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Calibration principles for flow injection analysis–capillary electrophoresis systems with electrokinetic injection

Petr Kuban[a], Kirsi Tennberg[b], Robert Tryzell[a], Bo Karlberg[a,*]

[a]*Department of Analytical Chemistry, Stockholm University, S-106 91 Stockholm, Sweden*
[b]*Laboratory of Analytical Chemistry, University of Helsinki, FIN-00014 Helsinki, Finland*

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Abstract

The utility of different calibration techniques in flow injection analysis–capillary electrophoresis systems based on electrokinetic injection has been studied in detail and compared. Best results were obtained with the internal standard method or by applying the conductivity corrected peak area method. These methods yielded a relative error of prediction of less than 6% and can be recommended for quantitative analysis. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Flow injection introduction of samples into capillary electrophoresis (CE) systems has been shown to have great practical applicability [1,2]. Sample pretreatment techniques such as dialysis [3] and gas diffusion [4] can be integrated in an automated fashion. Two types of flow injection analysis–capillary electrophoresis (FIA–CE) interfaces have been described for the accommodation of the capillary in the flow system; one with a horizontal channel [1] and one with a conical chamber [2]. Typically, a sample portion is injected into a stream of the CE electrolyte in the FIA system and carried towards the interface, into which one end of a capillary and a platinum electrode have been inserted. The other end of the capillary and a second platinum electrode are placed into a vial containing the same electrolyte solution. A constant high voltage is applied between the two platinum electrodes and a small sample fraction is electrokinetically injected when the sample plug passes by the capillary opening. The system allows for multiple sample injections in one uninterrupted electrophoretic run. Sample throughput rates can reach 170/h and the repeatability is typically 2% (R.S.D.) [5].

Electrokinetic (EK) injection in CE offers some interesting features, for instance instrumental simplicity, preconcentration potential through sample stacking [6] and injection selectivity. Nevertheless, EK injection has not yet been widely accepted, probably due to the problems arising in conjunction with quantitative analysis.

Bias phenomena occur when applying EK sample injection [7]. The first type of bias arises due to the fact that the amount of a particular ion, present in an electrokinetically injected sample portion, is influenced by its effective mobility. Ions with a high

*Corresponding author.

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mobility will be over-represented in the electropherogram. This type of bias can be corrected for by multiplying the areas of the peaks by their respective migration times [7]. The second type of bias occurs due to differences in sample conductivity since the totally injected amount of ions is largely influenced by the sample conductivity. Sample pH, complexing reactions and viscosity may also influence the injected amount. Application of a matrix correction factor to peak area (COPA) has been suggested by Leube and Roeckel [8]. The correction factors were derived from 12 different sample matrices which were supposed to represent certain biological matrices.

Detection bias, occurring both for EK and hydrodynamic injection, needs to be accounted for. This bias arises due to the differences in migration velocities of the analytes during their passage through the detector [9–11]. The first type of EK injection bias and the detection bias can, under certain conditions, cancel each other out [12,13].

The internal standard (I.S.) method has been widely applied in conjunction with hydrodynamic injection [14,15] but to a lesser degree with EK injection [16]. Dose and Guiochon [17] suggested that two internal standards should be added to the sample to accomplish an improved compensation for large migration time differences between the various analyte ions. Lee and Yeung [18] have derived a theoretical equation for correction of the EK injection bias without using internal standards whereby an accuracy of 5% R.S.D. could be achieved.

The FIA–CE approach for sample introduction differs markedly from that normally applied in any commercial or laboratory-made CE instrumentation. The electrolyte is always present in the FIA–CE flow system. The inserted sample temporally splits this electrolyte solution on injection into the FIA system. However, some mixing between the sample and the electrolyte cannot be completely avoided during the transport through the flow system up to the capillary opening. This is in contrast to the introduction method used for commercial instruments in which the sample vial physically replaces the electrolyte vial when injecting the sample. A further difference relates to the mode of the high voltage (HV) supply for the two system types; when applying the FIA–CE approach, the HV is uninterrupted, while for the conventional approach, a disruption occurs during the exchange of vials. Since the FIA–CE system with EK injection offers certain interesting features, as discussed above, we decided to address its quantitative aspects. Available calibration techniques are critically examined and compared in this paper.

2. Calibration and peak evaluation principles

Calibration and sample solutions can be introduced in an FIA–CE system (a) as they are, i.e., no addition of any constituents; (b) with background electrolyte added; (c) with one internal standard added; (d) with two or more internal standards added; (e) with both internal standard(s) and background electrolyte added; and (f) with application of analyte standard addition. There are numerous evaluation principles for the peaks in the resulting electropherograms. The most commonly used principles are: (i) peak height; (ii) peak area; (iii) migration time corrected peak area; (iv) normalised peak area, i.e., analyte peak area divided by the total area of all peaks in the electropherogram; and (v) conductivity corrected peak area (CCPA). By combining some of these principles further evaluation methods result. When internal standard (I.S.) methods are applied the analyte peak area (or peak height) is divided by the peak area (height) of the I.S. This normalised value can be used as it is for regression purposes or treated further according to any of the methods above. Thus, there are many approaches for univariate regression.

Multivariate calibration can also be applied. Measured entities, such as all individual peak heights and areas in an electropherogram, the total peak height and area, and the measured conductivity of the sample are assembled in a matrix, **X**, and corresponding true analyte concentration values in a second matrix, **Y**. Multivariate regression is then performed. Common methods in this context are multiple linear regression (MLR), principal component regression (PCR) or partial least squares (PLS) regression.

The practical applicability of the various calibration and regression methods available can be investigated in a number of ways. In this study, two approaches have been applied: (1) separate cali-

bration and validation test sets, utilising univariate peak data for calibration purposes and linear regression, and (2) one experimentally designed validation test set employing both univariate and multivariate peak data for linear and PLS regression methods, respectively, both performed with leave one out cross-validation [19].

Separate conductivity measurements are required for peak area correction according to approach (v) above. The total amount of injected ions depends on the conductivity of the sample. Thus, by dividing the total peak area, ΣP_j (j peaks), by the conductivity of the sample, λ_s, a specific correction factor for each sample is obtained. For each ion in that sample, the measured peak area, P_i, is the divided by this specific correction factor, $\Sigma P_j/\lambda_s$.

3. Experimental

3.1. Reagents and standard solutions

All reagents were of analytical grade and deionised water having a resistivity above 18 MΩ cm^{-1} was used. The carrier electrolyte consisted of 6 m*M* K_2CrO_4, 3 m*M* boric acid and 30 μ*M* cetyltrimethylammonium bromide (CTAB) at pH 8. Anion stock solutions of chloride, sulphate, nitrate and thiosulphate (internal standard, I.S.), 10 000 mg/l, were prepared from their respective sodium salts.

3.2. Instrumentation

The FIA–CE system is depicted in Fig. 1. The FIA part comprised a peristaltic pump (Gilson, Villier-les-Belles, France) and a programmable injector (V-100, FIAStar 5020, Foss Tecator, Höganäs, Sweden) with an interchangeable injection loop. The normal injection volume was 50 μl and the electrolyte flow-rate was 3.0 ml/min. The CE part of the system included a high voltage supply (Series 230, Bertan Associates, Hicksville, NY, USA) and a UV detector (CV^4, ISCO, Lincoln, NE, USA) at 372 nm (indirect detection). The FIA–CE interface consisted of a body made of Plexiglas that has been described elsewhere [4].

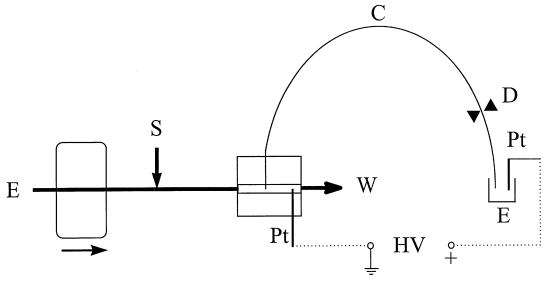

Fig. 1. Flow diagram of the complete FIA–CE scheme. E=Electrolyte; S=sample; C=capillary; Pt=platinum electrode; W=waste; D=detector; HV=high voltage.

All separations were performed in untreated fused-silica capillaries, 50 μm I.D. (Polymicro Technologies, Phoenix, AZ, USA). The total length of the capillaries was 70 cm. The distance between the injection and the detection sites was 50 cm. The electropherograms were registered with an ELDS Professional 1.0 laboratory data system (Chromatography Data System, Kungshög, Sweden). Conductivity measurements were performed using a conductometer (Model 120, Orion Research, Boston, MA, USA).

3.3. Operational principle of the FIA–CE system

The operational principle of the FIA–CE system has been described previously in detail [1]. The system allows repetitive sample injections in one electrophoretic run. One run might comprise up to 21 consecutive injections of samples containing the investigated ions. The limiting factor for a run is the electroosmotic flow (EOF) peak appearance at 12 min after the first injection. Sample and standard solutions were always injected in triplicate. The capillary was rinsed with electrolyte for 2 min after each completed run.

3.4. Anion solutions for calibration and validation

3.4.1. Separate calibration and validation test sets

The calibration range for the three selected anions, chloride, sulphate and nitrate, was 0–100 mg/l. Calibration set 1 contained equal concentrations of all three anions at six concentration levels. The conductivity values differed markedly between these calibration solutions, ranging from 86 to 764 μS, see Table 1. Calibration set 2 included varying concentration proportions of the three ions. The random-

Table 1
Composition of the calibration sets used for the classical approach

Set 1				Set 2			
Cl^- (mg/l)	SO_4^{2-} (mg/l)	NO_3^- (mg/l)	λ_s (μS)	Cl^- (mg/l)	SO_4^{2-} (mg/l)	NO_3^- (mg/l)	λ_s (μS)
10	10	10	86	100	20	10	415
20	20	20	160	20	70	70	379
40	40	40	322	10	40	100	327
70	70	70	544	40	100	20	422
100	10	100	764	70	10	40	340

ised amounts of ions in these calibration solutions gave a smaller range for the conductivity values, 327 to 420 μS (Table 1).

The validation set was designed as follows. Three concentration levels were used to cover the calibration range of 0–100 mg/l; these levels were 10, 50 and 100 mg/l. Thus, a full three-level design including three variables (chloride, sulphate and nitrate) will require $3^3=27$ experiments out of which every second combination was selected (13 experiments). Seven further experiments were added: (a) three experiments with two variables set at 75 mg/l and the third to 0, (b) three experiments with two variables set at 25 mg/l and the third to 0, and (c) one experiment with all concentrations set at 0 (blank solution). Thus, the total number of validation solutions amounted to 20.

3.4.2. Solution set for univariate and multivariate calibration with cross-validation

The same calibration range for the three anions was selected, 0–100 mg/l. As for the previous validation set, a full three-level design including three variables (chloride, sulphate and nitrate) will require $3^3=27$ experiments all of which were performed. Seven further experiments were added, as described above, in total 34 solutions.

3.5. Data treatment

The evaluation principles were peak height and peak area. When the I.S. method was applied, the peak area ratio, $P_{analyte}/P_{I.S.}$, was calculated for each analyte ion. The conductivity corrected peak area was obtained by dividing each individual peak area with the correction factor as described above.

Linear regression was applied for the univariate calibration approach. For multivariate regression using PLS, the **X** matrix comprised the conductivity (μS), the individual peak area values for the three anions, the total peak area, the individual peak heights for the three anions and the total peak height. When I.S. was added, the area and the height values of the I.S. peaks were included; these values were also added to the respective sums of peak area and peak height. The **Y** matrix entailed the concentration values for the anions. The cross-validation method used throughout the calibration was based on the leave-one-out approach [19].

The data were always autoscaled prior to loading into the software program, UNSCRAMBLER 6.1 (Camo, Trondheim, Norway).

4. Results and discussion

4.1. Classical calibration methods

Calibration sets 1 and 2 together with the validation set were introduced in the FIA–CE system according to the following principles: (i) directly, (ii) after background electrolyte addition, (iii) after I.S. addition and (iv) after addition of both background electrolyte and I.S.. The relative errors were calculated for each validation solution as the ratio between the predicted and the true value for all three anions and the observed ranges for the prediction are given in Table 2.

4.1.1. Direct injection

Fig. 2 shows calibration electropherograms resulting from the direct injection of calibration set 1. As can be seen, a non-linear relationship between the response and the analyte level is obtained which can

Table 2
Results of FIA–CE calibration, separate calibration and validation set

Evaluation method	Buffer	I.S.	Calibration set	Linearity (r^2)			Accuracy (relative error range)		
				Cl^-	SO_4^{2-}	NO_3^-	Cl^-	SO_4^{2-}	NO_3^-
(a) Peak area	No	No	1	0.9929[a]	0.9944[a]	0.9870[a]	0.140–[b]	0.048–[b]	0.101–[b]
	No	No	2	0.9888	0.9918	0.9902	0.600–3.180	0.600–3.940	0.557–4.290
(b) CCPA	No	No	1	0.9980	0.9978	0.9995	0.968–1.050	0.916–1.082	0.877–1.092
	No	No	2	0.9997	1.0000	0.9999	0.970–1.054	0.964–1.116	0.970–1.174
(c) Peak area	Yes	No	1	0.9953	0.9952	0.9894	0.835–2.311	0.827–2.789	0.816–1.231
	Yes	No	2	0.9916	0.9992	0.9976	0.895–2.220	0.925–2.608	0.870–1.177
(d) Peak area/I.S.	No	Yes	1	0.9997	0.9996	0.9993	0.944–1.017	0.940–1.044	0.944–1.019
	No	Yes	2	0.9996	0.9995	0.9998	0.944–1.017	0.940–1.044	0.944–1.019
(e) Peak area/I.S.	Yes	Yes	1	0.9995	0.9984	0.9990	0.818–1.031	0.839–1.057	0.848–1.106
	Yes	Yes	2	0.9966	0.9992	0.9983	0.818–1.031	0.839–1.057	0.848–1.106

[a] Evaluation based on a logarithmic curve fit.
[b] Relative error>10.

be attributed to the increasing conductivity of the calibration solutions for increasing concentrations of the anions (see Table 1). The stacking effect decreases when the conductivity values increase and this causes the observed curvature in the response function. On the other hand, calibration set 2 yielded a linear calibration plot since the conductivity values were almost equal. Nevertheless, neither of the two calibration methods is suitable for accurate quantitative analysis, as is evident from Table 2.

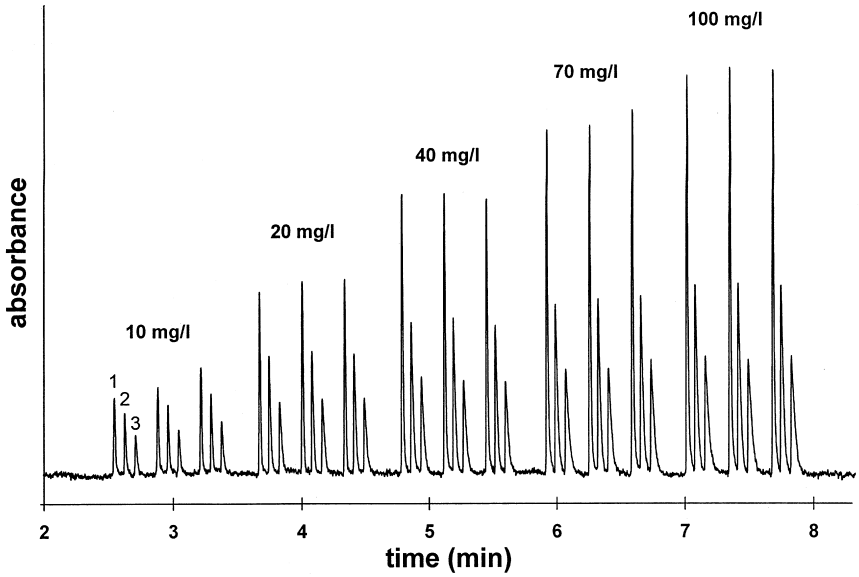

Fig. 2. Calibration electropherograms resulting from direct injection of calibration set 1. Conditions: HV 25 kV, chromate electrolyte, indirect UV detection at 372 nm, EK injection in FIA–CE system, consecutive, triplicate injections of each standard. Peaks: 1=chloride, 2=sulphate, 3=nitrate, concentrations are given above each set of triplicate injection.

4.1.2. Conductivity corrected peak area (CCPA)

By using this peak evaluation principle both the linearity of the calibration graphs and the prediction ability were significantly improved, see Table 2.

4.1.3. Addition of the background electrolyte

Addition of chromate electrolyte to the calibration solutions reduces the conductivity differences. However, the sensitivity decreases due to the absence of the stacking effect. Fig. 3 reveals a linear response, although a loss in signal-to-noise (S/N) ratio was also observed. Despite the linear response, relatively large errors were obtained, see Table 2. Improved accuracy would be expected if exact conductivity adjustment (e.g., conductivity titration) were to be performed. The imprecision in the peak area evaluation due to the decreased S/N ratio might be another factor contributing to the high relative error.

4.1.4. Addition of internal standard (I.S.)

The calibration electropherogram obtained after addition of 50 mg/l of thiosulphate (I.S.) to all solutions is presented in Fig. 4. As can be seen, the fractions of ions injected decrease as the conductivity of the solutions increases, however, the ratios between the peak areas for the I.S. and the analyte ions reflect the true concentration proportions. Excellent linearity is obtained when plotting peak area ratios, $P_{\text{analyte}}/P_{\text{I.S.}}$, and the relative errors for the predicted values do not exceed 6%.

4.2. Critical examination of the I.S. method

Results obtained with the I.S. method were superior to those of the other approaches. Some other interesting observations were made in this context.

(I) The calibration plots for the two calibration sets were identical. The peak area ratio used for the calibration seems to be independent of how the standard solutions are prepared.

(II) For a given electrolyte system and unchanged experimental conditions, such as HV magnitude, injection time and the amount of I.S. added, the calibration response is constant over a long time period. This was confirmed by injecting calibration sets 1 and 2 repeatedly over the course of one week. The calibration plot slopes and intercepts were identical within 1–3.5%. Thus, it would be possible to tabulate calibration responses for any given set of experimental conditions. Analysis of the samples

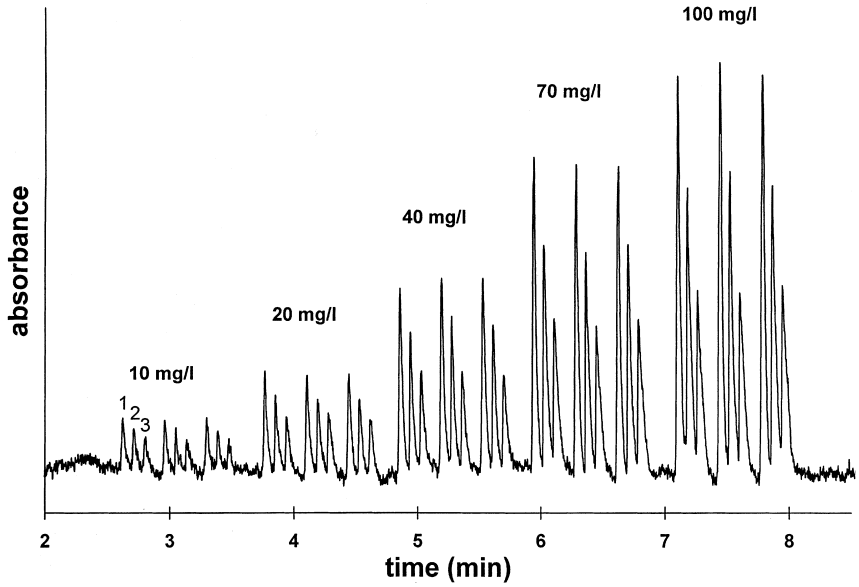

Fig. 3. Calibration electropherogram resulting from direct injection of calibration set 1 after addition of chromate electrolyte. Conditions: identical to those for Fig. 2.

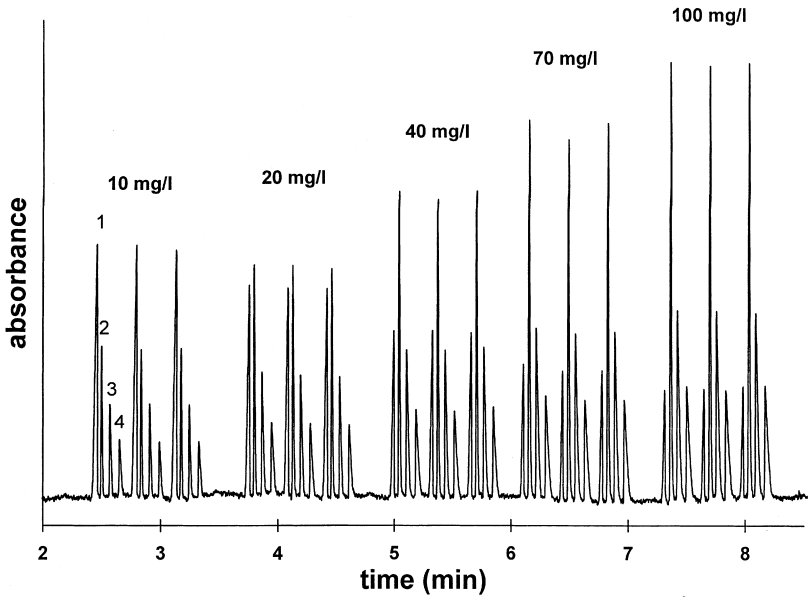

Fig. 4. Calibration electropherograms for calibration set 1. To each solution 50 mg/l of thiosulphate (I.S.) was added. Conditions as in Fig. 2. Peaks: 1=thiosulphate, 2=chloride, 3=sulphate, 4=nitrate.

could then be performed without repeating the entire calibration procedure as long as the experimental conditions remained unchanged.

(III) The calibration response does not change even if other ion constituents are present in the sample than those included in the calibration. This assumes that a complete resolution of the peaks in the electropherogram is maintained. A concentrated sodium carbonate solution was added to all validation solutions, resulting in a final carbonate concentration of 100 mg/l, and the quantitative evaluation of chloride, sulphate and nitrate concentration was performed based on the previous calibration sets with no carbonate added. The relative error remained unchanged.

4.3. Univariate and multivariate calibration methods using a designed standard set

The standard solution set comprising 34 different test solutions was employed and the results for univariate and multivariate calibrations are given in Table 3.

For the univariate calibrations entailing peak height, peak area, I.S. corrected peak area and CCPA, all the 34 response values and corresponding concentration values for each ion were used to determine the linear regression equation and the correlation coefficients. Leave-one-out cross-validation was applied by calculating the linear regression equation based on results obtained for 33 of the 34 solutions and predicting the concentration values for the excluded solution. This procedure was repeated until the concentration values of all solutions had been predicted. The correlation coefficient for this type of prediction was calculated based on the true, known, concentration values. The root mean standard error of prediction (RMSEP) values were calculated in the usual way [20].

When comparing the RMSEP for peak height and peak area evaluation, see Table 3, it is observed that the values are surprisingly high throughout, although when buffer is added they improve. It is interesting to note that the lowest RMSEP is obtained when the peak height is used for evaluation and when both I.S. and buffer are added. The RMSEP values for I.S. evaluation are lower than the corresponding peak height and peak area data, but still these values are

Table 3
Results from FIA–CE calibration

	Cl^-				SO_4^{2-}				NO_3^-			
I.S.	No	Yes	Yes	No	No	Yes	Yes	No	No	Yes	Yes	No
Buffer	No	No	Yes	Yes	No	No	Yes	Yes	No	No	Yes	Yes
Peak height												
r^{2a}	0.9018	0.9517	0.9908	0.9787	0.8505	0.9035	0.9807	0.9628	0.8828	0.9135	0.9729	0.9566
r^{2b}	0.9496	0.9756	0.9954	0.9893	0.9222	0.9506	0.9903	0.9812	0.9396	0.9558	0.9864	0.9781
RMSEP, mg/l	12.290	8.390	3.591	5.490	15.620	12.170	5.223	7.317	13.570	11.460	6.217	7.936
Peak area												
r^{2a}	0.9218	0.9612	0.9723	0.9771	0.8883	0.9398	0.9722	0.9708	0.9153	0.9260	0.9584	0.9570
r^{2b}	0.9601	0.9804	0.9861	0.9885	0.9425	0.9694	0.9860	0.9853	0.9567	0.9263	0.9790	0.9783
RMSEP, mg/l	10.850	7.480	6.286	5.706	13.210	9.426	6.298	6.457	11.330	10.350	7.762	7.898
Peak area/I.S.												
r^{2a}	–	0.9930	0.9822	–	–	0.9916	0.9821	–	–	0.9876	0.9815	–
r^{2b}	–	0.9965	0.9911	–	–	0.9958	0.9910	–	–	0.9938	0.9907	–
RMSEP, mg/l	–	3.128	5.016	–	–	3.428	5.029	–	–	4.1830	5.12	–
CCPA												
r^{2a}	0.9993	0.9987	0.9776	0.9985	0.9990	0.9967	0.9950	0.9984	0.9993	0.9973	0.9952	0.9975
r^{2b}	0.9993	0.9993	0.9988	0.9984	0.9990	0.9984	0.9975	0.9984	0.9993	0.9987	0.9976	0.9975
RMSEP, mg/l	1.407	1.358	1.813	2.077	1.632	2.132	2.628	2.089	1.417	1.930	2.591	2.631
PLS1												
No. of PCs	4	2	2	2	4	4	2	2	4	2	2	2
r^{2b}	0.9898	0.9856	0.9944	0.9958	0.9813	0.9903	0.9927	0.9921	0.9799	0.9972	0.9869	0.9861
RMSEP	5.313	6.305	3.945	3.408	7.184	5.180	4.483	4.690	7.435	7.906	6.013	6.197

[a] From linear regression.
[b] Between true and predicted.

quite high with no observable benefit to be gained when buffer is added to the solutions.

All the evaluations discussed above exhibit RMSEP values ranging from 3.5 to 15.6 mg/l keeping in mind that the total dynamic calibration range was 100 mg/l throughout. This could be accepted in some applications, but an improvement is desired. Consequently, conductivity measurements were performed to supplement the peak data. The conductivity was measured after the addition of I.S., when applied. As can be seen in Table 3 improved RMSEP values are obtained throughout with the CCPA method.

Using the multivariate approach, no dramatic amelioration of RMSEP values is obtained. Addition of more variables, such as CCPA and I.S. correction did not improve the quality of the prediction. Migration time corrected peak area and height data were tested but no significant advantage could be discerned.

5. Conclusions

It has been shown that when EK injection is applied in an FIA–CE system, quantitative analysis with satisfactory accuracy can be performed. However, the selection of sample introduction and evaluation principle is critical. The I.S method was found to provide good accuracy and long term stability of the calibration. Furthermore, it is insensitive to interferences which may be present in the sample solutions. A relative error of prediction of about 6% can be obtained. If the conductivity corrected peak area method a comparable precision can be attained.

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