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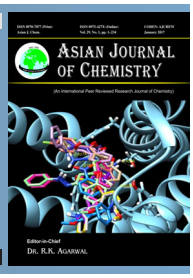

Determination of Yield of Enzymatic Hydrolysis of Vegetable Oils by Near-Infrared Spectroscopy

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In this work, a rapid and environmental friendly method to evaluate the yield of hydrolysis reactions of vegetable oils in relation to the production of free fatty acids is proposed, using near-infrared (NIR) spectroscopy and multivariate calibration. For calibration of the analytical method hydrolyzed samples of soybean oil with different free fatty acids contents were analyzed by partial least squares (PLS) regression. For the calibration and validation steps, values for root-mean-square error of 0.87 and 0.88, respectively were reported. The method was tested to predict the free fatty acids content in enzymatically hydrolyzed samples of different vegetable oils (soybean, macauba, sunflower and corn). It was observed from the results that the method presented is effective for predicting free fatty acids concentration in the range from 1.5 to 80.5 wt %, with prediction errors lower than 1.0 wt %. The correlation between the values observed for the reference methodology and those predicted by the NIR/PLS method presented coefficients of determination (R^2) > 0.97 for the all oils studied, demonstrating the effectiveness of the method to assess the progress of hydrolysis of vegetable oils with different fatty acid profiles. The results obtained demonstrate that the proposed method can be employed for monitoring and rapid screening analysis of free fatty acid production by the hydrolysis of vegetable oils.

Keywords: Free fatty acids, Hydrolysis, Near-infrared, Multivariate calibration.

INTRODUCTION

The production of free fatty acids has been applied industrially for many years in the production of soaps and other oleochemical products [1]. Currently, fatty acids are widely used as raw materials in the food, cosmetics and pharmaceutical industries [2], being mainly used for the synthesis of emulsifiers [3], obtaining bioaromas [4], production of biopolymers [5] and conjugated linoleic acid (CLA) [6]; for producing concentrates of free fatty acids for nutrition and supplementation [7] and for biofuel synthesis [8,9].

The main processes employed for free fatty acids production are chemical [10], enzymatic [11,12] and non-catalytic using water at pressurized conditions [13]. In this reaction, the formation of monoacylglycerols and diacylglycerols occurs as intermediates of three consecutive and reversible steps, free fatty acids being produced at every step with glycerol as a by-product.

The progress of the hydrolysis reaction is traditionally evaluated using the method of the American Oil Chemists' Society - AOCS [14]. It is considered simple, but it is laborious, lacks precision and requires a high consumption of reagents/solvents [15-17]. Techniques such as high-performance liquid chromatography and gas chromatography have also been used to determine free fatty acids [18], but these techniques are rather expensive and time-consuming. They require skilled operators and even they can have a high environmental impact. As an alternative to chemical methods to avoid the major drawbacks of these methodologies and according to the principles of green chemistry, near-infrared spectroscopy (NIR) has been investigated. It consists of a quick test procedure performed directly on the sample (no pre-treatment), with good repeatability and is more cost effective for analysis when compared with classical techniques [19,20]. Near-infrared has been widely applied to monitor reactions with vegetable oils and their derivatives [21-26]. However, monitoring the progress of the hydrolysis of vegetable oils using near-infrared spectroscopy is little explored in the literature. This, coupled with the increase in research related to obtaining free fatty acids, justifies the interest in the development of this study.

EXPERIMENTAL

Soybean oil (Soya/Brazil), macauba oil (Cocal/Brazil), sunflower oil (Cocamar/Brazil) and corn oil (Cocamar/Brazil) were used in the hydrolysis reactions with sodium phosphate buffer (Neon) and the immobilized enzymes of *Rhizomucor miehei* and *Thermomyces lanuginosus* as catalysts. Table-1 shows the chemical composition of the vegetable oils used in

TABLE-1
CHEMICAL COMPOSITION OF VEGETABLE OILS UTILIZED

Chemical composition	Soybean oil	Macauba oil	Sunflower oil	Corn oil
Palmitic acid (mass %)	10.8	11.07 ± 0.22	8.36 ± 0.14	14.03 ± 0.16
Stearic acid (mass %)	3.8	3.46 ± 0.03	5.03 ± 0.02	3.33 ± 0.08
Oleic acid (mass %)	27.7	19.74 ± 0.07	27.65 ± 0.11	35.08 ± 0.09
Linoleic acid (mass %)	50.8	62.33 ± 0.15	56.30 ± 0.12	44.40 ± 0.17
Linolenic acid (mass %)	4.4	0.33 ± 0.01	2.06 ± 0.06	1.98 ± 0.09
Other fatty acids (mass %)	–	3.07	0.65	1.20
Water content (mass %)	0.3 ± 0.03	0.7 ± 0.05	0.6 ± 0.05	0.4 ± 0.04
Free fatty acids content (mg free fatty acids 100 mg^{-1})	0.2 ± 0.01	23.0 ± 0.4	0.25 ± 0.01	0.26 ± 0.01

this work, as determined by gas chromatography. In the sample titrations analytical grade reagents and solvents: ethyl ether (Vetec), ethanol (Nuclear), potassium hydroxide (Nuclear) and phenolphthalein (Nuclear) were used.

For the calibration, validation and prediction steps, different experimental runs were carried at a temperature of 55 °C, in pH 8.0 buffer, with agitation at 400 rpm, using different water-to-oil mass ratios (1:1 to 1:20) and a catalyst percentage ranging from 5 to 20 % (relative mass of substrate) and reaction times of 4 to 10 h. The methodology for conducting the reactions is presented in the work of Raspe *et al*. [12]. After completion of the reaction, the catalyst was removed by filtration and the filtrate was centrifuged with *n*-hexane for 15 min at 3500 rpm, to remove the water and glycerol formed. The samples were transferred to sampling flasks and placed in an oven to evaporate the excess solvent.

The free fatty acids content in samples was determined by the conventional titration method AOCS (Ca5a-40), three times for each sample and the average values are reported. The free fatty acids content in the samples ranged from 1.5 to 80.5 wt %. The near-infrared spectra were acquired in a near-infrared spectrophotometer model N-200 (Büchi) at room temperature using a glass petri dish (height 1.7 cm and diameter 9 cm) in the spectral range 700 to 2500 nm. After obtaining the spectra, free fatty acids content information of each sample was assessed using the NIRCal software. Seventy spectra were used to create the calibration protocol and 30 spectra were used for the validation. Spectral treatments to avoid baseline fluctuations were applied and calibration models were constructed using the software NIRCal 4.01 (Büchi) and partial least squares (PLS) regression. The relationship between the data was assessed by a leave-one-out cross-validation procedure and optimised in terms of the appropriate number of factors and pre-treatments by minimizing the root-mean-square error (RMSE). The root-mean-square error is designated as RMSEC (%) for calibration and RMSEV (%) for validation.

The predictive capability was tested against samples obtained from the hydrolysis of soybean oil, macauba oil, sunflower oil and corn oil. The values predicted by the near-infrared method were compared with those obtained by the titration method by means of linear correlation, using the Statistica® 7.0 (Statsoft Inc) and the root-mean-square error of prediction (RMSEP) was evaluated.

RESULTS AND DISCUSSION

Method development: Fig. 1 shows the near-infrared spectra of the soybean oil and sample of reaction medium

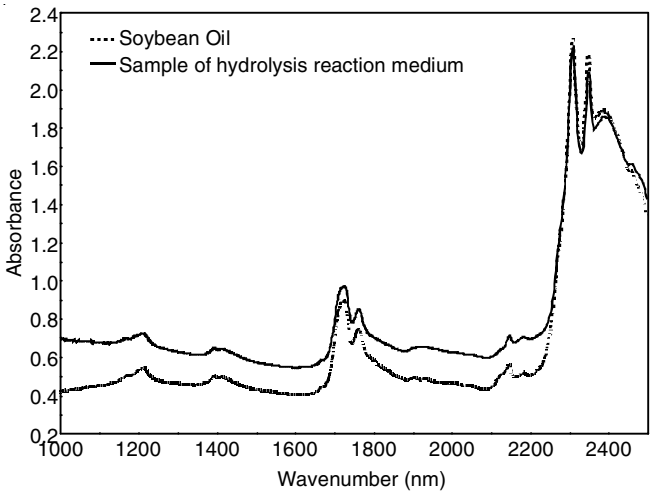

Fig. 1. Near-infrared spectra of soybean oil and sample of hydrolysis reaction medium (with free fatty acids content of 61.2 wt %) for spectral region between 1000 and 2500 nm

(with free fatty acids content of 61.2 wt %). Different regions of absorption are observed in the spectrum, with different intensities corresponding to the oil and the products of the hydrolysis reaction. The 1700-1800 nm region corresponds to the first overtone of C–H bonds [27] and the increase of the peak in this region indicates the presence of free fatty acids in the sample. The other regions 1400-1600 and 2000-2200 nm correspond to the regions of absorption of monoglycerides and diglycerides [27], respectively, which are present in the hydrolysis reaction samples since they are formed as intermediate products. The developed method used spectrum data obtained in the region between 1400 and 2200 nm, region selected because it indicated a response in the absorption corresponding to the progress of the hydrolysis reaction.

Table-2 shows the pre-treatments investigated for obtaining the most accurate and robust calibration model obtained using 7 (seven) partial least squares factors. For this table it was observed that the lowest RMSE obtained was 0.87 and 0.88 for calibration and validation of the method, respectively, with smoothing and first derivative obtained by the Savitzky-Golay method (9 points). Consistency is defined as the percentage of the standard error of estimation (SEE) divided by the standard error of prediction (SEP) and must be as close as possible to 100, with acceptable values between 80 and 110. The consistency value of > 98 found in this work supports the acceptable quality of the calibration curve. Fig. 2 shows the performance of the developed model for the representation of the experimental data used in the calibration and validation steps, with a strong linear relationship between the values and coefficient of determination (R^2) > 0.99.

TABLE-2
RESULTS FROM DIFFERENT PRETREATMENT METHODS OF DATA OBTAINED BETWEEN 1400 AND 2200 nm IN THE NIR/PLS METHOD ESTABLISHING FOR FREE FATTY ACIDS CONTENT (wt %)

Parameter	Baseline correction[a]	[a] + SG smothing[b]	[b] + Derivative[c]
R^2 calibration	0.996	0.998	0.999
R^2 validation	0.995	0.998	0.998
RMSEC (%)	1.615	1.080	0.870
RMSEV (%)	1.645	1.080	0.880
Consistency	98.21	99.6	98.03

[a]Obtained using as reference the spectra without pretreatment.
[b]Savitzky-Golay smoothing, 9 points.
[c]First derivative obtained by Savitzky-Golay method, 9 points.

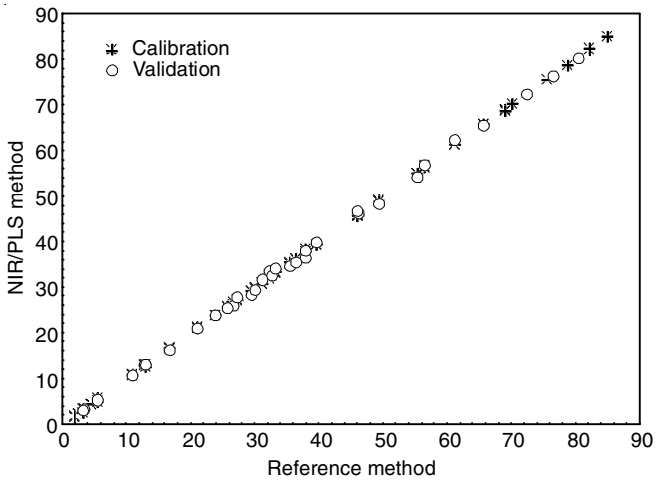

Fig. 2. Relationships between near-infrared-predicted and reference values for free fatty acids content (wt %)

The data presented in Table-2 are consistent with the RMSE and R^2 values reported in the literature for the determination of free fatty acids content in different vegetable oils and in oils and different reactions. For example, the correlation between the near-infrared absorption spectrum obtained between 1333-2175 nm and the acid value of peanut oil was examined by Rao *et al*. [28] and the reported values of 0.9725 and 0.308 for coefficient of determination and SECV (standard error of cross-validation), respectively. Mba *et al.* [29] reported values of 0.2 and 0.9973 for RMSEC and coefficient of determination, respectively, in the evaluation of the free fatty acids content in palm oil and canola blends by near-infrared and partial least squares. Blanco *et al*. [27] proposed the use of near-infrared spectroscopy to control the esterification reaction of glycerol with fatty acids and report an RMSEC of 0.5 for the method using spectral data obtained from 1100 to 2500 nm, with partial least squares regression and processing using the standard normal variate to determine the free fatty acids content from 0.1 to 31.3 %. For monitoring the enzymatic interesterification, Zhang *et al*. [30] developed a NIR/PLS method and obtained values of 0.173 and 0.982 for RMSEC and R^2, respectively, using the spectral range 832-2215 nm as a reference. Raspe and Silva [26] reported an RMSEC and RMSEV of 1.35 and 1.68 in the evaluation of free fatty acids consumption in the esterification reaction by near-infrared and utilising a spectral range of 1000 to 2500 nm.

Efficiency of method: In order to test the applicability of the developed method, its predictive capability was tested with independent samples of hydrolyzed soybean oil, macauba oil, sunflower oil and corn oil and the linear relationship between the free fatty acids content obtained by the reference methodology and the predicted by NIR/PLS as shown in Fig. 3. The RMSEP obtained for results reported in Fig. 3 were 0.88, 0.81, 0.88 and 0.82 for the reactions with soybean, macauba, sunflower and corn oil, respectively. Coefficients of determination (R^2) > 0.97 were reported for all the available vegetable oils, indicating good correlation and predictive performance. These results demonstrate that the methodologies are well correlated and the developed method efficiently predicts the yields of hydrolysis reactions of vegetable oils with different fatty acid profiles.

For the determination of free fatty acids in vegetable oils and animal fat using near-infrared spectroscopy and chemometric analysis by partial least squares, Moschner and Biskupek-Korell [31], Rao *et al*. [28], Adewale *et al*. [32] and Mba *et al*. [29] reported a strong correlation between the data obtained by the AOCS official method and those predicted by the developed methods. Comparing the RMSEP obtained in this work and results of the literature emphasises the efficiency of the developed method and its great potential for monitoring the production of free fatty acids. Houmøller *et al*. [33] reported the use of near-infrared for the determination of the degree of enzymatic interesterification of vegetable oils and obtained an RMSEP of 0.192 % using calibration in the range of 0.228 to 2.195 % free fatty acids content with data in the spectral region 1110 to 2220 nm. For the quantification of free fatty acids in samples of esterification reactions of oleic acid by near-infrared spectroscopy and the partial least squares method, Raspe and Silva [26] reported a coefficient of determination for the prediction of 0.999 and an RMSEP of 1.07 %. The NIR/PLS method developed by Blanco *et al*. [27] to evaluate the consumption of free fatty acids in esterification with glycerol presented an RMSEP of 0.7 %. Adewale *et al*. [32] developed an NIR/PLS method for absorbance data obtained between 1333 and 1639.88 nm using pre-treatment with the first derivative and reported an RMSEP of 0.477 % for the determination of free fatty acids in different animal fat wastes.

The results obtained can be compared to the application of other spectroscopic methods for the assessment of free fatty acids content. Mueller *et al.* [22] used infrared spectroscopy to quantify free fatty acids in enzymatic esterification reactions of glycerol and lauric acid and obtained an RMSEP of 1.84 %. In the same context, Khaskheli *et al.* [34] monitored lipase catalyzed hydrolysis of castor oil by infrared spectroscopy, obtaining strong correlation between the results predicted by the developed method and reported by the reference method, with a standard error of 0.019 % of free fatty acids. The use of infrared spectroscopy was shown to be efficient in determining free fatty acids in poultry feed lipid as reported by Sherazi *et al.* [35], with an R^2 of 0.9998 and a standard deviation of 0.15 between the predicted data by IR and obtained by the titration method.

The ^{1}H NMR spectroscopy has also been reported for the determination of free fatty acids content as reported in the work of Satyarthi *et al*. [17] and Skiera *et al.* [36] with strong

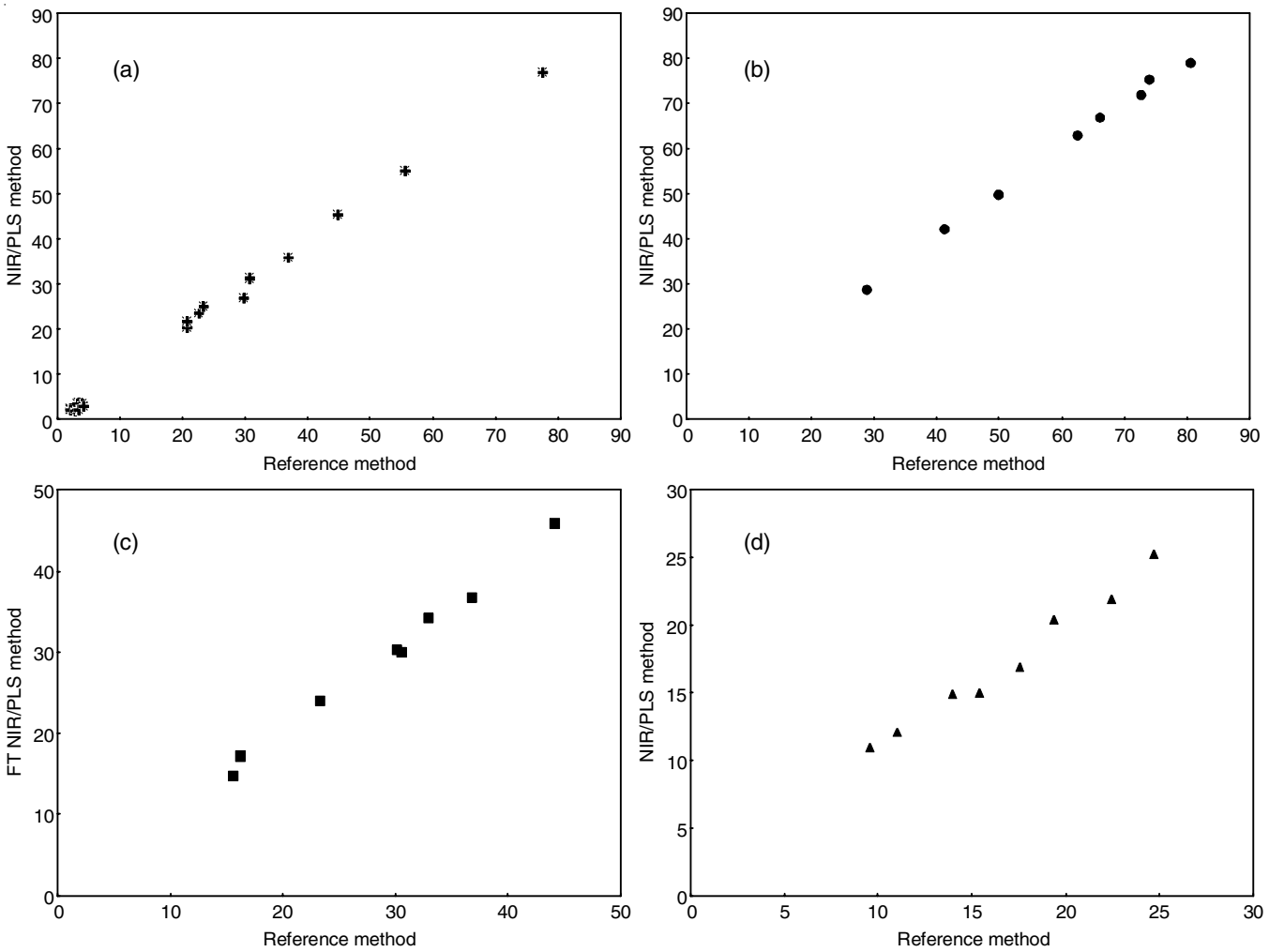

Fig. 3. Comparison of free fatty acids content (wt %) determined by the reference method and predicted by the near-infrared for sample of hydrolysis of: (a) soybean oil; (b) macauba oil; (c) sunflower oil and (d) corn oil

correlation (> 0.99) between the results from titration and ^{1}H NMR, but this method requires dilution of the sample in appropriate solvents prior to analysis.

Conclusion

The establishment of an accurate method for the assessment of the hydrolysis yield using near-infrared spectroscopy and partial least squares regression was proposed in this study. The NIR/PLS method used samples of hydrolysis reactions of soybean oil in the calibration step, however, the method demonstrated efficiency in predicting the free fatty acids content in samples obtained from various vegetable oils. The results presented RMSEP values of < 1 % and showed strong correlation (R^2 > 0.99) between the methodologies.

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