - k$$

[6]

X: Training set matrix (isotope ratios of FAMEs)

w: Weights of the variables

w_0: Bias value (should equal zero for standardized data)

x: Sample mean vectors, that describe the location of the centroids in

m: Dimensional space of each class

S: Pooled variance-covariance matrix

n_1: Number of objects in class A

n_2: Number of objects in class B

A: Data matrix of A isotope ratios

B: Data matrix of B isotope ratios

k: Number of classes

This leads to the following linear discriminant function (7), where x_1, x_2 and x_3 represent the standardized $^{13}C/^{12}C$ isotope ratios from each FAME peak ($w_0 = 0$). If d_i is greater than zero, the sample belongs to the class A, while a smaller linear discriminant than zero indicates membership of class B (Figure 7).

$$d_i = 2992x_1 + 1713x_2 + 699.3x_3$$

[7]

Conclusion

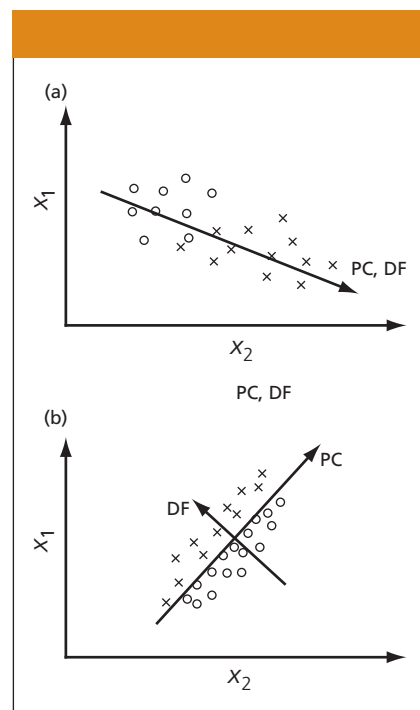The results show that classification between extra virgin olive oils of different origins is possible and applicable by isotope ratio mass spectrometry of the FAMEs. It was shown that by modeling three different FAME peaks, enhanced resolution of the geographic origin was obtained relative to using the total isotope ratio of the oil.

The major component, oleic acid, was so large relative to the other fatty acids, that it sometimes caused problems with the reduction oven. Selective extraction methods of the FAME headspace might prevent this FAME from saturating the reduction oven.

Acknowledgment

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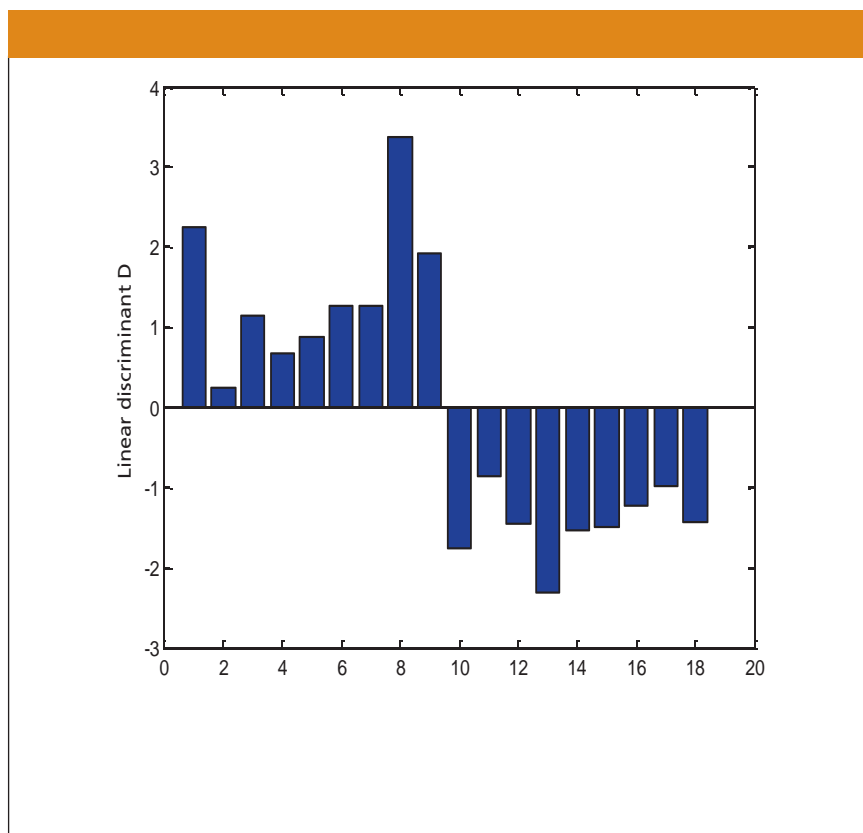(2) T. Galeano Diaz, *Food Control* **16,** 339–347 (2005).

Figure 7: Pattern recognition using Fisher LDA (Portuguese, 1–9; Turkish, 10–18).

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