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Electrophoretic Sample Pre-treatment Techniques Coupled On-line With Column Liquid Chromatography

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The on-line coupling of zone electrophoretic and isotachophoretic sample treatment with column liquid chromatography (LC) is described. The electrophoretic sample treatment methods are carried out in free solution. Zone electrophoretic sample treatment (ZEST) of 20 mm³ of sample takes about 20 min. The applicability of ZEST to environmental analyses is demonstrated with the determination of bentazone in water from the river Rhine. Preliminary results are presented for the on-line coupling of isotachophoresis (ITP) with LC, using a single switching valve. With ITP 30 mm³ sample volumes, which can be concentrated to an injection volume of 5 mm³, can be treated within 17 min.

Keywords: Column liquid chromatography; zone electrophoretic sample treatment; isotachophoretic sample treatment; on-line coupling; bentazone

Nowadays, sample treatment methods that can be coupled on-line with column liquid chromatography (LC) are receiving more attention than off-line methods, which generally are laborious, time consuming and subject to errors caused by, e.g., contamination and/or analyte losses. In environmental analysis, solid-phase extraction techniques are frequently used for the determination of compounds in water samples,[1] reversed-phase LC then being the preferred separation technique. These techniques are mainly used for the determination of apolar and medium-polarity compounds. If ionic compounds have to be isolated from aqueous samples, two or more extraction and/or sorption steps are often required. In order to simplify the sample treatment in such instances and to increase the selectivity, new methods have been developed that are based on electrophoretic principles, i.e., on differences in the migration velocities of charged analytes and other sample matrix constituents in an electric field. However, until now the applications have been restricted to the analysis of biological samples[2-4] and, in many instances, they are performed in an off-line mode.[5,6]

Zone electrophoretic sample treatment (ZEST) was described as an on-line sample treatment method coupled to LC for the treatment of biological samples.[2-4] Separation was achieved in free solution using open capillaries. A single-valve set-up allowed the treatment of 20 mm³ samples in about 15 min. In this work, on-line ZEST–LC was used for the analysis of water extracts from the river Rhine in order to demonstrate its applicability to environmental studies.

Isotachophoresis (ITP) was described as an off-line electrophoretic sample treatment method for LC.[5] Isotachophoresis was performed on gel slabs. After treatment of the sample, the zones of interest were cut out, the analytes were desorbed and the desorbates analysed by LC. Recoveries of 85–90% were achieved in experiments using aqueous buffers as sample solutions. In this paper, preliminary results are reported on on-line ITP–LC using a single switching valve.

Bentazone (3-isopropyl-1H-benzo-2,1,3-thiadiazin-4-one 2,2-dioxide) was used as a model compound in this study. It was selected because the low μg dm⁻³ determination of acidic

ionic compounds in surface waters is hampered by the high concentration (up to mg dm⁻³) of the complex humic and fulvic acids present in such samples.

Experimental

Chemicals

Sodium dihydrogen phosphate, disodium hydrogen phosphate, barium hydroxide, pentanoic acid, hydrochloric acid and tris(hydroxymethyl)methylamine (Tris) were purchased from Merck (Darmstadt, Germany). Morpholinopropanesulfonic acid (MOPS) and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) were purchased from Sigma (St. Louis, MO, USA). Water was de-mineralized with a Milli-Q water purification system from Millipore (Milford, MA, USA). Acetonitrile of HPLC-grade was purchased from FSA (Loughborough, Leicestershire, UK) and poly(vinyl alcohol) from Hoechst (Frankfurt, Germany). Bentazone was obtained from Riedel-de Haën (Seelze, Germany). As a blank water sample, 1 dm³ of river Rhine water was loaded on a C_{18} solid-phase cartridge. After elution with 1 cm³ of ethyl acetate, the extract was evaporated under a nitrogen atmosphere and the residue dissolved in de-mineralized water or a phosphate buffer for the ITP and ZEST experiments, respectively. In order to obtain a spiked sample, 1 dm³ of the same batch of river water was spiked with 0.3 μg of bentazone and was treated in the same way as the blank sample.

Column Liquid Chromatography

The LC equipment for the ITP experiments consisted of a Beckman (Palo Alto, CA, USA) Model 100 A LC pump and a Philips (Eindhoven, The Netherlands) PU 4225 variable-wavelength ultraviolet (UV) absorbance detector. The ZEST experiments were performed using an LKB (Bromma, Sweden) Model 2150 pump and a Kratos (Ramsey, NJ, USA) Spectroflow 757 UV absorbance detector. All chromatographic runs were performed at ambient temperature using a flow rate of 0.6 cm³ min⁻¹. The eluent was acetonitrile–5 × 10⁻³ mol dm⁻³ phosphate buffer (pH 3) (3 + 7, v/v). A 150 × 4.6 mm i.d. stainless-steel column, packed with 5 μm Zorbax 5-ODS (Dupont, Wilmington, DE, USA), was used for the

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ITP experiments whereas for the ZEST experiments 5 μm RoSil C_{18} (Research Separation Laboratories, Eke, Belgium) was used as packing material. For the ITP experiments, direct sample injections were made with a Valco (Schenkon, Switzerland) N-60 HPLC injection valve equipped with a 30 mm³ sample loop.

In the electrophoretic sample treatment experiments, UV detection was performed at 230 nm. The detector output was recorded with a BD41 potentiometric-type recorder (Kipp & Zonen, Delft, The Netherlands) and/or a Philips Type P3202 computer, equipped with a laboratory-built Multilab-TS interface (noise filter: RC time 1/15 s).

Coupling ZEST to LC

The on-line coupling of ZEST to LC was achieved with a laboratory-built switching valve.[4] A laboratory-built power supply was used in the constant-current mode (3 mA) to apply the electrical field. The ZEST valve which was placed between two laboratory-built electrode vessels consists of three discs, the sample, transfer and injection discs. Three main channels are drilled through the discs. By means of the sample disc the sample is introduced into the channel in which electrophoresis is performed. After the electrophoretic separation, the compounds of interest that have migrated into the injection disc via the transfer disc are injected onto the analytical column by switching the injection disc. The sample volume that could be treated by ZEST was 20 mm³ (volume of sample disc); the injection volume onto the analytical column was 40 mm³ (volume of injection disc). The complete set-up of the ZEST-LC system is shown in Fig. 1.

Coupling ITP to LC

The on-line ITP-LC set-up consisted of a laboratory-built ITP apparatus (Eindhoven University of Technology), equipped with an alternating current (a.c.) conductivity detector, as described previously,[7] and a laboratory-built rotary-type switching valve (Free University, Amsterdam). The rotary switching valve consists of a PVDF [poly(vinyl difluoride)] rotor and Arnite [poly(ether ethene terephthalate)] stators. Two channels are drilled through the rotor and stators, each with an inner diameter of 1.0 mm. The length of the rotor and
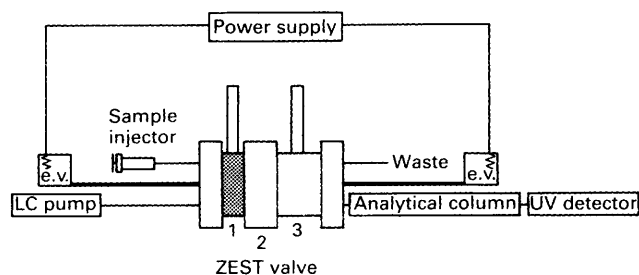

Fig. 1 Scheme of ZEST coupled on-line to LC: e.v., electrode vessel with flow-through chamber; 1, sample disc with volume of 20 mm³; 2, transfer disc with volume of 20 mm³; and 3, injection disc with volume of 40 mm³. For a detailed description, see ref. 4
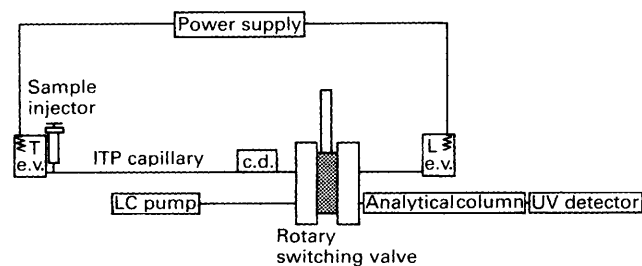

Fig. 2 Scheme of ITP coupled on-line to LC: e.v., electrode vessel; c.d., conductivity detector; T, terminating electrolyte; and L, leading electrolyte

hence the injection capillary is 6.0 mm. The injection volume will therefore be appoximately 5 mm³. The valve can be used without leaking with liquid pressures up to 55 bar.

A constant electrical current (245 μA) was delivered by a Brandenburg (Thornton Heath, UK) Type 807r power supply. The inner diameter of the 10 cm capillary used for isotachophoresis was 1.0 mm. Fig. 2 shows the scheme of the ITP-LC set-up.

Results and Discussion

ZEST Coupled to LC

ZEST can be performed in open capillaries filled with a buffer solution. The technique aims at the electrophoretic separation of charged analytes of interest from the sample matrix. In earlier studies, 0.05 mol dm⁻³ phosphate buffers were used for the treatment of plasma and urine samples. Using this buffer concentration, sample treatment took less than 20 min if a current of 4 mA was applied. At higher currents electrophoresis breakdown was observed. Large differences in conductivity between the sample and the buffer in which electrophoresis is carried out will lead to disturbances of the analyte zone profile, causing incomplete recovery and poor precision. Because of the high ionic strength of biological samples, the plasma and urine samples had to be diluted 2–3-fold.

Environmental samples generally have a relatively low ionic strength, and can therefore be analysed without prior dilution. Further, the low sample conductivity allows the use of dilute electrophoresis buffers to obtain comparable conductivities of the buffer and sample solution. This has the advantage that ZEST can be carried out in the same time as for the biological samples (approximately 20 min), but applying lower currents. Nevertheless, in order to compare the results obtained in this work with those achieved in earlier studies, electrophoresis was performed using a 0.05 mol dm⁻³ phosphate buffer (pH 7).

Determination of bentazone using ZEST

The dependence of analyte recovery on the time of electrophoresis was studied using bentazone, dissolved in a 0.05 mol dm⁻³ phosphate buffer, as a model compound. Recoveries were obtained by comparing the peak heights of bentazone after ZEST-LC with those obtained when the sample was injected directly onto the LC column. The results are shown in Fig. 3. If a phosphate buffer of pH 7 is used, bentazone ($pK_a \approx 3.2$) will be completely dissociated. As can be seen, quantitative recovery was obtained in 7 min, with plateau conditions being maintained for about 2 min. Although the time plateau is relatively short, acceptable results were obtained with respect to repeatability; for $n = 10$ the relative standard deviation (RSD) was 2.6%.

Bentazone was used as the model compound because solid-phase extraction methods show many interferences in

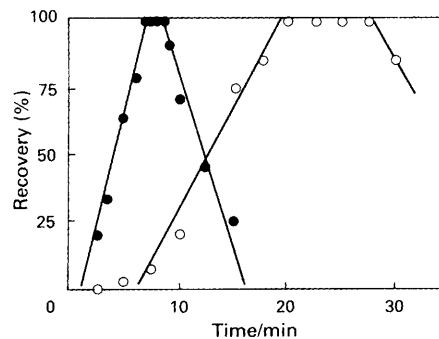**Fig. 3** Recovery versus time plots for 1×10^{-5} mol dm⁻³ bentazone in 0.05 mol dm⁻³ phosphate buffer solution (pH 7) (closed circles) and in 0.05 mol dm⁻³ phosphate buffer (pH 2) (open circles). UV detection, 230 nm; ZEST current, 3 mA
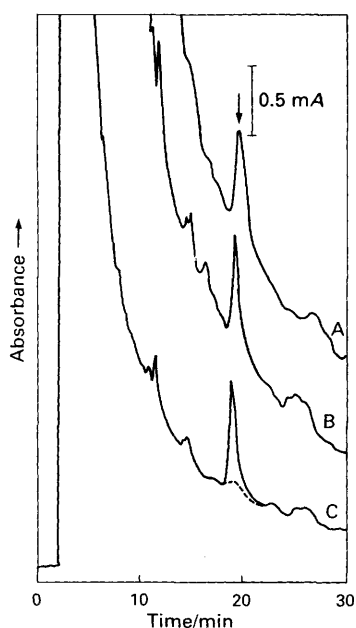
Fig. 4 LC–UV traces of spiked (bentazone, 0.3 µg dm⁻³) river Rhine water sample (dissolved in buffer). A, Direct injection of 20 mm³ of sample; B, injection of 20 mm³ of sample after ZEST, using a 0.05 mol dm⁻³ phosphate buffer (pH 7); and C, injection of 20 mm³ of sample after ZEST, using a 0.05 mol dm⁻³ phosphate buffer (pH 2). ZEST of 20 mm³ of blank Rhine river water, using a 0.05 mol dm⁻³ phosphate electrophoresis buffer (pH 2), is also shown (broken line). ZEST current, 3 mA; UV detection, 230 nm. For other LC conditions, see text. The arrow indicates the bentazone peak

Table 1 Analytical data for the determination of bentazone in Rhine water using electrophoretic sample treatment. For conditions, see text

Parameter	ZEST	ITP
Calibration graph*	$y = 1 \times 10^{-7} + 2.24x$	$y = 3 \times 10^{-7} + 5.12x$
Regression coefficient (r^2)	0.9996	0.9981
RSD (%) ($n = 10$)	1.3	6.3
Detection limit (ng dm⁻³) (S/N† = 3)	25	30

* x represents concentration (µg dm⁻³) and y the absorption signal (A).

† Signal-to-noise ratio.

carried out). The analytical data for the determination of bentazone using ZEST–LC are given in Table 1. The method was linear over three orders of magnitude of concentration, with a detection limit of 25 ng dm⁻³ of bentazone in Rhine water. In buffer solution a detection limit of 7 ng dm⁻³ could be achieved.

ITP Coupled to LC

With ITP only the anions or cations present in a sample can be separated within one run. When determining anions the cathode compartment is filled with an electrolyte, the terminating electrolyte, which contains an anion having a lower mobility than those of the sample anions. The electrolyte in the anode compartment and the separation chamber, the leading electrolyte, contains an anion that has a higher effective mobility than those of the sample anions. The sample solution is introduced at the zone boundary of the leading and the terminating electrolyte solutions. If an electrical field is applied, the charged sample constituents will be separated as a result of their different effective mobilities. When a steady state is reached, zones with sharp boundaries appear that all have the same migration velocity. In the steady-state situation the concentration of ions in each zone is determined by the concentration and effective mobility of the leading electrolyte and the effective mobility of the pertinent component, as has been shown by Kohlrausch.[10] According to the Kohlrausch equation, ITP of dilute samples will result in more highly concentrated sample ion zones. Contrary to expectations based on the above, the use of ITP as a pre-treatment technique for biological samples has been shown to give relatively low recoveries (60–75%) at low analyte concentrations.[5] Leaving aside the analyte–protein binding, which has also been observed in studies on ZEST but without any negative effect on analyte recovery at trace levels,[4] one can state that the high ionic strength of the samples apparently hinders the isotachophoretic process as a result of the decreased electric field strength prevailing after sample introduction. Because of the reduced field strength, part of the analyte is retarded in the terminating electrolyte solution during ITP and is consequently lost. Such a loss of analyte will, of course, be more significant at low concentration levels.

Instrumental development

In the previous off-line set-up,[5] ITP sample treatment was performed on a gel slab which acted as an anti-convective medium and was fixed between the terminating and the leading electrolyte compartments. In the present set-up, ITP was performed in an open capillary which connects the two electrolyte compartments (Fig. 2). Three steps can be distinguished in the procedure. First, the isotachophoresis capillary inserted between the sample injection device and the switching valve is filled with leading electrolyte, and the capillary inserted between the electrode vessel containing the terminating electrolyte and the injection device is flushed with terminating electrolyte. Next, the sample is introduced by

the LC–UV traces.[8] The LC–UV traces of preconcentrated samples of Rhine water (blank and spiked at 0.3 µg dm⁻³) are shown in Fig. 4. The samples were dissolved in 0.05 mol dm⁻³ phosphate buffer in order to prevent large differences in conductivity between the sample zone and the electrophoresis buffer, which could lead to disturbances in the zone profile during ZEST. The large number of interferences in the LC–UV trace on direct injection of a preconcentrated sample dissolved in phosphate buffer [Fig. 4(A)], will hamper the determination of compounds with capacity factors smaller than that of bentazone. In comparison with direct injection, a gain in selectivity is achieved when using ZEST performed in a pH 7 buffer [Fig. 4(B)]. However, the gain is disappointing as many interferences still show up in the chromatogram. Obviously many humic and fulvic acids have migration velocities that are close to that of bentazone and are therefore not separated from the analyte.

In order to decrease the amount of interfering compounds, as an alternative a 0.05 mol dm⁻³ phosphate buffer of pH 2 was used. At this pH bentazone is only partially dissociated and will have a lower effective electrophoretic mobility than at pH 7.[9] The recovery curve of bentazone dissolved in the pH 2 buffer is also shown in Fig. 3. Because of the lower effective mobility of bentazone, quantitative recovery is now observed only after about 20 min. Experiments carried out using an electrophoresis time of 23 min gave a satisfactory RSD of 1.3% ($n = 10$). Fig. 4(C) shows the LC–UV trace of the spiked Rhine river water sample after ZEST using the pH 2 buffer. Clean-up is seen to be distinctly more efficient than at pH 7. Apparently at pH 2 the differences in the effective mobilities of many interfering acidic compounds and bentazone are considerably larger than at pH 7; consequently, a better separation is achieved between bentazone and the matrix constituents.

As a means of comparison, Fig. 4 also shows the LC–UV trace of the blank Rhine water sample. The very small peak at $t_r = 19$ min corresponds to a concentration of 40 ng dm⁻³ of bentazone (no confirmation by an independent method was

Table 2 Operational buffer system for ITP coupled on-line to LC

	Leading electrolyte	Terminating electrolyte
Anion	1×10^{-2} mol dm^{-3} chloride	5×10^{-3} mol dm^{-3} HEPES
Counter ion	Tris	Tris
pH	7.5	8.5
Additive	0.05% m/m Mowiol	1×10^{-3} mol dm^{-3} Ba(OH)$_2$
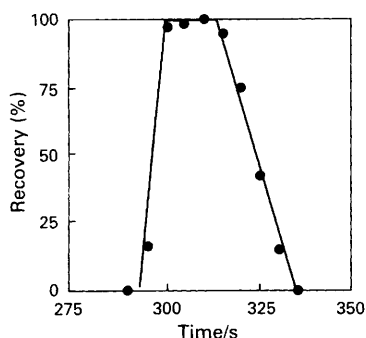
Fig. 5 Recovery *versus* time plot for 4.5×10^{-6} mol dm^{-3} bentazone in water. Leading electrolyte, chloride; terminating electrolyte, HEPES. UV detection, 230 nm; ITP current, 245 μA

Fig. 6 LC–UV traces obtained after: A, direct injection; and B, ITP treatment of 30 mm^3 of a bentazone-spiked Rhine river water sample (0.3 μg dm^{-3}). The chromatogram of an ITP-treated blank river Rhine water sample is also shown (broken line). ITP current, 245 μA; UV detection, 230 nm. For other LC conditions, see text. The arrow indicates the bentazone peak

means of a syringe at the boundary of the leading and the terminating electrolytes and an electrical current is applied. In order to monitor the ITP separation, a conductivity detector is positioned in-line, close to the rotary switching valve. Finally, after ITP, *i.e.*, when the analytes have migrated into the switching valve, the valve is switched and the analytes are flushed onto the analytical column by the LC eluent.

It is evident that the compounds of interest should be dissolved in a medium suitable for injection onto the LC column. In order to achieve this the operational buffer system for isotachophoresis shown in Table 2 was used. Using this system no chromatographic or detection (UV detection, 230 nm) problems were observed. Mowiol was added to the leading electrolyte to suppress the electro-osmotic flow and barium hydroxide was added in order to reduce the carbonate concentration.

Determination of bentazone using ITP

A recovery *versus* time plot (Fig. 5) was constructed in order to determine the optimum time of ITP, *i.e.*, the time elapsing between the moment the bentazone zone passes the conductivity detector and the moment the zone is inside the switching valve. Quantitative recovery was observed after 5 min. The total ITP procedure takes 17 min, because the sample zone passes the conductivity detector approximately 12 min after injection. In order to determine the repeatability, ten consecutive analyses of 5 mm^3 of a 4.5×10^{-6} mol dm^{-3} bentazone solution were carried out (Table 1). The high RSD of 6.3% is probably due to the short plateau on the recovery *versus* time curve, which adversely affects the repeatability.

In order to determine the maximum allowable injection volume, sample volumes of 2.5–45 mm^3 were treated. Although an injection of 45 mm^3 of a dye solution still gave sharp zone boundaries (visual observation), above about 30 mm^3 the repeatability became poor (RSD >10%). As the volume of the switching valve is 5 mm^3, with an injection volume of 30 mm^3 an enrichment factor of approximately 6 is achieved.

Because the concentrated Rhine water samples were dissolved in de-mineralized water, the ionic strength of the
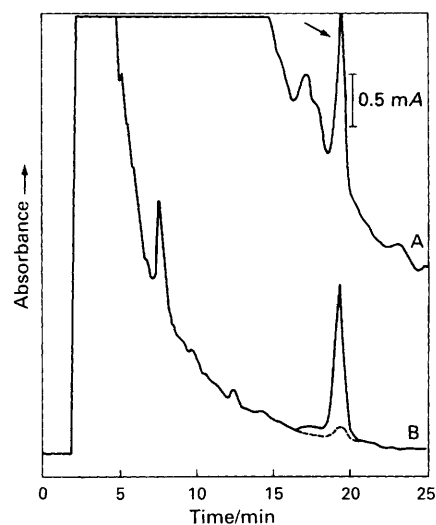
samples will be relatively low. Therefore, no disturbances as observed with the ITP treatment of biological samples are expected. In order to improve the separation of bentazone from matrix components, pentanoic acid and MOPS were used as spacers. Under the conditions given in Table 2 their effective mobilities are 14% larger and 33% smaller, respectively, than that of bentazone. Using these spacers less of the adjoining zones, which may contain matrix constituents, will enter the injection loop of the rotary switching valve.

Blank and spiked river Rhine water samples were analysed by injecting 30 mm^3 of sample with and without ITP sample treatment. To the 30 mm^3 samples subjected to ITP, 0.5 mm^3 of 0.1 mol dm^{-3} pentanoic acid and 5 mm^3 of 0.01 mol dm^{-3} MOPS were added as spacers. Fig. 6(A) shows the LC–UV trace for a direct injection of a spiked sample onto the LC column; Fig. 6(B) shows the trace obtained after on-line ITP sample treatment. A considerable gain in selectivity is observed, which is particularly important when determining trace amounts of bentazone such as were found in the blank river water sample. The bentazone concentration determined in this sample was approximately 50 ng dm^{-3}, which compared fairly well with the result obtained by ZEST. Table 2 gives the analytical data for the determination of bentazone using ITP. The method was linear over two orders of magnitude of concentration. With the present set-up, the detection limit of bentazone in Rhine water is approximately 30 ng dm^{-3}. In buffer solutions the detection limit is 8 ng dm^{-3}.

Conclusions

Electrophoretic sample treatment by means of both ZEST and ITP can be coupled on-line to LC, which helps to speed up sample treatment. Both ZEST–LC and ITP–LC are performed using single-valve configurations, which will facilitate the automation of these systems.

For ZEST, the selective clean-up of environmental samples takes about the same time (20 min) as previously reported for biological samples.[4] However, because of the relatively low ionic strength of environmental samples, no dilution is needed. Adjusting the pH of the electrophoresis buffers can help to increase the selectivity of the sample treatment procedure. For example, with the anionic bentazone the

number of interfering compounds observed in the LC–UV trace is considerably reduced if the electrophoresis buffer has a pH of 2 instead of 7. The selectivity is expected to be higher when cationic compounds have to be determined; the interfering humic and fulvic acids will then be completely separated from the basic analytes during electrophoresis.[11]

Using a rotary switching valve, which is similar to the valve used for ZEST, on-line coupling of ITP with LC can be achieved. With the present system 30 mm³ samples can be treated in 17 min. In contrast to high ionic strength biological samples, which yield diminishing recoveries at low analyte concentrations, with the present low ionic strength surface water samples the recovery remains constant even at low concentration levels. The selectivity of the ITP procedure can be enhanced by introducing suitable spacers.[12]

Finally, as regards future developments of on-line ITP–LC, attempts should be made to improve the geometry of the ITP set-up in order to obtain better reproducibilities and to allow the treatment of larger samples. Further, the coupling of ITP and micro-LC should be considered. With the present set-up, 30 mm³ samples can be concentrated to about 5 mm³. Full use of the ITP concentration effect should, in principle, allow concentration to even smaller volumes. This would make the combination with 0.3–1 mm i.d. micro-LC columns highly suitable.

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