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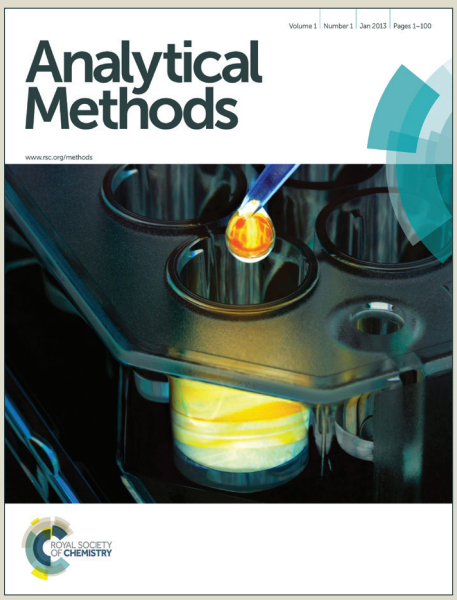

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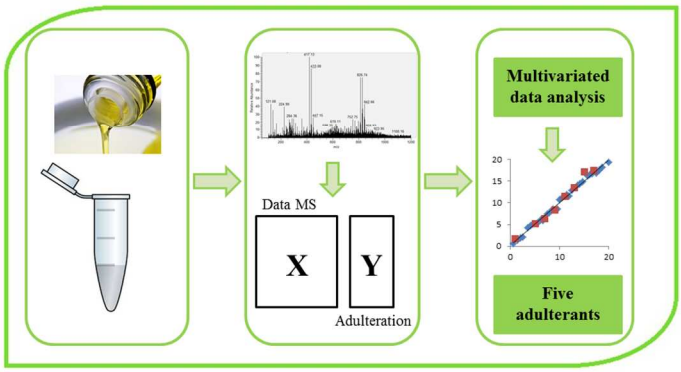
Fast method of multivariate calibration applied on ESI-MS data for quantification of adulteration of EVOO with cheaper edible oils.



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ARTICLE

Multivariate calibration applied on ESI mass spectrometry data: a tool to quantify adulteration in extra virgin olive oil with inexpensive edible oils

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A constant supervision is required to ensure the quality control in extra virgin olive oil, a quite expensive product with worldwide consumption. In this paper, a rapid, simple and efficient method based on the application of partial least squares (PLS) approach on electrospray ionization mass spectrometry (ESI-MS) data was developed for determining adulteration of extra virgin olive oil with four adulterant oils (soybean, corn, sunflower and canola). Each model was built with 40 adulterated samples (from 0.5 to 20.0 % w/w), which were prepared using commercial oils. These models presented root mean square errors of prediction of 1.73% w/w for soybean, 1.01% w/w for corn, 1.37% w/w for sunflower and 1.03% w/w for canola. The methods were submitted to a complete multivariate analytical validation in accordance with the Brazilian and international guidelines, and were considered accurate, linear, sensitive and unbiased. So, it can be envisaged that this methodology has potential to be applied in quality control of extra virgin olive oil samples.

1. Introduction

Extra virgin olive oil (EVOO) is obtained by cold-pressing the fruit of olive tree followed by cold centrifugation. It has been traditionally consumed in European countries, and more recently it has become increasingly popular due to its potential health benefits.[1,2] Extra virgin olive oil (EVOO) is particularly expensive and because of that unscrupulous producers stretch their profits by adulterating it with cheaper edible oils, including soybean (SO), corn (CO), sunflower (SF), and canola (CA).[1] The adulteration of EVOO with canola oil (CA) is considered sophisticated, because both raw materials are chemically very similar and have oleic acid as the major component.[3]

Quality assurance is a key issue in the modern food production. Food products are expected to have the right properties and should be safe, wholesome, authentic, and with a composition accordingly to specific regulations.[4] Much effort has been spent in the development of reliable analytical methods for the authentication of food products in general, and for olive oil in particular. These methods have not been limited to the identification of adulterants, but have been expanded to quantify adulteration. Most of them are based on gas chromatography (GC)[2,5,6] and high performance liquid chromatography (HPLC)[7,8] and have been successfully applied to identify and quantify adulterants in olive oil. Nevertheless, these methods have the drawbacks of being time-consuming and relatively expensive. Moreover, they are divergent with the principles of green chemistry as they demand large quantities of solvents and generate a lot of residues. Thus, there is a continuing requirement for rapid and accurate methods aiming at the complete characterization of adulterations in EVOO, which have lead some recent studies that combine vibrational techniques, such as mid (MIR)[9,10] and near infrared (NIR)[11] spectroscopy, with multivariate calibration.

Electrospray ionization mass spectrometry (ESI-MS)[12] is a soft ionization technique that is fast, versatile, reproducible, and sensitive. It is widely used for the analysis of polar and less polar compounds, requires little or no sample preparation and provides almost instantaneous information about the composition of a certain sample. In the last years, many studies have reported the employment of direct infusion ESI-MS and the treatment of these data with chemometric classification methods: unsupervised (*e.g.*,

principal component analysis – PCA, hierarchical cluster analysis – HCA) and supervised (*e.g.*, linear discriminant analysis – LDA, partial least squares discriminant analysis - PLS-DA). Several types of samples as diverse as vegetable oils,[13] alcoholic beverages,[14] coffee[15] and olive oil,[16] have been evaluated by this combined approach. Although its use has allowed the attainment of important set of information, it is restricted to qualitative analysis. In order to exploit the full potential of ESI-MS combined with chemometric methods, it seems to be natural to apply multivariate calibration in the development of quantitative methodologies. On the contrary of other techniques, such as NIR, MIR and UV/Vis spectroscopy, only few reports (a total of six) describes the application of multivariate calibration on ESI-MS data. Hence, the quantification of biodiesel in blends with petrodiesel,[17,18] surfactants in oil,[19] cholesterol and triglycerides in mouse plasma,[20] and the determination of protein charge–state distribution of myoglobin[21] as well as the blend composition of commercial robusta and arabica coffee[22] have been reported. Other authors have used direct matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and PLS for determining the concentration of milk of different origins in mixtures.[4]

In a previous report, we developed a qualitative PLS-DA model for detecting EVOO samples adulterated with cheaper oils.[23] In the present work, a similar PLS-DA method, based on direct infusion ESI-MS data, was coupled with specific PLS models and the methodology was extended for direct quantification of four adulterant oils (SO, CO, SF and CA) in EVOO samples. Other important issue addressed herein is the multivariate analytical validation, a fundamental requisite for the official recognition of new multivariate calibration methods. The extension of analytical validation for multivariate methods is not so trivial and this matter is absent in all of the previously cited papers.[17-22] The developed methods were validated by estimating the following figures of merit (FOM): analytical sensitivity, selectivity, linearity, trueness, precision, bias, residual prediction deviation (RPD), limits of detection (LOD) and quantification (LOQ).

2. Materials and methods

2.1 Sample oils

All oils were acquired at local stores. Six brands of EVOO of well-known trademarks were used to prepare a stock blend. One brand of each edible oil (SO, CO, SF, and CA) was used for adulterations. The adulterated samples (EVOO with the edible oils) were prepared by adding increasing proportions (from 0.5 to 20.0 % w/w, with increments of 0.5% w/w) of each adulterant to the EVOO sample. The masses of each sample (EVOO, SO, CO, SF, and CA) were measured on an analytical balance (Adventurer Pro, Ohaus Corporation, Pine Brook, NJ, USA) and forty adulterated samples of each class (EVOO with SO, CO, SF, and CA) were therefore prepared and analyzed. For the 40 admixtures of each class, 30 were selected for the calibration set whereas the other 10 were used for the validation set. For each adulterant, authentic replicates (six per sample) were also prepared at five levels (4.0, 6.0, 10.0, 14.0 and 16.0% w/w) in order to evaluate precision. Ten samples of commercial EVOO samples were also analyzed for confirming the ability of the method to detect unadulterated samples. Five EVOO samples adulterated with an ordinary olive oil (at 1.0, 5.0, 10.0, 15.0 and 20% w/w) were analyzed for confirming the ability of the PLS-DA model to detect other adulterants.

2.2 Sample preparation

Aliquots of 100 µL of each sample were transferred to 1.5 mL Eppendorf® tubes followed by the addition of 1.0 mL of a solution of methanol (HPLC grade, Merck, São Paulo, SP, Brazil)/deionized water (Mili-Q, Millipore Corporation, Bedford, MA, USA) 1:1 (v/v) with 0.1% of formic acid. The extraction was performed under vigorous stirring during 30 s in a vortex apparatus (Phoenix AP-56, Phoenix, Araraquara, SP, Brazil). All the extractions were performed in triplicate (or sextuplicate-see above) and individually analyzed. The aqueous-methanolic phase was then isolated and subsequently injected into the mass spectrometer ion source.

2.3 Instrumentation, software and data processing

The samples (aqueous-methanolic phase of each admixture) were injected with a microsyringe at a flow rate of 10 µL min^{-1} directly into the ESI ion source of the mass spectrometer (LCQ-Fleet, Thermo-Scientific, San Jose, CA, USA) operating in the positive ion mode. The conditions of the instrument were as following: inner capillary temperature at 300 ºC, inner capillary voltage of 35 V and cone voltage of 3 kV. The mass spectra were collected in the 100-1000 *m/z* range. Data processing was performed with the Matlab v. 7.9 (The Mathworks Inc., Natick, MA, USA) and PLS Toolbox v. 6.5 (Eigenvector Research Inc., Wenatchee, WA, USA) softwares. A homemade routine[24] was also employed for the detection of outliers.

The relative intensities of ions corresponding to each *m/z* value were used for building the data matrix. The data were collected from mean mass spectra recorded for each replicate. Initially, a PLS-DA

model (similar to the one described in our previous paper)[23] was built and applied to differentiate the adulterated from the unadulterated samples. In sequence, four specific quantitative PLS models (one for each type of adulteration) were built and validated.

2.4 Multivariate analytical validation

Multivariate analytical validation is a relatively recent issue that is not completely well-established. Since the application of multivariate calibration methods has grown very rapidly, the harmonization between the validation aspects of univariate and multivariate methods has become necessary. The first guidelines to address this topic were launched in 2000, describing standard practices for infrared multivariate quantitative analysis.[25] Since then, the analytical validation of multivariate methods has been discussed for applications in different areas, such as pharmaceuticals,[26] foods,[27] agricultural products,[28] and in other fields.[29] In almost all of the cases, this discussion is related to the use of vibrational techniques, mainly NIR spectroscopy. However, to the best of our knowledge, no investigations using mass spectrometry coupled to multivariate validation have been so far described. Concerning specifically food analysis, Brazilian and international official validation guidelines have completely ignored multivariate approaches.[30-33]

In this paper, robust validation procedures were adopted, including outlier detection, by the evaluation of abnormal values of leverage, spectral and prediction residuals;[24,28] linearity assessment, using statistical tests to verify the randomness of the fit residuals; accuracy evaluation (performed similarly to univariate calibration); estimate of selectivity, sensitivity, LOD and LOQ based on the concept of net analyte signal (NAS).[26,34] Other FOM, such as bias and residual prediction deviation (RPD) were also estimated. Particularly, the linearity was verified by applying the tests of Ryan–Joiner (RJ), Brown–Forsythe (BF), and Durbin–Watson (DW) to the model residuals, in order to evaluate their normality, homocedasticity and independency, respectively. A deeper discussion about multivariate analytical validation can be found elsewhere.[26-29]

3. Results and discussion

3.1 Mass spectra

The ESI(+) mass spectra of the EVOO stock blend and pure SO, CO, SF and CA oils are shown in Figure 1. These mass spectra were obtained from the same brands and lots of oils than those used in our previous work,[23] for which a PLS-DA model was developed. Consequently, these mass spectra displayed herein are practically identical than the ones shown in our previous work.[23] The most abundant ions for each type of oil are indicated in Figure 1, and some of them can be ascribed to specific components based on the literature data. For EVOO (Figure 1a), the ions of *m/z* 121, 137, 165 and 225 were attributed to tyrosol, hydroxytyrosol, coumaric and sinapic acids, respectively.[13,16] The ion of *m/z* 417, the most abundant in the MS of EVOO sample, was attributed to the protonated form of (1)-1-acetoxypinoresinol.[35] The ions in the region of *m/z* 800-900, such as 827 and 843, were generically attributed to triacylglycerols (TAGs).[36] Some abundant ions can also be attributed to specific TAGs in other oils, such as 1,2-dilinoleoyl-3-oleylglycerol (*m/z* 881) and 1,2-dilinoleyl-3-palmitoylglycerol (*m/z* 855), in the MS of SF oil (Figure 1d), and 2,3-dioleyl-1-linoleyglycerol (*m/z* 883), in the MS of CA oil (Figure 1e).[37] ESI-MS were also obtained in the negative mode. PLS-DA and PLS methods were also tested on the ESI(-)-MS data, but yielded models with much worse predictive ability. For this reason these results were not shown and discussed herein. In fact, for almost all the papers that deal with the development of PLS models[17-22] based on direct infusion ESI-MS data, the positive mode is by far predominant.

3.1 Chemometric modeling

The analytical strategy proposed herein was inspired in a paper that determined different adulterants in gasoline using MIR spectroscopy, the SIMCA (Soft Independent Modeling of Class Analogy) classification methodology and specific quantitative PLS models.[38] The present strategy is depicted in Figure 2, and the initial step is the building of a supervised PLS-DA (instead of SIMCA) classification models. Because these models are similar to the ones described (and deeply discussed) in our previous paper,[23] only a brief mention on them is made herein. A PLS2-DA model was built with the mass spectra of 175 samples, 40 from each aforementioned adulterated admixture, 10 from unadulterated EVOO samples and 5 from the EVOO admixtures (see above) adulterated with ordinary olive oil. All the samples were correctly classified in their respective classes. The PLS2-DA algorithm was employed because all samples are simultaneously predicted providing similar results than PLS1-DA. Unlike PLS1-DA, which builds separately a specific model for each class (a **y** vector is predicted for each class), PLS2-DA allows for the simultaneous prediction of all classes in a single model (a **Y** matrix is predicted with each column related to each class). Once a sample was detected as adulterated, it is subsequently forwarded to

the second step of this strategy, which consists of four PLS1 models aiming at determining the content of each adulterant. Samples classified as unadulterated do not need further analysis. The five samples of EVOO adulterated with ordinary olive oil were not attributed to any class, demonstrating the ability of the method to detect other unmodeled adulterant oils.

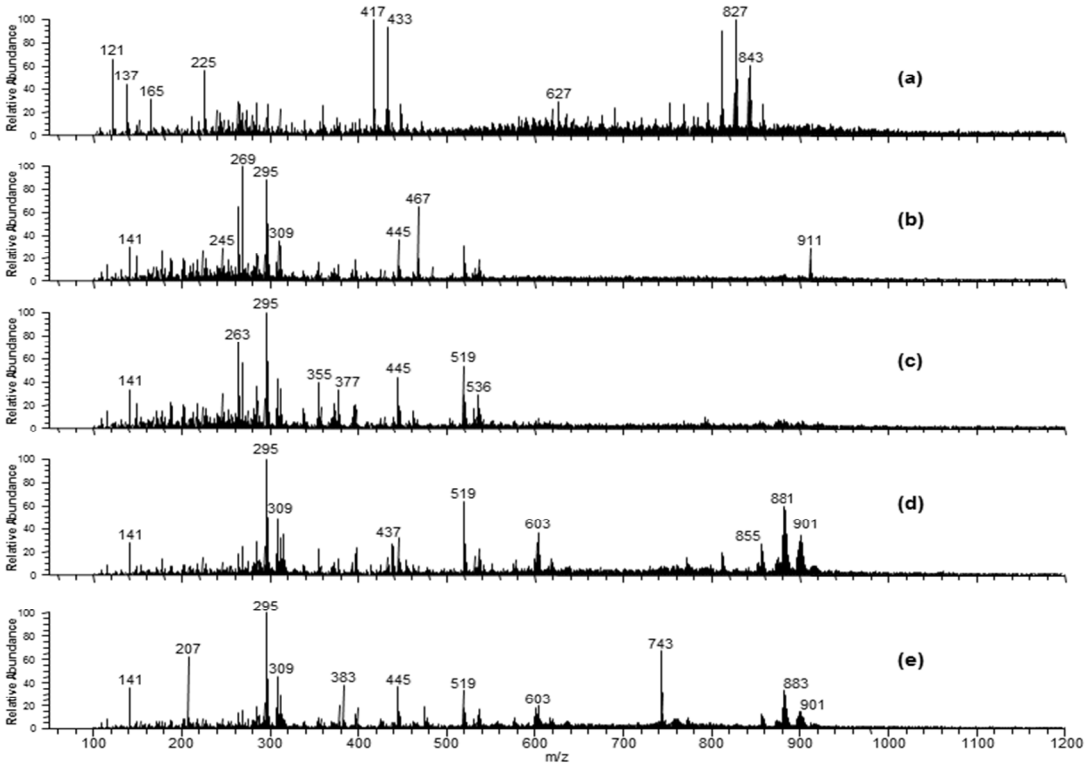

Figure 1 ESI(+)-MS of the following oils: (a) EVOO blend, (b) soybean (SO), (c) corn (CO), (d) sunflower (SF), (e) canola (CA).

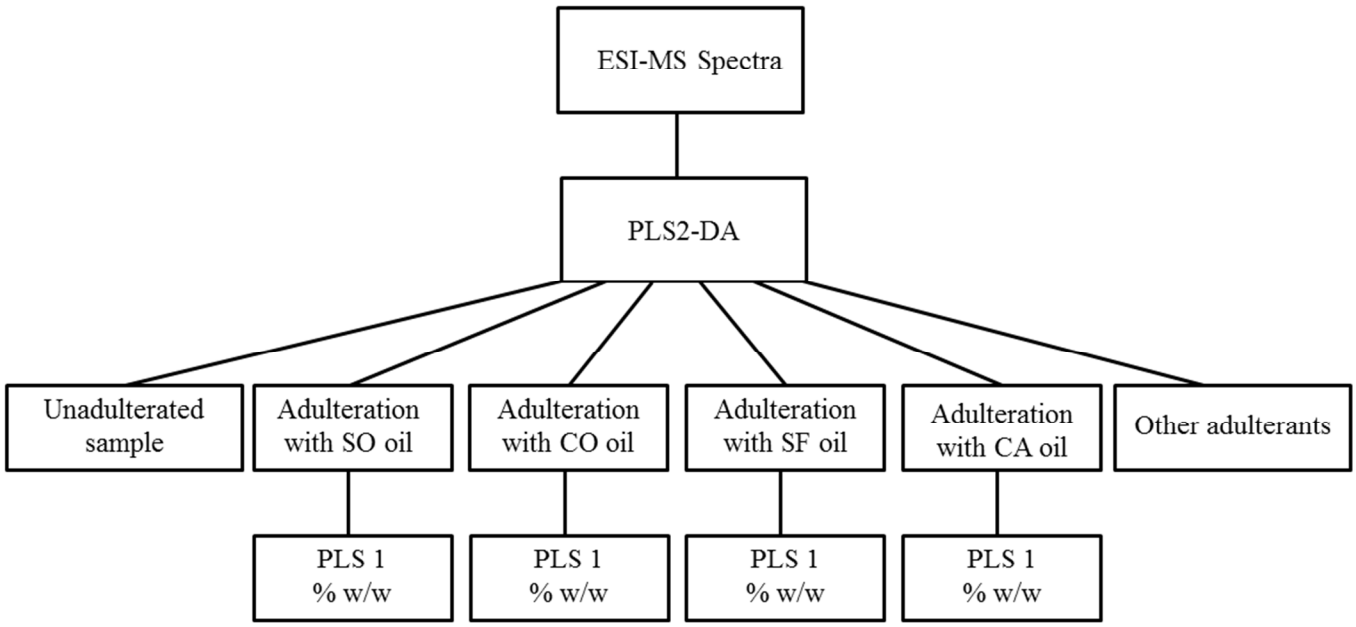

Figure 2 Chemometric strategy used with ESI(+)MS data for identification and quantification of adulterated EVOO samples.

For each adulterant model, the samples (a total of 40) were split into 30 and 10 for the calibration and validation sets, respectively. The calibration samples were chosen in order to homogeneously cover the analytical range, from 0.5 to 20.0% w/w of each adulterant. The initial attempts to build PLS models invariably showed a first latent variable (LV) that accounts for much more variance in the X (mass spectra) than in the Y (concentrations) blocks. This indicates variation in **X** that is unrelated to **Y**, which may disturb the multivariate modeling and cause imprecise predictions for new samples. This situation justifies the use of orthogonal signal correction (OSC),[39] a preprocessing technique for removing the information in **X** unrelated to **y** vector. The data were then mean centered. The number of LV in each model was chosen by contiguous cross-validation blocks (with nine splits), based on the lower values of root mean square errors of cross validation (RMSECV). As an alternative to OSC, discrete variable selection using the successive projection algorithm (SPA)[40] was also attempted, but it was not succeeded.

In sequence, the four PLS models were optimized using a procedure for the detection of outliers. This procedure was based on a robust methodology that identifies samples with extreme leverages, large residuals in the **X** block (spectral data) or large residuals in the **Y** block (concentration values)[24,28] at 95% confidence level. These outliers can be removed within a limit of 2/9 (22.2 %) of the total number of samples, as established by the Brazilian and international guidelines.[30,41,42] Outliers in the validation set were also detected by the jackknife (externally studentized residuals) test,[41] but this was only performed after finishing the optimization of the calibration set. The number of outliers detected varied from one to three for each set of adulterated samples. Particularly, the samples corresponding to the lowest level, 0.5 % w/w, were considered outliers in almost all the models, excepting for CA. The only sample excluded from this model was the one adulterated with 19.5% w/w. The number of LV used for building each model varied between three and five. The parameters for each model are shown in Table 1. The results describing the predictive ability of each model will be discussed later in this paper (analytical validation, section 3.3).

Table 1. Parameters for the optimized PLS models regarding samples of EVOO adulterated with four edible oils: SO, CO, SF and CA.

Parameter	SO	CO	SF	CA
Number of calibration samples	29	27	29	29
Number of validation samples	8	9	9	8
Number of LV	5	4	5	3

The development of these quantitative methods does not demand ion identification. Nevertheless, it is also possible a qualitative interpretation of the models by analyzing their regression coefficients shown in Figure 3. Ions with positive regression coefficients contribute for predicting each specific adulterant, while ions with negative coefficients are related to EVOO. It is noteworthy that some diagnostic ions for EVOO previously identified by other authors,[13,16] such as tyrosol (*m/z* 121), hydroxytyrosol (*m/z* 137) and coumaric acid (*m/z* 165), presented negative regression coefficients for all the PLS models. Other significant negative regression coefficients were observed for the ions of *m/z* 319, 361, 379, 443, 449, 505, 549, 811, 827 and 843. The last three ions were ascribed as TAGs, as observed in the ESI(+)-MS of EVOO (Figure 1a). The ions that most contribute for predicting SO, CO, SF and CA adulterant oils are indicated in Figures 3a-d.

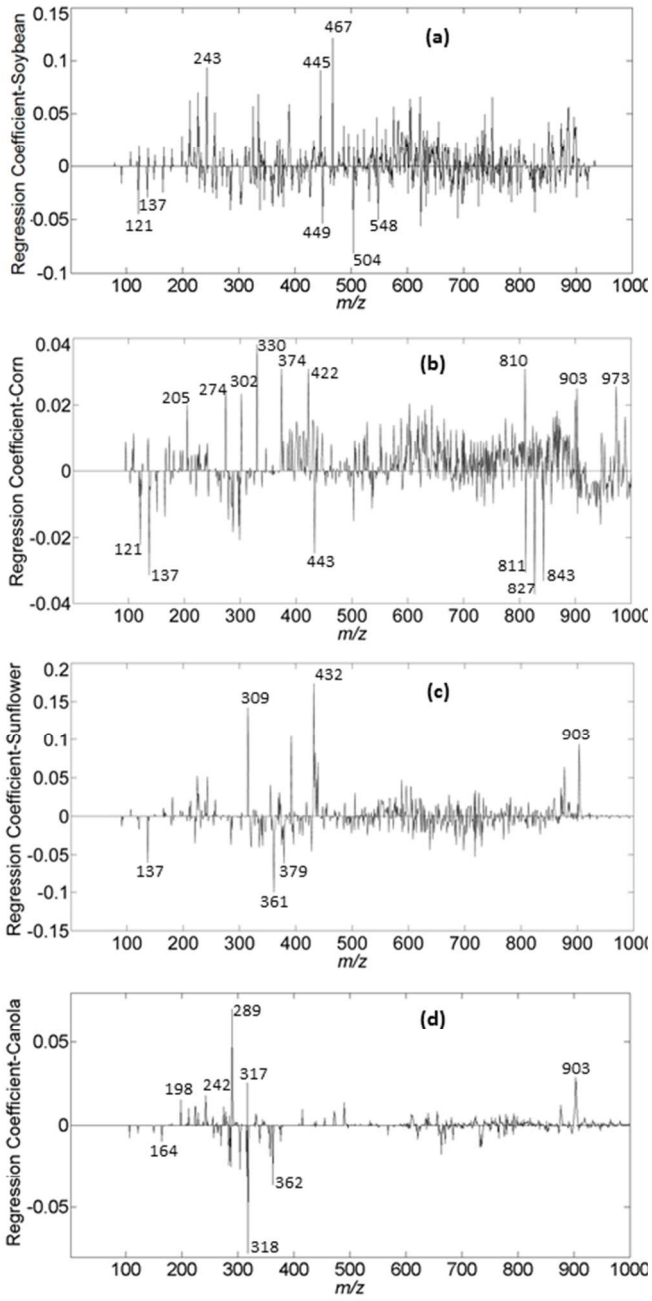

Figure 3 Regression coefficients for PLS models of the following oils: (a) SO, (b) CO, (c) SF and (d) CA.

3.3 Multivariate analytical validation

The four developed PLS methods were validated by the estimate of FOMs and the use of appropriate statistical tests. These results are summarized in Table 2 and discussed following in this paper. The plots of reference *versus* predicted values are shown in Figure 4.

The linearity of multivariate methods is estimated by the fit of the reference *versus* predicted values and the most used parameter to evaluate it is the correlation coefficient (r). As can be observed in Table 2, all of the models have a good linearity, expressed as r values above 0.993. Nevertheless, this evaluation cannot be grounded only on the r values and the random distribution of the residuals should obligatorily be verified. In this work, we used a methodology based on three statistical tests, originally prescribed for univariate methods.[41] The extension of this methodology for multivariate methods is simple and an in-depth discussion about these tests can be found in the paper by Souza and Junqueira[41] and the references therein. The first test to be applied is the RJ and the residuals would be considered normally distributed if the estimated R is above the critical R_{limit}, which is calculated as a function of the number of samples. At 95%, only the SF model does not meet this criterion. However, the residuals for this model were considered normal at a confidence level of 99 % (Table 2). In sequence, the homocedasticity of the residuals was evaluated with the BF test. The residuals should be considered homoscedastic if the estimated probability p is above the critical $p > 0.05$ (95%) value. As can be observed, all the models can be considered homoscedastic. Finally, the presence of auto-correlation in the residuals was evaluated with the DW test, at 95% confidence level. For three out of four models, the estimated DW values were within their respective acceptance ranges. Only the residuals of the CO oil cannot be considered independently distributed.

The trueness of the models can be evaluated by the absolute root-mean-square-errors of prediction (RMSEP) and calibration (RMSEC) parameters. These parameters varied between 1.0-1.7% w/w and 0.6-1.0% w/w, respectively, indicating that the methods produced results in good agreement with the reference values. The trueness can also be observed by the relative mean errors for individual samples, between 3.6% and 8.2%. These values are in accordance with Brazilian guidelines, which establish acceptance limits of relative errors within −20/+10%.[30] The precision was evaluated at the level of repeatability by estimating the mean relative standard deviation (RSD) for six replicates obtained at five concentration levels (4.0, 6.0, 10.0, 14.0 and 16.0% w/w). This is in accordance with the ASTM E-1655 regulation,[25] which prescribes the estimation of the average precision by the pooled standard deviation obtained from a number of samples at least equal to the number of LV used in the PLS model (five in our case), with six replicates per sample. The RSD varied between 1.5% and 7.5%. The combined results of trueness and precision allow attesting therefore the accuracy of the developed models.

For univariate methods the requirement of 100% of selectivity is usual, but for multivariate approaches there is no practical meaning in establishing this requisite. Unlike for univariate methods, low values of selectivity can be observed even for accurate PLS methods. For the present models, the estimated values of selectivity only indicated that only a small fraction (from 14% to 39%) of the analytic signal was used for predicting adulteration. The sensibility is dependent on the analytical technique employed and, therefore, not adequate for comparisons with other methods. Thus, the analytical sensitivity (γ) was estimated by dividing the sensibility by the estimate of the instrumental noise (ε = 0.178), which was obtained from the pooled standard deviation of 15 replicates of a blank sample. The inverse of γ provides an estimate of the minimum concentration difference that can be discriminated by the models, considering the random instrumental noise as the only source of errors. This value also indicates the number of decimal places (two) to be used in the expression of the results. However, we decided to express the final results with only one decimal place as a more realistic option.

LOD and LOQ were estimated as 3.3 and 10 times of the inverse of γ, respectively.[26] The results are presented in Table 2 and together with the accuracy and linearity evaluations allow establishing the analytical ranges from 1.0 % to 20.0 % w/w for all the developed methods. The bias was calculated only with the validation samples, according to the ASTM normative.[25] This calculation consists in a t-test that employs the estimate of bias and the standard deviation of the validation errors, with the number of degrees of freedom equals to the number of validation samples. As can be seen in Table 2, all the models presented no significant bias, assuring the absence of systematic errors.

Finally, RPD,[43] the ratio of natural variation in the calibration or validation samples to the size of probable errors occurring during the prediction, was estimated. It represents how well the calibration model predicts a specific set and it is more appropriate for evaluating the performance of a model in absolute terms. According to the literature,[27,43] good calibration models must have RPD higher than 2.4. For our models, RPD values varied between 3.1 and 5.6, indicating their good quality.

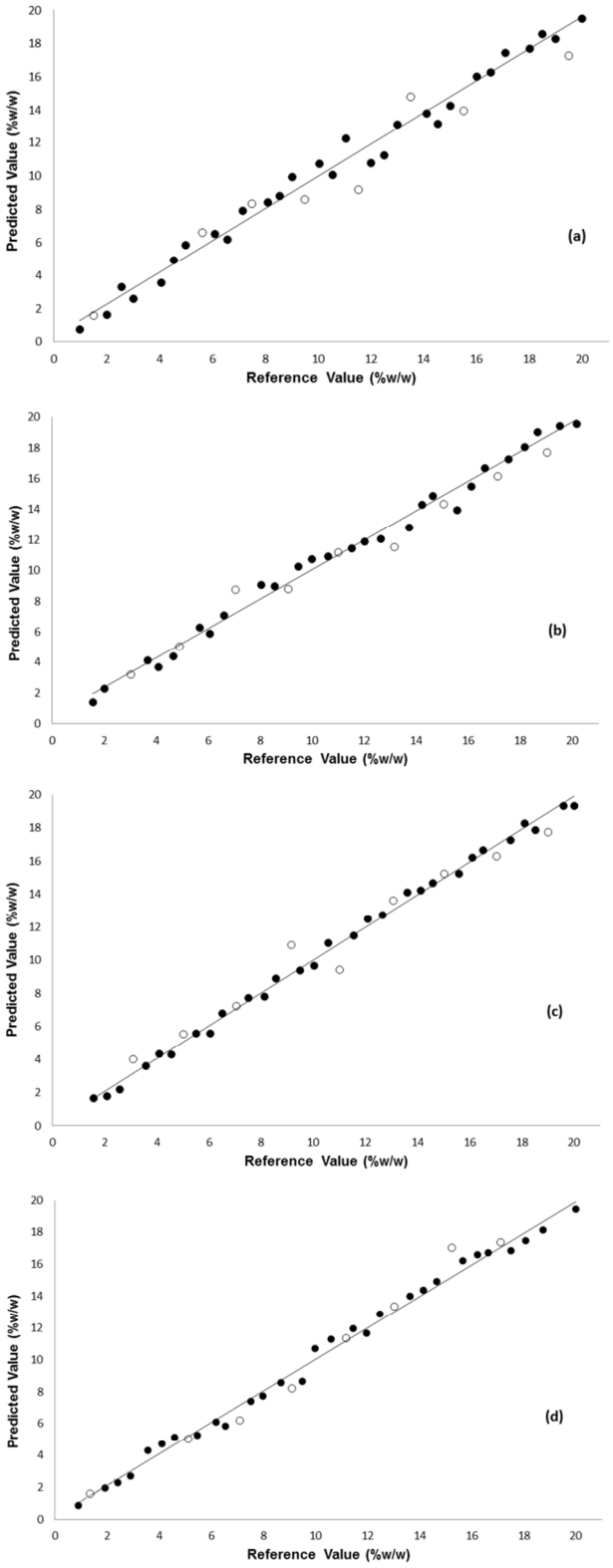

Figure 4 Plots of the reference *versus* predicted values for the PLS models obtained from the ESI(+)-MS data of samples of EVOO adulterated with the following edible oils: (a) SO, (b) CO, (c) SF, (d) CA. Full and empty circles refer to calibration and validation samples, respectively.

4. Conclusions

In this paper it was developed a chemometric methodology based on direct infusion ESI(+)-MS data for detecting four types of cheaper oils, soybean, corn, sunflower and canola, used as adulterants in EVOO samples. The level of adulteration was determined by the PLS method and the four obtained models were thoroughly validated, being considered accurate, linear, sensitive and unbiased in the range of 1.0% to 20.0% w/w. All this methodology is experimentally simple and rapid and demands less than one minute for its execution. The present strategy can be extended to incorporate more sample variability and to include other possible adulterants. This possibility is underway in our laboratory. Finally, this work demonstrated the utility of several multivariate tools, which has been commonly used for evaluating infrared PLS methods but has never been previously applied to quantitative mass spectrometric data.

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Table 2: Parameters estimated for the PLS models obtained from the ESI(+)-MS data recorded for the samples of EVOO adulterated with SO, CO, SF and CA oils.

FOM	Parameter (unit)	Soybean (SO)		Corn (CO)		Sunflower (SF)		Canola (CA)	
		Value	Limit	Value	Limit	Value	Limit	Value	Limit
Linearity	Normality	R = 0.9829	R > 0.9696	R = 0.9860	R > 0.9689	R = 0.9609	R > 0.9580[a]	R = 0.9706	R > 0.9696
	Homocedasticity	p = 0.6193	p > 0.05	p = 0.3357	p > 0.05	p = 0.5683	p > 0.05	p = 0.8622	p > 0.05
	Independency	2.19	1.53 – 2.58	1.42	1.53 – 2.58	1.72	1.54 – 2.57	1.76	1.53 – 2.58
	r[b]	0.9931		0.9951		0.9984		0.9966	
Bias	...	t = 0.86	t < 2.31	t = 0.84	t < 2.26	t = 0.29	t < 2.26	t = 0,57	t < 2.31
Trueness	RMSEC (% w/w)	1.0		0.9		0.6		0.7	
	RMSEP (% w/w)	1.7		1.0		1.4		1.0	
	Relative mean error (%)	8.2		5.4		3.6		5.5	
Precision	DPR (%)	6.6		7.5		6.4		1.5	
Selectivity	...	0.14		0.27		0.20		0.39	
Sensitivity	[c]	1.96		4.90		2.13		6.28	
Analytical Sensitivity (γ)	$(\%w/w)^{-1}$	11.0		27.5		12.0		35.3	
1/ γ	(%w/w)	0.09		0.04		0.08		0.03	
LOD	(%w/w)	0.3		0.1		0.3		0.1	
LOQ	(%w/w)	0.9		0.4		0.8		0.3	
RPD	RPD Calibration	3.1		5.6		4.3		5.6	
	RPD Validation	3.3		5.5		4.0		5.2	

[a] Estimated at 99% confidence level. All the other limit values were estimated at 95 % confidence level [b] Values for the line fitted to the calibration samples. [c] Values expressed as the ratio between the relative abundance and % w/w.

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