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How to achieve higher repeatability and reproducibility in capillary electrophoresis

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Abstract

The influence of experimental parameters on precision of migration times and accuracy of integration has been studied. The repeatability of migration times strongly depends on the proper selection of the rinse steps between each run of a CE system. The rinse steps have to be optimized additionally with each separation system. This is especially important for systems providing an anodic electroosmotic flow. The errors introduced by the integration software were studied by transferring the same data sets to different commercial available integration softwares. A strategy for the transfer of raw data files between several softwares is described. By using systems with identical peak areas or with identical peak heights it could be shown that the newly introduced softwares can cope better with the leading or tailing peaks. The RSD of quantitation strongly depends on the signal-to-noise ratio. At S/N ratios larger than 35 no differences between the various softwares studied could be observed. At lower S/N ratios the newly released softwares are to be preferred. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Repeatability; Reproducibility; Migration times; Peak areas; Software, integration; Metal cations

1. Introduction

Capillary electrophoresis (CE) has become a powerful separation technique due to the high achievable separation efficiency and short analysis time [1] in the field of biochemical separations [2,3], inorganic ions [4–7] or chiral [8,9] separations. However, in pharmaceutical quality control it is used only to a minor extent. One reason for this might be that it is still difficult to validate CE methods using the criteria in quantitation and accuracy posed by regulated bodies for application to HPLC or GC methods [10].

During the last years, important efforts have been made to increase the precision of CE methods. Guidelines for improved method validation [11,12] or precise quantitative CE [13] have been described. One of the factors controlling precision in CE is injection [11,14]. Possible sample loss from the capillary due to volume expansion [15] can be avoided by the use of voltage ramps at the beginning of the separation or via the co-injection of a buffer slug. This possible source of error can also be controlled by the use of internal standards [10,16].

Another important factor affecting the precision in CE is temperature control [14]. This factor has a major impact on the repeatability of migration times as they are dependent on the viscosity of the buffer solution, which has an influence on the linear velocity of the electroosmotic flow (EOF) [17]. Problems in temperature control are also encountered when one method is transferred from one instrument to another, because different instruments possess different cooling capacities [18]. Migration time

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precision depends additionally on the pretreatment of the inner capillary surface before and during analysis. The surface properties (zeta potential) are responsible for the generation of the EOF and its velocity. The standard rinsing procedure applied in most separation systems is flushing the capillary for several minutes with the applied buffer before each separation [19–21]. On the other hand for some systems more complex rinse procedures, such as combinations of sodium hydroxide and buffer [16,19], methanol, hydrochloric acid and buffer [23] or methanol and buffer [24], have been described. Ehmann et al. [25] recently published a systematic investigation about the effect of different pre-rinsing techniques on migration times and corrected peak area precision. The application of these more complex rinse procedures was found to stabilize and improve the reproducibility of the migration times of the analytes, as the capillary surface is reconditioned or adsorbed solutes are removed.

A third factor, which also influences the precision of CE, is accuracy and reproducibility of peak integration. The problems stem from the often asymmetric and triangular shaped peaks commonly observed in CE, especially with indirect detection systems. Not too many papers have been published in this field. Schirm and Wätzig [26] recently presented a modification of an algorithm that showed lower values for the relative standard deviation (RSD) of the peak area than commercial integration softwares. Whatley et al. [27] compared the Beckman System Gold Software to the new Beckman P/ACE System MDQ Software and found that the new software reduces the RSD value for peak areas.

This paper is focused on two factors influencing the precision of CE: In the first part, the reproducibility of migration times is studied by applying different rinse steps. Secondly, the influence of different commercial available softwares on the accuracy and precision of peak area measurements is compared.

2. Experimental

2.1. Instrumentation

All experiments were carried out on a capillary electrophoresis system Beckman P/ACE 2050 (Beckman Instruments, Munich, Germany), equipped with a UV detector with wavelength filters of 200, 214, 254 and 280 nm. The part of the capillary where separation takes place was kept at a constant temperature (25°C) by immersion in a cooling liquid circulating in the cartridge with a detection aperture of 100 μm×200 μm. Solutes were injected in the hydrodynamic mode by applying an external pressure of 0.035×10^5 Pa (0.5 p.s.i.). The detector time constant was 0.1 s and the data aquisition rate was 20 Hz.

2.2. Capillaries

Fused-silica capillaries (75 μm I.D.×375 μm O.D.) were purchased from Polymicro Technologies (Phoenix, Az, USA). The polyimide coating at the detection window was removed by electric heating. Capillaries were preconditioned by rinsing with 1 *M* sodium hydroxide, water from a Milli-Q unit (Millipore, Bedford, MA, USA) and buffer solution for 15 min each. Approximately 2 mm of the polyimide coating at the ends of the capillaries was removed by heating to obtain a plain surface of the capillary ends.

2.3. Materials

All buffer solutions were prepared using water from a Milli-Q unit (Millipore). The pH of each solution was verified on a WTW 537 pH meter (WTW, Weilheim, Germany). All chemicals were purchased with the highest available degree of purity. Pyridine, sodium chromate, histamine dihydrochloride, 2-aminobenzimidazole, 18-crown-6, glycolic acid, hexamethonium hydroxide (HMOH), hexadecyltrimethylammonium bromide (CTAB) and sodium dihydrogenphosphate were purchased from Fluka (Neu-Ulm, Germany). Hexadimethrine bromide (HDB, Polybrene) was purchased from Aldrich (Milwaukee, WI, USA).

2.4. Rinse procedures

The performance of the migration time was studied by 30 consecutive repetitions of each separation system. Between each run, different combina-

tions of rinse steps were tested. The capillary was flushed with every solution for 3 min [pressure: 1.38×10^5 Pa (20 p.s.i.)]. If an electroconditioning-step was performed, separation voltage was applied to the buffer-filled capillary after preconditioning for 1 h, before each series. As no replenishment system was available, the electrolytes had to be changed frequently in order to prevent changes in buffer composition due to electrolysis. The separation vials were changed after every third or fourth separation. The total run time of each separation was 10 min.

2.5. Integration softwares

The following softwares were used in this comparison: Beckman System Gold V 7.12 (Beckman Instruments), Beckman P/ACE System MDQ V 1.2 (Beckman Instruments), HP 3D-CE ChemStation Rev.A.05.02 (Hewlett-Packard, Waldbronn, Germany) and CEPLOT (software written in our laboratory).

The electropherograms were measured with a Beckman P/ACE 2050 instrument and the data were analyzed by using the Beckman Gold software. Data were exported in the data interchange format (*.dif) into the CEPLOT software and analyzed.

In order to transfer the GOLD-data files into the P/ACE System MDQ format, the following procedure had to be applied: (1) The P/ACE Station V1.0 was installed on the same computer as the P/ACE System MDQ software; (2) The Gold files are transformed by the program convert.exe in the Pace directory. (This step is not necessary, when Gold-files from the Version 8.1 or newer are used); (3) The P/ACE Station V1.0 is loaded via the Program-Menu of the Windows 95/98 desktop. This Window should be kept at the OK-prompt; (4) Before pushing the OK button, the Gold-File Converter is loaded via the Program-Menu and the files can be converted. (This does not work if the P/ACE Station is not left at the OK-prompt as in point (3).) The resulting file format can be read by the P/ACE Station and P/ACE System MDQ, which are identical softwares concerning the data analysis. Upon analysis in the Beckman P/ACE System MDQ software, the data files were converted into the Analytical Instruments Association (AIA) format (*.cdf). This format can be read by the HP 3D-CE ChemStation software.

Following this routine, one set of raw data files could be analyzed with several different softwares. Thus the only detail which is different in this comparison is the software that was used.

For every software an integration method has been set up with integration parameters adapted to the peaks in the electropherogram. Nevertheless, it was sometimes necessary for some peaks to adjust the integration parameters manually.

2.5.1. Accuracy of true value

For this purpose a calibration curve with four standard solutions was measured: 5.1, 10.6, 20.4 and 40.7 ppm of NH_4^+ in solution with an internal standard (Ca^{2+}, 20.0 ppm). For every point four repetitions were made. A solution with a known concentration (24.7 ppm) was injected for quantitation. As a separation system, a system for indirect UV-detection of cations with pyridine was used [28]. Comparison criteria were the deviation from the ''true'' value, the standard deviation of the result by including the error of the calibration curve [29], the correlation coefficient (r) and the slope of each calibration curve.

2.5.2. Reproducibility of integration

For this investigation ten repetitions of every separation were made and the data files were analyzed by all five softwares. The comparison of the softwares was done by the use of the RSD of the corrected peak areas.

3. Results and discussion

3.1. Repeatability of migration times

3.1.1. Systems with cathodic EOF

The proper selection of the rinse step on the migration time is demonstrated first with a rather simple separation system: the reproducibility of the migration time of histamine in an acidic phosphate buffer was investigated and the results are summarized in Fig. 1. The selection of the intermediate rinse steps has a serious impact on the consistency of the migration time. Here four different combinations

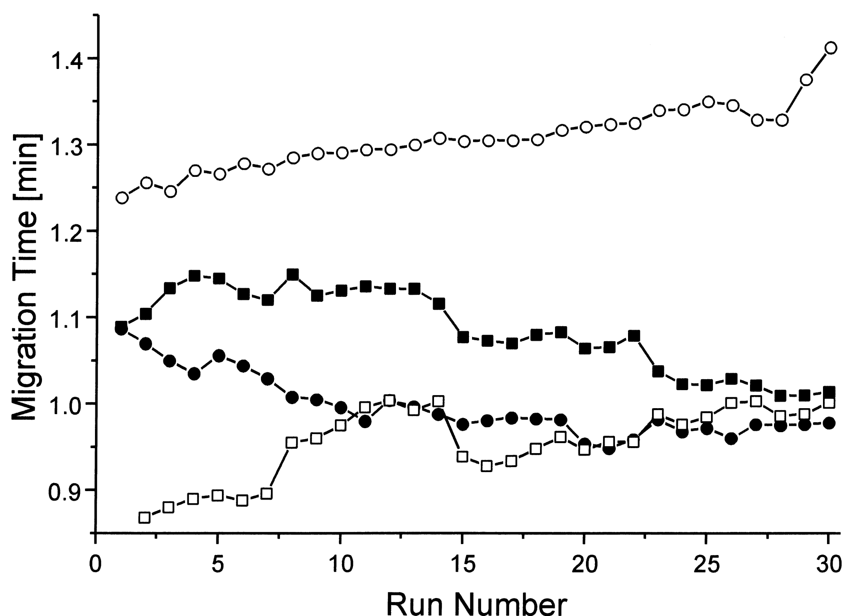

Fig. 1. System with cathodic EOF. (●) buffer; (■) 0.1 *M* sodium hydroxide, buffer; (O) 0.1 *M* hydrochloric acid, buffer; (□) water, buffer. Conditions: buffer=25 m*M* phosphate, pH=4.5; capillary=37 cm (effective length 30 cm); separation voltage=20 kV; detection, UV=254 nm; marker=histamine (75 ppm); injection time=5 s.

have been examined: rinsing by buffer only, by 0.1 *M* sodium hydroxide and buffer, by 0.1 *M* hydrochloric acid and buffer and by plain water and buffer. Hydrochloric acid and sodium hydroxide were chosen because here the surface of the capillary has been cleaned in between each run. Possible memory effects, caused by adsorption of analyte or buffer components should be reduced by the use of such solutions. As known these adsorbed molecules may influence the surface properties of the capillary and affect the velocity of the EOF. As can be seen in this figure, the combination of hydrochloric acid and buffer leads to a very stable series of migration times. In all the other cases, the values for the migration time show a much higher fluctuation. It should be mentioned that after every fourth injection the buffer vials had been changed to eliminate the possible influence of buffer pH changes caused by electrolysis. The figure shows, that the choice of the flushing buffer solutions can have a major impact on both, the absolute value and possible tendencies in migration time.

3.1.2. Systems with anodic (reversed) EOF

The influence of the proper selection of the rinse step is more important in the case of systems with reversal of the electroosmotic flow. Such systems are commonly used for the analysis of inorganic anions in order to be able to separate anions with high and low mobilities in a single run [30–32]. The reversal of the EOF can be achieved by the use of positively charged compounds as buffer additives that are adsorbed on the silica surface. This layer of adsorbed positive molecules yields in an overall positively charged surface which causes the reversal of the direction of the EOF. Three commonly used EOF modifiers were studied here: HMOH, HDB and CTAB. Their structures are given in Fig. 2.

These modifiers are adsorbed on the surface due to electrostatic interactions, consequently the effect of the rinse steps is of great importance. Several effects are possible: the coating layer on the surface may be destroyed when sodium hydroxide is used to flush the capillary. When using acidic mixtures, the proton can displace the coating cation via an ion-exchange mechanism. It has to be experimentally determined whether a rinse step leads to a higher or lower repeatability of migration times. All three adsorptive coatings were studied by using the same buffer systems. As can be seen in Fig. 3, the results are

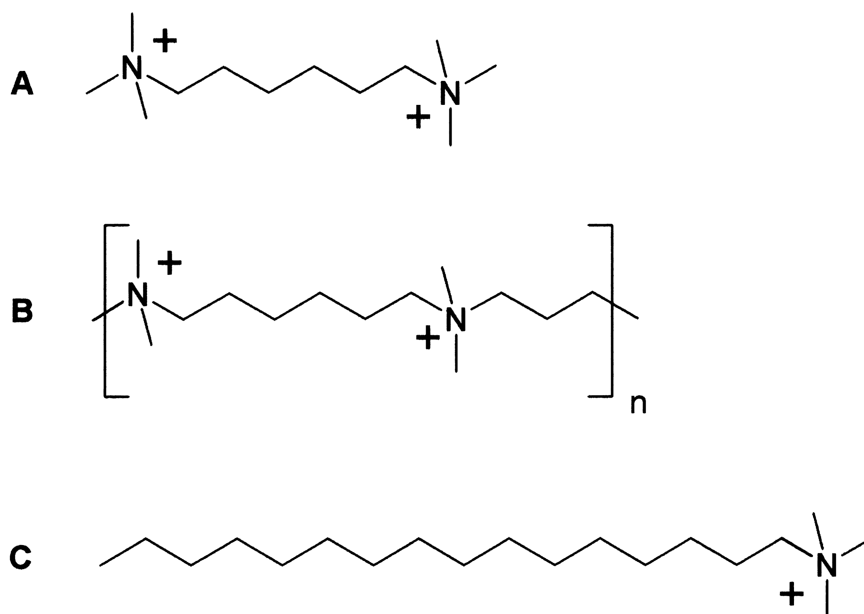

Fig. 2. Structures of EOF modifiers. A=hexamethonium hydroxide (HMOH); B=hexadimethrine bromide (HDB); C=hexadecyltrimethylammonium bromide (CTAB).

very different despite the structural similarity of the modifiers. In the case of the HMOH modifier (Fig. 3A), rinsing with buffer only leads to strong fluctuations of the migration times, which can be related to the changes of the separation buffer vials. The fluctuation might be caused by an inhomogenous capillary surface before each run. When rinsing with a combination of hydrochloric acid and buffer, stable values of the migration times have been observed. The protons of the acidic solution may displace adsorbed solutes on the coating layer and retransform the surface after each run in the original state. An electroconditioning step [11,13,14] was applied additionally to the optimized system (hydrochloric acid and buffer) because especially at the beginning of each series the migration times showed a strong drift. By applying the voltage intended for the separation on the capillary for 1 h, the capillary is rinsed additionally by the electroosmotic flow. As this flow is generated at the surface of the capillary it has a distinct rinse effect close to the surface and can, therefore, have an important influence on the equilibria at the capillary surface. This step leads to a further increase of the stability of the migration times.

In Fig. 3B the results for the HDB coating are shown. In this case, rinsing with buffer gave the best result. All the other rinse combinations like sodium hydroxide or hydrochloric acid and buffer gave poorer stability. With the combination of sodium hydroxide and buffer a strong drift in the migration time was observed. Also here it was found that an additional electroconditioning step resulted in better repeatability of migration times with the optimized system.

With the third modifier, CTAB (Fig. 3C), it was found that rinsing with buffer gave the best repeatability. For the rinse combinations with sodium hydroxide or hydrochloric acid, a drift in migration time was observed. By an electroconditioning step, the repeatability of the migration time could be improved additionally. The RSD values for the migration time for the different rinse procedures are summarized in Table 1. Amelioration by a factor up to three has been possible by applying an electroconditioning step.

3.2. Reproducibilty of the corrected peak area

CE systems are usually purchased with a corresponding software for system control and data analysis. Only a few CE systems can be operated by

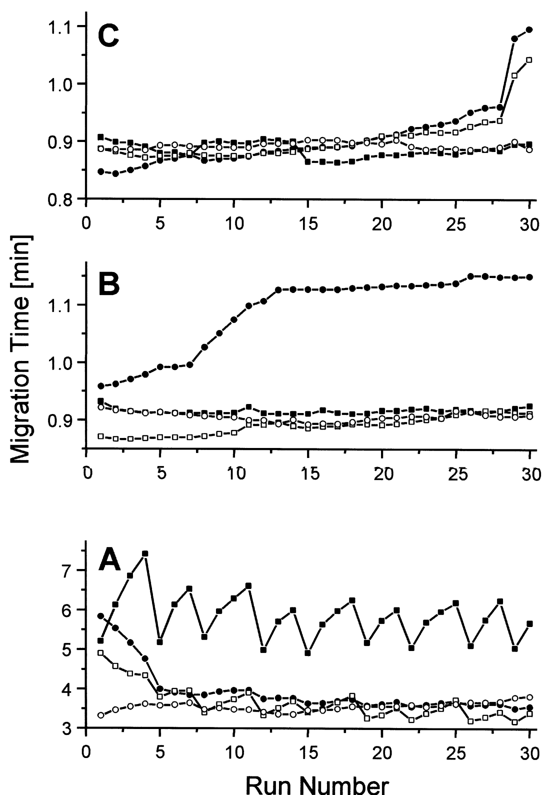

Fig. 3. System with anodic EOF. (■) Buffer; (●) 0.1 *M* sodium hydroxide, buffer; (□) 0.1 *M* hydrochloric acid, buffer; (O) electroconditioning plus optimized system. Conditions: buffer=5 m*M* chromate, pH=10.5 (sodium hydroxide); capillary=37 cm (effective length 30 cm); separation voltage=−20 kV; detection, UV=214 nm; marker=thiocyanate (80 ppm); injection time=5 s; modifier=(A) 0.75 m*M* HMOH, (B) 0.001% (w/v) HDB, (C) 0.02 m*M* CTAB.

different softwares such as the Beckman P/ACE 2000 and Beckman P/ACE 5000 series. In this part, softwares from different manufacturers have been compared by exchanging several packages of raw data sets acquired with one system. Significant differences were found for softwares from different manufacturers or of different age of production. In Table 2 all softwares compared are gathered together with the corresponding CE instruments usually operated with. The used softwares can be divided into two main categories. The so-called "older" softwares were originally designed for control and data aquisition of HPLC systems. A CE option was adapted to them later. Therefore, some special requirements for CE have not been initially included in their programming. Considerations essential for CE are the usual small signal heights, as one is always working close to the detection limit due to the short optical path length. In contrary to HPLC, in CE triangular peak shapes are observed when working with low buffer concentrations. The Beckman System Gold V7.12 software and the CEPLOT software are named "older" softwares in this article. Whereas the so-called "newer" softwares have been designed originally for the use with CE systems. Integration and the peak identification algorithms have been especially designed [27] for the use with CE. The Beckman P/ACE System MDQ and the Hewlett-Packard HP 3D-CE ChemStation will be referred as "new" softwares in this article. In Table 2 the Beckman P/ACE Station V1.0 is listed additionally, as the data analysis is identical to the Beckman System MDQ software. It can be used to control the Instruments of the P/ACE 2000 and 5000 series.

For the laboratory-written software CEPLOT two different integration algorithms were programmed (see Fig. 4). Usually in quantitative CE the total peak area is divided by the migration time at peak maximum, i.e. to use corrected peak areas [13,14,22,33,34]. This measurement is required be-

Table 1
RSD values of the different rinse steps

Rinse steps	RSD (%)		
	CTAB	HMOH	HDB
Buffer	1.44	3.04	0.55
0.1 *M* NaOH, buffer	6.53	7.36	6.29
0.1 *M* HCl, buffer	4.42	5.18	1.85
Optimized system	Buffer	0.1 *M* HCl, buffer	Buffer
Electroconditioning plus optimized system	0.65	2.23	0.83

Table 2
Softwares used in the comparison

Software	Manufacturer	Corresponding CE system
Gold V7.12	Beckman Instruments	Beckman P/ACE 2000/5000
P/ACE System MDQ V1.2	Beckman Instruments	Beckman P/ACE MDQ
(P/ACE Station V1.0)	–	(Beckman P/ACE 2000/5000)
HP 3D-CE ChemStation Rev. A 05.02	Hewlett-Packard	HP 3D CE System
CEPLOT and CEPLOT (Integral)	Self programmed software	Modular CE Systems

cause in CE the analytes are passing the detector cell at different velocities. Therefore, when the migration time of a compound is subject to variations, the peak area will change. When working at low buffer concentrations tailing or leading peaks with triangular shape are often observed. With heavily tailing peaks as shown in Fig. 4, it is obvious that the migration time at peak maximum should no longer be representative for the total peak area. Therefore, an integration algorithm named CEPLOT (Integral) has been developed which divides every signal point by its corresponding migration time and then calculates the sum over all fractions. This algorithm should show lower RSD values in the case of triangular peaks observed when there are significant mobility differences between the buffer ions and the analyte ions. The advantage of this algorithm can be determined by comparing the RSD values to the CEPLOT algorithm. Both algorithms have been incorporated in one software and are using the same peak boundaries and baseline values.

3.2.1. Accuracy

First, the accuracy of the softwares to find the "true" value has been checked. With the same data set calibration curves have been calculated, and with

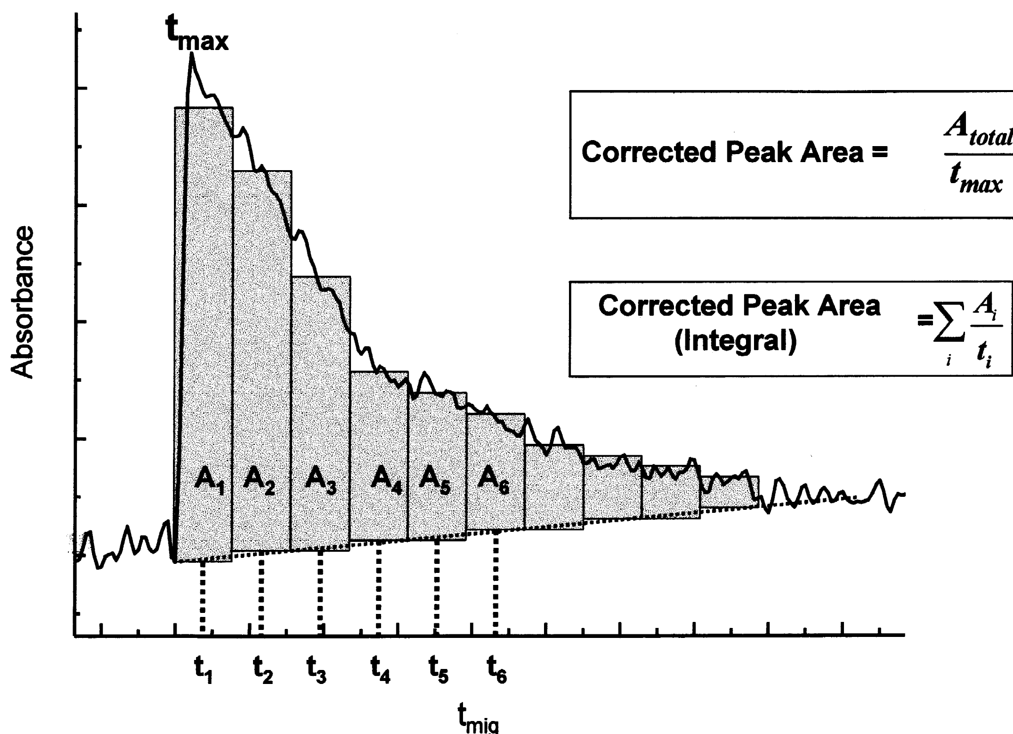

Fig. 4. Integration modes of CEPLOT and CEPLOT (Integral).

Table 3
Comparison of different softwares: accuracy[a]

Software	Value found (ppm)	SD (ppm)	r	Slope
Gold V7.12	25.6	1.4	0.9999	0.0616
CEPLOT	22.3	3.1	0.9988	0.0647
CEPLOT (Integral)	23.2	2.6	0.9958	0.0657
Beckman P/ACE System MDQ	25.7	1.7	0.9987	0.0627
HP 3D-CE ChemStation	25.7	1.7	0.9987	0.0627

[a] The concentration of the unknown solution was 24.7 ppm.

an ‘‘unknown’’ sample the quantitation has been performed. The results are summarized in Table 3. All softwares are able to find the correct value of the injected solution within the standard deviation. The standard deviation with the ‘‘new’’ softwares are better than with the ‘‘old’’ softwares. All calibration curves showed good correlation coefficients. The slopes for all calibration curves have almost identical values.

This result shows that by the exchange of raw data files no major errors are introduced into the data sets. Therefore, the method can be used to compare the performance of different softwares.

3.2.2. Reproducibility

First a system for the indirect UV detection of eight different cations was used. The concentration of each analyte was chosen in such a way, that peak areas were identical for each analyte. The electropherogram used for these measurements is shown in Fig. 5. According to Horwitz [35] the RSD should increase when the measured values decrease. In order to minimize errors introduced by injection, calcium was chosen as the internal standard. The calculated RSD are summarized in Fig. 6. In general, with the ‘‘old’’ softwares higher values for the RSD are observed than with the ‘‘new’’ softwares. Espe-

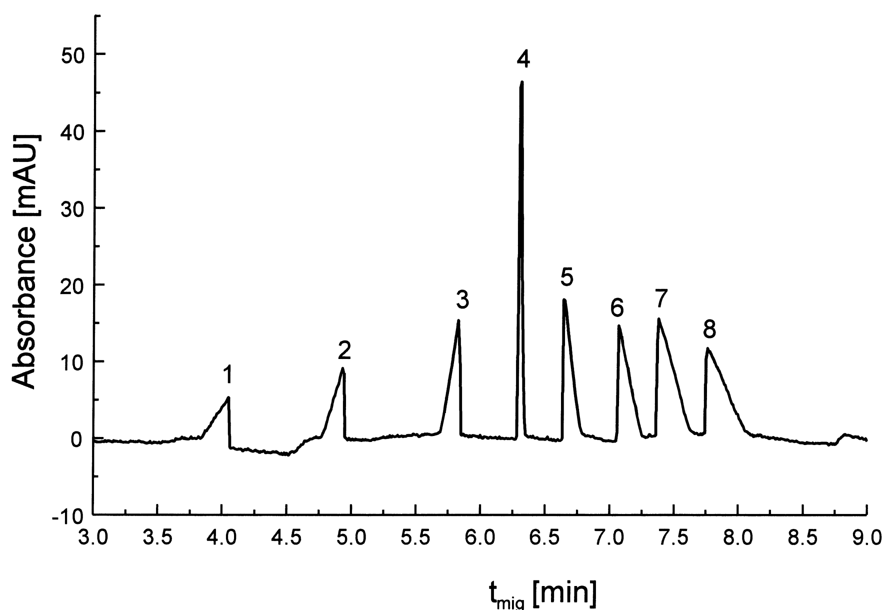

Fig. 5. Separation of eight cations with identical peak area. Conditions: buffer=9 m*M* pyridine, 12 m*M* glycolic acid, 5 m*M* 18-crown-6, pH=3.6 (sulfuric acid); capillary=67 cm (effective length 60 cm); separation voltage=25 kV; detection, UV=254 nm. Peak assignment: 1=ammonium, 2=potassium, 3=sodium, 4=calcium, 5=magnesium, 6=strontium, 7=lithium, 8=barium.

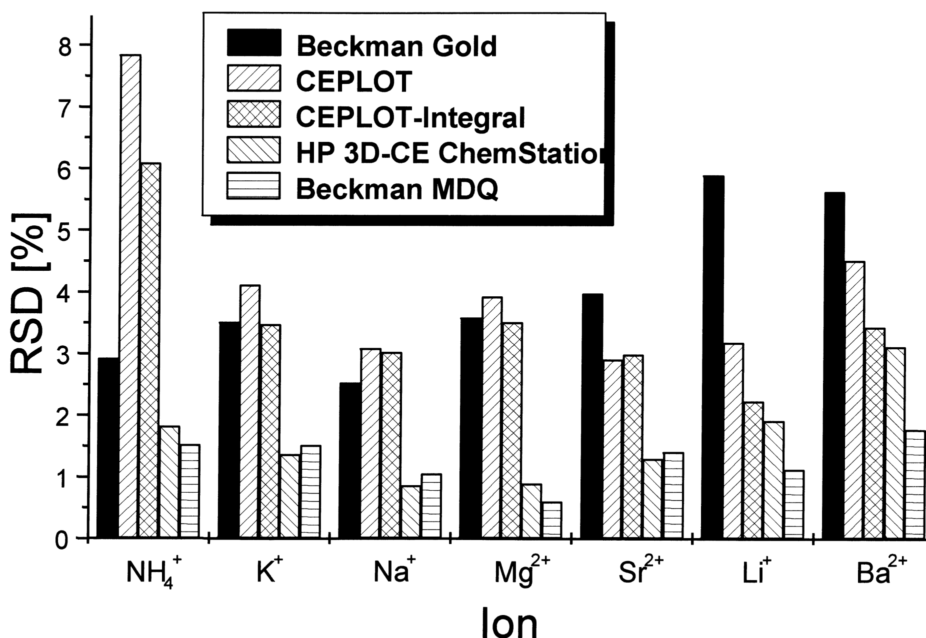

Fig. 6. RSD values of different softwares by integrating peaks with identical area.

cially strongly tailing peaks at lower S/N ratios cause problems. Between the ''new'' softwares no significant difference could be observed whether strongly leading or tailing peaks are integrated. The difference between the CEPLOT and the CEPLOT (Integral) demonstrates one of the reasons: for the strongly tailing or leading peaks, the CEPLOT (Integral) gives the better RSD values. Whereas, when the peaks are more symmetrical differences are negligible. Therefore, by applying an integration algorithm which uses a weighted integration, lower RSD values can be achieved.

In a second set of experiments a separation was chosen where all peaks had been of identical height. The selected concentration gave peak heights with a signal-to-noise ratio of around ten. The RSD with this set of quantitative analysis are around 6% for the ''new'' integration softwares, whereas the ''old'' softwares yield values up to 16% (see Fig.7). Interestingly, the CEPLOT (Integral) algorithm always gives better RSD values than the CEPLOT algorithm. The gain in reproducibility by using the CEPLOT (Integral) algorithm is usually in the range of 2%.

Additionally, the influence of the signal-to-noise ratio on accuracy of quantitation was studied. Because of the higher dynamic range, direct UV-detection was used in this case. Quantitation was done between peak heights with a signal-to-noise ratio of 5 and 168. The results are summarized in Fig. 8. Significant differences between the different softwares can only be observed at S/N ratios below 35. Here, the ''old'' softwares produce higher values for the corrected peak areas than the ''new'' softwares. The RSD values close to the detection limit are 10% or more. At higher S/N values (similar to those which are achieved in HPLC) no significant differences between the different softwares can be observed. The error here is minimized to a value of approximately 1%. The RSD value for the ''new'' softwares such as the Beckman System P/ACE MDQ seems almost to be independent from the S/N ratio. Only the ''old'' softwares show variations as a function of the S/N ratio. Again the comparison of both CEPLOT algorithms is interesting; the CEPLOT (Integral) algorithm yields lower RSD values again than the CEPLOT algorithm.

One of the reason for these differences might be

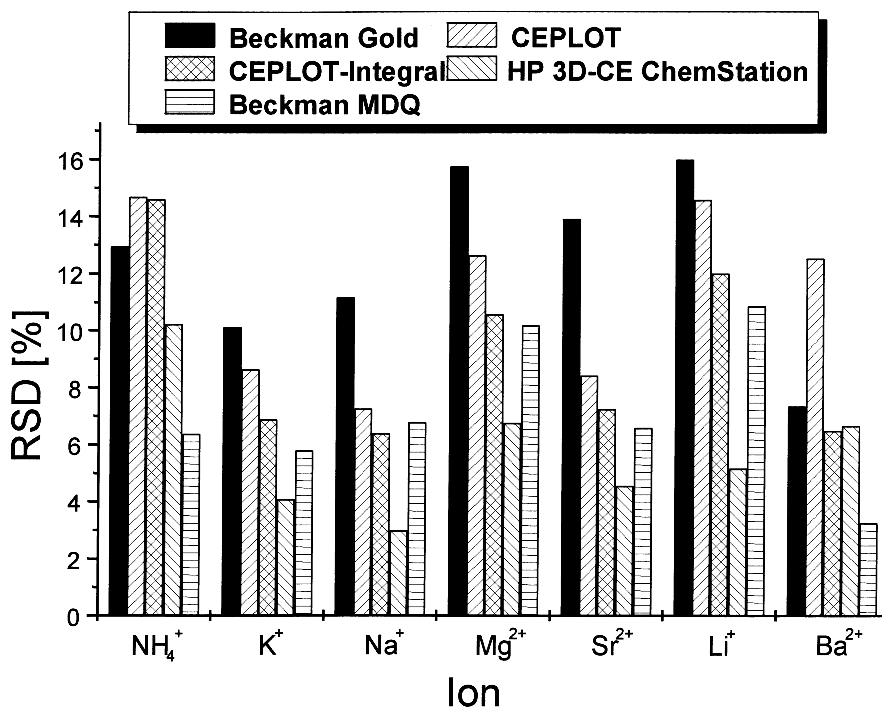

Fig. 7. RSD values of different softwares by integrating peaks with identical height.

the problem of correctly finding the baseline. Only the ''newer'' softwares are able to identify correctly the start and end of peaks close to the detection limit. ''Older'' softwares may have greater problems to identify correctly peak start and end point. Therefore, they are not determining the total actual peak area or they are adding noise of the baseline to it. This causes the higher RSD values at low S/N ratios. As the S/N ratio, and so the peak area, rises these small mistakes of the older softwares upon peak start and end detection can be neglected. Therefore the performance of these softwares is identical to the ''new'' ones at higher S/N ratios.

4. Conclusion

The precision of capillary electrophoresis, and hence its use in routine analysis, depends on the repeatability of migration time and the accuracy of quantitation. The proper selection of rinse steps has an important influence on the repeatability of migration times. The inner surface of the capillary is reconditioned and adsorbed molecules are eliminated by the use of the rinse steps. For systems with an anodic electroosmotic flow, it could be demonstrated that even structurally very similar EOF modifiers require totally different combinations of rinse steps. The effect of a fluctuation in migration times on the peak area can be reduced by the use of corrected peak areas. To identify peaks by their retention time in qualitative analysis, it is essential to work with systems providing constant migration times. The selection of the various rinse steps has to be an essential and additional optimization parameter in CE method development.

In order to determine the errors introduced by the software upon peak integration, commercial available softwares have been compared with different sets of identical raw data files. It was found that all the softwares tested are able to determine correctly

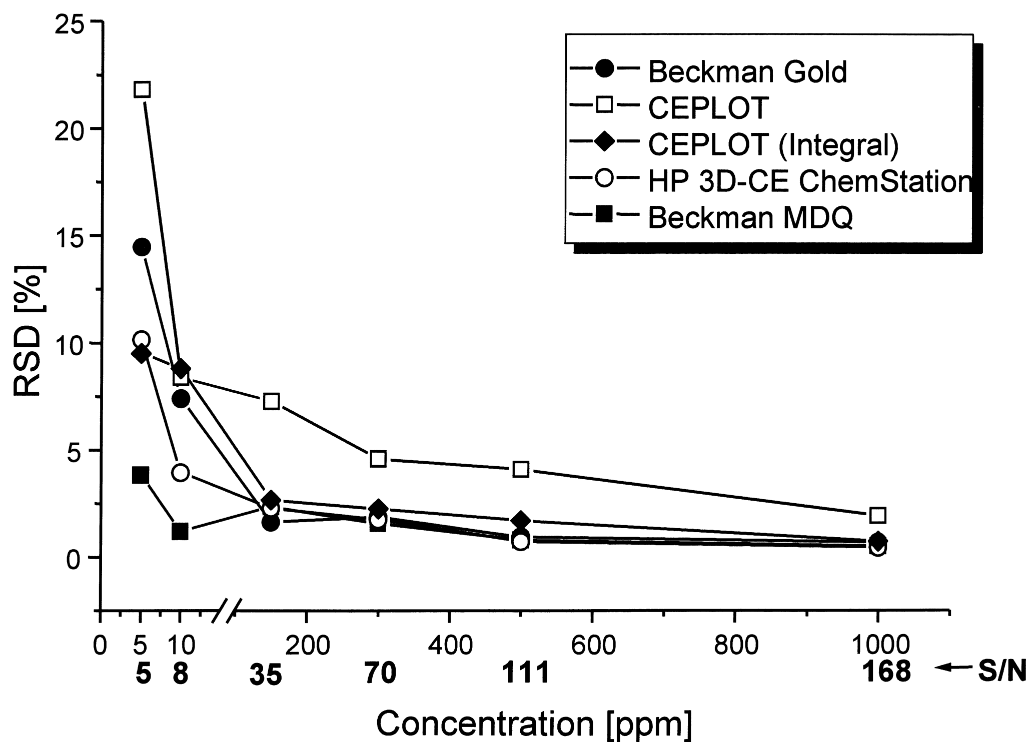

Fig. 8. RSD values of different softwares as a function of the signal-to-noise ratio.

the true value of an unknown solution within the limit of the standard deviation. Concerning the reproducibility of integration, it was found that the RSD values are a function of the S/N ratio. All softwares give identical RSD values around 1% at S/N ratios above 35. When the S/N ratio decreases to lower values, significant differences between the individual softwares could be observed. In general, softwares of the ‘‘newer’’ generation produce lower RSD values than the ‘‘older’’ softwares still in use. Algorithms working with a weighted integration system can provide lower RSD values than the usual algorithms working with corrected peak areas. This is especially recognizable for peaks which show a strong leading or tailing. In order to reduce the error introduced by the integration software it is either possible to import the data files into newer software versions or to work at higher S/N ratios by injecting higher concentrated solutions or using stacking effects. However, this can not always be selected due to analytical requirements preset by the analyte concentrations.

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