effect of even a strong φ–m interaction on the three differences will be only minor because of a large degree of compensation.

2.3. Solute–mobile phase fluid interaction (a–m) within a stationary phase

In Eq. 7, the quantities directly affected by the title interaction are $(\partial\mu_{af}^{\infty}/\partial w_{mf})$ and $(\partial\mu_{ag}^{\infty}/\partial w_{mg})$. A strong and/or specific a–m interaction will shift both ratios from the values typical of non-polar systems. The two ratios appear in Eq. 7 with opposite signs although not as a plain difference. Therefore, the effects of the a–m interaction on $(\partial\mu_{af}^{\infty}/\partial w_{mf})$ and $(\partial\mu_{ag}^{\infty}/\partial w_{mg})$ will largely counterbalance each other, at least in systems where none of the a–φ, a–γ, φ–m and γ–m interactions is much stronger than the rest.

The a–m interaction also has indirect, second-order effects on the transfer properties $\Delta H_{ta}^{f\to g}$ and $\Delta V_{ta}^{f\to g}$. As both stationary phases f and g are swollen with the absorbed mobile phase fluid, the effects of the a–m interaction on $\Delta H_{ta}^{f\to g}$ and $\Delta V_{ta}^{f\to g}$ will be minor.

Finally, therefore, the most important effects of a particular pairwise interaction within a chromatographic system on the properties involved in temperature-driven selectivity shifts may be summarized as follows:

Interaction Property

a–φ, solute–stationary polymer

$(\partial\mu_{af}^{\infty}/\partial w_{mf})$, $\Delta H_{ta}^{f\to g}$, $\Delta V_{ta}^{f\to g}$

φ–m, stationary polymer–mobile phase fluid

$\Delta S_{tm}^{m\to f}/\Delta V_{tm}^{m\to f}$, $(\partial w_{mf}/\partial P)_{T,\sigma}$

a–m, solute–mobile phase fluid

These interaction–property correspondences confirm the importance of stationary phase swelling in the temperature-induced selectivity shifts at a constant density of the mobile phase fluid. The quantities $(\partial\mu_{af}^{\infty}/\partial w_{mf})$, $\Delta S_{tm}^{m\to f}/\Delta V_{tm}^{m\to f}$ and $(\partial w_{mf}/\partial P)_{T,\sigma}$ are direct reflections of the swelling. The effects of swelling on the transfer properties $\Delta H_{ta}^{f\to g}$ and $\Delta V_{ta}^{f\to g}$ are less direct but definite. Interestingly, none of the properties appearing in Eq. 7 depends solely on the interaction between a solute and a pure stationary polymer.

In the systems investigated by Chester and Innis [1], the observed shifts in selectivity most likely originate from variations in the ratios $(\partial\mu_{af}^{\infty}/\partial w_{mf})$ for different solutes in different polymer–fluid systems. Further, as $(\partial w_{mf}/\partial P)_{T,\sigma}$ and $\Delta S_{tm}^{m\to f}/\Delta V_{tm}^{m\to f}$ vary from one polymer–fluid system to another, the variations in these solute-independent quantities serve to amplify those in the composition derivatives of the solute chemical potential (cf., Eq. 7).

3. Conclusion

A model-independent treatment has been presented of selectivity in temperature-programmed, constant-density, capillary SFC. Thermodynamic analysis confirms the expected importance of stationary phase swelling in determining the temperature-driven selectivity shift for a pair of solutes between two stationary phases. It appears that the thermodynamic background of the shift does not involve any property that would depend only on the interaction between a solute and a stationary polymer. Instead, the selectivity shift involves properties that depend either on the interaction between the stationary polymer and the mobile phase fluid or on all the three possible pairwise interactions within the solute–stationary polymer–supercritical fluid system. The only other thermodynamic derivative that contributes to the selectivity shift is the thermal pressure coefficient of the pure mobile phase fluid. It follows from these findings that swelling of the stationary phase plays an essential role in determining the temperature-induced shifts in selectivity among different stationary phases at a constant density of the mobile phase fluid.

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Statistical evaluation of various qualitative parameters in capillary electrophoresis

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Abstract

Various qualitative migration parameters for capillary electrophoresis have been evaluated. A theoretical discussion of the relative merits of the parameters is presented. The parameters were experimentally determined under a series of conditions to ascertain their dependence on such factors as the temperature and applied field. Statistical analysis of the results was used to elucidate the relative importance of the experimental conditions on the variance in the measured parameters. It was found that the relative parameters provide more reproducible results and are independent of experimental conditions, although they require the addition of at least one migration standard to the sample. Parameters which use the relative mobilities of the analyte and reference standard are most reproducible.

1. Introduction

The rate of migration for a given compound in capillary electrophoresis (CE) is determined by its effective molecular charge and hydrodynamic radius. Fast and highly efficient separations have been achieved over the past decade for a variety of water soluble compounds [1,2].

However, there still are several limitations to the approach. These include the need for reproducible, stable and reliable surface-coating technologies and the reproducibility of quantitative (amount) and qualitative (identity) information.

Solutions to many of these problems are the subject of intense study, and there have been several reports concerning qualitative reproducibility [3–11] and on-line monitoring of electroosmotic flow to improve qualitative repeatability [12]. Migration time remains the qualitative parameter most often reported (although occasionally relative migration times [8–10,13] or electrophoretic mobilities are used). As the separations performed with CE become more complex, and as the technique becomes more generally applied in regulatory environments, where the reliable transfer of methodology is essential, the need for a dependable and reproducible qualitative parameter will become more acute. Such a parameter would also be very helpful in the development of new methodology, especially when several instruments, laboratories, or conditions are employed.

An ideal qualitative parameter should depend only on the properties of the analyte and those conditions of analysis which are completely

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under the control of the analyst. Given these properties, and careful work by the analyst, such a parameter would also yield high reproducibility. The capacity factor in isothermal gas chromatography, for example, represents such a qualitative parameter. Capacity factors can be reproduced with different instruments and different laboratories with relative ease, provided that the same stationary phase and temperature are used.

In CE, even with state-of-the-art commercial instrumentation, the operator really only has complete control of the buffer composition and the applied field (or current). With most instruments the temperature can also be controlled, but the temperature control capabilities vary widely with manufacturer. Temperature has been shown to rise due to the effects of Joule heating even in thermostated systems [14–17]. Additionally, the portion of the capillary which is under temperature control varies between instruments, and with a given instrument the proportion of the total capillary length which is under temperature control varies with the length of the capillary itself. The variability between the temperature control capabilities of various instruments may also become apparent in their ability to offset the effects of Joule heating, resulting in different optimum field strengths for different instruments. Thus, it would be best if the parameter were also independent of the applied potential.

The capillary dimensions are not always completely under the control of the operator. Certainly, the inner diameter of the capillary, as reported by the manufacturer, is only a mean value and can vary significantly [18]. While the operator can in principle set the capillary length very accurately and precisely, in practice capillaries are often cut to length after they have been installed in a cartridge. Additionally, changes in capillary length, either large or small, are often part of the method development process.

Perhaps the most difficult factor to control is the electroosmotic flow. Electroosmotic flow directly affects the migration time of every analyte. The dependence of electroosmotic flow on the chemistry of the silica surface (zeta potential) makes it very difficult to control or reproduce. Potential solutions to this problem include the development of new capillary materials with more reproducible surfaces, or the development of reproducible and stable surface modification procedures for fused-silica surfaces which eliminate the problems associated with the zeta potential. In the meantime, however, a qualitative parameter should be independent of the zeta potential, so that it can be employed with present technology.

The result of the problems presented above is that the duplication of separation conditions, between different laboratories, or different instruments, or indeed different capillaries, is difficult. Previous reports of inter-laboratory validation studies [8,10] have shown excellent intra-laboratory results for relative migration time, but inter-laboratory results have shown 1.3 [8] to 4.5 [10] percent R.S.D. A qualitative parameter which is independent of the problematic factors discussed above is necessary to permit method development and transfer of methodology. The qualitative parameter should depend only on the buffer composition and the analyte properties, and should be independent of the temperature, the applied field, the capillary dimensions, and the electroosmotic flow.

Lee and Yeung [3] have reported the use of a migration index and adjusted migration index for improving qualitative precision, but this approach requires that the internal diameters of the capillaries be known to within 0.5%. This presents a significant problem in light of current capillary manufacture technology [19]. Vespalec et al. [5] have reported a method which permits the determination of the "actual effective mobility" of the unknown. This can be determined from its migration time and the migration times of two migration reference standards in the same electrophoretic run. The actual mobilities of the two reference standards, under a given set of conditions, must be known. This approach produces reproducible results under a variety of conditions, and is reported to be independent of temperature. The limitation of this approach is the requirement that the electrophoretic mobilities of the two standards be known. Others have

shown the utility of relative migration times, in which the migration time of the analytes is divided by the migration time of an internal standard, for improving the qualitative results [8–10,13]. Recently, the utility of a retention index [19,20] and migration indices [11] in micellar electrokinetic chromatography have been investigated.

In this work a comparative study of the migration time, relative migration time, electrophoretic mobility, actual mobility, and relative mobility has been performed for a series of test analytes. The repeatability, reproducibility and practicality of the various parameters are compared.

2. Theory

Capillary electrophoresis separates ions by differences in their electrophoretic mobility. The effective migration rate of a given analyte is determined by its mobility and the electroosmotic flow through the capillary.

The electrophoretic mobility of a given compound can be most simply expressed in terms of its radius, r, the charge on the molecule, q, and the viscosity of the separation medium, η, as follows:

$$\mu_{ep} = \frac{q}{6\pi r\eta} \tag{1}$$

This simple equation is limited to fully ionized species, and does not account for non-ideality. More complete treatments, which account for the effects of ionic strength, can be found elsewhere [6,21–23]. In this work the ionic strength is assumed to be under the control of the analyst, and the effects of ionic strength are not investigated in detail.

The electrophoretic mobility is dependent on the properties of the compound. The temperature of the separation medium is also important, as it affects the viscosity (the viscosity of water changes by approximately 2%/°C in the range from 20 to 40°C [23]). In practice, the electrophoretic mobility (μ_{ep}) is calculated from experimental results as follows:

$$\mu_{ep} = \frac{lL}{V}\left(\frac{1}{t_{mig}} - \frac{1}{t_0}\right) \tag{2}$$

where l is the effective length of the capillary (inlet to detector), L is the total length of the capillary, V is the applied potential and t_{mig} and t_0 are the migration time of the analyte and the migration time of an uncharged solute, respectively. Thus, in order to accurately determine the electrophoretic mobility t_0 must be measured, and the capillary dimensions must also be known accurately. In practice, the determination of electrophoretic mobility requires accurate knowledge and control of the capillary dimensions.

The migration time is easily determined experimentally. It can be expressed as:

$$t_{mig} = \frac{lL}{V(\mu_{ep} + \mu_{eo})} \tag{3}$$

where μ_{eo} is the electroosmotic mobility, which is defined as

$$\mu_{eo} = \frac{\epsilon\zeta}{\eta} \tag{4}$$

where ϵ is the dielectric constant of the separation medium and ζ is the zeta potential of the capillary surface. Looking at Eqs. 1, 3 and 4, one can see that the migration time is affected by the capillary dimensions, the zeta potential (ζ) of the silica surface, and the temperature of the system (affecting the viscosity, the dielectric constant and the zeta potential). Thus, it can be seen that migration time is affected by all of the problematic factors.

The use of relative migration time (RMT),

$$t_{rel} = \frac{t_{mig}}{t'_{mig}} \tag{5}$$

where primed values are for a reference standard run with the analyte, does eliminate the effects of the viscosity and the capillary dimensions, as can be seen by combining Eqs. 1 and 3 through 5:

$$t_{rel} = \frac{\epsilon\zeta + \frac{q'}{6\pi r'}}{\epsilon\zeta + \frac{q}{6\pi r}} = \frac{a + \frac{q'}{r'}}{a + \frac{q}{r}} \tag{6}$$

where a ($a = 6\pi\epsilon\zeta$) is independent of analyte characteristics, but is dependent on the zeta potential. This assumes that the solution–analyte viscosity is identical to the solution–surface viscosity, which is justified under most operating conditions [3]. It also assumes that the analyte and the reference material experience the same viscosity, which is true unless there is temperature drift or gradient in buffer composition which causes one to encounter a higher or lower average viscosity than the other. Relative migration time does depend on the zeta potential, although this appears in the numerator and denominator of the equation and thus its effects should be limited. Still, this could impair the ability of this parameter to permit the transfer of methodology. Temperature effects on RMT should be limited to the consequent changes in the zeta potential and dielectric constant.

Analysis of Eq. 6 by differentiation with respect to the electroosmotic flow leads one to the conclusion that variations in RMT caused by changes in the electroosmotic flow can be minimized by selecting a reference material with similar electrophoretic mobility to that of the analyte. This would also help with the effects of gradients or temperature drift during the run.

Consider the relative electrophoretic mobility (RM). This is most simply defined as:

$$\mu_{rel} = \frac{\frac{q}{r}}{\frac{q'}{r'}} = \frac{qr'}{q'r} \tag{7}$$

and is calculated from the experimental parameters:

$$\mu_{rel} = \frac{t'_{mig}(t_0 - t_{mig})}{t_{mig}(t_0 - t'_{mig})} \tag{8}$$

Eq. 7 reveals that this parameter depends only on the properties of the analyte and the reference standard. Eq. 8 reveals that no experimental parameters such as column dimensions etc. are required for the determination of the RM. The migration times of a nonionic solute, a reference standard, and the analyte must be determined. The chemical (pK_a) and electrophoretic properties (q) of the analyte and the reference materials should match as closely as possible to negate the effects of non-ideality on the electrophoretic mobility. The temperature must be constant during the run so that both the analyte and the reference material experience the same average temperature. Likewise, electroosmotic flow must either be large enough to permit measurement of t_0, or small enough that t_0 can be eliminated from Eq. 8.

With either Eq. 6 or Eq. 8 it can be shown that when electroosmotic flow is minimal (i.e. t_0 is very large) the relative migration time (t_{rel}) is the inverse of the relative electrophoretic mobility (μ_{rel}).

In the approach of Vespalec et al. [5], the actual mobility of the analyte, μ_x, is calculated from the known mobilities of two reference standards and the experimentally determined migration times:

$$\mu_x = \mu_{ep}'' + (\mu'_{ep} - \mu_{ep}'')\frac{t'_{mig}(t_{mig}'' - t_{mig})}{t_{mig}(t_{mig}'' - t'_{mig})} \tag{9}$$

Due to differences in the effects of ionic strength on the mobility of compounds with different charge, this approach should work best when the reference materials have the same charge as the analytes and/or when the standard electrophoretic mobilities are determined under conditions nearly the same as those used for the analysis. This parameter may, in fact, perform better than the relative mobility, since the reference standards can be chosen such that the analyte falls between them in mobility and migration time. It is certainly a better choice when electroosmotic flow is significant (preventing t_0 from being eliminated from Eq. 8) but not measurable, since no determination of electroosmotic flow (or t_0) is necessary.

Eq. 9 essentially reduces to Eq. 8 when μ_{ep}'' is set to 0, and t_{mig}'' is thus equal to t_0. This is not entirely true, since in Eq. 9 μ'_{ep} refers to the known electrophoretic mobility of the reference compound under a given set of standard conditions, and the calculated μ_x is thus the mobility of the analyte under the same set of standard conditions. In practice, this difference amounts only to μ_x being directly proportional to μ_{rel}.

When two migration reference materials with mobility are used μ_x is a unique parameter. An advantage of this parameter is that when the reference materials and the analytes have similar chemical and electrophoretic properties (same charge) the value for a given compound should not depend on the reference materials used, whereas relative mobility is specific to a given analyte and reference material.

3. Experimental

A Spectra-Physics Model 1000 CE was used for all studies. The instrument was controlled with an IBM PS/2 Model 70 386 PC equipped with Spectraphoresis version 1.03 data acquisition and control software. All electropherograms were collected at both 210 and 255 nm.

All chemicals were purchased in the highest grade possible. Sodium hydroxide, sodium chloride, sodium tetraborate, boric acid, and acetone were obtained from E. Merck (Darmstadt, Germany). Anthraquinone-2-sulfonic acid, *p*-toluene-sulfonic acid, salicylic acid and phthalic acid, which were used as test analytes, were obtained from Aldrich (Steinheim, Germany).

The fused-silica capillary was purchased from LC Packings (Emmen, Netherlands), and was sourced from Polymicro Technologies, USA. The capillary had a 75 μm internal diameter, and a total length of 70 cm (effective length of 63 cm). The capillary was rinsed when installed with 1 *M* NaOH for 5 min at 50°C, 20 min at 40°C with 0.1 *M* NaOH, 20 min at 40°C with millipore water, and 30 min at 30°C with the run buffer. Before each run, the capillary was rinsed for 3 min with the buffer at the run temperature, and after each run the capillary was washed for 2 min with the run buffer at the run temperature. At the end of each day, the capillary was rinsed for 30 min at the run temperature with millipore water. At the beginning of each day, the capillary was rinsed first for 10 min at 40°C with 0.01 *M* NaOH, followed by millipore water for 10 min at 40°C, and finally by run buffer for 30 min at the run temperature. Each day, a blank run was performed with the run buffer before any actual runs were performed.

A 0.02 *M* borate buffer, pH 8.2, was prepared in millipore water as follows: a 0.02 *M* boric acid solution was titrated to pH 8.2 using a 0.005 *M* solution of sodium tetraborate (0.02 *M* in borate). The buffer was vacuum filtered through a 0.45-μm filter before use. For higher ionic strength measurements, sodium chloride was added to the buffer at the indicated concentrations. No further pH adjustment or filtrations were made after addition of the sodium chloride.

Stock solutions of the test analytes were prepared by dissolving 15 to 20 mg of the compound in 10.00 ml of the borate buffer. These stock solutions were stored at 4°C when not in use. Run solutions were prepared by adding 10 μl of the stock solution of interest and 20 μl of pure acetone to 1.2 ml of buffer solution in a sample vial. The final concentration of test analytes was thus 12–16 ppm.

Electrophoretic measurements were made using applied potentials of 10, 20, or 30 kV and temperature settings of 30, 40, or 50°C. At least three measurements were made at each condition. At each new condition the migration order of the test analytes was reconfirmed by consecutive addition of the analytes to the run sample vial or by the relative intensities of the peaks at the two wavelengths. Measurements at a given potential and applied temperature were not all made consecutively. In most cases a given condition was returned to after several runs at other conditions, and/or on different days. A total of 40 electropherograms were collected at the various conditions over a period of 9 days.

4. Results and discussion

A typical electropherogram of a mixture of the four test analytes is presented in Fig. 1, showing the 210-nm trace. The migration order is indicated. Acetone, used as a marker for the electroosmotic flow, does not appear in Fig. 1 as it is not detected at 210 nm. Electropherograms run at the same conditions, on consecutive injec-

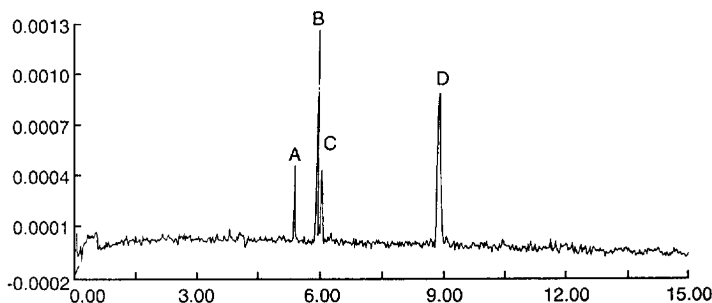

Fig. 1. Typical electropherogram, monitored at 210 nm. Peaks: A = anthraquinone-2-sulfonic acid, B = p-toluene sulfonic acid, C = salicylic acid, D = phthalic acid.

tions, are presented in Fig. 2. This figure illustrates limitations in the migration repeatability.

The electropherogram of these compounds provides the necessary features to test the utility of the various qualitative parameters. All of the compounds have pK_a values less than 5.5 (pK_{a2} for phthalic acid) and are fully ionized at pH 8.2. *p*-Toluene-sulfonic acid and salicylic acid have similar migration times, testing the ability to distinguish between compounds with similar migration characteristics. Phthalic acid has a charge of −2 and much greater mobility than anthraquinone-2-sulfonic acid which has a charge of −1, allowing the parameters to be tested with respect to migration time and testing their range of applicability. The effects of non-ideality on the electrophoretic mobility are expected to show for compounds with different charge. For relative migration time and relative mobility, anthraquinone-2-sulfonic acid was employed as the migration reference material. For actual mobility measurements, anthraquinone-2-sulfonic acid and phthalic acid were used as the reference migration materials. Because of the −2 charge on phthalic acid, its utility as a reference material for the analytes with −1 charge is questionable. We have tested the significance of this problem by repeating some of the studies using anthraquinone-2-sulfonic acid and salicylic acid as reference materials. The average electrophoretic mobility for these compounds at 30°C and 30 kV was used for the actual mobility calculations.

4.1. Migration time

Plotted in Fig. 3 are the migration times for a series of runs in which the applied potential was kept constant at 30 kV and the temperature was varied from 30 to 50°C. It can be seen from this plot that the migration time varies significantly with temperature, as expected. It can also be

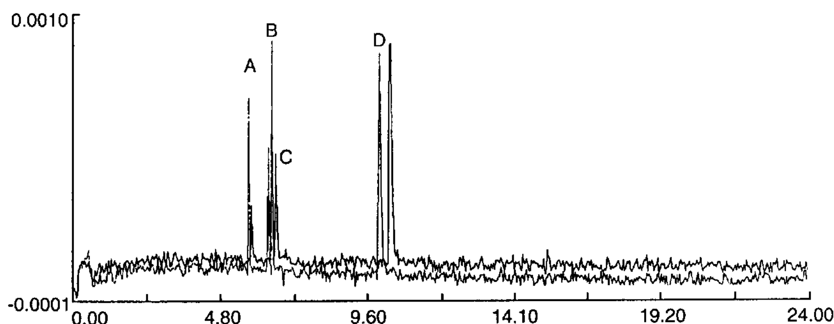

Fig. 2. Consecutive electropherograms at 20 kV applied potential and 30°C, monitored at 210 nm. Peaks: A = anthraquinone-2-sulfonic acid, B = *p*-toluene-sulfonic acid, C = salicylic acid, D = phthalic acid.

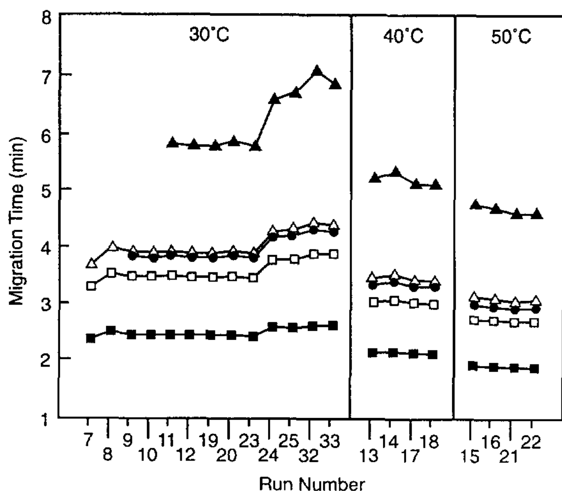

Fig. 3. Migration times as a function of the run number for the four analytes and acetone (t_0) at 30 kV and 30, 40, and 50°C. (■) acetone; (□) anthraquinione-2-sulfonic acid; (●) p-toluene-sulfonic acid; (△) salicylic acid; (▲) phthalic acid.

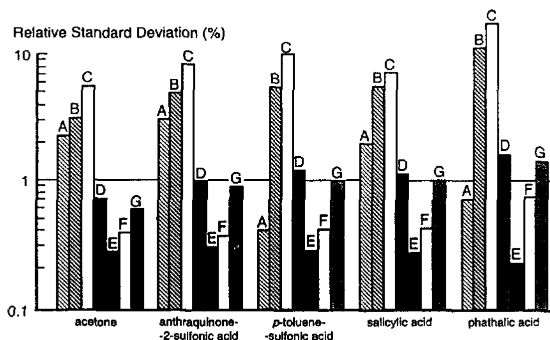

Fig. 4. Relative standard deviations in migration time for each analyte under the seven sets of analysis conditions. A = 30 kV, 30°C; B = 30 kV, 40°C; C = 30 kV, 50°C; D = 20 kV, 30°C; E = 20 kV, 40°C; F = 10 kV, 30°C; G = 10 kV, 40°C. Plotted on a semilogarithmic scale.

seen that there is significant variability in the migration times within a given temperature range. In fact, it would be impossible to determine whether a single peak in an unknown sample represented p-toluene-sulfonic acid or salicylic acid using migration time alone. It is important to notice the pattern in the variations. The fluctuations are always in the same direction, although they are not of the same magnitude. This implies that a common phenomenon, probably the electroosmotic flow, is causing these variations.

The data presented in Fig. 4 confirm the observations made from Fig. 3. There is a lot of variability in these results within a given condition, with the worst cases being 10–20% R.S.D. On the other hand, in the best cases, the precision was very good (<0.2% R.S.D.).

The poor precision is aggravated by the fact that the data were collected on different days and by the fact that the runs within a day were not made consecutively, but runs with different conditions fell between them. Often, the R.S.D. for a series of runs made consecutively at the same condition was very good (below 0.2%), but it was observed to be as high as 2%. The average migration time was observed to change by as much as 11% between days, when the analyses were otherwise run at the same conditions.

Analysis of covariance for the logarithm of the migration time (the logarithm was used to equalize the variance between analytes) for all of the separations run at 30 kV indicated that the type of analyte, the temperature and the run number all make highly significant contributions to the variance. Including the 10 and 20 kV data indicated that the applied potential also makes a highly significant contribution.

For a given temperature and applied potential, the inverse of the migration time was found to be highly correlated ($r^2 \geq 0.980$, as high as 0.9998) with the inverse of t_0. This is the expected behavior when the variations in migration time are caused solely by variations in electroosmotic flow.

An interesting result emerging from the statistical analysis is that the slopes of the plot of the logarithm of the migration time vs. the temperature are not significantly different for the five analytes. The slope of these plots (-0.0109 ± 0.0004) is similar to the slope of a plot of the logarithm of the viscosity of water with respect to the temperature (−0.0082) in the range from 30 to 50°C (data obtained from Ref. [24]). Changes in the viscosity appear to be the dominant factor, but do not sufficiently account for the observed slope. The difference may be the result of changes in the dielectric constant,

zeta potential or solvation brought on by changes in the temperature.

4.2. Electrophoretic mobility

Fig. 5 shows the data for electrophoretic mobility for the same data set as presented in Fig. 3. It can be seen that the electrophoretic mobility is also temperature dependent, but that it is in general more precise than migration time for a given temperature setting. The electroosmotic mobility is observed to vary significantly, with a pattern similar to that for the migration times (Fig. 3). This indicates that the common phenomenon which causes the poor precision in migration time for a given condition is the electroosmotic flow, probably due to zeta potential effects. These variations in electroosmotic mobility are not mirrored by variations in the electrophoretic mobilities.

Again, these observations are confirmed with study of Fig. 6. It can be seen that the relative standard deviations for electroosmotic mobility are often greater than one. The high variability in the electroosmotic flow is not reflected in the electrophoretic mobility values, which generally have an R.S.D. of less than one.

Analysis of covariance of the electrophoretic

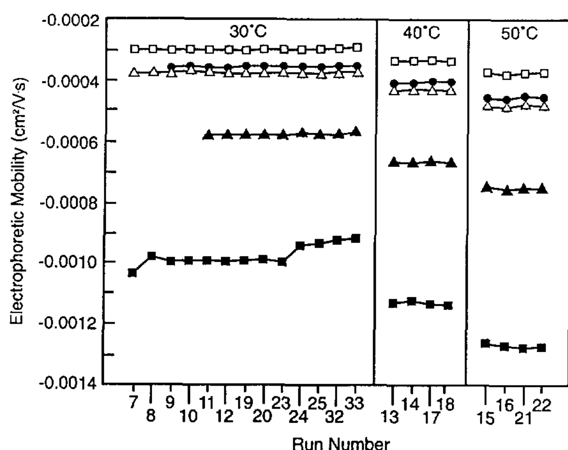

Fig. 5. Electrophoretic and electroosmotic mobilities at 30 kV and 30, 40, or 50°C, as a function of run number. (■) acetone; (□) anthraquinione-2-sulfonic acid; (●) p-toluenesulfonic acid; (△) salicylic acid; (▲) phthalic acid.

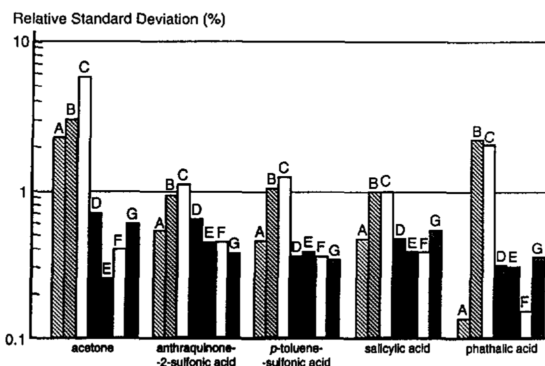

Fig. 6. Relative standard deviations in electrophoretic mobility for each of the analytes under seven sets of analytical conditions. Study conditions as in Fig. 4. Plotted on a semilogarithmic scale.

mobility indicated that the temperature and the analyte make the greatest contributions to the variance. The applied potential also makes a significant contribution, but its absolute contribution is minor. This may be the result of Joule heating, which is a function of the applied potential and affects the working temperature in the capillary. The run number also makes a minor, but significant, contribution. No reproducible correlation was observed between the electrophoretic mobility and t_0 or $1/t_0$, indicating that the electrophoretic mobility is unaffected by electroosmotic flow.

As was observed for migration time, there were differences between the mobilities measured on different days when the same conditions were used which affected the results reported in Fig. 6. These differences are not statistically significant, and since the data was collected on only two days no analysis of variance could be performed to determine the relative contributions to the variance. However, the changes in electrophoretic mobility between the two days are in the same direction and of nearly the same relative magnitude for all of the analytes. This must be caused by a real phenomenon, and is most likely due to differences in actual temperature between days. Although the temperature setting and reading were the same, these data indicate that there were still differences in temperature that affected the mobilities.

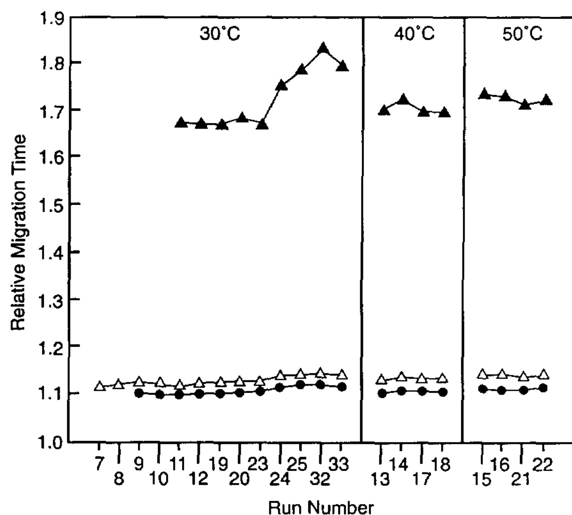

Fig. 7. Relative migration time at 30 kV and 30, 40, and 50°C, as a function of the run number. (●) *p*-Toluene-sulfonic acid; (△) salicylic acid; (▲) phthalic acid.

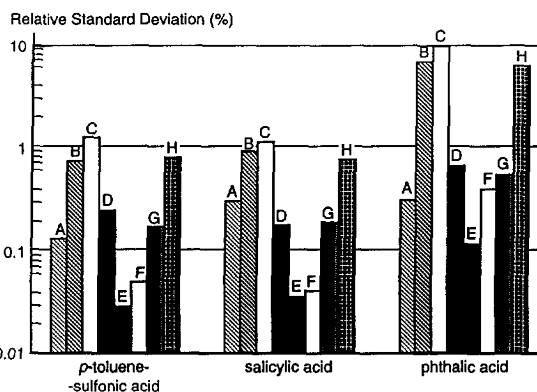

Fig. 8. Relative standard deviations in relative migration time for three analytes. Conditions of analysis as in Fig. 4. H = All conditions combined. Plotted on a semilogarithmic scale.

4.3. Relative migration time

Fig. 7 shows the results for relative migration time (RMT) using anthraquinone-2-sulfonic acid as a reference standard, for the same data as presented in Fig. 3. What can be seen here is that the RMT is not affected to a high degree by the temperature of the system. This was predicted by Eq. 6. Additionally, it can be seen that there remains some variation in the RMT within a given condition, especially for phthalic acid.

Fig. 8 presents the R.S.D. data for all the conditions. In this case we can also report an overall data set which takes into account the results for all conditions on all days, since the RMT should be independent of the conditions. The R.S.D. values are quite good for salicylic acid and *p*-toluene-sulfonic acid ($\leq$1%), but rather poor for phthalic acid (up to 10%). This is probably the result of the dependence, discussed in the Theory section, of the reproducibility of this parameter on the difference between the mobility of the analyte and the reference material. Phthalic acid has the greatest difference in mobility, and shows the poorest results. Also, when the electrophoretic mobility of the analyte is similar in magnitude but opposite in direction to the electroosmotic flow, small changes in the electroosmotic flow have a large effect on the migration time. The effect may also be caused by temperature drift, since this would cause phthalic acid to experience a different average temperature than the other analytes. It is also notable that the pattern of the plots follows that for electroosmotic mobility (Fig. 5) for each of the analytes. This indicates that the RMT is not completely free from the effects of variations in electroosmotic flow, as seen in Eq. 6.

Analysis of covariance for the data set collected at 30 kV indicates that only the type of analyte contributes significantly to the variance in the data set. The temperature and the run number do not significantly contribute. For a given applied potential and temperature, the inverse of the natural logarithm of the RMT was found to be highly correlated ($r^2 \geq 0.92$, as high as 0.999) with the inverse of t_0. This is as one would expect when the variation in RMT is caused solely by variations in electroosmotic flow.

The zeta potential and dielectric constant do depend on the temperature, so one might expect that the RMT would be affected to a greater extent by changes in temperature. This is a further indication that the effects of changes in the temperature are dominated by viscosity influences.

The ratio of the migration time of the analyte

to t_0 was also evaluated. In spite of earlier reports [13], this parameter behaved very poorly, showing poor repeatability within a given condition, and poor overall reproducibility. This probably results from the large difference in mobility between the analyte and the reference standard when this approach is used.

4.4. Relative electrophoretic mobility

Presented in Fig. 9 are the results for relative electrophoretic mobility, using anthraquinone-2-sulfonic acid as the migration standard. It can be seen from this figure that the RM is independent of temperature, and that it is reproducible over a range of analytical conditions. This is confirmed when the data are presented in Fig. 10. The R.S.D.s for this parameter are very good (≤1.4%), even for cases where all data are included. The R.S.D.s do appear to increase from salicylic acid to phthalic acid, which is not unexpected, since the difference in mobility and migration time between the reference standard and the analyte also increases. This may also be the result of variations or drift in the temperature of the system during the analytical runs. The between-day or between-condition variations

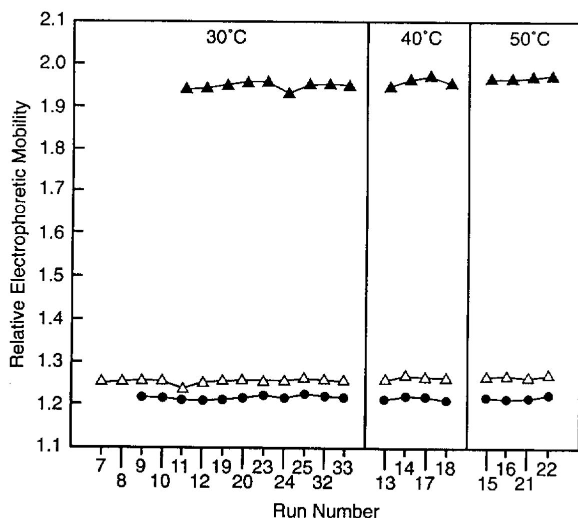

Fig. 9. Relative electrophoretic mobilities at 30 kV and 30, 40 and 50°C, as a function of the run number. (●) p-Toluene-sulfonic acid; (△) salicylic acid; (▲) phthalic acid.

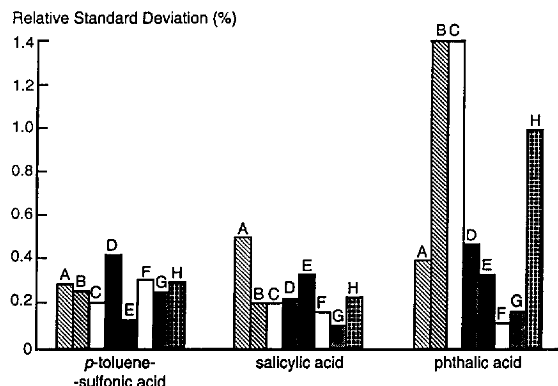

Fig. 10. Relative standard deviations in relative electrophoretic mobilities for three analytes. Conditions as in Fig. 4. H = All conditions combined. Plotted on a linear scale.

which were problematic for the other parameters were not observed for the RM.

Analysis of covariance indicated that all of the parameters (analyte, applied potential, temperature and run number) make significant contributions to the variance. The analyte contributes by far the greatest amount to the total variance. The temperature can not be considered significant with 95% confidence, but is significant at the 90% confidence level. However, these results must be considered with respect to the trivial overall variance in the data set, which results in high significance being assigned to only minor variations. No correlation between RM and t_0 or $1/t_0$ was observed.

4.5. Actual mobility

The actual mobility, calculated using anthraquinone-2-sulfonic acid and phthalic acid as reference standards, also provides very good results, as can be seen from Figs. 11 and 12. The precision of these results is equivalent to that for the relative electrophoretic mobility results for these analytes. There is no evidence that they are better, as might be expected since the mobilities of the two analytes are between those of the two reference standards.

Because phthalic acid has a charge of −2, it may be expected that it would not perform well as a reference standard for compounds such as p-toluene-sulfonic acid and salicylic acid, which

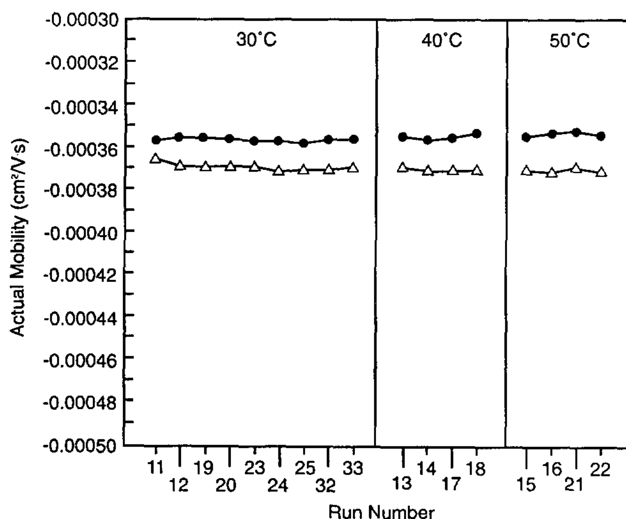

Fig. 11. Actual mobility for *p*-toluene-sulfonic acid and salicylic acid at 30 kV and 30, 40 and 50°C, plotted as a function of the run number. (●) *p*-Toluene-sulfonic acid; (△) salicylic acid.

each have a charge of −1. To investigate this, we have calculated the actual mobility of *p*-toluene-sulfonic acid using anthraquinone-2-sulfonic acid and salicylic acid as reference materials. The overall average actual mobility and reproducibility using these two reference materials ($3.56 \pm 0.02 \cdot 10^{-4}$ $cm^2 V^{-1} s^{-1}$) were not significantly different from the results obtained with anthraquinone-2-sulfonic acid and phthalic acid as reference materials ($3.58 \pm 0.02 \cdot 10^{-4}$

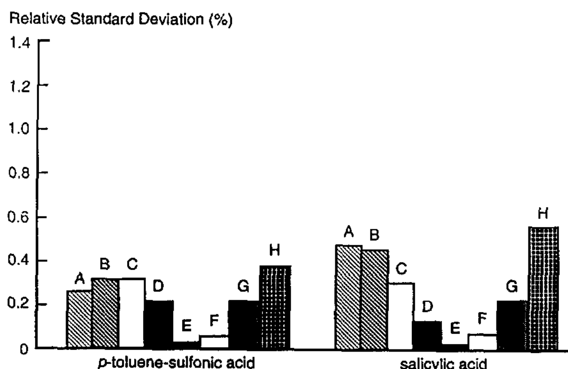

Fig. 12. Relative standard deviations in actual mobility for *p*-toluene-sulfonic acid and salicylic acid. Analysis conditions as in Fig. 4. H = All conditions combined. Plotted on a linear scale, at the same scale as Fig. 11.

$cm^2 V^{-1} s^{-1}$). This indicates that, under the conditions of this study, when the ionic strength of the analysis buffer is not significantly different from the ionic strength of the buffer used for determination of the standard mobilities, the identity and even the charge of the reference materials is not a significant factor in determining the actual mobilities of the analytes.

Analysis of covariance for this data set was not performed. Due to the small variance in the data set the results were expected to mirror those of the relative migration time. No correlation was observed between the actual mobility and t_0 or $1/t_0$.

4.6. Ionic strength

It remains a question what the effect of the ionic strength of the separation buffer, and differences between the ionic strength of the sample and the buffer, would have on these parameters. Ionic strength directly affects the electroosmotic flow, and thus is expected to have a direct effect on the migration times and an indirect effect on the relative migration times. Additionally, the ionic strength may affect the charge or solvation of the analytes and has an effect on the electrophoretic mobility [6,21–23]. Differences in the ionic strength of the run buffer might be expected to have an effect on the relative parameters if the standards and the analytes react differently to the change in solvation. Additionally, a difference between the sample and run buffers might be expected to affect these parameters since immediately after injection the electric field in the sample and separation regions of the capillary will be different.

We have conducted only preliminary studies, in which the conductivity of the run buffer was doubled by addition of 5 m*M* sodium chloride. While the migration times were affected drastically and failed to stabilize, the other parameters showed no statistically significant change. This represents only preliminary work, and a more complete study of the effects of ionic strength should be conducted.