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Sample injection in capillary electrochromatography by heart-cut technique

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Abstract

The splitter working in heart-cut regime was used for sample injection in capillary electrochromatography. The principle was implemented in an automated microgradient system allowing to inject from microlitre down to nanolitre volumes with high repeatability and minimal extra-column band broadening. The apparatus is able to deliver discrete volumes of liquids at a preset volumetric flow rate and to stop and restore the flow at any moment. This brings a high degree of liquid manipulation flexibility. An extremely low split ratio is sufficient during the analysis, which saves mobile phase consumption substantially. The key parameters influencing the function of the heart-cut splitter were characterised. The function of the apparatus was demonstrated under isocratic, preconcentration and gradient capillary electrochromatography separation conditions. In all cases the statistic evaluation of the main parameters was performed, showing that high repeatability of retention times, peak heights and areas was achieved.

Keywords: Electrochromatography; Injection methods; Sample handling; Gradient elution; Automation

1. Introduction

Significant progress could be seen in capillary electrochromatography (CEC) in recent years [1–3]. The number of applications grows steadily [4–6] and improvement of column technology, particularly monolithic, is well documented [7,8]. Two main groups of instruments are being used, namely commercial capillary electrophoreographs and home made set-ups of various construction [9–11]. Specialised commercial CEC instrumentation is scarce [11–13], which negatively influences wider exploitation and further development of CEC. The reason of such a situation could be, among others, a seemingly peripheral problem, which is sample introduction into CEC column. The requirements for an ideal CEC sample injection system are challenging. It should be capable to inject extremely small (nanolitres, nl) and also moderate (microlitres, μl) sample volumes with high repeatability and reliability. The contribution to extra-column band broadening should be minimised. It should be readily made automatic. And also, it should be compatible with electrokinetic and pressure (hydrodynamic) mode of injection/elution and mobile phase gradient.

Currently used sample injection procedures fulfil most, but not all of the above demands. The most often exploited method is a simple dip of the end of the CEC column into the sample vial, followed by electrokinetic or pressure sample introduction and plunge back into the mobile phase vial. This method could be automated easily and therefore it is used in commercial capillary electrophoreographs. Stepwise mobile phase gradient is applicable [14], but the main disadvantage is that this method is incompatible with continuous gradient elution [11]. Another recently published injection method suitable for capillary electrophoresis (CE) and CEC is a miniaturised rotary-type injector [15,16]. Significant limitation of this concept is due to the impossibility to change sample volume, given strictly by the injector internal design. The third approach, widely applied in laboratory-made set-ups, utilises a splitter creating the flow interface between a sample/mobile phase delivery device and a CEC separation column. The published functions of the splitter are three-fold: it works as a sampling device [17,18], as a means of creating microflow in liquid

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chromatography [19] and as part of sample/gradient delivery systems in capillary electrophoresis (CE) and CEC [20–23].

The splitting could effectively serve for all of the above purposes, but in case of sampling it suffers from one inherent limitation. The function of the splitter occurs between two extremes. In one extreme the volume of the original sample plug is high and the split ratio is low. Substantial part of the original sample plug enters the CEC column. The original sample concentration could be easily attained in the injection part of the column, which is desirable, but peak tailing occurs due to the slow washing of the sample from the splitter. In the other extreme it is vice versa, the original sample plug volume is low and the split ratio is high. Here, only minute part of the original sample plug reaches the column. Because of much more intensive mixing, the original sample concentration cannot be reached in the inlet of the column. At the same time, high volumetric flow given by the high split ratio washes the splitter quickly, effectively suppressing the peak tailing and decreasing the extra-column band broadening. Although the dispersion processes are widely influenced by the volume of the original sample plug, by geometry of the transfer line and the splitter, and by the volumetric flow rate, the general problem remains: the original sample concentration, the peak shape or part of both will be sacrificed.

The above described problem could be solved by time-resolved function of the splitter, it is splitting in heart-cut regime. The sample injection by heart-cutting consists in the introduction of a very narrow sample cut, preferably of the original concentration, into the separation column (by virtue of pressure or voltage), while the rest of the original sample plug before and after the cut is wasted. For the first time the sample injection by heart-cut was described by McGuffin and Novotny [24] in microcolumn liquid chromatography and by Jorgenson and Guthrie in open tube liquid chromatography [25]. This injection technique was later applied, e.g. in CEC [26] and CE [27], although not named explicitly.

Recently we have described an automated microgradient system, in which sample injection based on heart-cut principle was integrated [28]. We have shown high short-term repeatability of retention times ($R.S.D. \sim 0.1\%$), documenting high uniformity of gradient operations. In the present paper, we will characterise and study the key parameters influencing processes occurring in the splitter during sample injection in heart-cut mode. In the second part of the paper, we will show repeatability of the quantitative CEC analysis, accomplished on our instrumental set-up comprising the heart-cut sampling splitter under isocratic, preconcentration and gradient conditions.

2. Experimental

2.1. Reagents and materials

Fused silica capillaries were supplied by Polymicro Technologies (Phoenix, AZ, USA) and by CACO (Bratislava, Slovakia). Acetonitrile (ACN) Supragradient HPLC grade was from Scharlau Chemie (Barcelona, Spain), Tris [tris(hydroxymethyl) aminomethane] and boric acid were purchased from Fluka (Buchs, Switzerland), poly(ethylene glycol) M_r 10 000, alkylphenones, tetramethoxysilane, tetraethoxysilane and octyldimethylchlorsilane for sol–gel column preparation were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium nitrate and reversed-phase sorbent Silasorb SPH C_{18} 5 μm were obtained from Lachema (Brno, Czech Republic). All solutions were prepared in deionised water. The mobile phase was 10 mM Tris, 15 mM boric acid (pH 8.3) with given content of acetonitrile. This stock solution was degassed and filtered each day before filling reservoirs.

2.2. Column preparation

For all experiments we have used laboratory made capillary electrochromatographic columns. The packed columns were filled by a slurry packing method with Silasorb SPH C_{18} 5 μm particles. The sol–gel monolithic columns were prepared according to the method described in the literature [29] and octyldimethylchlorsilane was used for silica surface modification.

2.3. Instrument

The automated laboratory-made sample introduction/gradient system, described previously in detail [28], is schematically represented in Fig. 1. It consists of three main parts: a liquid handler, a splitter, and a CEC separation unit. The liquid handling device/gradient generator includes a liquid distribution block with five inlets for sample injection, a weak and strong mobile phase feed, a waste outflow and a sample/mobile phase transfer line to the splitter, and a 75 μl glass syringe with a stainless steel needle. The needle has a side hole (0.15 mm in diameter), which can be joined to one of the five inlets. All operations are executed by means of two computer controlled stepping motors according to an appropriate algorithm [28]. The liquid handler is connected to the splitter by the transfer line (fused silica capillary, 215 mm × 50 μm i.d.). The splitter is the injection part of the CEC separation unit and serves as grounded anode. The grounding is essential to avoid electrical interference with the liquid handler microprocessor. The CEC column is inserted between the splitter and the outlet electrolyte chamber. The high-voltage power supply (HVS) from Spellman (Plainview, NY, USA) is used to generate electric fields and it is computer controlled by a custom-made digital switch by EldeSys (Brno, Czech Republic).

The sample pulses from the liquid handler are scanned on the end of the transfer line (detection point 1) and CEC separations are monitored on the column (detection point 2). In both cases, UV detection (UV-980 Jasco, Tokyo, Japan) via optical fibers (300 μm core/360 μm o.d., Polymicro Technologies) is performed. DataApex (Prague,

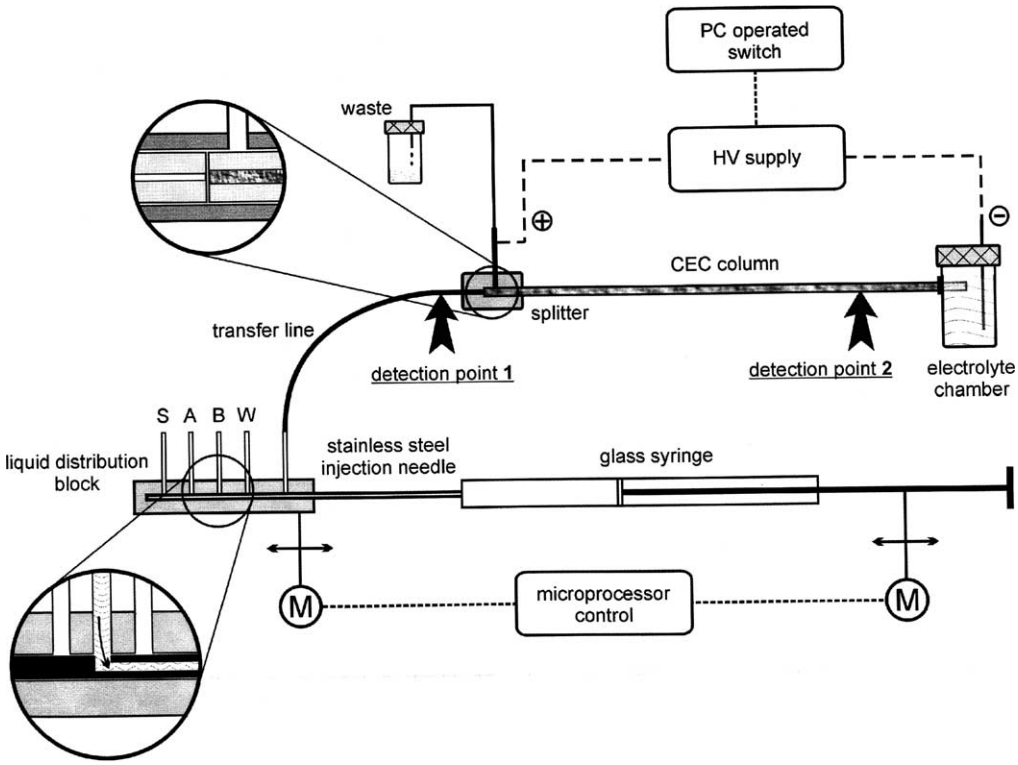

Fig. 1. Schematic diagram of the CEC instrument with detailed illustration of the splitter and the liquid distribution block. Letters S, A, B, W represent connections to the vials with sample, weak and strong mobile phases and waste, respectively.

Czech Republic) integration software was used for data collection.

2.4. Instrument function

The electrochromatograph operates according to a preset algorithm, which is controlled by microprocessor. Generally, at first the syringe is filled with appropriate mobile phase. In case of gradient elution, after the strong mobile phase is sucked into the syringe, the weak mobile phase follows and the gradient is formed by turbulent mixing on their boundary [28].

After that the selected sample volume (0.1–1 μl) is being sucked at specified speed into the needle. The sample plug is then displaced via transfer line into the splitter and there stopped. The course and the shape of the sample plug are monitored by UV detection at detection point 1. Afterwards, the heart-cut injection is carried out by switching on the high voltage supply so that the defined part (few nl) of the original sample plug is electrokinetically introduced into the CEC column. The other possibility is not to switch the HV supply on so that the sample is introduced merely by diffusion. The last step of the sample introduction process is the flushing of the splitter by the preset volume of mobile phase. Finally, the excess of the fresh mobile phase, which was sucked into the syringe before, is continuously fed into the splitter, the high voltage supply is turned on and the CEC analysis begins.

Analytes are monitored by UV detection behind the frit (packed capillaries) or through the packing (monolithic capillaries) at detection point 2. For various types of CEC analyses (isocratic or gradient elution, sample enrichment) the appropriate algorithms were compiled and applied. All experiments were performed at ambient room temperature without any thermostating. The electrochromatographic analyses were performed without any pressurisation.

3. Results and discussion

3.1. Character of sample plugs entering the splitter

The ideal shape of the sample plug entering the CEC column is a rectangle, i.e. the steep increase from zero to the original sample concentration followed by steep decrease back to zero concentration. Moreover, if we consider non-focusing conditions, the width of this sample plug should be very small, not to overload the column volumetrically. As a rule of thumb it should be less than about 0.5% of the column volume [11,30], which is several nl at the typical CEC column volume of about 1 μl. If there is any focusing mechanism at work (chromatographic preconcentration or electrophoretic stacking), the sample volume could be very large, but for practical reasons a maximum of one column volume is injected. In this case, the electrochromatographic conditions (column packing chemistry,

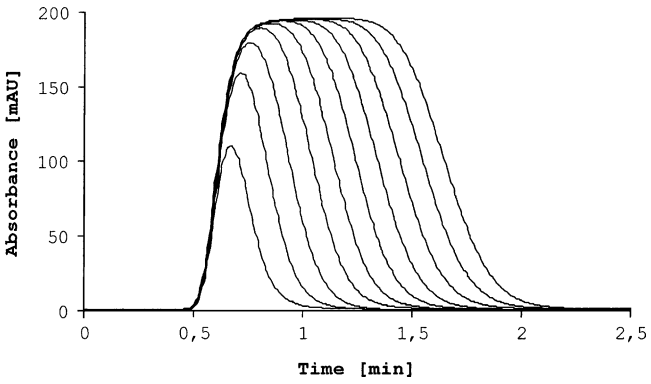

Fig. 2. Influence of the sample plug volume (0.1–1 μl) on the original plug shape. Conditions—transfer line: 215 mm × 50 μm i.d.; flow rates of sucking and displacement 1 μl/min for all experiments; detection: UV absorbance at 210 nm (detection point 1, Fig. 1); sample: 10 mM nitrate in water; transport liquid: deionised water.

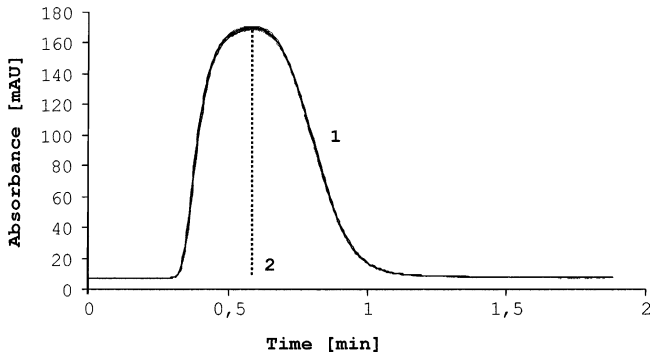

Fig. 4. Repeatability of the sample plug shape (1) (nine subsequent injections overlaid) with schematic illustration of the heart-cut injected zone (2). Conditions—transfer line: 215 mm × 50 μm i.d.; sample: 10 mM nitrate, volume: 0.8 μl; flow rates of sucking and displacement: 2 μl/min; other conditions are as presented in Fig. 2.

mobile phase composition, sample matrix) must be adjusted very thoroughly. Thus the sampling device has to inject low nanolitre volumes under non-focusing conditions and also high nanolitre to microlitre volumes under focusing conditions. The splitter interface is a convenient device for this purpose. Moreover, the splitter is ideally compatible with gradient elution and problems with buffer depletion are eliminated. Nevertheless, as we have already mentioned in the Introduction, the splitter suffers from one important limitation. If we try to decrease the sample volume entering the column inlet (it could be done either by volume reduction of the original sample plug before splitting or by higher splitting ratio), at the same time we increase the dispersion and effectively decrease the sample concentration.

We have accomplished the set of experiments (Figs. 2–4), which, on one hand, illustrate the above reasoning about splitter limitations and, on the other hand, serve as a starting point for optimisation of key parameters influencing the function of the splitter in heart-cut mode. The following parameters were examined: (i) the volume of the original sample plug before splitting, generated by the automated sample introduction/gradient system (Fig. 2), (ii) the volumetric rate of sample sucking and displacement (Fig. 3), and (iii) the repeatability of sample plugs entering the splitter (Fig. 4). The above experiments were performed on the set-up depicted in Fig. 1 and the shape of the sample plugs was monitored in detection point 1, just before the splitter. Another key parameter, which is transfer line geometry (215 mm × 50 μm i.d.), was dictated by the apparatus design and was kept constant.

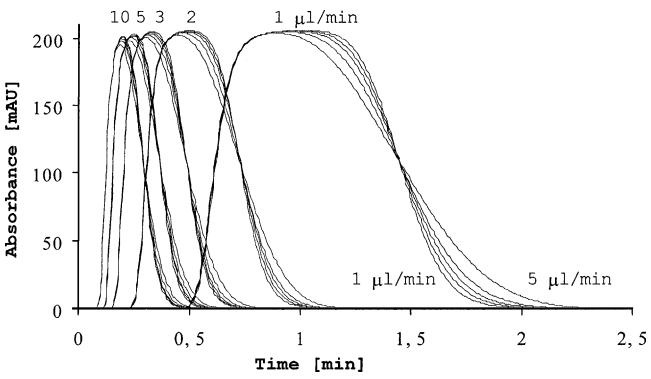

Fig. 3. Choice of the optimal combination of sucking and displacement flow rates. The upper values show the displacement flow rates and the lower display the sucking flow rates. Conditions—sample volume: 0.8 μl, other conditions are as presented in Fig. 2.

The influence of the sample pulse volume on its resulting shape is shown in Fig. 2. The volumes of 0.5–1 μl give the clearly visible plateau corresponding with the original sample concentration. At the volumes of less than 0.5 μl the mixing prevails so that the original sample concentration cannot be reached in the splitter. The changing shape of the sample pulse at various sucking (1–5 μl/min) and displacement (1–10 μl/min) flow rates is shown in Fig. 3. The concentration plateau gradually disappears due to the intensified mixing at a growing flow rate and the net result is the same as in Fig. 2—the original sample concentration will not be reached. In Fig. 4, the repeatability of the sample pulse shape is shown as the first prerequisite of high repeatability of the whole injection process. Nine consecutive runs were overlaid in Fig. 4 and the concentration in maximum was measured (R.S.D. 0.49%).

Considering the demands on the ideal shape of the sample plug entering the CEC column and the results shown in Figs. 2 and 3, we can see two general solutions of the problem. Either the original sample plug volume will be decreased to the desired degree (nl) or this small volume will be cut out from the relatively large (μl) original sample plug. In the first case, we have to fight with flow dependent mixing, we can decrease the flow by decrease of the split ratio, and in the limit we get to a miniaturised rotary-type injector. The second case is the sample introduction by the splitter in heart-cut regime. The heart-cut injected zone is depicted in Fig. 4 as a dotted line.

The heart-cut sample injection consists of several steps, which should be optimised. The maximum aims of such

optimisation could be defined as the introduction of (i) the undiluted sample plug of (ii) the desired volume into the CEC column in (iii) the shortest possible total time and (iv) the negligible contribution to extra-column band broadening. (i) The first condition requires to reach the concentration plateau in the splitter at the expense of time, the expense of the original sample volume or both (Figs. 2 and 3). (ii) The desired injection volume could be cut from the original sample plug electrokinetically or hydrodynamically. In the first case, the exact injection volume corresponds to the product of injection time and voltage; in the second case, it is the product of injection time and pressure applied. (iii) The total time of injection process comprises the time needed for generation of the original sample plug in the sample delivery device (valve, liquid handler), its transport through the transfer line into the splitter, injection of a small portion by heart-cutting, and for washing out the sample rest from the splitter. After that, the analysis can start. The influence of the key parameters, which are the original sample volume and the volumetric flow of sample sucking and displacement, is shown in Figs. 2–4. Another key parameter potentially influencing the total injection time is the transfer line geometry. Although not supported experimentally here, we can presume that the decrease in the transfer line internal diameter and length can lead to substantial time and/or sample volume savings.

The set-up we have used [28] is especially suitable for the above described task. It is capable of automatic delivery of discrete volumes of liquids with resolution of 10 nl and a variable volumetric flow rate, both with high repeatability. This means that the liquid flow could be stopped and the split ratio could be changed at any moment. Because the negligible mobile phase overflow is necessary during the analysis in the splitter, the split ratio of 1:2 or even lower is sufficient. This substantially saves mobile phase consumption. Moreover, the instrument is capable to generate continuous mobile gradients easily.

3.2. Use of heart-cut sample injection for various modes of CEC analyses

3.2.1. Calibration of heart-cut injection device

An important factor for the real use of any analytical separation method is linearity of sample injection. In Fig. 5, the calibration curves of diffusion and electrokinetic injection of thiourea are plotted. In case of the diffusion injection, the sample plug was transported into the splitter and left there for a defined period of time (1–20 s) so that the analytes could enter the column. Subsequently the splitter was cleaned and the analysis began. When the electrokinetic injection was performed, the plug was stopped in the splitter, HVS was turned on (1–13 s) and the sample was injected. The measured peak area represents the total volume of the sample introduced into the column and it is proportional to the product of applied voltage and time. It is obvious that in this case the total amount of the injected sample is the sum

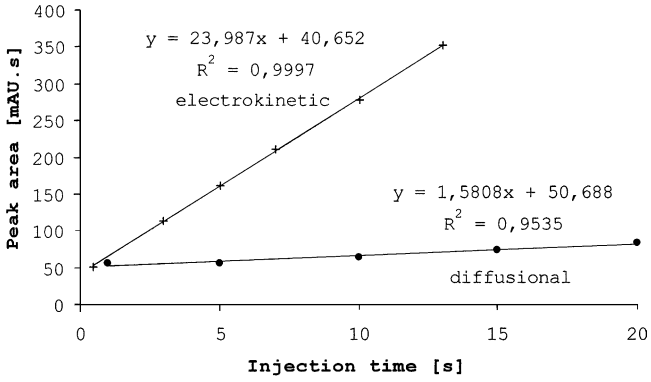

Fig. 5. Calibration dependence of the peak area (hence the amount of sample) on the injection time, the sample introduction by mere diffusion and electrokinesis. Conditions—capillary: monolithic sol–gel C_8 column, 180 mm (140 mm effective length) × 100 μm i.d.; mobile phase: 40% acetonitrile in 10/15 mM Tris–borate buffer; applied voltage: 8 kV; injection: 1 kV; detection: UV absorbance at 240 nm (detection point 2); sample: thiourea.

of that introduced both elektrokinetically and by diffusion. However, the effect of diffusion is partially eliminated by the subsequent washing of the splitter. High linearity of both events, i.e. diffusion injection and electrokinetic injection, was achieved (correlation coefficients 0.95–0.99).

3.2.2. Isocratic analysis

Fig. 6 shows the overlay of twelve successive isocratic analyses of test mixture containing thiourea, acetophenone and propiophenone. These separations were performed on the packed capillary and 65% acetonitrile was used as mobile phase. High short-term repeatability of retention times (R.S.D. < 0.2%), peak heights and areas (R.S.D. < 1.5%) was achieved, as it is shown in Table 1. It gives evidence of the high functional repeatability of our experimental set-up. Also high symmetry of peaks was achieved. It

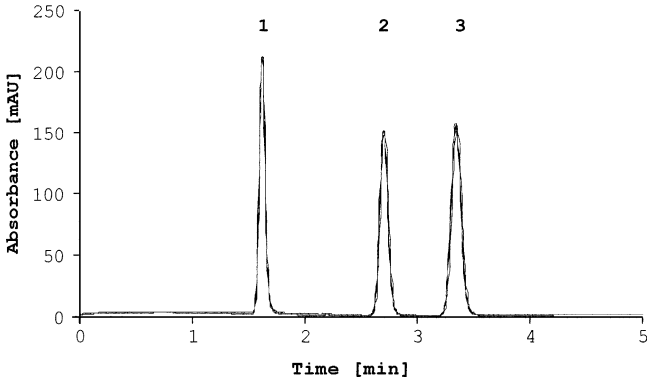

Fig. 6. Short-term repeatability of isocratic separations (n = 12). Conditions—capillary: 125 mm (90 mm effective length) × 100 μm i.d. packed with Silasorb SPH C_{18} 5 μm; mobile phase: 65% acetonitrile in 10/15 mM Tris–borate buffer; applied voltage: 7 kV; injection: 7 kV, 1 s (injected volume: 5 nl); detection: UV absorbance at 240 mm (detection point 2); sample: 1 = thiourea, 2 = acetophenone, 3 = propiophenone (each 10 mM).

Table 1
Statistical evaluation of isocratic and preconcentration analyses of two alkylphenones

	Thiourea	Acetophenone	Propiophenone
Isocratic analysis			
t_r (min)			
Mean	1.61	2.70	3.35
R.S.D. (%) ($n = 12$)	0.11	0.12	0.13
Peak area (mAU/min)			
Mean	800.44	797.80	989.16
R.S.D. (%) ($n = 12$)	0.64	1.14	1.42
Peak height (mAU)			
Mean	208.09	148.88	154.72
R.S.D. (%) ($n = 12$)	0.58	1.15	1.37
Asymmetry			
Mean	1.29	1.14	1.13
R.S.D. (%) ($n = 12$)	5.77	6.93	3.47
Sample preconcentration			
t_r (min)			
Mean	n.a.	2.96	3.87
R.S.D. (%) ($n = 6$)		0.25	0.32
Area (mAU/min)			
Mean		20.60	24.41
R.S.D. (%) ($n = 6$)		1.74	0.78
Peak height (mAU)			
Mean		4.07	4.12
R.S.D. (%) ($n = 6$)		1.05	0.66
Asymmetry			
Mean		1.18	1.14
R.S.D. (%) ($n = 6$)		7.97	5.72

n.a.: not applicable.

indicates that the use of the heart-cut injection technique effectively eliminates the contribution to the extra-column band broadening.

3.2.3. Preconcentration

The introduction of large volumes was also examined. The diluted mixture of thiourea, acetophenone and propiophenone was elektrokinetically loaded into the column. The sample plug represented 28% of the dead column volume. The chromatograms of six successive analysis are depicted on Fig. 7 and the repeatability of this process is shown in Table 1. It is apparent that the repeatability is not as high as with isocratic analysis because of longer injection period. During this process the resulting sample plug can be affected by the changing electrokinetic conditions. This influenced especially the peak heights and areas.

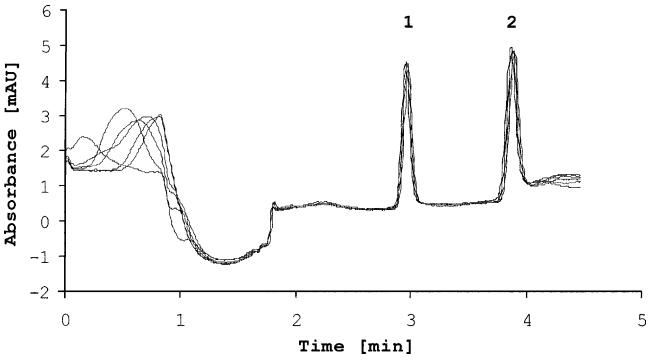

Fig. 7. Short-term repeatability of on-column preconcentrations ($n = 6$). Conditions—capillary: 125 mm (90 mm effective length) × 100 μm i.d. packed with Silasorb SPH C_{18} 5 μm; mobile phase: 65% acetonitrile in 10/15 mM Tris–borate buffer; applied voltage: 6 kV; injection: 6 kV, 30 s (injected volume: 140 nl, 28% of column volume); detection: UV absorbance at 240 mm (detection point 2); sample: 1 = acetophenone, 2 = propiophenone (both 10 μM, dissolved in 1% acetonitrile).

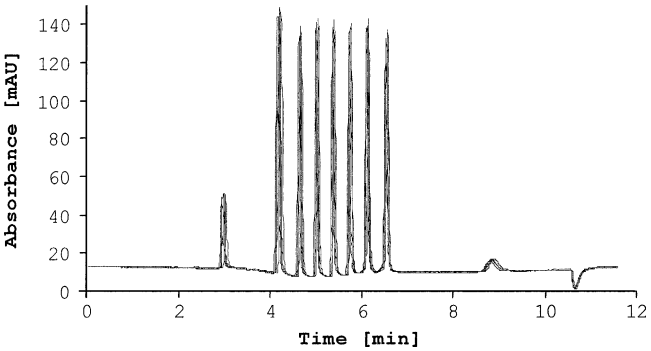

Fig. 8. Short-term repeatability of gradient CEC separations ($n = 5$). Conditions—capillary: monolithic sol–gel C_8 column, 180 mm (140 mm effective length) × 100 μm i.d.; mobile phase: S-shaped gradient, 42–80% acetonitrile/water – 10/15 mM Tris–borate buffer; applied voltage: 8 kV; injection: 8 kV, 1.5 s (injected volume: 6 nl); detection: UV absorbance at 240 mm (detection point 2); peak order: thiourea, acetophenone, propiophenone, butyrophenone, valerophenone, hexanophenone, heptanophenone, octanophenone (10 mM each).

Table 2
Statistical assessment of five successive gradient analysis of alkylphenones mixture

Gradient analysis ($n = 5$)	t_r (min)			Peak area (mAU/min)			Peak height (mAU)		
	Mean	S.D.	R.S.D. (%)	Mean	S.D.	R.S.D. (%)	Mean	S.D.	R.S.D. (%)
Thiourea	2.98	0.03	0.98	151.2	1.6	1.0	38.63	0.54	1.4
Acetophenone	4.19	0.03	0.64	548.9	10.2	1.9	136.90	2.27	1.7
Propiophenone	4.65	0.02	0.52	440.7	7.8	1.8	127.98	2.16	1.7
Butyrophenone	5.02	0.02	0.40	423.1	6.4	1.5	132.34	2.05	1.5
Valerophenone	5.38	0.02	0.35	415.7	6.4	1.5	130.99	2.09	1.6
Hexanophenone	5.75	0.02	0.33	412.2	8.0	1.9	128.22	2.23	1.7
Heptanophenone	6.13	0.02	0.30	472.9	9.5	2.0	130.31	2.47	1.9
Octanophenone	6.55	0.02	0.26	486.0	17.4	3.6	123.38	2.97	2.4

3.2.4. Gradient analysis

The ability of the instrument to perform gradient analysis, the principle of gradient mixing, and the particular algorithm were described previously [28]. High repeatability of retention times (R.S.D. 0.1%) was also presented. In this work we carried out the gradient analysis with respect to the repeatability of peak heights and areas. Fig. 8 shows five successive separations of mixture of homologous alkylphenones (acetophenone–octanophenone) and thiourea as a marker of electro osmotic flow (EOF). The analytes were separated on the monolithic sol–gel column and S-shaped gradient (40–82% of ACN in water) was used for their elution. The obtained results are shown in Table 2, R.S.D.s for peak heights and areas were in the range of 1–2%. We assume that these values are greatly affected by worse repeatability of processes in the monolithic column during the CEC analysis in comparison with the function of the injection device.

4. Conclusions

The sample injection represents a challenging problem in capillary electrochromatography. The methods currently used for the purpose do not meet all demands laid on an ideal injection system, namely: the capability to inject extremely small (nl) and also moderate (μl) sample volumes with high repeatability and reliability, minimisation of contribution to extra-column band broadening, easy automation, compatibility with electrokinetic and hydrodynamic mode of injection/elution and mobile phase gradient. We have employed the splitter working in heart-cut regime as a sampling device potentially fulfilling all the above requirements. An automated liquid handling device was used for delivery of the sample plug and mobile phase into the splitter. Key parameters influencing the function of the heart-cut splitter were characterised. To demonstrate the possibilities of the instrumental set-up used, the quantitative isocratic, preconcentration and gradient electrochromatographic analyses of test mixtures were performed. Repeatability of all instrument functions was characterised by typical R.S.D. values of 0.2–0.5% for retention times and 1–2% for peak heights and areas, respectively.

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