An alternative method for rapid quantitative analysis of majority cis–trans fatty acids by CZE

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Total Trans Fatty Acid Analysis in Spreadable Cheese by Capillary Zone Electrophoresis

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An alternative method for determination of total trans fatty acids expressed as elaidic acid by capillary zone electrophoresis (CZE) under indirect UV detection at 224 nm within an analysis time of 7.5 min was developed. The optimized running electrolyte includes 15.0 mmol L^{-1} KH_2PO_4/Na_2HPO_4 buffer (pH ~ 7.0), 4.0 mmol L^{-1} SDBS, 8.0 mmol L^{-1} Brij35, 45%v/v ACN, 8% methanol, and 1.5% v/v *n*-octanol. Baseline separation of the critical pair C18-9cis/C18:1-9t with a resolution higher than 1.5 was achieved using C15:0 as the internal standard. The optimum capillary electrophoresis (CE) conditions for the background electrolyte were established with the aid of Raman spectroscopy and experiments of a 3^2 factorial design. After response factor (R_F) calculations, the CE method was applied to total trans fatty acid (TTFA) analysis in a hydrogenated vegetable fat (HVF) sample, and compared with the American Oil Chemists' Society (AOCS) official method by gas chromatography (GC). The methods were compared with an independent sample *t* test, and no significant difference was found between CE and GC methods within the 95% confidence interval for six genuine replicates of TTFA analysis (*p*-value > 0.05). The CE method was applied to TTFA analysis in a spreadable cheese sample. Satisfactory results were obtained, indicating that the optimized methodology can be used for trans fatty acid determination for these samples.

KEYWORDS: Capillary electrophoresis; total trans fatty acid; spreadable cheese

INTRODUCTION

Fat consumption and its effects on human health have been the main area of research in nutrition. A considerable increase has been recently observed in the consumption of trans fats coming from industrial processes. Since the 1930s, the hydrogenation process of vegetable oils has become popular due to the emergence of margarine (*1*). The change in physical−chemical properties of oils allows them to be used as raw material for fat frying, margarine, shortenings, and spreadable cheese (a daily product manufactured in Brazil obtained by curd mass fusion, washing, and whey free, which is obtained by milk acid and/or enzymatic coagulation) (*2*). It uses hydrogenated vegetable fat (HVF) from soybean oil and/or cotton oil, and/or babassu oil, in order to improve consistency and to reduce production costs. Partially hydrogenated fats change plasma lipid levels in negative ways. They calcify cells and cause inflammation of the arteries, which are well-known risk factors for heart disease. Trans fats inhibit cyclooxygenase (COX-2), which is an enzyme that converts arachidonic acid into an eicosanoid which is necessary to prevent blood clots in the arteries and veins. A blood clot in the coronary arteries can result in sudden death (*3*). On the other hand, cis monounsaturated and polyunsaturated fatty acids have not been found to promote the development of cardiovascular disease (*4*, *5*). In the hydrogenation process, heated oil is exposed to hydrogen under pressure in the presence of a catalyst. The result of hydrogen addition of the fatty acid (FA) molecule is a change from liquid to a semisolid state, and greater oxidation stability (*5*). During this process, the linoleic acid (C18:2 cis-9, cis-12) results in oleic (C18:1, cis-9), elaidic (C18:1 trans-9), and stearic (C18:0) acids as main byproducts (*1*). The elaidic acid is the most common trans fatty acid (TFA) reported in nutrition tables, being responsible for 80−100% of total TFA contained in processed foods (*6*, *7*). Several studies have been carried out in the last few decades evaluating the effects of trans fatty acids on the body. However, only in 2003 the Brazilian nutritional food labeling was put in place enabling the control of trans fatty acids consumption by consumers (Brazil, Agência Nacional de Vigilância Sanitária - ANVISA - RDC 360/03). However, the information on trans fatty acids does not express the percentage of the daily value (% DV), since the recommendation of relevant organizations, such as the WHO, is that consumption should not exceed 1% of

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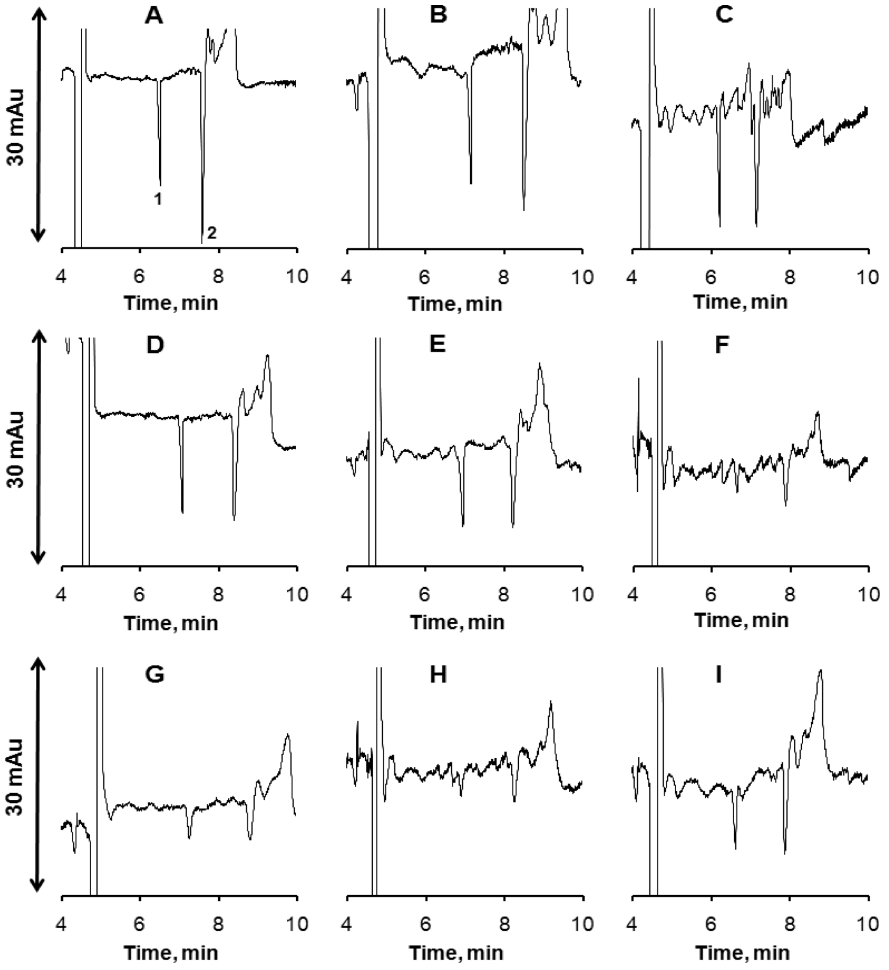

Figure 1. Standard fatty acid electropherograms of (1) C18:1 9t, (2) C13:0 (internal standard - IS), all with a concentration of 0.50 mmol L^{-1} using different buffers 15.0 mmol L^{-1}. (A) NaH_2PO_4/Na_2HPO_4, (B) KH_2PO_4/K_2HPO_4, (C) $NH_4H_2PO_4/(NH_4)_2HPO_4$, (D) KH_2PO_4/Na_2HPO_4, (E) $KH_2PO_4/(NH_4)_2HPO_4$, (F) $NaH_2PO_4/(NH_4)_2HPO_4$, (G) NaH_2PO_4/K_2HPO_4, (H) $NH_4H_2PO_4/Na_2HPO_4$, (I) $NH_4H_2PO_4/K_2HPO_4$. All electrolytes were added with 4.0 mmol L^{-1} of SDBS, 10.0 mmol L^{-1} Brij 35, 2.0% *n*-octanol, and 45.0% acetonitrile. Conditions of analysis: injection 5 s 12.5 mbar, voltage +28 kV, indirect detection at 224 nm and at 25 °C temperature inside the cartridge.

the caloric value of daily diet. In Brazil, there are no studies estimating the consumption of trans fatty acids (*8*). Therefore, the development of alternative analytical methods to quantify trans fat in food matrices has been the issue of intense research in order to improve quality monitoring of processed foods.

Common techniques used to determine TFA levels require total lipid extraction and/or methylation of the FA. Silver ions are used for TFA quantification in various chromatographic techniques, such as high performance liquid chromatography (Ag^+-HPLC) (*9*), thin-layer chromatography (Ag^+-TLC) (*10*), and solid phase extraction (Ag^+-SPE) (*11*). These measurements are based on the formation of a complex through the transfer of charge between silver *d* orbitals and π electrons of the FA double bonds. The separation of cis−trans homologues is based on the number of double bonds, position, and configuration (*12*). Fourier transform infrared (FT-IR) is a nondestructive technique widely used for TFA analysis, in which the vibrational mode at 966 cm^{-1} is considered. However, TFA quantification in a matrix whose trans content is less than 5% of the total fat is not possible with this technique. Furthermore, FT-IR does not provide details on the nature of the isomers, such as the length of the chain, or the insaturation number (*12*−*15*). Infrared methods with a second derivative (2D-ATR) allow for quantification of trans total fat content in matrices (*15*, *16*). TFA quantification by gas chromatography (GC) involves lipid phase extraction of the sample followed by transformation into fatty acid methyl esters (FAMEs) (*14*, *17*, *18*).

In the past decade, capillary electrophoresis (CE) has been used as an alternative technique for analysis of fatty acids in oils and fats (*19*−*21*). Long chain fatty acids (above 10 carbons) are weak acids with $pK_a \sim 5$. Therefore, the analysis is performed in counter-electroosmotic mode (electrophoretic and electroosmotic mobilities in opposite directions) by using alkaline buffers (the species are analyzed as anions), where fatty acids with longer chains elute first under indirect UV detection (*22*). The most common electrolyte systems are composed of buffers, chromophore agents (*p*-anisato (*23*), sodium dodecyl benzenesulfonate (SDBS) (*19*, *24*, *25*)), and other additives such as organic solvents (methanol (*23*), ethanol (*26*), ACN (*27*), 1-octanol (*19*, *27*)), surfactants (sodium dodecyl sulfate (SDS) (*28*), polyoxyethylene 23 lauryl ether (Brij 35) (*19*, *24*, *25*)), and cyclodextrins (*26*, *28*). However, based on the current analytical scenario, there is room for improvements allowing cis−trans fatty acid separations in food matrices by CE. Despite the importance of reported CE methodologies for trans fatty acids analysis, in general they presented resolution lower than 1.5 for the critical pair elaidic and oleic acids. Therefore, this work proposes the optimization of an alternative capillary zone electrophoresis (CZE) methodology under indirect UV detection at 224 nm for analysis of TTFA expressed in elaidic acid in HVF and Brazilian spreadable cheese

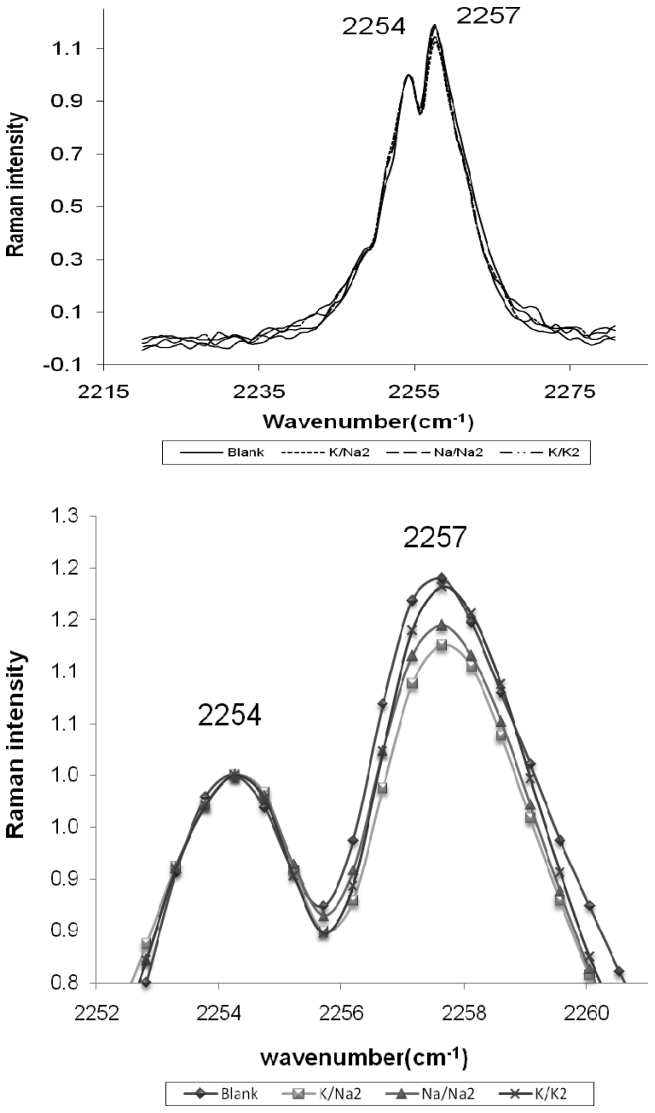

Figure 2. Raman spectra of the C≡N stretching mode of acetonitrile in different electrolyte solutions. Blank refers to the electrolyte formed by all the additives listed in the text, except for phosphate buffer.

with an analysis time of 8.0 min and resolution of 1.50 for the critical pair of elaidic and oleic acids.

MATERIAL AND METHODS

Materials. All reagents used were analytical grade, and water was purified by deionization (Milli-Q system; Millipore, Bedford, MA, USA). Methanol (MeOH), ethanol (EtOH) and acetonitrile (ACN) were purchased from Vetec (Rio de Janeiro, Brazil); and 1-octanol was purchased from Fluka (Germany). Polyoxyethylene 23 lauryl ether (Brij 35) and sodium dodecyl benzenesulfonate (SDBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fatty acid methyl esters (FAME) was 13:0 purchased from Sigma-Aldrich (St. Louis, MO, USA). Monobasic sodium phosphate (NaH_2PO_4), dibasic sodium phosphate (Na_2HPO_4), monobasic potassium phosphate (KH_2PO_4), dibasic potassium phosphate (K_2HPO_4), monobasic ammonium phosphate ($NH_4H_2PO_4$) and dibasic ammonium phosphate ($(NH_4)_2HPO_4$) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Standard pentadecanoic (C15:0), palmitic (C16:0), stearic (C18:0), oleic (C18:1 9c), and elaidic acids (C18:1 9t) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Individual 20.0 mmol L^{-1} fatty acid stock solutions were prepared by dissolving appropriate amounts of the selected standards in methanol, and they were stored in a freezer until analysis. A mixture of all standards was prepared at 0.5 mmol L^{-1} each by appropriate dilution in MeOH.

Vegetable fat samples were acquired from the product manufacturer, and spreadable cheese was purchased from Vereda Alimentos Ltda.

Table 1. 3^2 Factorial Design Matrix with Genuine Triplicate in the Central Point and Responses[a]

experiments	1	2	3	4	5	6	7	8	9	10	11
BRIJ35 (mM)	−1	−1	−1	0	0	0	1	1	1	0	0
n-octanol (%)	−1	0	1	−1	0	1	−1	0	1	0	0
N/*R* (10^4)	101.5	69.8	67.8	29.3	32.3	54.8	20.8	32.2	0.0	27.1	28.6

[a] Brij 35: (−1) 8.0 mmol L^{-1}, (0) 10.0 mmol L^{-1}, (1) 12.0 mmol L^{-1}, *n*-octanol: (−1) 1.5%, (0) 2.0%, (1) 2.5%.

Methods. Aqueous Brij 35 stock solution was prepared by weighing and dissolving mass corresponding to 50.0 mmol L^{-1} in volumetric flask of 100.0 mL. Mass corresponding to 0.5 mol L^{-1} of sodium hydroxide (NaOH) was weighed and dissolved in a volumetric flask of 100.0 mL, and the volume was completed with MeOH. Aqueous SDBS stock solution was prepared by weighing and dissolving mass corresponding to 100.0 mmol L^{-1} in a volumetric flask of 100.0 mL. Aqueous buffer stock solutions at 100.0 mmol L^{-1} used in the present work were prepared as follows: 1- Mass corresponding to 50.0 mmol L^{-1} NaH_2PO_4, and 50.0 mmol L^{-1} Na_2HPO_4, were weighed and dissolved in volumetric flask of 250.0 mL. 2- Mass corresponding to 50.0 mmol L^{-1} KH_2PO_4, and 50.0 mmol L^{-1} K_2HPO_4, were weighed and dissolved in volumetric flask of 250.0 mL. 3- Mass corresponding to 50.0 mmol L^{-1} $NH_4H_2PO_4$, and 50.0 mmol L^{-1} $(NH_4)_2HPO_4$, were weighed and dissolved in volumetric flask of 250.0 mL. (4) Mass corresponding to 50.0 mmol L^{-1} KH_2PO_4 and 50.0 mmol L^{-1} Na_2HPO_4 was weighed and dissolved in a volumetric flask of 250.0 mL. (5) Mass corresponding to 50.0 mmol L^{-1} KH_2PO_4 and 50.0 mmol L^{-1} $(NH_4)_2HPO_4$ was weighed and dissolved in a volumetric flask of 250.0 mL. (6) Mass corresponding to 50.0 mmol L^{-1} NaH_2PO_4 and 50.0 mmol L^{-1} $(NH_4)_2HPO_4$ were weighed and dissolved in a volumetric flask of 250.0 mL. (7) Mass corresponding to 50.0 mmol L^{-1} NaH_2PO_4 and 50.0 mmol L^{-1} K_2HPO_4 was weighed and dissolved in a volumetric flask of 250.0 mL. (8) Mass corresponding to 50.0 mmol L^{-1} $NH_4H_2PO_4$ and 50.0 mmol L^{-1} Na_2HPO_4 was weighed and dissolved in a volumetric flask of 250.0 mL. (9) Mass corresponding to 50.0 mmol L^{-1} $NH_4H_2PO_4$ and 50.0 mmol L^{-1} K_2HPO_4 was weighed and dissolved in a volumetric flask of 250.0 mL. Phosphate buffers and Brij 35 stock solutions were kept in a freezer to prevent mold formation. The working electrolyte solution was prepared by appropriate dilution of stocks and incorporation of solvents.

The experiments of separation optimization were performed in a capillary electrophoresis system (HP3d CE, Agilent Technologies, Palo Alto, USA) equipped with a DAD set at 224 nm, a temperature control device maintained at 25 °C, and data acquisition and treatment software (HP ChemStation, rev A.06.01). Samples were injected hydrodynamically (12.5 mbar 5 s) and the electrophoretic system was operated under normal polarity and constant voltage conditions of +28 kV. In all experiments, a fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) 48.5 cm (40 cm effective length) × 75 μm ID × 375 μm OD was used. Two centimeters of the tip of the capillary was removed to avoid adsorption problems (*29*).

FAMEs, prepared by means of the Hartman and Lago (*30*) procedure, were analyzed by GC on a Shimadzu gas chromatograph (GC 17A model), with a flame ionization detector (FID), using a capillary fused silica column with a cyano propyl polysiloxane stationary phase (CP-7420^{TM}, 100 m × 0.25 mm id, 0.25 μm film thickness, Varian, USA). The chromatographic conditions were those established in the AOCS Ce 1 h-05 method (*31*): isothermal column temperature at 180 °C, injector and detector temperature at 250 °C, carrier gas is hydrogen, and column pressure at 170 kPa. The compounds were identified by standard coinjection and relative retention time to FAME 13:0 (internal standard). Appropriate response factors were employed to convert area percent of FAME into true weight percent. The correct response for each FAME was calculated theoretically and expressed in terms of the methyl palmitate response (*32*). Fatty acids were determined by FAME 13:0 addition as internal standard and expressed in g/100 g of sample. The method precision was evaluated by relative standard deviation (%RSD) (six genuine replicates).

Fourier transform Raman spectroscopy was carried out using a Bruker RFS 100 instrument, equipped with a Nd^{3+}/YAG laser operating at 1064 nm and CCD detector cooled with liquid N_2. Good signal-to-noise

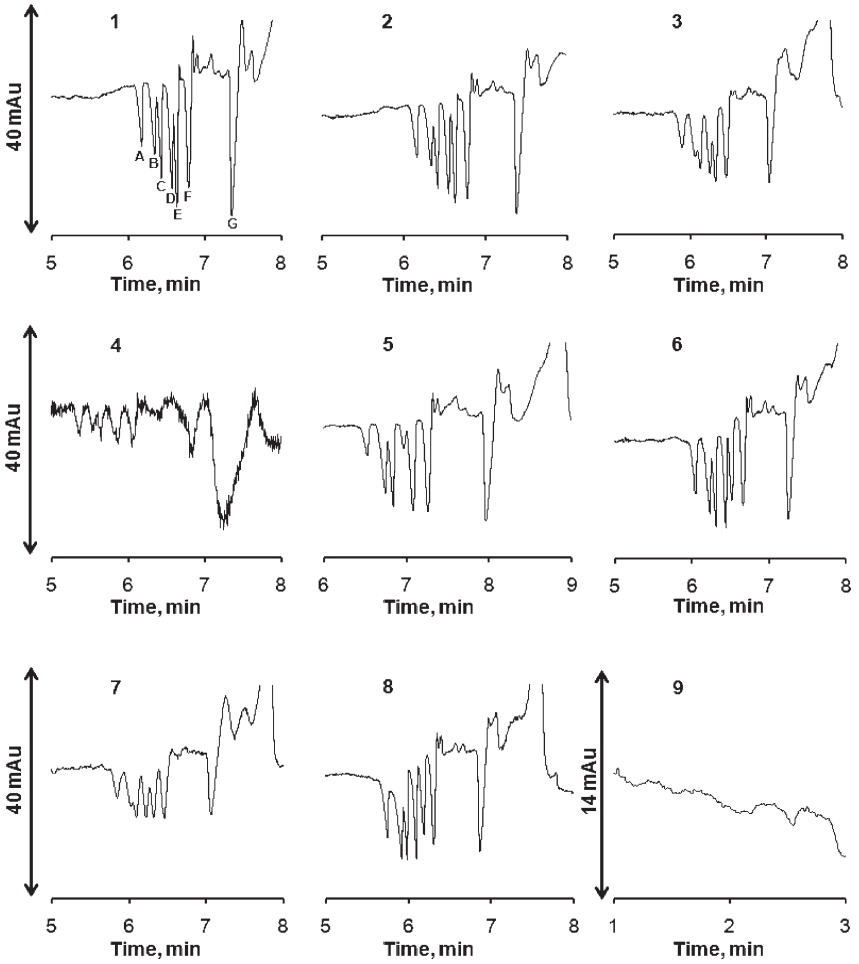

Figure 3. Standard electropherogram 0.5 mmol L^{-1} (A) C18:0, (B) C18:1 9t, (C) C18:1 9c, (D) C16:0, (E) C18:2 cc, (F) C15:0, (G) C13:0. Electrolytes consisted of 15.0 mmol L^{-1} KH_2PO_4/Na_2HPO_4; SDBS 4.0 mmol L^{-1}, acetonitrile 45% v/v. Concentration of Brij 35 and *n*-octanol proportion according to experimental design. Analysis conditions of injection 5 s, 12.5 mbar, voltage +28 kV, indirect detection at 224 nm, and at 25 °C temperature inside the cartridge.

ratios were obtained from 1000 scans accumulated over a period of about 30 min and 100 mW of laser power, using 4 cm^{-1} as spectral resolution.

When a new capillary was used, it was conditioned by a pressure flush of 1.0 mol L^{-1} NaOH solution (30 min), deionized water (5 min), and electrolyte solution (10 min). In between runs, the capillary was replenished with 0.2 mol L^{-1} NaOH solutions (2 min), deionized water (2 min), and fresh electrolyte solution (3 min, pressure flush). This conditioning procedure was found to be critical to ensure peak area and migration time reproductibility, and to prevent deleterious solute adsorption to the capillary wall (*29*).

Approximately 250 mg of vegetable fat and 400 mg of spreadable cheese samples were saponified separately with 2 and 3.0 mL, respectively, of a methanolic NaOH solution (0.5 mol L^{-1}) at 75−80 °C for 25 min by an aqueous heating bath. Before injection, samples were diluted in methanol 1:25 and 1:5, respectively, for CE analysis. Fatty acid methyl esters (FAMEs), prepared by means of the Hartman and Lago (*30*) procedure, were used for GC analysis.

Statistical Analysis. Microsoft Excel and SPSS 8.0 for windows statistical program were used for data processing. Shapiro−Wilk normality test, test F, independent sample *t* test, priori test hypothesis, multiple regression, least-squares method, ANOVA, and surface response methodology were used for statistical evaluation.

RESULTS AND DISCUSSION

Preliminary Study. Preliminary studies were performed with different electrolytes based on the following salt and acid buffers: (1) NaH_2PO_4/Na_2HPO_4; (2) KH_2PO_4/K_2HPO_4; (3) $NH_4H_2PO_4/(NH_4)_2HPO_4$; (4) KH_2PO_4/Na_2HPO_4; (5) $KH_2PO_4/(NH_4)_2HPO_4$, (6) $NaH_2PO_4/(NH_4)_2HPO_4$, (7) NaH_2PO_4/K_2HPO_4, (8) $NH_4H_2PO_4/Na_2HPO_4$, (9) $NH_4H_2PO_4/K_2HPO_4$. In all these electrolytes, the concentration was 15.0 mmol L^{-1} with 4.0 mmol L^{-1} of SDBS, 10.0 mmol L^{-1} of Brij 35, 2.0% of *n*-octanol, and 45.0% of acetonitrile (*19*). The investigation was carried out in order to evaluate the behavior of different sets of cations as alternative counterions for phosphate buffer. The analysis of CE measurements of these nine different buffers indicated that electropherograms A, B, and D in **Figure 1** are satisfactory upon consideration of variables such as resolution between the critical pair C18:1cis-9/C18:1trans-9, noise, and baseline stability.

Raman spectroscopy investigation was carried out for buffers in experiments A, B, and D of **Figure 1** in order to understand the cation effect on the separation profile. The Raman band corresponding to the C≡N stretching mode is observed at 2254 cm^{-1} in pure acetonitrile. In acetonitrile/water mixtures, another band at a higher frequency is observed, which becomes stronger with an increasing amount of water, and a double peaked pattern is clearly seen for the molar fraction of acetonitrile below ca. 0.5 (see Figure 3 in ref *33*). The cation effect on the microenvironment formation is revealed by the Raman spectra shown in **Figure 2**. The spectra have been normalized by the intensity of the 2254 cm^{-1} band, so that the relative intensity of the high frequency component changes with the electrolyte. The high frequency component (2257 cm^{-1}) is assigned to acetonitrile molecules bonded to water molecules (*33*), so that the enhancement of the ACN band in the water molecule neighborhood can be compared among the electrolytes. Therefore, changes in the intensity

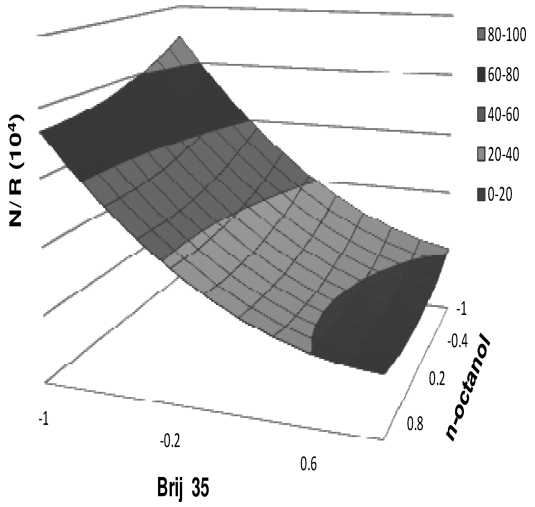

Figure 4. Factorial response surface for 3^2 experiment design.

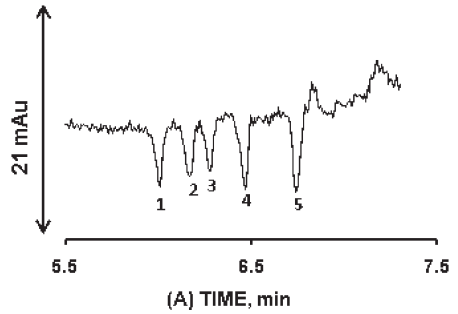

Figure 5. Standard fatty acid electropherograms of (1) C18:0, (2) C18:1 9t, (3) C18:1 9c, (4) C16:0, (5) C15:0 (IS), all with a concentration of 0.50 mmol L^{-1}. Operational conditions: injection 5 s, 12.5 mbar, +28 kV applied voltage, 25 °C cartridge temperature and indirect detection at 224 nm. Electrolyte: 15 mmol L^{-1} KH_2PO_4/Na_2HPO_4 at pH 6.86, 4 mmol L^{-1} SDBS, 8 mmol L^{-1} Brij 35, 45% v/v ACN, 8% v/v MeOH, and 1.5% *n*-octanol.

corresponding to the water molecule's neighborhood indicate more organic solvent incorporation in the running electrolyte, which in turn can improve the critical pair C18:1 9t/C18:1 9c. The results indicate that KH_2PO_4/Na_2HPO_4 buffer should be the most appropriate electrolyte for producing a less intense acetonitrile CN band in the water molecule's neighborhood, that is, more organic solvent incorporation in the running electrolyte.

Electrolyte Optimization. A design of experiment (DOE) was performed in order to optimize the critical pair C18-9cis/C18:1-9t separation using KH_2PO_4/Na_2HPO_4. The selected variables were Brij35 and *n*-octanol, which present a positive effect for cis−trans separation as suggested by Oliveira et al. (*19*). Fatty acid standards of C18:0, C18:1 9t, C18:1 9c, C16:0, C18:2 cc, C15:0, and C13:0 were used for the separation performance study. C15:0 and C13:0 were investigated as possible internal standards (IS) because they are not common in real samples. Other variables such as voltage, cartridge temperature, capillary dimensions, wavelength, and other electrolyte constituents were kept constant. **Table 1** shows a complete factorial design matrix of 3 levels and 2 factors (3^2) with a genuine triplicate in the central point (*34*), and a response for proportion between theoretical plate number (*N*) for stearic acid and normalized noise (*R*) (proportion between signal standard deviation and signal mean). **Figure 3** shows electropherograms obtained from nine experiments.

Table 2. Results of a Priori Hypothesis Test

$$Y\ (\pm 0.0.25) = 0.642(\pm 0.023)\ [C18:1t]/[C15:0] + 0.003(\pm 0.026)$$

$$R^2 = 0.9841$$

[C18:1t]/[C15:0]	signal 1st replicate	signal 2nd replicate	signal 3rd replicate
0.6	0.392	0.380	0.382
0.8	0.496	0.525	0.489
1	0.649	0.686	0.670
1.2	0.760	0.790	0.821
1.4	0.901	0.868	0.882

Table 3. Statistical Analysis of the TTFA Quantification by GC and CE[a]

sample (genuine replicate)	GC method (%m/m)	CE method (%m/m)
1	27.51	29.0
2	27.85	31.12
3	27.44	29.84
4	29.51	28.57
5	30.39	25.52
6	31.4	27.88
medium	*29.02*	*28.65*
standard deviation	*1.67*	*1.90*

[a] Normality test *p*-values (Shapiro−Wilk test): 0.25 for GC and 0.92 for CE. Test F *p*-value: 1.29. Independent sample *t* test *p*-value: 2.23.

In **Table 1**, the *n*-octanol variation level (−1,0,+1) for fixed Brij35 values in the low level (−1,−1,−1) or high level (+1,+1,+1) indicates that noise increases, and the theoretical plate number decreases, when Brij35 passes from low (experiments 1, 2, and 3) to high level (experiments 7, 8, and 9). Concerning *n*-octanol level variations (−1,0,+1) within the intermediary Brij35 level (0,0,0), no significant variations were found. However, interaction between Brij35 and *n*-octanol is always negative, because major C18:0 peak enlargement and minor pair cis−trans resolution occur when the factors are in levels +1 and −1 alternately (experiments 3 and 7). Moreover, when Brij35 and *n*-octanol were kept at high levels (experiment 9), microbubble formation and current drop occurred, making the experiment impracticable, so that zero response was assigned to it. Therefore, the highest response was obtained when Brij35 and *n*-octanol were simultaneously kept at low levels. Finally, after the electropherogram profile was analyzed, C15:0 was selected as the IS proper for more suitable features.

After multiple regression through the least-squares method was performed, the model $y = 32.7(\pm 1.4) - 31.0(\pm 1.1)x_1 - 4.8(\pm 1.1)x_2 + 13.1(\pm 1.7)x_1^2 + 4.2(\pm 1.7)x_2^2 + 3.1(\pm 1.3)x_1x_2$ was obtained. The analysis of variance was carried out and, as the calculated *F* value (70.29) was minor compared to the tabled *F* value (99.16), a lack of fit evidence in the 99% significance interval was not found. Moreover, the results indicate Brij35 (variable x_1) as the most significant factor for the response considered. The response surface obtained from experiments is shown in **Figure 4**, which indicates a region with a major response for Brij35 at a concentration of 8.0 mmol L^{-1} and 1.5% *n*-octanol.

After Brij35 and *n*-octanol optimization, the aim of separation was not fully achieved because critical pair C18-9cis/C18:1-9t resolution equal to or higher than 1.5 was not obtained. Therefore, based on the Raman study, the next step was to carry out tests adding ethanol and methanol in the optimized electrolyte, taking into account features such as dilution effect and polarity. Different ethanol amounts in the electrolyte resulted in peak enlargement and noise increase (not shown). However, after some

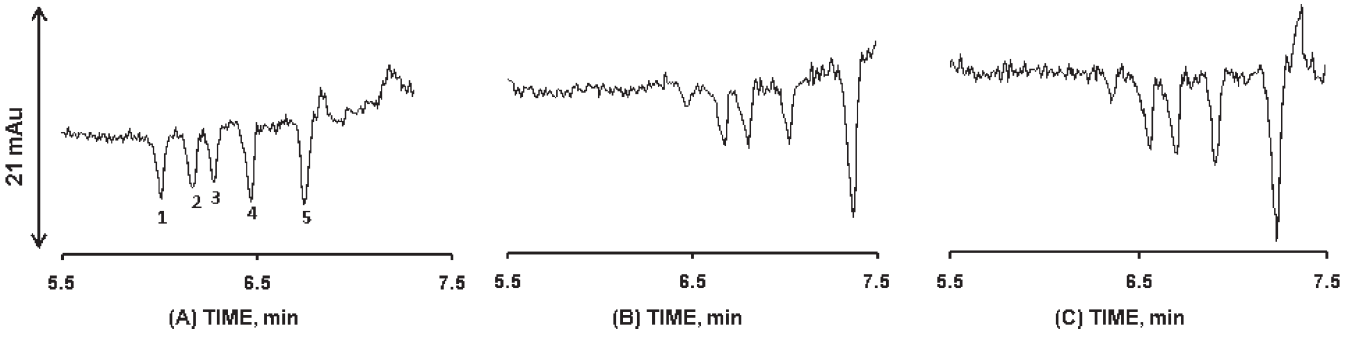

Figure 6. (A) Standard fatty acid electropherogram of (1) C18:0, (2) C18:1 9t, (3) C18:1 9c, (4) C16:0, (5) C15:0 (PI), all with a concentration of 0.50 mmol L^{-1}. (B) GVH sample electropherogram, (C) spreadable cheese sample electropherogram. Operational conditions: injection 5 s. 12.5 mbar, +28Kv applied voltage, 25 °C cartridge temperature and indirect detection at 224 nm. Electrolyte: 15 mmol L^{-1} KH_2PO_4/Na_2HPO_4 at pH 6.86, 4 mmol L^{-1} SDBS, 8 mmol L^{-1} Brij 35, 45% v/v ACN, 8% v/v MeOH, and 1.5% *n*-octanol.

tests, the addition of 8% methanol in the running electrolyte consisting of 15.0 mmol L^{-1} KH_2PO_4/Na_2HPO_4, 4.0 mmol L^{-1} SDBS, 8.0 mmol L^{-1} Brij35, 45% v/v ACN, and 1.5% v/v *n*-octanol allowed baseline separation of the critical pair C18-9cis/C18:1-9t with a resolution higher than 1.5. **Figure 5** shows the electropherogram obtained for the optimized condition.

Response Factor (R_F) Calculation and TTFA Determination. The proposal of trans fatty acid quantification in the spreadable cheese sample was based on a statistical study including the response factor (R_F) calculation by using C15:0 as the internal standard (IS) (*29*). In order to calculate R_F, a random experiment in genuine triplicate using an elaidic acid standard solution with varying concentrations at 0.30, 0.40, 0.50, 0.60, and 0.70 mmol L^{-1}, and fixed pentadecanoic acid (IS) concentration at 0.5 mmol L^{-1}, was performed by means of the least-squares method (LSM) through a priori test hypothesis (eq **1**). **Table 2** shows the values used in the regression model. The regression model diagnosis (*35*, *36*) was satisfactory with no lack of fit because the value of $F_{0.05,\ 3;\ 10}$ calculated (3.46) was lower than $F_{0.05,\ 3;\ 10}$ critical (3.71), and regression significance was higher ($F_{sign} = 637.73$).

$$F_{\text{calculado}} = \frac{s^2_{y,x}}{s^2_y} = \frac{\sum_{i=1}^{p} m_i(\bar{y}_i - \hat{y}_i)^2/(p-2)}{\sum_{i=1}^{p}\sum_{j=h}^{m_i}(y_{ij} - \bar{y}_i)^2/(m-p)} \quad (1)$$

The quantification procedure involved the calculation of R_F as described by the following mathematical expression:

$$\frac{A_{C18:1t}}{[C18:1t]} = R_f \frac{A_{C15:0}}{[C15:0]} \quad (2)$$

where $A_{C18:1t}$ is the elaidic acid area, $A_{C15:0}$ is the pentadecanoic acid area, [C18:1t] is the elaidic acid concentration in mmol L^{-1}, and [C14:0] is the pentadecanoic acid concentration at 0.5 mmol L^{-1}.

Since the regression model diagnosis was satisfactory, the slope can be used as a response factor in eq **1**, and as long as the internal standard C15:0 at 0.5 mmol L^{-1} is used, the concentration of C18:1t remains hidden. TTFA percentage in the sample was carried out through eq **3**, obtained after rearranging eq **2**:

$$\%\text{trans} = \frac{A_{C18:1t} \cdot [C_{15:0}] \cdot v \cdot MW_{C18:1t}}{R_f \cdot A_{C15:0} \cdot m} \cdot 100 \quad (3)$$

where $A_{C18:1t}$ is the area of trans fatty acids in the sample, V is the volume in liters, $A_{C15:0}$ is the internal standard area, m is the sample mass in milligrams, R_F is the response factor (fitted model slope), [C15:0] is IS concentration at 0.5 mmol L^{-1}, and $MW_{c18:1t}$ is C18:1t molecular weight.

Sample Analysis. In order to evaluate the performance of the optimized methodology in real samples, TTFA concentration (expressed as elaidic acid) was determined in HVF and spreadable cheese. In case of HVF, the CE method was compared with the AOCS GC method (*31*) in six genuine replicates. **Table 3** shows statistical results (Shapiro−Wilk normality test, test F, and independent sample *t* test) for CE and GC, where no evidence of significant differences between the two methodologies was observed in the 95% confidence interval (*p*-value > 0.05) (*37*). Thus, the CE method was applied for the analysis of spreadable cheese sample, in which 3.89 (±0.08) % m/m of TTFA has been obtained on average of authentic duplicates. **Figure 6** shows electropherograms obtained from HVF and spreadable cheese samples. The results are satisfactory, indicating that the optimized methodology can be successfully used for TTFA determination in these samples.

Summing up, the method was successfully applied for the analysis of total trans fatty acid expressed in elaidic acid in HVF and spreadable cheese without a derivatization step during sample preparation. It has been shown that the methodology is suitable for monitoring trans fatty acids ("screening") in HVF and spreadable cheese samples. Important advantages of the method are the simple sample preparation procedure, simple running electrolyte, short analysis time (about 7.5 min), and low cost.

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