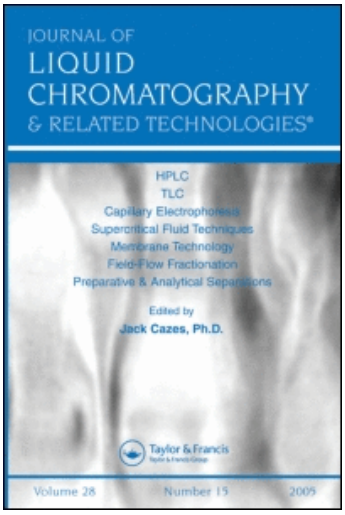

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The Repeatability of the Quantitative Analysis in Electrochromatography

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THE REPEATABILITY OF THE QUANTITATIVE ANALYSIS IN ELECTROCHROMATOGRAPHY

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ABSTRACT

The effect of a number of injection and separation parameters on the repeatability of quantitative analyses on a home-constructed instrument for electrochromatography was studied. The performance of electrokinetic and hydrostatic sample injection methods was compared. Peak area and peak height values were applied as the evaluation parameters. The best quantitative repeatability for charged and uncharged compounds, obtained in this study was in the range of 1% to 5% of relative standard deviation.

INTRODUCTION

Electrochromatography covers a group of separation techniques for neutral and charged compounds as well, applied in a strongly increasing

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number of application areas. At present these techniques are leaving the more academic research areas and find their way to the routine laboratories. In spite of the fact that the second generation of electro-chromatographic equipment is already available, a number of these apparatus are not always meeting the specific demands of the user with respect to the flexibility and educational needs.

Therefore, for basic research and also for educational purposes a home-designed electrochromatographic instrument was constructed. In this report an evaluation of this equipment with respect to the quantitative analysis results is presented.

A number of different injection and separation parameters may strongly influence the quantitative repeatability in electrochromatography. In principle, the quality of the sample introduction into the separation capillary depends only on the applied injection method. But also, both the data acquisition and handling determine the final quantitative results. To find out the optimal quantitative repeatability for a specific instrument it is necessary to check a number of sample introduction and separation parameters. A number of reports are dealing with the quantitative repeatability in electrochromatography, which may vary over a wide range [1-8,11,12]. The most significant parameters influencing the sample introduction are the applied voltage and the injection time at an electrokinetic sample introduction or the height difference of the sample and buffer solution levels and the injection time when hydrostatic sample introduction is applied. For a set of specific experimental conditions i.e., length, inner diameter and material of the capillary; the density, viscosity and composition of the sample and of the separation buffer, these parameters determine the lengths of the sample plug and of the zones of the sample components injected into the capillary; see Figure 1. Furthermore, the quantities of the sample components introduced into the separation capillary can be calculated, when their concentrations in the sample solution are known.

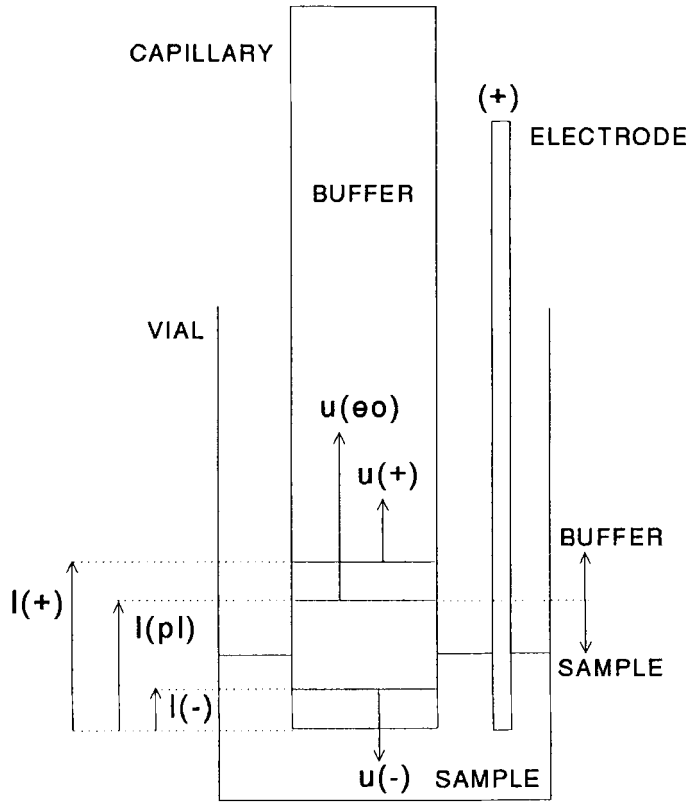

FIGURE 1

Electrokinetic injection: u(eo), electroosmotic velocity of buffer; u(+), electrophoretic velocity of a positively charged sample component; u(-), electrophoretic velocity of a negatively charged sample component; l(pl), sample plug length; l(+), zone length of a positively charged sample component; l(-), zone length of a negatively charged sample component.

In electrokinetic sample introduction, the inlet end of the capillary is immersed into the sample solution. In the next step, a high voltage is applied across the capillary for a certain interval of time forcing the sample to enter the capillary. At this injection technique sample introduction takes place by electrophoretic migration of charged sample compounds and by electroosmotic flow of the sample solution containing both charged and uncharged sample components [3]. Therefore, this sample introduction method is principally a discriminating technique.

As the electrophoretic mobilities of uncharged compounds are zero the length of the sample plug l_{pl}, introduced by electrokinetic injection, equals:

$$l_{pl} = \int_0^{t_{inj}} u_{eo}(t)dt = u_{eo} \cdot t_{inj} = (\mu_{eo} \cdot \frac{V}{L}) \cdot t_{inj} \qquad (1)$$

where:

- $u_{eo}(t)$ is the electroosmotic velocity of the buffer; $u_{eo}(t)$ is assumed to be constant and equal to u_{eo} during the injection step; $u_{eo} > 0$ for all sample components,
- t_{inj} is the injection time,
- μ_{eo} is the electroosmotic mobility,
- V is the voltage applied across the capillary during sample introduction, and
- L is the total capillary length.

In addition to that, the zone length l_i of a specific charged sample component i for electrokinetic injection equals:

$$l_i = \int_0^{t_{inj}} [u_{ep,i}(t) + u_{eo}(t)]dt = (u_{ep,i} + u_{eo}) \cdot t_{inj} = \\ = (\mu_{ep,i} \cdot \frac{\rho_S}{\rho_B} + \mu_{eo}) \cdot \frac{V}{L} \cdot t_{inj} \qquad (2)$$

where:

$u_{ep,i}(t)$ is the electrophoretic velocity of the sample component i; $u_{ep,i}(t)$ is supposed to be constant and equal to $u_{ep,i}$ during the injection step; $u_{ep,i} > 0$ for positively charged components and $u_{ep,i} < 0$ for negatively charged components if the polarity of the inlet electrode is positive,

- $\mu_{ep,i}$ is the electrophoretic mobility of the component,
- ρ_S is the electric resistivity of the sample solution,
- ρ_B is the electric resistivity of the buffer solution.

It is mentioned, that the value of $u_{ep,i}$ may be positive or negative depending on the polarity of the applied voltage. The ration ρ_S/ρ_B in equation (2) represents, that at decreasing sample conductivities, higher electric field strengths are induced in the sample solution. This is discussed in more detail in [13-15].

In the case of the injection of charged compounds, the zone lengths of the sample components differ from the sample plug length in equation (1). Because these charged compounds additionally move into or from the inlet end of the capillary tube during injection as schematically outlined in Figure 1.

In addition to that, a possible sample stacking process, which may occur on the boundary between buffer and sample solutions, also determines the final concentrations of charged components in the capillary tube after the injection. This stacking process of sample components may occur during electrokinetic injection, when the conductivities of the separation buffer and the sample solution differ from each other. However, the quantities of the individual sample components injected into the capillary will not be effected by the sample stacking process. If the polarity of the inlet electrode is positive, only the positively charged ions of the sample can reach the boundary between buffer and sample solutions during the injection procedure; see Figure 1. These positively charged cations will stack on the boundary already during the

injection procedure, when the conductivities of the separation buffer and the sample solution are different from each other. This stacking process of the cations may influence only their local concentrations in the boundary region but not their amounts introduced into the capillary tube. On the other hand, the negatively charged ions of the sample will not stack during the injection procedure, if the polarity of the inlet electrode is positive. These negatively charged anions may stack on a new boundary between the sample and buffer solutions. This new boundary arises in the capillary tube by replacing of the sample solution located in the inlet vial by the buffer solution after the injection step was finished. There is evidence, that this stacking process of the anions on this new boundary may take place, when the separation step was already started. This stacking process of the anions will also not effect their amounts injected into the capillary as well.

From these facts it may be concluded, that the stacking process influences only the local concentrations of the sample components but not their final quantities injected into the capillary tube. Consequently, an amount of a specific sample compound introduced into the capillary under a set of experimental conditions will be the same for both cases with or without the occurrence of a sample stacking process. It is obvious, that the higher the concentration of a compound achieved by sample stacking, the shorter the zone length of it in the capillary tube after the injection procedure.

The situation is less complicated for hydrostatic injection methods, because no discriminating effects occur when charged and uncharged compounds are injected. In hydrostatic sample introduction, the inlet end of the capillary is immersed into the sample solution. Subsequently, the sample reservoir is raised vertically to a specified height for a certain interval of time, creating a height difference between the liquid levels of the sample reservoir and the buffer reservoir at the detector side of the capillary. This height difference results in a hydrostatic pressure across the capillary, so forcing a flow of sample solution into the inlet of the capillary [3].

The resulting injection plug length of the sample solution and the zone length of a specific sample component i are equal for charged and uncharged components and equals:

$$l_{pl} = l_i = u_{hd} \cdot t_{inj} = (d \cdot g \cdot r^2 \cdot \frac{\Delta h}{8 \cdot \eta \cdot L}) \cdot t_{inj} \qquad (3)$$

where:

- u_{hd} is the average hydrodynamic velocity of the sample/buffer solution,
- d is the density of the buffer solution,
- g is the constant for gravitational acceleration,
- r is the capillary inner radius,
- Δh is the height difference between the liquid levels,
- η is the viscosity of the buffer solution.

Generally if the concentration of a sample component i at a position z is $C_i(z)$, the amount of the component injected into the capillary equals:

$$Q_i = \pi \cdot r^2 \cdot \int_0^{l_i} C_i(z)dz \qquad (4)$$

It was shown already, that the sample stacking process will change only the local concentrations of sample components but not their amounts introduced into the capillary during injection. Considering this fact and assuming that $C_i(z)$ is constant and equal C_i for the whole length of the zone l_i, the following equations for the injected amounts can be derived:

$$Q_i = l_i.\pi.r^2.C_i = (\mu_{eo}.\frac{V}{L}).t_{inj}.\pi.r^2.C_i \qquad (5)$$

$$Q_i = l_i.\pi.r^2.C_i = (\mu_{ep,i}.\frac{\rho_S}{\rho_B} + \mu_{eo}).\frac{V}{L}.t_{inj}.\pi.r^2.C_i \qquad (6)$$

$$Q_i = l_i.\pi.r^2.C_i = (d.g.r^2.\frac{\Delta h}{8.\eta L}).t_{inj}.\pi.r^2.C_i \quad (7)$$

where:

- C_i is the concentration of a specific sample component i in the sample solution.

For electrokinetic injection, equations (5) and (6) have to be applied for uncharged and charged sample compounds, respectively. Expression (7) must be used when hydrostatic injection is applied. Similar expressions have been given by others previously [3,4,6].

When the inlet end of a separation capillary filled with a buffer solution is immersed into a sample solution, immediately some amounts of the sample components may enter into the capillary by diffusion, hydrostatic flow, displacement and/or convective flows [9,10]. This unwanted but unavoidable sample introduction is superimposed on the regular injection procedure and will complicate the repeatability of the injection. It might be clear, that the ratio between the regular and the uncontrolled part of the injection step will strongly determine the repeatability of the injection method. This ratio may be improved by using a lower sample concentration with a higher injection voltage over a longer injection time at electrokinetic injection or with a higher distance between the solution levels combined with a longer injection time in the case hydrostatic injection is applied. However, these injection conditions will have a negative effect on the efficiency, because the lengths of the sample plug and of the sample component zones will increase.

Besides these injection parameters also other parameters may influence the quantitative repeatability of the analysis. The frequency of the electronic sampling of the peak and the separation voltage are the most important of them.

In this study we investigated the performance of a home-designed electrochromatographic instrument especially with respect to the quantitative results of the hydrostatic and electrokinetic injection methods.

EXPERIMENTAL

Instrumentation

The home-constructed instrument for the electrochromatographic experiments was of Perspex. In Figure 2 a schematic outline of the equipment is presented. Both the injection unit for the sample introduction and a holder with a vial as the buffer reservoir on the detector outlet side were situated in the box. The Perspex box was equipped with an interlock safety system, to prevent contact with the high-voltage components. An open, polyimide-clad fused silica capillary (350 μm o.d., 50 μm i.d., Siemens, FRG) of a total length of 64.5 cm was applied as the separation column. The capillary was provided with an optical window at 50 cm from the inlet side. Detection was accomplished by using an on-column UV absorbance detector (Unicam Analytical Systems, Cambridge, UK) operating at a wavelength of 230 nm. The cell containing part of the detector was also situated in the box. A $\pm$35 kV dc power supply (HCN 140-35 000, FUG, Elektronik GmbH, FRG), used in the positive voltage mode, was used for both the electrochromatographic separation and electrokinetic sample injection. The injection unit was connected to the positive electrode and the buffer reservoir at the detector outlet side to the ground electrode by using platinum wires. The injection unit, schematically presented in Figure 3, was constructed to allow the application of two different methods of sample introduction, electrokinetically and hydrostatically from a vial of a volume of 1 ml or from a conical reservoir of a volume of 4 ml. The latter reservoir was provided with three microvalves, which were used to flush the reservoir either with sample or buffer solutions. A detailed explanation of

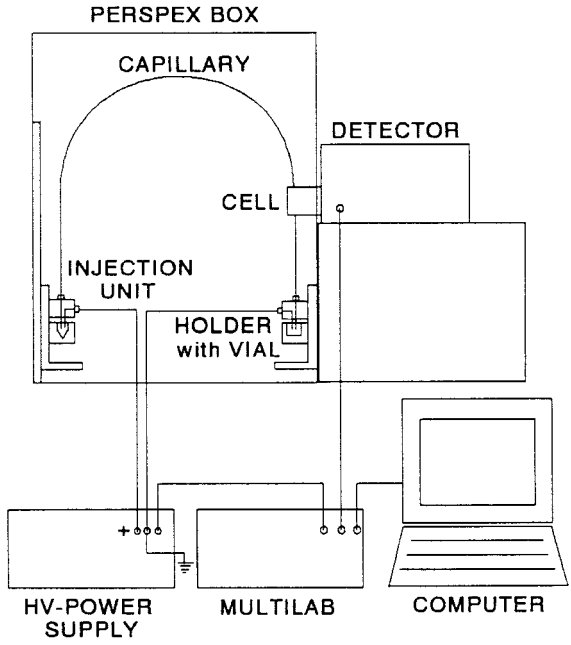

FIGURE 2

Schematic outline of the electrochromatographic equipment.

these two introduction methods will be given in the Procedures chapter. A Tulip AT compact 3 computer ('s-Hertogenbosch, The Netherlands) together with a home-constructed interface Multilab-TS allowed the control of both the electrokinetic injection and separation. The data acquisition and calculation were performed with the software program Caesar (B-Wise Software, Geleen, The Netherlands).

Chemicals

The buffer solution used consisted of a 20 mM sodium phosphate solution (pH 6.76) prepared from Sodium dihydrogen phosphate dihydrate,

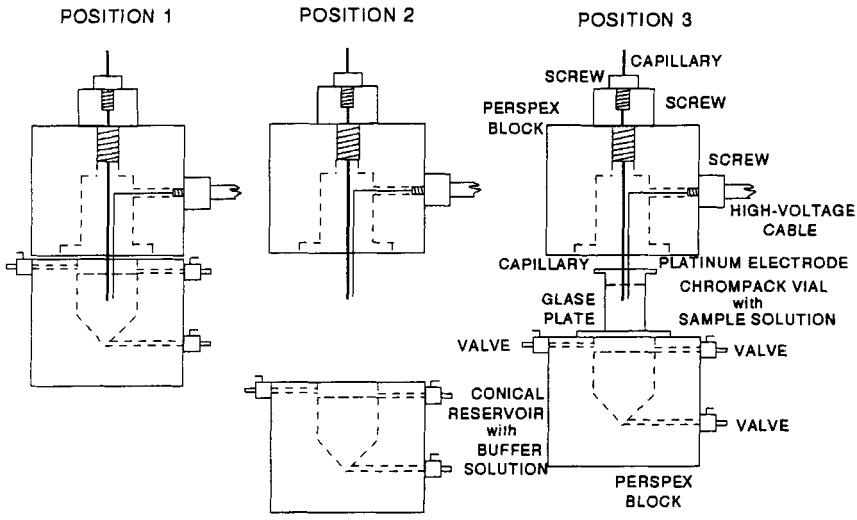

FIGURE 3

Schematic outline of the injection unit.

extra pure (Merck, Darmstadt, Germany) and di-Sodium hydrogen phosphate dihydrate, p.a. (Merck). A mixture of tyramine, mesityl oxide and naphthalene-2-sulphonic acid was applied as the sample. Tyramine was selected as a positively, mesityl oxide as a neutral and naphthalene-2-sulphonic acid as a negatively charged sample compound. For the preparation of the sample, Tyramine hydrochloride, 98% (Janssen Chimica, Belgium), Naphthalene-2-sulphonic acid sodium salt, H.P.L.C. grade (Fisons Scientific Apparatus, England), and Mesityl oxide, p.a. were dissolved in water or the buffer. Subsequently, these solutions were diluted to the concentration of 1 mM or 0.1 mM. The water used for the preparation of the solutions was purified by a Milli-Q Water Purification System (Millipore Corp., USA) prior to use.

Procedures

About the preparation and storage of capillary tubes in electrochromatography, contradictory results have been reported [1,4,16-19]. In this study a standard procedure was selected for the preparation and use of the capillaries.

Before using a capillary, a flushing procedure with 0.1 M sodium hydroxide solution for 10 minutes and subsequently with the buffer solution for another 5 minutes was repeated three times. In between the performance of the experiments, no washing of the capillary with sodium hydroxide solution was carried out. Every morning, the capillary was flushed with the buffer solution for 10 minutes and after that a voltage of 30 kV was applied for another 10 minutes.

The capillary inlet was immersed into the buffer solution located in the conical reservoir of the injection unit, when a separation was carried out; see Figure 3, Position 1. That position was also the initial position of the conical reservoir for each sample introduction.

The first method of sample introduction (procedure 1) was started by moving down the conical reservoir filled with the buffer solution; Figure 3, Position 2. In this step, the inlet end of the capillary was surrounded only by air. Subsequently, a vial containing the sample solution was put on the glass plate. After that the capillary end together with the platinum electrode were immersed into the sample solution; Figure 3, Position 3. To introduce the sample into the capillary, an injection voltage was applied for a specific period of time for electrokinetic injection. Alternatively, the whole injection unit, both the upper and lower Perspex blocks, was raised vertically to a specific height for a selected interval of time, when hydrostatic injection was applied. In the next step, the capillary inlet together with the platinum electrode were moved *out from the* sample reservoir. Subsequently, both the vial and the glass plate were removed; Figure 3, Position 2. In this position, the inlet end of the

capillary was surrounded only by air again. After that, the capillary inlet together with the platinum electrode were immersed into buffer solution by moving up the conical reservoir; Figure 3, Position 1. Then the separation was started by turning on the voltage.

The second method of sample introduction (procedure 2) was started by moving down the conical reservoir filled with the buffer solution; see Figure 3, Position 2. In this position, the inlet end of the capillary was surrounded only by air. In the next step, the buffer solution in the conical reservoir was substituted by the sample solution using the three valves, shown in Figure 3. Subsequently, the capillary end together with the platinum electrode were immersed into the sample solution by raising of the conical reservoir; Figure 3, Position 1. To introduce the sample electrokinetically into the capillary, an injection voltage was applied for a specific time or alternatively the whole injection unit was moved up vertically to a specific height for a time interval, when hydrostatic injection was applied. After the sample was introduced into the capillary, the sample solution was washed out from the conical reservoir by flushing it with 20 ml of buffer solution using the three valves. This step made it possible to change from the sample to the buffer solution without moving up and down the conical reservoir. In this way, both the capillary inlet and the electrode remain immersed in a liquid and a possible hydrostatic flow will be prevented; Figure 3, Position 1. Then the separation was started by turning on the voltage.

RESULTS AND DISCUSSION

The duration of individual injection steps differ from injection to injection, when an injection method is carried out manually. E.V.Dose et al. [9] reported, that immediately after a buffer-filled capillary is immersed into a sample solution, a rapid diffusion of sample components into the capillary will start. The rate of entry decreases with the time and in the absence of

hydrodynamic flow the amount in the capillary is proportional to the square root of time. When there is a hydrodynamic flow into the capillary inlet, more sample will enter the capillary and the rate of entry approaches a constant value until ultimately the sample reaches the opposite end of the capillary. On the other hand, when a hydrodynamic flow out of the capillary inlet takes place, the entry starts very rapidly, but the amount of sample entered into the capillary approaches a limiting value. This unwanted but ubiquitous sample entry will especially decrease the repeatability of a manual injection method as the duration of the individual injection steps from injection to injection will vary.

As a result of computer simulations it was reported by E.V.Dose et al. [9], "diffusional problems with CZE quantitation are worst for short injections, electrokinetic "concentrating" injections, or long delays during the injection sequence". Taking into account the above considerations in this study, a number of injection methods differing from each other in the injection voltage, the distance between the solution levels, the injection time, and the sample concentration were investigated.

The results are summarized in the Tables 1-4. The relative standard deviation (R.S.D.) of both the peak areas and peak heights from 5 repeated measurements for each injection method are presented. A sample concentration of 1 mM was used for the injection programmes of 0.5 kV/2s, 5 kV/2s, 4 cm/5s, and 2 cm/10s. For the other injection programmes, a sample concentration of 0.1 mM was applied. All injections were performed from a vial according to procedure 1. The separation was carried out by a potential drop of 30 kV across the capillary and electropherograms were recorded with a sampling frequency of 5 Hz. As an example an electropherogram is presented in Figure 4.

An improved repeatability of the injection compared to the other ones was achieved, when the injection programmes 5kV/10s, 10kV/5s, and 2cm/60s in combination with the sample concentration of 0.1 mM were applied. This

TABLE 1

R.S.D.-values of area and height measurements of electrokinetic sample injection from a vial; sample, 1 or 0.1 mM tyramine, mesityl oxide and naphthalene-2-sulphonic acid solution in water; number of analyses, n=5; separation voltage, 30kV; sampling frequency, 5Hz.

Injection programme (sample conc.)	Tyramine		Mesityl oxide		Naphthalene-2-sulphonic acid	
	area	height	area	height	area	height
0.5kV/2s (1mM)	24.8%	14.8%	24.6%	22.0%	40.7%	40.4%
5kV/2s (1mM)	18.7%	15.3%	13.6%	8.8%	28.1%	27.6%
5kV/10s (0.1mM)	44.6%	31.3%	10.8%	5.2%	57.8%	60.1%
10kV/5s (0.1mM)	39.1%	19.3%	6.6%	5.6%	39.8%	55.8%

TABLE 2

R.S.D.-values of area and height measurements of electrokinetic sample injection from a vial; sample, 1 or 0.1 mM tyramine, mesityl oxide and naphthalene-2-sulphonic acid solution in buffer; number of analyses, n=5; separation voltage, 30kV; sampling frequency, 5Hz.

Injection programme (sample conc.)	Tyramine		Mesityl oxide		Naphthalene-2-sulphonic acid	
	area	height	area	height	area	height
0.5kV/2s (1mM)	27.2%	36.7%	51.5%	56.2%	23.6%	24.8%
5kV/2s (1mM)	9.1%	16.7%	9.4%	5.1%	13.1%	27.8%
5kV/10s (0.1mM)	13.8%	9.1%	12.1%	4.4%	10.2%	6.7%
10kV/5s (0.1mM)	10.3%	9.3%	4.2%	3.8%	10.1%	6.3%

TABLE 3

R.S.D.-values of area and height measurements of hydrostatic sample injection from a vial; sample, 1 or 0.1 mM tyramine, mesityl oxide and naphthalene-2-sulphonic acid solution in water; number of analyses, n=5; separation voltage, 30kV; sampling frequency, 5Hz.

Injection programme (sample conc.)	Tyramine		Mesityl oxide		Naphthalene-2-sulphonic acid	
	area	height	area	height	area	height
4cm/5s (1mM)	14.9%	12.3%	30.7%	12.6%	19.2%	19.5%
2cm/10s (1mM)	36.3%	26.8%	32.5%	25.8%	52.3%	56.2%
2cm/60s (0.1mM)	23.3%	21.8%	4.8%	3.1%	5.4%	5.3%

TABLE 4

R.S.D.-values of area and height measurements of hydrostatic sample injection from a vial; sample, 1 or 0.1 mM tyramine, mesityl oxide and naphthalene-2-sulphonic acid solution in buffer; number of analyses, n=5; separation voltage, 30kV; sampling frequency, 5Hz.

Injection programme (sample conc.)	Tyramine		Mesityl oxide		Naphthalene-2-sulphonic acid	
	area	height	area	height	area	height
4cm/5s (1mM)	17.4%	15.4%	38.5%	33.8%	36.3%	32.9%
2cm/10s (1mM)	34.8%	30.6%	21.5%	23.1%	45.7%	47.7%
2cm/60s (0.1mM)	19.6%	8.5%	5.5%	5.0%	3.4%	3.8%

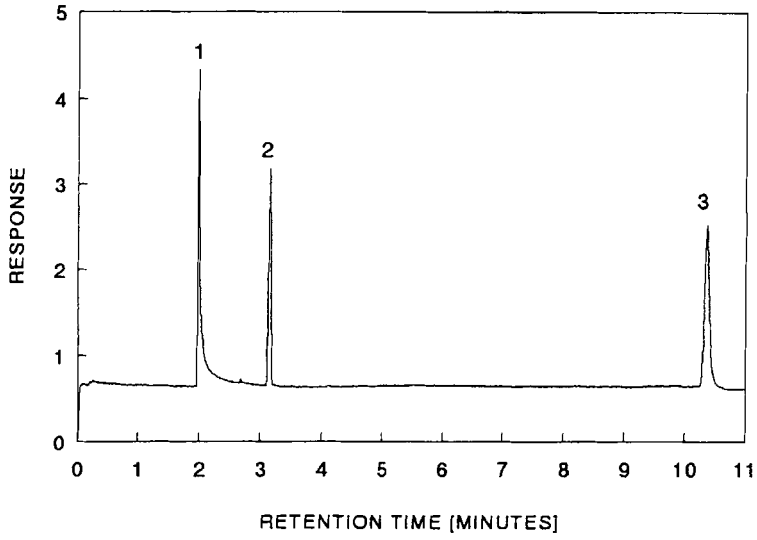

FIGURE 4

Electropherogram of a mixture of 1, tyramine; 2, mesityl oxide; and 3, naphthalene-2-sulphonic acid: electrokinetic injection, 5kV/2s; sample, 1 mM solution in water; separation voltage, 30kV; separation buffer, 20 mM phosphate buffer, pH=6.76; sampling frequency, 5Hz; detection, UV at 230 nm.

can be attributed to the fact, that the ratio between the regular and the uncontrolled part of a specific injection procedure might be improved by using these programmes in combination with a lower concentration of the sample. Also applying these injection programmes, a significantly better repeatability of the injections was observed for the uncharged mesityl oxide for both the hydrostatic and electrokinetic injection methods. Moreover under the proper conditions for the hydrostatic injection, an improvement of the repeatability for the negatively charged naphthalene-2-sulphonic acid was observed. The injection repeatability for the positively charged tyramine in these measurements was effected only slightly. Based on these results, the three

TABLE 5

R.S.D.-values of area and height measurements of electrokinetic sample injection from a vial; injection programme, 10kV/5s; sample, 0.1 mM tyramine, mesityl oxide and naphthalene-2-sulphonic acid solution; number of analyses, n=5; separation voltage, 30kV; sampling frequency, 5Hz.

Injection from	Tyramine		Mesityl oxide		Naphthalene-2-sulphonic acid	
	area	height	area	height	area	height
WATER	39.1%	19.3%	6.6%	5.6%	39.8%	55.8%
BUFFER	10.3%	9.3%	4.2%	3.8%	10.1%	6.3%

above mentioned injection programmes 5kV/10s, 10kV/5s, and 2cm/60s were selected for the next investigations.

In practice, the sample solution may vary in between water and an ionic solution. In this part of the study, the influence of the composition of the sample solution with respect to the ionic strength on the injection repeatability was investigated. Therefore, a number of samples either consisting of water or the buffer solution of the three compounds were separately injected. The results of the influence of the sample solution ionic strength on the quantitative injection repeatability are presented in the Tables 5-10. All the injections were performed from a vial (procedure 1) with exception of the injections given in Table 9. For the electrokinetically injected charged compounds, significant differences could be observed between water and buffer as the sample solvent. For charged compounds injected from the buffer as the sample solvent, a significant improvement of the repeatability of injections was observed compared to water. In addition to that, for the electrokinetically injected uncharged mesityl oxide small differences between water and buffer as the sample solvent were observed. Also for the results of the hydrostatic injection procedures, it was noticed that the selection of water or buffer as the sample

TABLE 6

R.S.D.-values of area and height measurements of electrokinetic sample injection from a vial; injection programme, 5kV/10s; sample, 0.1 mM tyramine, mesityl oxide and naphthalene-2-sulphonic acid solution; number of analyses, n=5; separation voltage, 30kV; sampling frequency, 5Hz.

Injection from	Tyramine		Mesityl oxide		Naphthalene-2-sulphonic acid	
	area	height	area	height	area	height
WATER	44.6%	31.3%	10.8%	5.2%	57.8%	60.1%
BUFFER	13.8%	9.1%	12.1%	4.4%	10.2%	6.7%

TABLE 7

R.S.D.-values of area and height measurements of hydrostatic sample injection from a vial; injection programme, 2cm/60s; sample, 0.1 mM tyramine, mesityl oxide and naphthalene-2-sulphonic acid solution; number of analyses, n=5; separation voltage, 30kV; sampling frequency, 5Hz.

Injection from	Tyramine		Mesityl oxide		Naphthalene-2-sulphonic acid	
	area	height	area	height	area	height
WATER	23.3%	21.8%	4.8%	3.1%	5.4%	5.3%
BUFFER	19.6%	8.5%	5.5%	5.0%	3.4%	3.8%

TABLE 8

R.S.D.-values of area and height measurements of electrokinetic sample injection from a vial; injection programme, 10kV/5s; sample, 0.1 mM tyramine, mesityl oxide and naphthalene-2-sulphonic acid solution; number of analyses, n=5; separation voltage, 30kV; sampling frequency, 40Hz.

Injection from	Tyramine		Mesityl oxide		Naphthalene-2-sulphonic acid	
	area	height	area	height	area	height
WATER	15.2%	13.7%	3.9%	2.8%	60.5%	49.2%
BUFFER	8.3%	5.7%	2.3%	3.0%	11.9%	6.2%

TABLE 9

R.S.D.-values of area and height measurements of hydrostatic sample injection from conical reservoir; injection programme, 2cm/60s; sample, 0.1 mM tyramine, mesityl oxide and naphthalene-2-sulphonic acid solution; number of analyses, n=5; separation voltage, 10kV; sampling frequency, 5Hz.

Injection from	Tyramine		Mesityl oxide		Naphthalene-2-sulphonic acid	
	area	height	area	height	area	height
WATER	15.9%	12.2%	3.7%	2.7%	1.5%	2.4%
BUFFER	11.3%	4.4%	3.5%	2.2%	1.8%	1.1%

TABLE 10

R.S.D.-values of area and height measurements of electrokinetic sample injection from a vial; injection programme, 10kV/5s; sample, 0.1 mM tyramine, mesityl oxide and naphthalene-2-sulphonic acid solution; number of analyses, n=5; separation voltage, 30kV; sampling frequency, 40Hz.

Injection from	Tyramine		Mesityl oxide		Naphthalene-2-sulphonic acid	
	area	height	area	height	area	height
WATER	15.2%	13.7%	3.9%	2.8%	60.5%	49.2%
BUFFER	8.3%	5.7%	2.3%	3.0%	11.9%	6.2%

solvent only slightly influenced the repeatability of injection for charged and neutral sample compounds as well. The observations with respect to the electrokinetic injection procedures can be explained by a change of the mutual position between the inlet end of the capillary and the platinum wire electrode; see Figure 3. The change of their mutual position may be caused by the manipulation with the injection unit, performed during the sample introduction. The change of the capillary to electrode position may change the electric field

TABLE 11

R.S.D.-values of area and height measurements of electrokinetic sample injection; injection programme, 5kV/10s; sample, 0.1 mM tyramine, mesityl oxide and naphthalene-2-sulphonic acid solution in water; number of analyses, n=5; separation voltage, 30kV; sampling frequency, 5Hz.

Injection from	Tyramine		Mesityl oxide		Naphthalene-2-sulphonic acid	
	area	height	area	height	area	height
VIAL	44.6%	31.3%	10.8%	5.2%	57.8%	60.1%
RESERVOIR	25.0%	22.6%	10.3%	5.1%	45.8%	49.7%

in the sample solution. The intensity of this electric field is higher in a water-solution of the sample compared to a buffer-solution. The intensity change of the electric field in the sample solution plays an important role, when charged compounds are injected electrokinetically.

The injection unit of the equipment, used in this study, allowed the replacement of the buffer/sample solutions in two different ways, the introduction from a vial or from the conical reservoir. The procedures of these introduction methods are described in detail in the Procedures section. The results of the influence of these two methods of solution replacement at the capillary inlet on the quantitative repeatability are summarized in the Tables 11-14. It is obvious, that the sample introduction from the conical reservoir showed a higher quantitative repeatability compared to the introduction from a vial. This can be explained by the different injection steps in these two procedures.

In the case of a sample introduction from the conical reservoir, both the capillary inlet and the electrode are during the whole injection procedure immersed in a liquid. In contrast to that, during the sample introduction procedure from a vial there will be an increased risk of a hydrostatic flow in

TABLE 12

R.S.D.-values of area and height measurements of hydrostatic sample injection; injection programme, 2cm/60s; sample, 0.1 mM tyramine, mesityl oxide and naphthalene-2-sulphonic acid solution in water; number of analyses, n=5; separation voltage, 30kV; sampling frequency, 5Hz.

Injection from	Tyramine		Mesityl oxide		Naphthalene-2-sulphonic acid	
	area	height	area	height	area	height
VIAL	23.3%	21.8%	4.8%	3.1%	5.4%	5.3%
RESERVOIR	19.0%	10.2%	7.5%	4.1%	6.0%	3.0%

TABLE 13

R.S.D.-values of area and height measurements of hydrostatic sample injection; injection programme, 2cm/60s; sample, 0.1 mM tyramine, mesityl oxide and naphthalene-2-sulphonic acid solution in water; number of analyses, n=5; separation voltage, 10kV; sampling frequency, 5Hz.

Injection from	Tyramine		Mesityl oxide		Naphthalene-2-sulphonic acid	
	area	height	area	height	area	height
VIAL	12.5%	7.4%	7.0%	5.6%	4.6%	4.3%
RESERVOIR	15.9%	12.2%	3.7%	2.7%	1.5%	2.4%

TABLE 14

R.S.D.-values of area and height measurements of electrokinetic sample injection; injection programme, 10kV/5s; sample, 0.1 mM tyramine, mesityl oxide and naphthalene-2-sulphonic acid solution in water; number of analyses, n=5; separation voltage, 10kV; sampling frequency, 5Hz.

Injection from	Tyramine		Mesityl oxide		Naphthalene-2-sulphonic acid	
	area	height	area	height	area	height
VIAL	7.6%	4.8%	3.3%	2.5%	2.7%	3.2%
RESERVOIR	4.3%	4.3%	2.5%	1.9%	2.4%	3.9%

TABLE 15

R.S.D.-values of area and height measurements of electrokinetic sample injection from a vial; injection programme, 5kV/10s; sample, 0.1 mM tyramine, mesityl oxide and naphthalene-2-sulphonic acid solution in water; number of analyses, n=5; sampling frequency, 5Hz.

Separation voltage	Tyramine		Mesityl oxide		Naphthalene-2-sulphonic acid	
	area	height	area	height	area	height
30 kV	44.6%	31.3%	10.8%	5.2%	57.8%	60.1%
10 kV	32.1%	22.5%	3.7%	2.9%	33.5%	10.1%

the capillary. This and other unwanted effects like the change of the mutual position of the capillary and the electrode in the latter procedure make the sample introduction from the conical reservoir more favourable.

As was already mentioned, the applied voltage during the separation and the sampling frequency during peak recording might also effect the quantitative repeatability. The influence of both the separation voltage and the sampling frequency was also investigated and the results are summarized in the Tables 15-17. In many cases, the quantitative repeatability increased when a lower separation voltage or a larger peak sampling frequency was used. From the results it is obvious, that the application of a lower separation voltage and/or a higher sampling frequency improves the precision of the peak integration.

In addition to that, a comparison between the applications of a lower separation voltage in combination with a smaller sampling frequency or a higher separation voltage together with a larger sampling frequency is summarized in the Tables 18 and 19. The results show, that the application of a lower separation voltage in combination with a possibly largest sampling frequency improves the quantitative repeatability. However, the separation

TABLE 16

R.S.D.-values of area and height measurements of hydrostatic sample injection from conical reservoir; injection programme, 2cm/60s; sample, 0.1 mM tyramine, mesityl oxide and naphthalene-2-sulphonic acid solution in water; number of analyses, n=5; sampling frequency, 5Hz.

Separation voltage	Tyramine		Mesityl oxide		Naphthalene-2-sulphonic acid	
	area	height	area	height	area	height
30 kV	19.0%	10.2%	7.5%	4.1%	6.0%	3.0%
10 kV	15.9%	12.2%	3.7%	2.7%	1.5%	2.4%

TABLE 17

R.S.D.-values of area and height measurements of electrokinetic sample injection from a vial; injection programme, 10kV/5s; sample, 0.1 mM tyramine, mesityl oxide and naphthalene-2-sulphonic acid solution in water; number of analyses, n=5; separation voltage, 30kV.

Sampling frequency	Tyramine		Mesityl oxide		Naphthalene-2-sulphonic acid	
	area	height	area	height	area	height
5 Hz	39.1%	19.3%	6.6%	5.6%	39.8%	55.8%
40 Hz	15.2%	13.7%	3.9%	2.8%	60.5%	49.2%

TABLE 18

R.S.D.-values of area and height measurements of hydrostatic sample injection from a vial; injection programme, 2cm/60s; sample, 0.1 mM tyramine, mesityl oxide and naphthalene-2-sulphonic acid solution in water; number of analyses, n=5.

Separation voltage/ Sampling frequency	Tyramine		Mesityl oxide		Naphthalene-2-sulphonic acid	
	area	height	area	height	area	height
30kV/5Hz	23.3%	21.8%	4.8%	3.1%	5.4%	5.3%
10kV/5Hz	12.5%	7.4%	7.0%	5.6%	4.6%	4.3%
30kV/40Hz	20.7%	12.9%	5.5%	3.2%	7.7%	5.0%

TABLE 19

R.S.D.-values of area and height measurements of electrokinetic sample injection from a vial; injection programme, 10kV/5s; sample, 0.1 mM tyramine, mesityl oxide and naphthalene-2-sulphonic acid solution in buffer; number of analyses, n=5.

Separation voltage/ Sampling frequency	Tyramine		Mesityl oxide		Naphthalene-2-sulphonic acid	
	area	height	area	height	area	height
30kV/5Hz	10.3%	9.3%	4.2%	3.8%	10.1%	6.3%
10kV/5Hz	7.6%	4.8%	3.3%	2.5%	2.7%	3.2%
30kV/40Hz	8.3%	5.7%	2.3%	3.0%	11.9%	6.2%

voltage cannot be arbitrary lowered because its decreasing rapidly increases the analyses time.

The better results obtained at the lower separation voltages compared to the results at higher electric fields may be explained by the less heat development in the capillary tube during the separation at lower voltages.

The above mentioned data showed clearly the influences of some injection and separation parameters on the quantitative repeatability. From this, the optimal injection conditions were selected and compared. The results of the quantitative repeatability of these selected electrokinetic and hydrostatic injection programmes are presented in the Tables 20-22. The highest repeatability of injection was obtained, when the buffer was used as the sample solvent, the injection was performed from the conical reservoir, and the separation was carried out by the potential drop of 10 kV across the capillary; Table 22. Further more, only small differences between the three injection programmes were noticed at these injection and separation conditions. However, the positively charged tyramine, which had the shortest retention time, exhibited more poor quantitative repeatability in comparison with the

TABLE 20

R.S.D.-values of area and height measurements of sample injection from a vial; sample, 0.1 mM tyramine, mesityl oxide and naphthalene-2-sulphonic acid solution in water; number of analyses, n=5; separation voltage, 30kV; sampling frequency, 5Hz.

Injection programme	Tyramine		Mesityl oxide		Naphthalene-2-sulphonic acid	
	area	height	area	height	area	height
10kV/5s	39.1%	19.3%	6.6%	5.6%	39.8%	55.8%
5kV/10s	44.6%	31.3%	10.8%	5.2%	57.8%	60.1%
2cm/60s	23.3%	21.8%	4.8%	3.1%	5.4%	5.3%

TABLE 21

R.S.D.-values of area and height measurements of sample injection from a vial; sample, 0.1 mM tyramine, mesityl oxide and naphthalene-2-sulphonic acid solution in buffer; number of analyses, n=5; separation voltage, 30kV; sampling frequency, 5Hz.

Injection programme	Tyramine		Mesityl oxide		Naphthalene-2-sulphonic acid	
	area	height	area	height	area	height
10kV/5s	10.3%	9.3%	4.2%	3.8%	10.1%	6.3%
5kV/10s	13.8%	9.1%	12.1%	4.4%	10.2%	6.7%
2cm/60s	19.6%	8.5%	5.5%	5.0%	3.4%	3.8%

TABLE 22

R.S.D.-values of area and height measurements of sample injection from conical reservoir; sample, 0.1 mM tyramine, mesityl oxide and naphthalene-2-sulphonic acid solution in buffer; number of analyses, n=5; separation voltage, 10kV; sampling frequency, 5Hz.

Injection programme	Tyramine		Mesityl oxide		Naphthalene-2-sulphonic acid	
	area	height	area	height	area	height
10kV/5s	4.3%	4.3%	2.5%	1.9%	2.4%	3.9%
5kV/10s	4.2%	4.9%	3.8%	2.8%	1.7%	3.0%
2cm/60s	11.3%	4.4%	3.5%	2.2%	1.8%	1.1%

repeatability obtained for the other compounds. This can be explained by the mutual interactions between the negatively charged capillary wall and the positively charged tyramine. The tailing of the tyramine peak in Figure 4 refers to this mutual interactions as well. Finally, the results show that the hydrostatic injection procedure yield good quantitative repeatability for naphthalene-2-sulphonic acid; Tables 20 and 21.

Moreover, it was found out that generally the peak height showed better reproducible quantitative results compared to the peak area. This may be explained by linear relationships between the sample concentration and the peak height and on the other hand between the injected sample amount and the peak area. The unwanted but unavoidable sample introduction, superimposed on the regular injection procedure, will more modify the injected sample amount and consequently the peak area than the sample concentration.

Further more, three other parameters were also tested as the evaluation parameters for the calculation of the repeatability, namely:

i) the area of a peak divided by the retention time of the peak, $A_{(i)}/t_{R(i)}$;

ii) the area of a peak divided by the peak area of mesityl oxide, $A_{(i)}/A_{(MO)}$;

iii) the ratio of the area of a peak divided by the retention time of the peak to the peak area of mesityl oxide divided by the retention time of mesityl oxide, $(A_{(i)}/t_{R(i)})/(A_{(MO)}/t_{R(MO)})$.

However, no improvement of the quantitative repeatability was observed, when these three parameters as the evaluation parameters were applied. The parameter $A_{(i)}/t_{R(i)}$ may significantly improve the quantitative repeatability in the case, when it is subject to an error possibly originating from the irreproducible retention times. But this was not the case, because in all measurements for all sample components the retention time RSDs from 5 repeated analyses were less than 3%. Some workers [1,5,11,12] could improve the quantitative repeatability by use of an internal standardization method with

one or two internal standards. The application of mesityl oxide as an internal standard by the parameter $A_{(i)}/A_{(MO)}$ in our case had no positive influence on the quantitative repeatability as well. The different charges of the three sample component may be one of possible explanations for that. Finally, the parameter $(A_{(i)}/t_{R(i)})/(A_{(MO)}/t_{R(MO)})$ which combines the two above mentioned parameters did also not improve the quantitative repeatability.

CONCLUSIONS

The effects of a number of injection and separation parameters on the repeatability of the quantitative analyses in electrochromatography were studied on a home-constructed equipment. Especially parameters like injection program, concentration and composition of the sample solution, method of injection, separation voltage, peak sampling frequency and the evaluation parameters were investigated. Two different sample introduction methods based on hydrostatic and electrokinetic injection were studied and compared. Tyramine, mesityl oxide and naphthalene-2-sulphonic acid as negatively, neutral and positively charged substances respectively, were selected as the sample components. The compounds with different charges were applied in order to study the effect of the sample component charge on the repeatability of the analyses.

Both the peak area and the peak height were used as the evaluation parameters for the quantitative repeatability. It was found out that generally the peak height yielded better repeatable quantitative results compared to the peak area. Further more, three other parameters $A_{(i)}/t_{R(i)}$, $A_{(i)}/A_{(MO)}$, and $(A_{(i)}/t_{R(i)})/(A_{(MO)}/t_{R(MO)})$ were also tested as the evaluation parameters for the calculation of the repeatability of the quantitative date. However, no improvement of the quantitative repeatability was observed, when these three parameters as the evaluation parameters were applied.

The injection programs based on a higher injection voltage or on a higher distance between the liquid levels and/or on a longer injection time like

5kV/10s, 10kV/5s and 2cm/60s in combination with lower, 0.1 mM concentration of a sample showed more repeatable results compared to the other injection programs. When charged compounds were injected electrokinetically, the sample introductions carried out from buffer-solutions exhibited significantly better quantitative repeatability in comparison with sample injections performed from water-solutions. For neutral compounds and for hydrostatic injections as well, only small differences between water and buffer as the sample background were noticed. The injections from the conical reservoir, decreasing the risk of a reverse hydrostatic flow in the capillary during an injection procedure, yielded much better quantitative results compared to the injections from a vial. Finally, applications of a lower separation voltage and/or a larger peak sampling frequency made also quantitative results more repeatable.

The highest quantitative repeatability of manual injections achieved in the presented study on a home-constructed electrochromatographic equipment was in the interval of 1% to 5% of relative standard deviation.

In this study it was shown that a carefully optimization of the injection parameters in electrochromatography is necessary to obtain optimal quantitative results. A number of the conclusions resulting from this study are valid not only for the optimization of manual injection methods but also for the optimization of automatic injections performed on commercial instruments.

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