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# Sequential injection titration method using second-order signals: Determination of acidity in plant oils and biodiesel samples

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ABSTRACT

A new concept of flow titration is proposed and demonstrated for the determination of total acidity in plant oils and biodiesel. We use sequential injection analysis (SIA) with a diode array spectrophotometric detector linked to chemometric tools such as multivariate curve resolution-alternating least squares (MCR-ALS). This system is based on the evolution of the basic specie of an acid–base indicator, alizarine, when it comes into contact with a sample that contains free fatty acids. The gradual pH change in the reactor coil due to diffusion and reaction phenomenona allows the sequential appearance of both species of the indicator in the detector coil, recording a data matrix for each sample.

The SIA-MCR-ALS method helps to reduce the amounts of sample, the reagents and the time consumed. Each determination consumes 0.413 ml of sample, 0.250 ml of indicator and 3 ml of carrier (ethanol) and generates 3.333 ml of waste. The frequency of the analysis is high (12 samples $h^{-1}$ including all steps, *i.e.*, cleaning, preparing and analysing). The utilized reagents are of common use in the laboratory and it is not necessary to use the reagents of perfect known concentration.

The method was applied to determine acidity in plant oil and biodiesel samples. Results obtained by the proposed method compare well with those obtained by the official European Community method that is time consuming and uses large amounts of organic solvents.



## 1. Introduction

Free fatty acid (FFA) content is one of the most frequently determined quality indices in food quality control [1,2]. It also needs to be determined in biodiesel since it is one of the main factors that affects the transesterification process and if the lipid contains more than 0.5% FFA, soaps can be formed and the efficiency of the catalyst can be compromised [3].

Official methods for determining FFA content are based on non-aqueous titrimetry [4]. The procedure is time-consuming and involves high volumes of organic reagents and manual operations which are subject to personal error. To overcome these drawbacks an automated procedure is highly desirable.

Flow injection (FI) analytical methods, developed in 1975 by Ruzicka and Hansen [5], and sequential injection analysis developed later by Ruzicka [6], have proven to be extremely versatile for the precise and rapid automated analysis of samples for a vast number of analytes of interest, both species or chemical parameters [7–11]. These systems are highly versatile because they can be adapted to most analytical instruments [12] and enable data of different dimensions to be obtained that allows the use of various data treatments to get the required information [13].

Various flow and sequential injection titration procedures have been developed since the late 1970s. Wójtowicz [14] indexes a series of pioneering works in this area in a paper on novel approaches to analysis by flow injection gradient titration. Most total acidity determination is carried out in oil samples [15,16], but it is also of interest in such other matrices and as fruit juices [17–19], vinegar [20–22], soft drinks [22] and metallurgical solutions [23]. Flow-titrations have also been developed to determine such other analytical indexes as the antioxidant potential in wines [24] or basic index in lubricants [25] or concentrated hydrochloric acid [26].

The aim of this study is to develop a new concept of titration using sequential injection analysis (SIA) with a diode array UV–visible detector to obtain second-order data that permits to resolve the components of a sample when unknown interferents are present.

The method is based on introducing sequentially into the system a solution of an acid–base indicator, whose basic and acidic species have different spectra in the UV–vis range, and the solution sample. If the absorbance is recorded in a range of wavelengths at different

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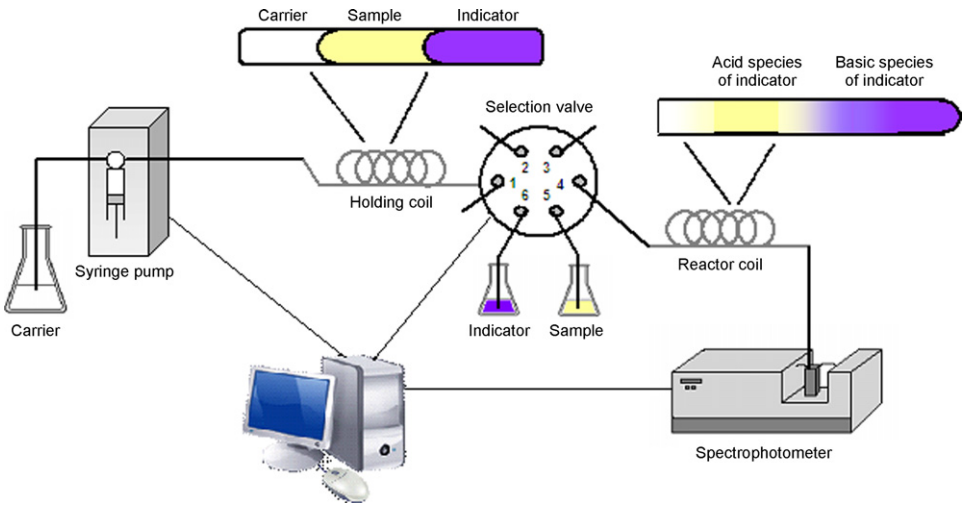


**Fig. 1.** Sequential injection analyzer and analytical process in the coils

times, a data matrix is obtained when the sample-indicator reaches the detector. The number of rows of this matrix corresponds to the number of spectra and the number of columns corresponds to the number of wavelengths [13]. When the data matrix has been obtained, multivariate curve resolution-alternating least squares (MCR-ALS) can be used to resolve the species present in a sample to obtain the spectra and the concentration profiles [27,28]. These profiles will correspond to the acidic and basic species of the indicator and to any other species contained in the sample that gives response in the UV–visible zone.

When a sample with free fatty acid is injected into the system, the concentration of the acidic species of indicator increases and the basic species decreases, so it is possible to establish a calibration relating the above mentioned indicator concentration profiles (areas) to the acidity.

Although nowadays there are numerous applications of second-order data to quantitative analysis [29–32] and among them there are those which use SIA to generate second-order data [33–35], we have found no references in which this sort of data was used to do an indirect determination of a quality index, as acidity, with a wide application area.

This method has all the advantages of flow systems: high frequency of analysis, automatization, low consumption of reagents and samples, and low production of waste. Moreover, it does not need a reagent of known concentration and the reagents are easy to obtain in any laboratory. It also has the "second-order advantage" [36] that permits to quantify the components of a sample when unknown interferents are present, which is a characteristic of high interest in the analysis of complex samples.

The novelty of this work with regard to other methodologies that uses second-order data is that the employed signal is indirectly related with the analyte of interest, what increases the application possibilities of these techniques.

## 2. Experimental

### 2.1. Procedure

#### 2.1.1. SIA method

The carrier, sample and indicator solution are sequentially aspirated towards the syringe and then pushed towards the detector through the reactor coil. During this operation, the zone undergoes some mutual dispersion. Due to the sample contains free fatty acids, a pH gradient is created between the pH of the acid solution and the basic pH of the indicator. When the solution reaches the detector, the first species of the indicator detected is the basic species and then the acidic species. In one zone both species are present, as well to other UV–vis sensitive species. This process is schematized in the enlargement of the holding and reaction coils in Fig. 1. As response we obtain a data matrix whose columns are the SIA peaks at a specific wavelengths and whose rows are spectra recorded at a specific time (Fig. 2a and b).

#### 2.1.2. Data treatment

The aim of the MCR-ALS method [37] is the bilinear decomposition of experimental data set **D** in order to obtain matrices **C** and $\mathbf{S}^T$, which have real chemical significance, according to Eq. (1):

$$\mathbf{D} = \mathbf{C}\mathbf{S}^T + \mathbf{E} \quad (1)$$

where the dimensions of the matrices are: **D** ($n \times m$), **C** ($n \times c$), $\mathbf{S}^T$ ($c \times m$), **E** ($n$ x $m$); $n$ is the number of spectra o times in which the signal has been obtained, $m$ is the number of wavelengths in which the signal has been obtained and $c$ is the number of components considered (chemical species contributing to the signal). **C** is the matrix that describes the concentration profiles of the species in the system and can be used to obtain the areas of each species, which are directly related to the concentration. $\mathbf{S}^T$ is the matrix that contains the response profiles of these species (spectra profiles) and **E** is the matrix of the residuals.

The first step in MCR-ALS is to analyse the rank of the data matrix to determine how many species are present in the sample; the second step is to make an initial estimation of the concentration profiles or of the pure spectra. The final step is to perform alternating least squares optimization to calculate new matrices **C** and $\mathbf{S}^T$ from initial estimates of **C** or $\mathbf{S}^T$. In this optimization process, we imposed the constraint of non-negativity for the concentrations (**C**) and spectral ($\mathbf{S}^T$) profiles and the constraint of unimodality for the concentration profiles (**C**) [38]. The resolution can be improved by treatment with what are known as augmented matrices [27], appending the spectra of the pure acid and basic species of the indicator.

The right side of Fig. 2 shows the concentration matrix **C** and the spectra matrix $\mathbf{S}^T$ profiles obtained by MCR-ALS. As an example, in Fig. 2c and d it is shown the results obtained from the resolution of an oleic acid standard matrix. If the analysed sample was complex and there were more absorbent species, the number of components would be superior to two, but it would continue having the same information with regard to the indicator signals (the called second-order advantage).

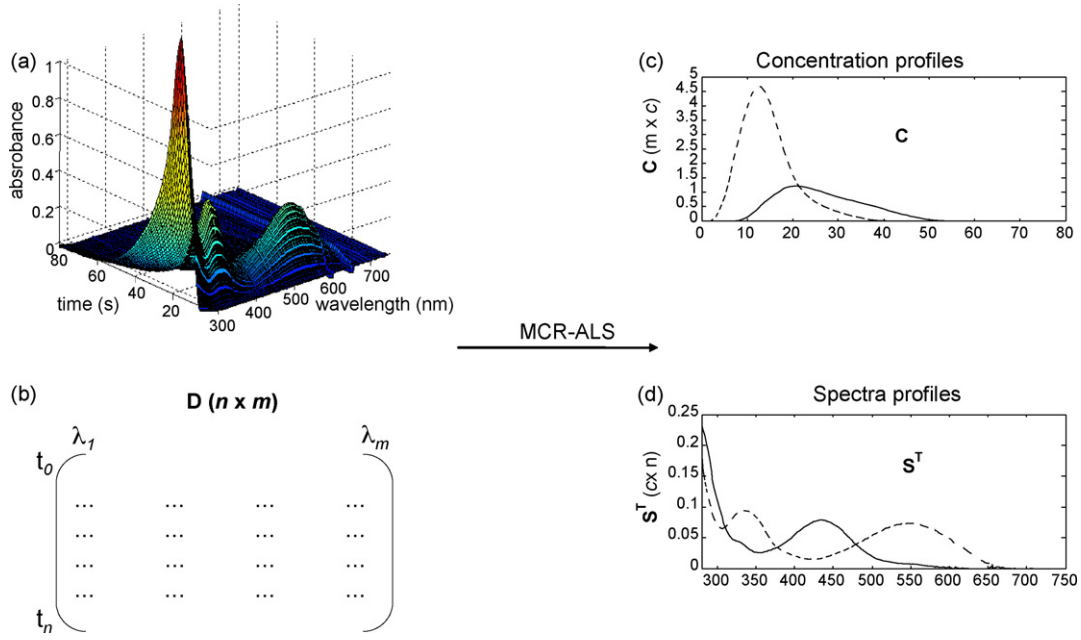


**Fig. 2.** (a and b) Data matrix resulting from the analytical process applied to an oleic acid standard, (c) matrix of concentration profiles (**C**) and (d) spectra profiles ($\mathbf{S}^T$). (- - -) Acid species of the indicator and (—) basic species of the indicator

To evaluate the quality of model fit we considered the percentage of variance explained by the product $\mathbf{CS}^T$, which is given by

$$R^2\ (\%) = 100 \times \sqrt{\frac{\sum_{i,j} d_{ij}^{*2}}{\sum_{i,j} d_{ij}^2}} \quad (2)$$

Another parameter that we used to determine the quality of the resolution process is the lack of fit, which is expressed as

$$lof\ (\%) = 100 \times \sqrt{\frac{\sum (d_{ij}^* - d_{ij})^2}{\sum d_{ij}^2}} \quad (3)$$

where $d_{ij}$ are each of the elements of the experimental matrix **D** and $d^*_{ij}$ are each of the elements of the reproduced data matrix **D**, obtained by the MCR-ALS decomposition.

The correlation coefficient of the spectra obtained in the resolution step ($X$) and the pure spectra ($Y$), calculated from (4), for the two species of the indicator was also used to assess the quality of the MCR-ALS results.

$$r = \sqrt{\frac{ss_{xy}^2}{ss_{xx} ss_{yy}}} \quad (4)$$

where $ss$ is the sum of squared values of a set of $n$ points.

### 2.1.3. Calibration model

To establish a calibration model we prepared a series of standard calibrations whose concentrations of free acid are known. The areas of the acidic and basic species of the indicator, obtained in the resolution process (Fig. 2c), are the analytical signal that we use to establish a univariate calibration versus the concentration of free acid.

The samples were analysed by the same procedure and the concentration of acid in the samples was obtained from its corresponding response value (areas of the species of the indicator) and the calibration parameters.

Subsequently, the acidity value is obtained from expression (5)

$$\text{Acidity} = c\frac{V}{P} \quad (5)$$

where $c$ is the concentration of free acid obtained by the proposed method, $P$ is the weight of the oil and $V$ is the volume to which the sample has been diluted before the analysis by SIA-MCR-ALS.

### 2.1.4. Official method

The Official European Community method [4] involves the following steps: (a) weighing 2.5–20 g of oil (according to expected acidity); (b) solubilization in 50–150 ml of an 1:1 ethanol–diethylether solvent and (c) titration with a 0.1000 M ethanolic KOH solution using phenolphthalein indicator.

The acidity, expressed as the percentage of oleic acid, is calculated according to the following expression

$$\text{Acidity} = \frac{V_{\text{KOH}} MN}{10 P_{\text{oil}}} \quad (6)$$

where $V$ is the volume in ml of KOH solution, $M$ is the molecular weight of oleic acid (282 g mol$^{-1}$), $N$ is the normalized concentration of KOH solution (mol l$^{-1}$) and $P$ is the weight in g of the sample.

## 2.2. Reagents and samples

In all analyses, we used analytical grade chemicals. Oleic acid was purchased from SIGMA-ALDRICH, sodium hydroxide from PROLABO and Alizarine from PANREAC. The carrier stream was absolute ethanol provided by Scharlab S.L. The oil and biodiesel samples were obtained from Bionet [39].

Oleic acid standards and samples were prepared by weighing the required amount of oleic acid (or sample) and dissolving in absolute ethanol. Indicator solutions were prepared by weighing the appropriate amount of alizarine and dissolving in NaOH solution.

The samples and reagent volumes used for the SIA analysis are detailed in Table 1.

## 2.3. Instrumental and software

The sequential injection analyzer (see Fig. 1) comprised a Cavro XL 3000 syringe pump (5 ml) equipped with a six-port multiposition automatic selection valve (Eurosas EPS 1306 BPB) and a

**Table 1**
Protocol and selected operating conditions in the SIA system.

| SIA method | | |
|---|---|---|
| Steps | Parameter | Volume (ml) |
| *Preparation cycle* | | |
| 1 | Sample aspiration from reservoir to waste | 0.33 |
| 2 | Indicator aspiration from reservoir to waste | 0.33 |
| 3 | Carrier aspiration from reservoir to syringe | 4.670 |
| *Analytical cycle* | | |
| 4 | Sample aspiration from reservoir to syringe | 0.083 |
| 5 | Indicator aspiration from reservoir to syringe | 0.250 |
| 6 | Expulsion to detector | 2.333 |
| 7 | Carrier aspiration from reservoir to syringe | 2.000 |
| *Cleaning cycle* | | |
| 8 | Carrier aspiration from reservoir to detector valve<br>Flow rate in step 6: 0.5 ml $min^{-1}$<br>Flow rate in the rest of steps: 5 ml $min^{-1}$ | 1.000 |

HP8452A diode-array spectrophotometer with a Hellma 178.711QS flow-through cell. All tubes connecting the various components of the flow system were made of Omnifit PTFE with and i.d. of 0.8 mm. The lengths of the holding and reaction coils were 2.0 and 0.7 m, respectively. The syringe pump, the automatic valve and the data acquisition provided by the spectrophotometer were controlled by a personal computer via an RS-232 interface, a PCL-711S PC Lab-Card and an HP-IB IEEE488 interface for communications.

The spectra were recorded between 280 and 750 nm in 2 nm steps. As each sample passed through the detector, 80 measurements were taken (one every 0.7 s). The data were acquired and monitored by the spectrophotometer using the HP89531A software. The instrumentation was controlled by customised software.

All calculations for multivariate curve resolution with alternating least squares (MCR-ALS) were performed with MATLAB 6.5 [40]. The software used in this study was written by the Chemometrics Group of Barcelona University and can been downloaded from the Web page [41].

## 3. Results and discussion

To do the calibrate it could have been used as standard any fatty acid since the parameter of interest is an index (acidity) that measures the non specific content in acids presents in the sample. Among different possibilities, the oleic acid was chosen because it is the acid to which one refers in the official method to determine the acidity.

Like in case of the acid, the choice of the alizarine as indicator is not an indispensable requirement to carry out the proposed method. Due to the acid-basic properties of alizarine ($pK_a$ is 5.7), it is a suitable indicator to the analysis because it does not need a too acid medium in order to evolve from its basic species, as is injected into the system, to the acidic species. Moreover, its spectral characteristics (the acid solution is yellow and the basic solution is violet) make alizarine a suitable indicator. Other indicators can fulfil the exposed conditions.

To establish an initial analytical sequence, we choose the working conditions (flow rate and volume of carrier, indicator and sample) in accordance with operational restrictions (length of tubes, volume of syringe, etc.). In this stage the essential requirements are that both species of indicator are present and the acid area increases when the acid concentration in the sample is higher.

The conditions selected for the SIA method and the analysis protocol are summarized in Table 1. The steps 2 and 3 of preparation cycle are performed at the beginning of a series of measures and after the first analysis only step 1 is repeated to clean the sample tube and to fill it with the following one.

In the application of MCR-ALS it has been proven that the resolution does not suffer significant changes if the data was resolved with two or three components, due to the poor sensitive signal of the oleic acid in the UV–visible zone. The iterative method of optimization was applied working with augmented matrices, adding to the response matrix **D**, the vectors related to the spectra of the two species of the indicator and of the oleic acid. The product of the **C** matrix and $\mathbf{S}^T$ matrix accounts for 98.5042% of the variance associated with the experimental data and the lack of fit is 1.58%, which in quantitative terms means that it explains practically all the variability of the experimental data. The goodness of the spectra profiles recovered by MCR-ALS for the chemical species was evaluated quantitatively by calculating the similarity coefficients between the recovered spectra and the pure spectra recorded for both species (acid and basic species), respectively. The values were 0.9851 for the acid species and 0.9909 for the basic species, which indicates that the recovered profiles have a high degree of concordance with the original profiles.

In the second step, we optimised the analytical sequence in order to work in the conditions that provide the best parameters (work range, slope and correlation coefficient) of the calibration graph. Two experimental factors were selected as the main variables involved in the response of the SIA-MCR-ALS method: (a) concentration of indicator and (b) concentration of NaOH solution. The concentration of indicator is important because the higher concentration, the greater global response. But it is also necessary to control that it stays within the linearity range, so the response of the matrix obtained can be decompose according to the minimum squares criteria. When the indicator solution, which contains NaOH, comes into contact with the sample, which contains free acids, a gradual pH change takes place in the reactor coil due to the diffusion phenomenon and to the neutralization. Thus, both phenomena will be more or less marked depending on the level of NaOH concentration in the indicator, so it has an influence on the concentration profiles of the two species of indicator.

We evaluated the influence of these factors with a full factorial design $2^2$, which has the experimental domain showed in the first two columns of Table 2. The levels of indicator concentration have been selected in order to obtain absorbance values between 0.2 and 1. The levels of NaOH concentration have been selected for the purpose of obtaining both indicator species at the expected free acidity. In each experiment, 12 oleic acid standards solutions were analysed in triplicate in the range between 0.0 and 128 mg $l^{-1}$. The average of the areas found for every experiment is shown in the last columns in Table 2. In all cases, and even when the standard of 0 mg $l^{-1}$ was aspirated, both species of the indicator were present. This is due to the acid character of the ethanol used as a carrier, which provides enough acidity for there to be a small amount of

**Table 2**
Experimental conditions of the $2^2$ factorial design and average response.

| Experiment number | [alizarina] $10^{-4}$ (mol $l^{-1}$) | [NaOH] $10^{-4}$ (mol $l^{-1}$) | Acid area | Basic area |
|---|---|---|---|---|
| 1 | 2.7 | 2.5 | 18.4795 | 22.5421 |
| 2 | 4.0 | 2.5 | 182.7724 | 35.3814 |
| 3 | 2.7 | 7.5 | 15.4252 | 71.7880 |
| 4 | 4.0 | 7.5 | 40.5479 | 126.6145 |

**Table 3**
Some calibration parameters evaluated in the different experimental conditions.

| Parameters | Acid calibrate | Basic calibrate |
|---|---|---|
| *Experiment 1* | | |
| Working range (mg l$^{-1}$) | 0–20.5 | 0–5.13 |
| Slope | 8.44 | −16.13 |
| Intercept | 9.24 | 33.53 |
| Correlation coefficient | 0.9684 | 0.7675 |
| *Experiment 2* | | |
| Working range (mg l$^{-1}$) | 0–12.8 | 0–25.6 |
| Slope | 13.16 | −10.36 |
| Intercept | 170.97 | 47.18 |
| Correlation coefficient | 0.9424 | 0.9741 |
| *Experiment 3* | | |
| Working range (mg l$^{-1}$) | 0–25.6 | 0–25.6 |
| Slope | 10.84 | −13.75 |
| Intercept | 1.55 | 89.08 |
| Correlation coefficient | 0.9924 | 0.9842 |
| *Experiment 4* | | |
| Working range (mg l$^{-1}$) | 0–10.2 | 0–12.8 |
| Slope | 22.57 | −31.87 |
| Intercept | 13.03 | 164.32 |
| Correlation coefficient | 0.9347 | 0.9876 |

the acidic species. When the high level of indicator concentration is considered, higher areas are obtained in the total concentration profiles. When the high level of NaOH concentration is considered, the area of the basic species is larger than the area of the acidic species. There is an interaction between the two factors: the response does not depend exclusively on the indicator concentration, but it is also affected by the relation between the acid and basic area. This might be because the two species have different spectral sensitivities.

For the results obtained, which present different sensitivity to the acidic and the basic signal, 4 calibration graphs were constructed for each experiment. The response was considered to be the area of the acidic species, the area of the basic species and the relative area (area of the acidic or basic species relative to the total area of indicator). These last two calibrations have been built to verify if some randomness of the data is corrected and better precisions are obtained.

Applying the ANOVA test to each calibration graph, we observed that in the overall calibration line there is a loss of linearity in the most concentrated standards. Table 3 shows some parameters of the calibration curves obtained from the acidic and the basic species, which passed the ANOVA test for a 0.05% level of significance and the corresponding degrees of freedom. The results obtained for the calibrations curves using relative areas are not shown because the precision of the regression line does not improve, which indicates that the variability in the total area of the indicator is negligible in comparison to other sources of variability in the measures.

**Table 4**
Figures of merit for the selected calibration.

| | |
|---|---|
| Working range (mg l$^{-1}$) | 0–26.0 |
| Slope | 10.8359 |
| Intercept | 1.5488 |
| *R* | 0.9924 |
| *n* | 30 |
| lod (mg l$^{-1}$) | 1.49 |
| RRMSC | 0.3488 |
| Standard error | 1.2756 |
| Standard deviation of the slope | 0.4754 |
| Standard deviation of the intercept | 0.5600 |
| ANOVA test | |
| *F* calculated | 2.16 |
| *F* tabulated (0.05, 8, 20) | 4.00 |

The table shows that there are any experience that jointly provides the best work range, the best slope and the best correlation coefficient. Considering the benefit of each parameter, we selected experiment 3 because it has the highest work range. This enables samples of different acidities to be used without doing previous dilutions. We used the absolute response of the acidic species because its correlation coefficient was best, which led to lower uncertainty in the results. Figures of merit for the calibration model selected are shown in Table 4. The limit of detection (LOD) was calculated by taking into account the uncertainty of the regression line [42] with 95% confidence. RRMSC is the relative root mean square error of the calibration values and it was calculated to evaluate the accuracy of the curve calibration.

Five oil samples and four biodiesel samples were analysed in triplicate under the selected conditions (0.083 ml sample volume aspiration, 0.5 ml min$^{-1}$ flow rate, $2.7 \times 10^{-4}$ mol l$^{-1}$ indicator concentration and $7.5 \times 10^{-4}$ mol l$^{-1}$ NaOH concentration). After the acid area had been obtained for every sample, the concentration was calculated in mg l$^{-1}$ by interpolation in the calibration curve. Subsequently, the acidity was determined by expression 5. In Table 5 it is possible to observe the analysed samples, the experimental conditions for each sample, the results obtained from SIA-MCR-ALS in mg l$^{-1}$ and acidity values and the acidity values obtained from the official method.

The accuracy of the SIA-MCR-ALS method was validated by comparing its results with those obtained by the official titration method. We constructed a regression graph where the *Y* axis represented the results from the SIA method and the *X* axis represented the results from the official titration method. The regression parameters were 0.9591 for the slope and 0.0096 for the intercept. Using the elliptic joint confidence region (EJCR) test [42], statistical comparison was carried out. The critical value of the Snedecor–Fisher statistic at a 95% confidence level was 4. The *F*-value of 2.48 (20 and 8 freedom degrees) was obtained. This indicates that the point (1, 0) lies within the EJCR. Therefore, the ellipse includes the theoretically expected value of (1, 0), and the method is reliable.

**Table 5**
Acidity values in oil and biodiesel samples obtained by the Official method and the SIA-MCR-ALS method.

| Samples | Weight (g) | Acidity value (s) | | |
|---|---|---|---|---|
| | | SIA method (mg l$^{-1}$) | SIA method | Official method |
| Refined sunflower oil | 0.4150 | 1.577 | 0.095 (0.012) | 0.080 (0.007) |
| Refined soybean oil | 0.3752 | 1.681 | 0.112 (0.012) | 0.0827 (0.0009) |
| Crude soybean oil | 0.1773 | 4.589 | 0.647 (0.014) | 0.697 (0.001) |
| Refined palm oil | 0.2230 | 2.542 | 0.285 (0.009) | 0.270 (0.004) |
| Refined waste oil | 0.1081 | 5.06 | 1.17 (0.02) | 1.19 (0.03) |
| Sunflower biodiesel | 0.3680 | 1.62 | 0.11 (0.01) | 0.11 (0.01) |
| Soybean biodiesel | 0.6330 | 1.519 | 0.06 (0.01) | 0.06 (0.05) |
| Palm biodiesel | 0.2990 | 1.722 | 0.144 (0.031) | 0.158 (0.006) |
| Waste biodiesel | 0.3730 | 1.64 | 0.11 (0.02) | 0.12 (0.2) |

The volume of each sample was 25 ml.

## 4. Conclusions

A new concept of flow titration was proposed and demonstrated for the determination of total acidity in plant oils and biodiesel samples. The advantages of using this method are that automatization is easy and the amounts of sample, reagents and waste are reduced. It is also less time-consuming and it is not necessary to use reagents of known concentration.

The use of second-order chemometric techniques make it possible to obtain the concentration profiles without having to do any sample treatment, except dilution process, even if other absorbent species are present in the sample.

The acidity results for various oil and biodiesel samples are statistically comparable to those obtained by the official method.

It should be possible to adapt the proposed system to study other acids or other substances involving reactions that produce or consume acids or bases and other types of samples.

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