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Optimization in sample stacking for the measurement of short chain organic acids in serum of natural rubber latex by capillary electrophoresis

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Abstract

It is of interest to measure the short chain organic acid content in natural latex serum, and capillary electrophoresis (CE) has proved to be a good tool for this study. However, for some acids, a higher sensitivity than that provided by the standard methods was needed. Therefore, a stacking effect was optimised and applied both for standards and real samples. Large-volume injection of sample solutions prepared in low conductivity matrices containing 50% acetonitrile (v/v) and 0.5% NaCl (w/v) gave the best results with enhancement factors over 17 and recoveries ranging from 83 ± 14 to 122 ± 4%.

Keywords: Stacking; Organic acids; Capillary electrophoresis; Latex; Quantification

1. Introduction

During our previous work on the development and validation of a capillary electrophoresis (CE) method for the measurement of short chain organic acids in serum of natural latex [1], we could observe the normal stacking mode (NSM) on the analytes when the serum was obtained by freezing the latex instead of by adding concentrated phosphoric acid which provides an increase of ionic strength in the samples.

Through the application of the method developed to real samples, we could see that lower limit of quantification (LOQ) were needed for some of the analytes (mainly formic and fumaric acids) and we decided to investigate different ways of stacking useful for this type of real samples, applicable to conventional CE equipment and bearing in mind the quantitative aspects.

Different authors have previously pointed out the need for the application of stacking effect to real samples and to perform reproducibility or validation studies [2,3]. Quantitative data, such as R.S.D. values of repeated injections or calibration graphs, are reported for the analysis of phenolic compounds in standards, opiate drugs in hair, and three anions (bromide, nitrate, bromate) in water [4]. Yang et al. reported R.S.D.

Abbreviations: AcN, acetonitrile; AcN–NaCl, acetonitrile–NaCl mixture; BGE, background electrolyte; C_{inj}, injected solute concentration; CTAB, cetyltrimethylammonium bromide; EOF, electroosmotic flow; FASS, field-amplified sample stacking; IC, ionic chromatography; ITP, isotacophoresis; KRF, Kohlrausch regulation function; LOQ, limit of quantification; LVSS, large-volume sample stacking; NSM, normal stacking mode; S_c, corrected peak area; Spl, sample; Std, standard

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data for migration time and peak area for three anions (chloride, nitrate and sulphate) in snow samples. Only two samples were compared with IC and data were found consistent, but no statistical test was applied. Nevertheless, few works have been found with accuracy studies. A validated assay for the determination of the anti-arrhythmic amiodaron and its main metabolites in human serum has been described [5]. Authors say that because of the variations in peak size obtained in head-column field-amplified sample stacking (FASS) and in extraction, typical intra- and inter-day imprecisions observed with external calibration were 10–20% and this is not to be recommended.

Several reviews [6–10] describe the existing stacking methods. Related to organic acids, He and Lee have described the stacking of maleic and fumaric acids and bromide and nitrate anions by large-volume sample stacking (LVSS) at acidic pH [11], but these conditions do not permit the resolution of all the organic acids present in the latex samples. The pH-mediated stacking has been applied to four phenolic acids in a physiological sample [12]. Field-amplified stacking injection has been applied for the analysis of released methacrylic acid from dental composites [13].

The objective of the present work was the optimization of injection conditions to obtain sample stacking for the measurement of short chain organic acids in serum of natural rubber latex by CE including the precision and accuracy evaluation of the method.

2. Experimental

2.1. Instrumentation

The separation was performed on a capillary electrophoresis P/ACE 5500 (Beckman) with UV detection at 200 nm. The injection was by pressure (0.035 bar) for 5 s or different intervals when indicated. The separation was carried out with an uncoated fused-silica capillary (57 cm × 50 μm i.d.) and was operated at −10 kV potential. Temperature was maintained at 25 °C. The background electrolyte was prepared with 0.5 M H_3PO_4, 0.5 mM cetyltrimethylammonium bromide (CTAB), as cationic surfactant to avoid electroosmotic flow (EOF), and pH adjusted by adding NaOH to 6.25.

Capillary was flushed between runs with 0.1 M NaOH for 3 min, H_2O for 3 min and the background electrolyte for 5 min.

2.2. Chemicals

Standards were obtained from Sigma (St. Louis, MO, USA). Phosphoric acid 85% was from Merck (Darmstadt, Germany), sodium hydroxide from Panreac (Madrid, Spain) and organic solvents from Scharlau (Barcelona, Spain).

2.3. Sample preparation

Natural rubber latex samples, directly imported, came from Thailand and were double centrifuged latex. They were kindly provided by Tecnilatex S.A. and they were representative of commercial material.

For coagulation to obtain the serum, latex samples were simply kept at −20 °C overnight. The following day, after samples reached room temperature, the serum was filtered and measured after being diluted as is explained in each experiment.

2.4. Injection linearity

In the first instance, the injection linearity was tested by injecting separately the standard and the sample during 5, 10, 15, 25, 35, 45 and 55 s. A stock standard containing: 0.25 mM nitrate, 0.5 mM oxalate, 4 mM formate, 0.25 mM fumarate, 0.25 mM aconitate, 2.0 mM succinate, 2.0 mM malate, 2.0 mM glutarate, 2.0 mM citrate, 3.0 mM acetate, 3.0 mM glycollate, 2.0 mM propionate and 2.0 mM quinate was kept at −4 °C and on the day of the assay it was diluted 1:1 (v/v) with water to prepare the working standard.

2.5. Stacking effect

Three sample matrices were quantitatively tested for their stacking effect: water, aqueous acetonitrile, and aqueous acetonitrile containing sodium chloride (AcN–NaCl). For this purpose the stock standard and the sample were diluted with purified water 1:1 (v/v) and measured with 5 s of injection time each, the same as in the reference method. Then, the stock standard and the sample were diluted with purified water 1:0.25, 1:1.5, 1:4 (v/v) and again each one of these was diluted

by mixing 1:1 (v/v) with water, AcN or AcN–NaCl. In this way the final dilution with relation to the reference were 2.5, 5 and 10 times higher. These solutions were injected for 12.5, 25 and 50 s, respectively, and therefore, the amount of analyte inside the capillary was constant.

2.6. Optimization of acetonitrile and sodium chloride proportion

The proportion of acetonitrile and sodium chloride employed in the matrices was optimised. Factorial designs are experimental designs involving simultaneous alteration of all parameters according to a predefined matrix of experiments. They are well adapted to the determination of the relative importance of each variable in comparison to the estimated responses. In this study, an experimental factorial design with two quantitative factors (acetonitrile and sodium chloride percentages) and three levels was evaluated to optimise the proportions of these components. For acetonitrile the levels were 45, 55 and 65% (v/v) and 0.5, 1 and 2% (w/v) for sodium chloride. It was applied both in standards and samples with a final dilution of the analytes corresponding to 1:5 (v/v) in each case and injected for 25 s. Analysis of these response factors, determined by the peak area for each acid, was carried out by Statgraphic® plus for Windows 4.1 (Rockville, MD, USA) program.

2.7. Accuracy and precision

For the evaluation of the accuracy of the method five independent samples coming from the same pool were diluted 1:1 (v/v) with water, following the standard method, and injected in duplicate for 5 s. Samples were quantified with the corresponding external standards with the same dilution and injection time. These values were considered to be the right value, because the method was previously validated. Another five samples coming from the same pool were processed as corresponding to the best conditions obtained in the assay, that is diluted 1:1.5 (v/v) with 1% NaCl in water (w/v) and then 1:1 (v/v) with acetonitrile and injected for 25 s. They were quantified with the corresponding external standards treated in the same way. Accuracy was evaluated as percentage recovery and precision with R.S.D. (%). The same experiment was repeated on a second day and all the values were pooled to obtain the intermediate precision.

2.8. Electroosmotic flow (EOF)

EOF was measured as Williams and Vigh [14] broadly described. The method relies on the accurate determination of both the differential spacing of the solute bands and the mobilization velocity. Measurements were performed in triplicate and CTAB ranged from 0 to 1.2 mM, while all the other components in the buffer remained constant.

3. Results and discussion

3.1. Injection linearity

In a preliminary assay injection linearity was tested for standards and samples, as described above, and a linear correlation was found for all the organic acids in both cases with correlation coefficients over 0.99. Nevertheless, at 15 s of injection time, malic and citric acids saturated the detector and then, oxalic and formic peaks overlapped. Therefore, only increasing the injection time was not a solution to increasing sensitivity.

3.2. Stacking effect

Different ways of single-column stacking were tentatively tested and discarded prior to the assay, mainly those related to electrokinetic injection, with or without a short plug of water first injected hydrodynamically in the capillary, FASS. Focusing of all sample ions did not occur to the same extent in electrokinetic injection and it could be observed that sample conductivity greatly affected accuracy and precision. Moreover, the approach of adding a constant amount of a non-interfering anion to normalize the ionic strength of both standards and samples is not attractive for samples of high ionic strength such as latex serum.

In sample stacking with hydrodynamic injection, long injections of sample solutions prepared in low conductivity matrices or water (LVSS) are better in order to maximise the obtainable sensitivity enhancement [2]. The stacking procedures bring the accumulation of trace analytes from large volumes of a sample injected into a narrow band (stack),

which then serves as an "ideal" sample for further electrophoretic separation.

Several sample matrices were tested; namely, pure water, aqueous acetonitrile, and aqueous acetonitrile containing high concentrations of sodium chloride.

In order to have a constant amount of analytes in the capillary and therefore to be able to evaluate the actual increase in signal, standards and samples were increasingly diluted in the same proportion as the injection was increased. Nevertheless, it is only true when comparing water; this is because the presence of acetonitrile modifies the viscosity and therefore, if a different volume were injected with the same time and pressure it would make comparisons difficult.

Fig. 1 shows the results obtained in standards and samples with the three matrices described above in Section 2 (stacking effect) for one of the organic acids giving the smaller peaks, formic acid. As can be observed, the area increase was higher in standards than in samples in all the cases giving acetonitrile, alone or with NaCl, better results than water, both in standards and samples. Acetonitrile, besides inducing stacking, has the advantage of removing serum proteins, which may be an interesting effect mainly in biological samples.

Instability of the current and, eventually, loss of current for longer injections were observed. This may be caused by the change in the charge of the capillary wall where the sample is found, because the CTAB provided only in a dynamic coating, and so 25 s was established as an acceptable injection time.

The exact focusing mechanism of acetonitrile stacking is unknown and could probably be due to the change in electrophoretic velocity caused by the change in viscosity as the sample ions move from the acetonitrile zone to the BGS zone [6]. Shihabi, the first to introduce this mode of stacking, proposed two factors [15]. In the absence of salts a limited stacking occurs due to the low conductivity of acetonitrile. However, when salts are added to the sample a further stacking occurs due to a different and more complex mechanism. Because of the limited solubility of inorganic ions in acetonitrile, they migrate rapidly with some water, leaving behind a more concentrated and narrower segment of acetonitrile. Weakly ionized organic compounds migrate, briefly, behind in the acetonitrile zone. The mechanism behind stacking acetonitrile-salt is similar to that of transient isotacophoresis (ITP). In a recent work [16], the same author states that acetonitrile can function as a terminating ion in transient isotacophoresis. It is suggested that this method could be termed transient "pseudo-isotacophoresis" (pseudo-ITP). Salts, when present in such samples act briefly as leading ions, migrating rapidly in the organic solvent until they are slowed at the interface of the separation buffer.

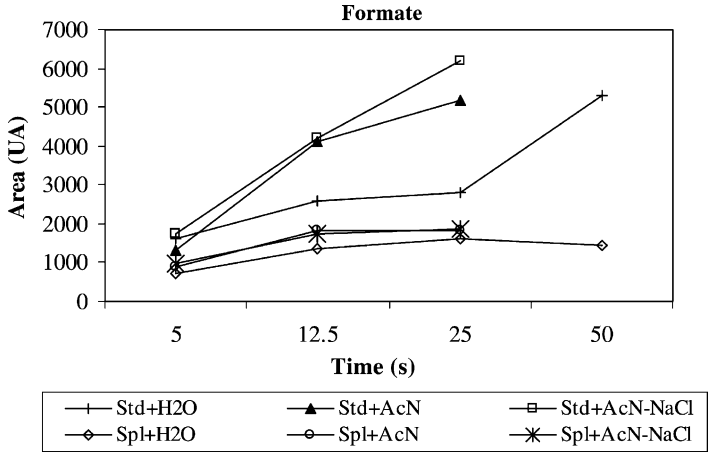

Fig. 1. Peak enhancement for formate. The stock standard and the sample were diluted with purified water 1:0.25, 1:1.5, 1:4 (v/v) and again each one of these was diluted 1:1 (v/v) with water, acetonitrile or acetonitrile and sodium chloride. These solutions were injected for 12.5, 25 and 50 s, respectively. For CE conditions, see the text. Std, standard; Spl, sample.

The fundamental principle of stacking is adjustment of concentration in accordance with the KRF. Procedures creating transient ITP are advantageously used [10].

3.3. Optimization of acetonitrile and sodium chloride proportion

As acetonitrile seemed crucial for the stacking effect, previous to the quantification we decided to optimise the proportion of AcN and NaCl with an experimental design. Analysis of the areas was carried out by Statgraphic®plus for Windows 4.1 program (Rockville, MD, USA). Pareto plot obtained with the data permits us to get the "size effect" of each of the parameters investigated upon the peak areas. In this treatment a parameter is deemed to have a significant influence if the size of the effect is greater than 2. Fig. 2 shows the Pareto plot for formate in standards and samples as an example. None of the parameters show a significant effect on the areas in the assayed ranges, therefore they could be varied in these limits without a significant effect. The response surfaces study gave the same results. Since different optimum conditions were obtained for different acids and with differences in standards and samples, finally, 50% AcN (v/v) and 0.5% NaCl (v/v) concentrations were chosen for the following assays.

In such conditions the electrophoregrams in Fig. 3 for standards and Fig. 4 for samples were obtained. The mean enhancement factor value (Table 1), for all the compounds was 17.4 ± 1.2 for standards and 16.4 ± 1.0 for samples, calculated as defined by Albert et al. ($F = (S_c/S_{c\,(standard)}) \times (C_{inj\,(standard)}/C_{inj})$) [17]. There was no statistical difference (*t*-test, $P >$ 95%) between the mean enhancement factor obtained in standards and samples. The sensitivity enhancement (SE) has also been calculated. It is similar to F but

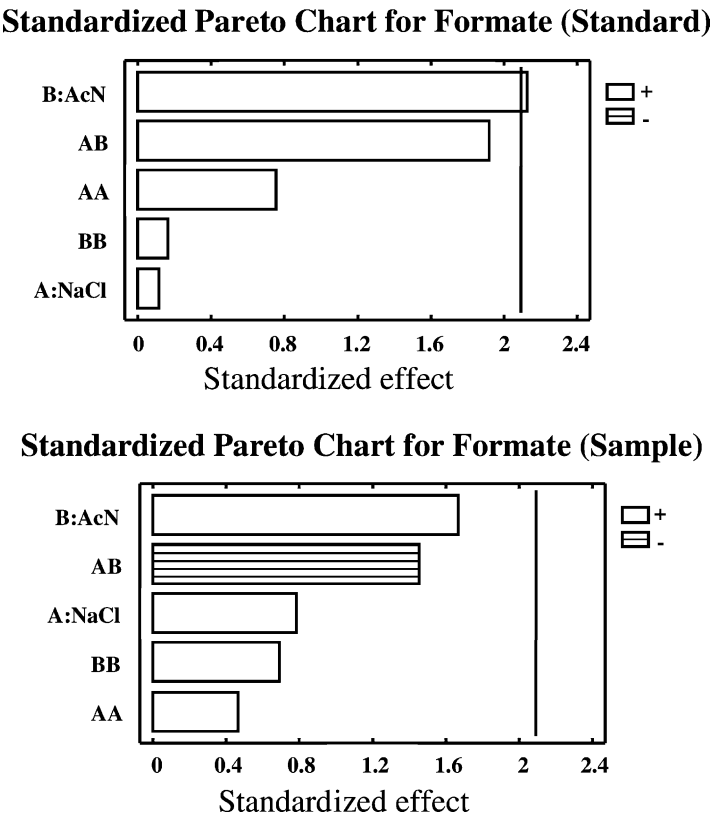

Fig. 2. Standardized Pareto plot for formate areas in standards and samples. (A) Acetonitrile; (B) NaCl.

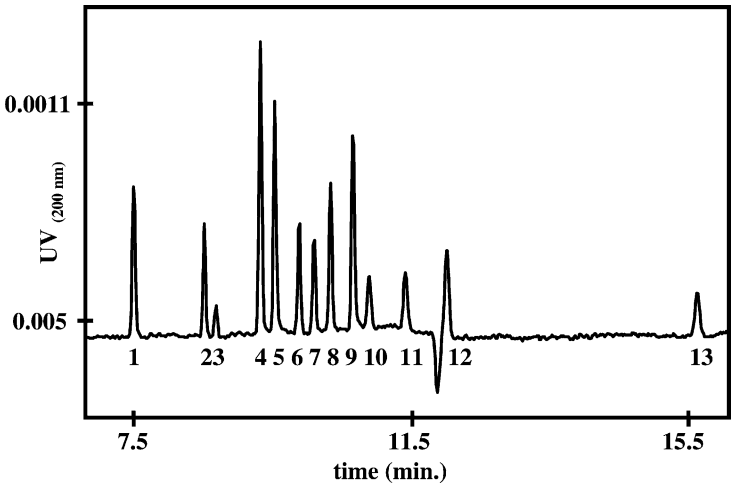

Fig. 3. Electropherogram of standards. Peaks in order correspond to: (1) nitrate, (2) oxalate, (3) formate, (4) fumarate, (5) aconitate, (6) succinate, (7) malate, (8) glutarate, (9) citrate, (10) acetate, (11) glycollate, (12) propionate, (13) quinate. Standards + AcN–NaCl 1:4 (v/v) and injection by pressure (0.035 bar) for 25 s. Buffer, 0.5 M H_3PO_4–0.5 mM CTAB pH 6.25. UV detection at 200 nm. Uncoated fused-silica capillary (57 cm × 50 μm i.d.), −10 kV potential. Temperature 25 °C.

related to peak heights instead of areas. Results are slightly lower when expressed in heights and it shows a small loss of efficiency due to large injection or dispersion during the stacking process [17].

Previously it was pointed out that caution is necessary when interpreting the enhancement factors reported in the literature because, for the comparisons, "standard" conditions with very short injection times and not operating under optimised conditions are often used [8]. The present work makes reference to a previously optimised method. As mentioned above, higher injection times in the initial conditions

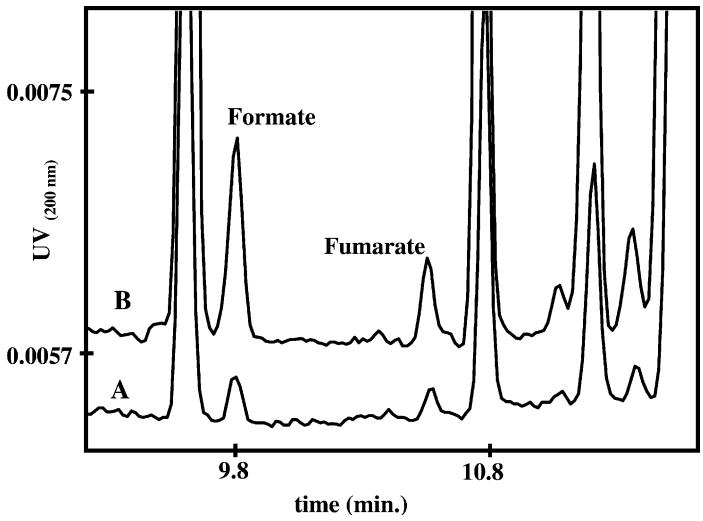

Fig. 4. Electropherogram of serum obtained by freezing. A: Sample + H_2O 1:1 (v/v) and injection by pressure (0.035 bar) for 5 s. B: Sample + AcN–NaCl 1:4 (v/v) and injection by pressure (0.035 bar) for 25 s. Peaks in order correspond to: sample + H_2O 1:1 (v/v) and injection by pressure (0.035 bar) for 5 s. Sample + AcN–NaCl 1:4 (v/v) and injection by pressure (0.035 bar) for 25 s. Buffer, 0.5 M H_3PO_4–0.5 mM CTAB pH 6.25. UV detection at 200 nm. Uncoated fused-silica capillary (57 cm × 50 μm i.d.), −10 kV potential. Temperature 25 °C.

Table 1
Stacking factors for standards and sample

Anion	Area		Height	
	Standard	Sample	Standard	Sample
Oxalate	17.2	16.2	15.8	14.1
Formate	16.7	16.1	14.2	13.6
Fumarate	17.0	15.1	15.4	15.3
Aconitate	19.7	17.8	18.8	16.0
Succinate	16.8	16.3	15.1	14.7
Acetate	17.1	16.8	14.7	16.1
Mean	17.4 ± 1.2	16.4 ± 1.0	15.7 ± 1.7	15.0 ± 1.1
I.S.	16.6	16.3	14.9	14.4

produced overlapping in some peaks. This is because, as the width of the peaks is significantly reduced by stacking procedure, much larger sample volumes may be injected without losing separation efficiency.

Furthermore, as the reference method was validated, quantitative results can be compared to study the accuracy in the new conditions, which was a great concern when it could be observed in the first assays that standard and sample areas did not increase to the same extent.

3.4. Accuracy and precision

We decided to include an internal standard (I.S.), because it could be possible that the organic acids in serum of natural rubber latex were associated in different ways with the matrix and they were freed to a different extent depending on the solvent. With an organic acid being externally added it could be controlled if the enhancement of the signal occurred to the same extent. Moreover, solvent volatilisation was observed at a certain degree when acetonitrile was employed, because CE vials are not hermetically sealed. Different compounds were tested (methylmalonate, malonate, ketoglutarate, ketoisovalerate, etc.) and finally glutarate was chosen because it does not interfere with any other peak in the electrophoregrams and it was not present in the samples.

There was no statistically significant difference (*t*-test, $P > 95\%$) between the enhancement factor found in the organic acids present in serum of natural rubber latex and that found for the internal standard, both in areas (16.4 ± 1.0 versus 16.3) and in heights (15.0 ± 1.1 versus 14.4) in the optimised conditions. Therefore, the matrix does not influence the liberation of the short chain organic acids from the latex serum.

Recoveries calculated with and without the internal standard are shown in Table 2 with the corresponding R.S.D.s. Recoveries calculated without internal standard are adequate on day 1 and low on day 2, that provides mean recoveries including 100% in all cases, but with low precision, since R.S.D.s ranged from 12 to 26%. As was to be expected, precision improved when calculus was performed including the internal standard. Recovery for acetate exceeded 100%, but R.S.D.s ranged from 3 to 12% for the media, which are acceptable values. Table 3 includes more exhaustive precision data for standards and samples on two different days. It can be observed that R.S.D.s without internal standard increased with stacking conditions, due to the factors explained above, but they were in the very acceptable ranges (1–8% on day1, 1–11% on day 2 and 1–14% intermediate) when employing the internal standard.

Table 2
Recoveries of organic acids in serum of natural rubber latex employing the stacking technique

Anion	Recovery ± R.S.D. (%)					
	Without I.S.			With I.S.		
	Day 1	Day 2	Mean	Day 1	Day 2	Mean
Oxalate	110 ± 6	68 ± 13	88 ± 24	104 ± 4	88 ± 7	95 ± 8
Formate	98 ± 6	82 ± 14	90 ± 12	94 ± 5	104 ± 3	99 ± 9
Fumarate	118 ± 10	63 ± 15	87 ± 26	112 ± 8	83 ± 14	94 ± 12
Aconitate	113 ± 6	77 ± 12	94 ±21	107 ± 5	99 ± 7	103 ± 7
Succinate	108 ± 8	78 ±13	92 ±20	103 ± 4	100 ± 1	101 ± 3
Acetate	107 ± 5	95 ± 11	101 ± 13	101 ± 4	122 ± 4	112 ± 8

Table 3
Precision for standards and samples

Anion	Validated conditions						Stacking conditions					
	R.S.D. (%) without I.S.			R.S.D. (%) with I.S.			R.S.D. (%) without I.S.			R.S.D. (%) with I.S.		
	Day 1	Day 2	Intermediate	Day 1	Day 2	Intermediate	Day 1	Day 2	Intermediate	Day 1	Day 2	Intermediate
Oxalate												
Std	8	6	13	10	7	8	10	10	11	4	6	10
Spl	4	9	12	4	3	4	14	6	20	1	3	4
Formate												
Std	6	6	13	6	8	7	13	7	18	3	3	10
Spl	12	10	14	10	11	10	15	9	25	3	3	3
Fumarate												
Std	5	7	13	3	3	3	11	5	8	3	4	6
Spl	21	12	16	20	22	12	18	14	28	8	11	10
Aconitate												
Std	7	11	16	8	5	6	11	6	12	3	7	14
Spl	4	12	15	6	6	6	12	10	16	4	2	11
Succinate												
Std	5	6	11	2	5	4	12	8	11	2	1	1
Spl	4	10	13	3	4	4	16	9	25	3	1	2
Acetate												
Std	7	5	10	6	7	7	16	5	18	2	4	11
Spl	9	12	16	8	6	7	17	8	27	4	1	4
I.S.												
Std	5	7	13				12	8	11			
Spl	4	8	14				15	9	24			

Citrate and malate are not included in Table 2 because they saturate the signal in these conditions; other compounds included in standards, but not in samples do not appear in these particular samples.

3.5. Electroosmotic flow

As previously recorded for different analytes [18,19], the use of a flow modifier, as CTAB, already included in the background electrolyte (BGE), eliminates the troublesome polarity switching step sometimes employed for stacking large sample volumes and that was not possible in the equipment employed for the analysis. When the capillary is primarily filled with sample dissolved in water and a reversed polarity is applied to the inlet, the EOF pushes the sample plug out of the capillary. As this happens, BGE from the detector side of the capillary is pulled into the column. The CTAB present in the BGE coats the capillary and reverses the direction of the EOF, eliminating the need for polarity switching.

Therefore, we decided to measure the electroosmotic flow following the procedure of Williams and Vigh [14] which seems more precise and accurate, mainly for low mobility values. Results are represented in the Fig. 5. At 0.5 mM, the working concentration for CTAB, EOF was $1.10^{-5}\,cm^2\,V^{-1}\,s^{-1}$ towards the detector which was placed in the anode. This can be considered a low value [20], but the EOF was not reverted towards the inlet in this range of CTAB concentrations. Lower values of CTAB were also tested (0.05 mM). They produced a flow towards the inlet, but migration times over 35 min were obtained without any further increase in the enhancement factor.

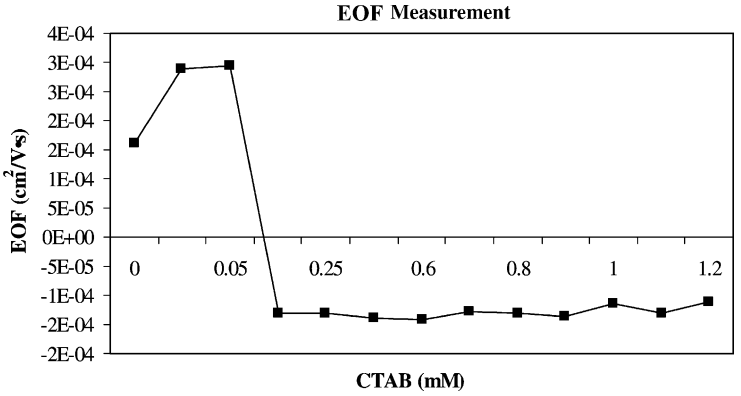

Fig. 5. EOF measurement for different CTAB concentrations in the separation buffer (0.5 M H_3PO_4 made up pH 6.25).

4. Conclusion

The sample stacking for short chain organic acids measurement in serum of natural rubber latex by CE has been optimised. Enhancement factors over 16 can be obtained with 50% acetonitrile (v/v) and 0.5% NaCl (w/v) and 25 s as injection time. That allows us the measurement of acids with low content in samples such as formic and fumaric acids. Accuracy and precision can be considered adequate mainly when employing an internal standard.

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