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Some possibilities of combining high-performance liquid chromatography with isotachophoresis for the trace determination of ionogenic compounds present in complex matrices

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ABSTRACT

The use of reversed-phase high-performance liquid chromatography (RP-HPLC) for sample preparation in capillary isotachophoresis (ITP) was investigated. Asulam (a pre- and post-emergent herbicide) served as a model analyte and soil extracts represented complex ionic matrices. Very efficient clean-up of the samples was achieved by using the ion-suppression mode of the chromatographic separation and hence, positive systematic errors in the ITP determination of asulam could be reduced to levels equivalent to 8–12 ppb (10^9) of the analyte depending on the extraction procedure employed. As the number of sample handling steps was small, high recoveries (95–105%) of the complete analytical procedure were typical. The proposed combination of the separation methods seems very promising for the trace determination of ionogenic compounds present in complex biological and environmental matrices.

INTRODUCTION

In the determination of trace ionogenic constituents present in complex ionic matrices by capillary isotachophoresis (ITP), suitable sample preparation procedures may become essential for achieving desired detection limits and/or for decreasing systematic analytical errors due to co-migrating matrix constituents. Liquid–liquid extraction (*e.g.*, refs. 1–3), solid-phase extraction (SPE) on suitable chromatographic

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sorbents (*e.g.*, refs. 4 and 5), coprecipitation[6,7] and the use of ion-exchange and chelating resins (*e.g.*, refs. 8–10) have mainly been proposed for sample clean-up and/or for trace enrichment before the final ITP analysis. Special attention was paid to the use of affinity sorbents in a combination with ITP[11]. Derivatization combined with these sample preparation procedures is also convenient, especially when selective detectors are employed for the evaluation of the analysis[12,13].

The above simple sample preparation procedures are less effective when the analyte and some of the matrix constituents have similar physico-chemical properties. For example, problems are encountered in practice when relatively hydrophilic ionogenic compounds present in aqueous sample solutions are of analytical interest. Preparative ITP[4,15], ITP on columns packed with granulated gels or with "inert" particular materials[16] and capillary zone electrophoresis[17,18] have been proposed for solving these problems in high-performance liquid chromatography (HPLC). On the other hand, gel chromatography was convenient for the fractionation of uraemic sera before analysis by ITP and by other high-performance separation methods[19,20]. Also, ITP determination of peptides in HPLC fractions is useful in the control of peptide synthesis (*e.g.*, ref. 21). In spite of a widespread use of HPLC, however, this technique was not considered for sample preparation in trace ITP analysis. This is surprising when the following facts are taken into account: (a) as the separation principles of ITP and HPLC are different it is reasonable to expect that in their sequential use working conditions giving non-correlated qualitative characteristics of the separands (retention time *vs.* effective mobility) can be found easily; (b) for example, by using various modes of reversed-phase (RP) HPLC, gel chromatography and ion-exchange chromatography on low-capacity sorbents, the mobile phases employed are often compatible with ITP electrolyte systems; (c) for many types of ionogenic compounds it is possible to use various alternatives of their liquid chromatographic separations so that an appropriate possibility for the sample preparation can be found easily; and (d) as a simple configuration of the chromatographic instrument can be sufficient for this purpose there are hardly any economic restrictions concerning the routine use of HPLC for sample preparation in ITP.

The aim of this work was to investigate some practical aspects of the use of RP-HPLC for sample preparation in ITP. The results presented were obtained with asulam [methyl (4-aminosulphonyl)carbamate] as a model analyte, and soil extracts served as typical complex ionic sample matrices. Asulam was chosen as in previous work[15] it served as a model analyte in illustrating the practical capabilities of preparative ITP as a sample preparation method for analytical ITP and HPLC. Hence the present results obtained with the sequence HPLC–ITP may in some respects be considered as complementary to the reverse sequence (ITP–HPLC) in order to compare their practical advantages and disadvantages.

EXPERIMENTAL

Instrumentation

A CS Isotachophoretic Analyzer (VVZ PJT, Spišská Nová Ves, Czechoslovakia) was assembled in the column-coupling configuration of the separation unit[22,23] using modules provided by the manufacturer. The analytical column was provided with an on-column UVD1 photometric detector (VVZ PJT) and detection was carried out at

254 nm. The samples were injected with the aid of a 30-μl sampling valve. An HP 3390A reporting integrator (Hewlett-Packard, Avondale, PA, U.S.A.) was used for signal evaluation.

An isocratic liquid chromatographic system consisting of an HPP 4001 pump (Laboratorní přístroje, Prague, Czechioslovakia), a Valco sampling valve provided with a 30-μl sample loop (Valco, Houston, TX, U.S.A.) and an LCD 2563 UV–VIS detector (Laboratorní přístroje) set at 254 nm was used. The signal from the detector was registered by a TZ 4200 line recorder (Laboratorní přístroje) and was evaluated by a Minigrator (Spectra-Physics, Santa Clara, CA, U.S.A.). Compact glass columns (150 × 3.3 mm I.D.) (Tessek, Prague, Czechoslovakia) were laboratory packed with 10-μm irregular Silasorb-phenyl sorbent (Lachema, Brno, Czechoslovakia). The columns were kept at 25 ± 0.5°C as described[24]. Water acidified with formic acid to pH 3.3 and mixed with methanol (75:25) served as the mobile phase in the chromatographic experiments at a flow-rate of 0.5 ml/min.

Chemicals

Chemicals used for the preparation of the leading and terminating electrolyte solutions were obtained from Serva (Heidelberg, F.R.G.), Sigma (St. Louis, MO, U.S.A.) and Lachema. Some were purified by conventional methods. Discrete spacers for ITP analyses as proposed by Madajová *et al.*[25] were employed. Hydroxyethylcellulose 4000 obtained from Sigma was purified on a mixed-bed ion exchanger (Amberlite MB-1; BDH, Poole, U.K.).

Water delivered by a Rodem-1 two-stage demineralization unit (OPP, Tišnov, Czechoslovakia) was further purified by circulation through laboratory-made polytetrafluorethylene (PTFE) cartridges packed with Amberlite MB-1. Only freshly recirculated water was employed for the preparation of the mobile phase, ITP electrolyte solutions and sample solutions. Doubly glass-distilled methanol of analytical-reagent grade (Lachema) was used throughout.

Asulam and sulphanilamide were kindly provided by the Residue Laboratory of the Research Institute of Chemical Technology (Bratislava, Czechoslovakia). Soil samples were obtained from the Centre of Soil Fertility (Bratislava, Czechoslovakia).

HEMA-cart DEAE and Silica-cart C_{18} disposable minicolumns were obtained from Tessek.

Soil extraction procedures and preparation of the extract for analysis

Methanolic extraction. A 50-g amount of air-dried soil was shaken with 100 ml of methanol for 5 h. The extract was filtered through a dense filter-paper and then through a 0.45-μm PTFE membrane filter (Schleicher and Schüll, Dassel, F.R.G.). An aliquot of the filtrate was concentrated to one third of the original volume under a stream of nitrogen and the residue was made up to this volume with water before the analysis. Soil samples fortified with asulam were treated in the same way.

A 50-ml aliquot of the filtered extract was evaporated to dryness under a stream of nitrogen to determine the amount of extracted material. For the soil samples used in this work and extracted with methanol it was *ca.* $2.5 \cdot 10^{-3}$% of the amount of the sample.

Extraction with aqueous borate buffer. A 50-g amount of air-dried soil was shaken with 100 ml of 10^{-2} *M* aqueous disodium tetraborate solution (pH 9.05) for 90

min. The extract was acidified with formic acid to pH $\approx$ 4.5 (part of the colloidal material coagulated) and filtered as above. In this way 0.9% of the sample taken for the analysis was extracted into the aqueous solution.

The extract obtained with borate buffer was further cleaned up by using the following procedure[26] to protect the HPLC columns from losses of their performance characteristics: (i) 17 ml of the filtered extract were percolated through a HEMA-cart DEAE minicolumn (the sorbent bed was washed successively with 5-ml volumes of 10^{-2} *M* aqueous disodium tetraborate solution and water immediately before use); (ii) the sorbent was washed with 2 ml of water to remove the unretained part of the sample; (iii) the analyte was eluted with 6 ml of $5 \cdot 10^{-2}$ *M* sulphuric acid; (iv) asulam from the desorbate was retained on a Silica-cart C_{18} column (the sorbent bed was washed successively with 5-ml volumes of methanol and water before sample application); (v) the sorbent bed was washed with 2 ml of $5 \cdot 10^{-2}$ *M* sulphuric acid to remove unretained matrix constituents; and (vi) asulam was backflushed with 1.2 ml of water–methanol (2:1, v/v).

RESULTS AND DISCUSSION

ITP and HPLC conditions

The composition of the operational (electrolyte) system used for the ITP experiments is given in Table I. The pH of the leading electrolyte was chosen in such a way that the number of isotachophoretically migrating matrix constituents was suppressed while the analyte having pK_a = 4.82 migrated spaced by *n*-propyl succinate and butyrate (see Fig. 1) to achieve favourable conditions for its quantitation by the spike method[27]. A higher content of methanol in the terminating electrolyte was necessary to avoid losses of the injected samples containing methanol due to different densities of the stacked solutions (vertical alignment of the ITP separation compartment).

Under these ITP working conditions we could detect and determine with confidence 200 pg of asulam (see Fig. 1). The detection limit for a signal-to-noise ratio

TABLE I

OPERATIONAL SYSTEM EMPLOYED IN THE ITP ANALYSES

The driving currents were 150 and 30 μA for the pre-separation and analytical columns, respectively.

Parameter	*Electrolyte*	
	Leading	*Terminating*
Solvent	Water–methanol	Water–methanol
Proportions	90:10	75:25
Anion	Cl^-	*n*-Caproate
Concentration (m*M*)	5	5
Counter ion	BALA[a]	BALA[a]
pH	3.85	$\approx$ 4
Additive	HEC[a]	–
Concentration (%, w/v)	0.1	–

[a] BALA = β-Alanine; HEC = hydroxyethylcellulose.

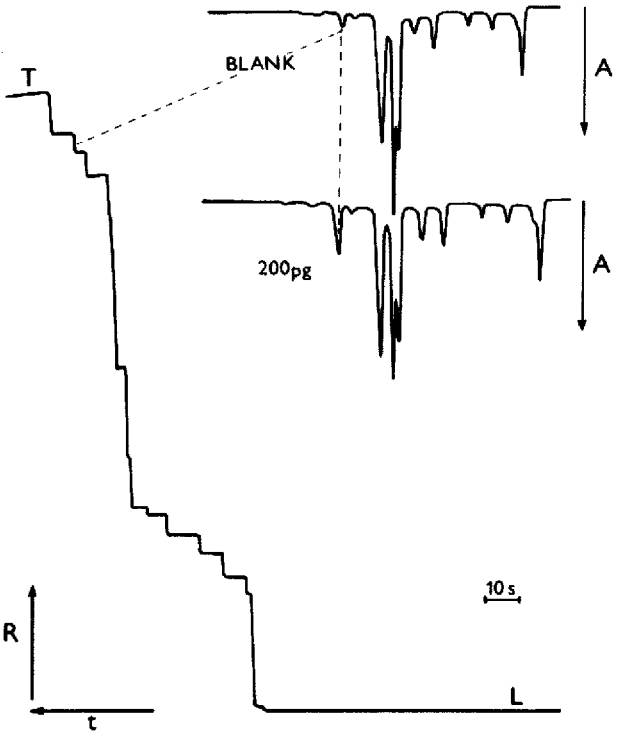

Fig. 1. Isotachopherograms from the analysis of 200 pg of asulam. In addition to the analyte, the injected sample solution contained 10^{-4} *M* sodium pyrophosphate (to eliminate losses of the analyte from adsorption on the surface of the sample handling devices) and a mixture of discrete spacing constituents (each at *ca.* 10^{-4} *M* concentration). In the blank run the same sample without the analyte was injected. The migration position of asulam was spaced by *n*-propyl succinate (a front spacing constituent in the direction of migration) and by butyrate (a rear spacer) as marked by dashed lines. The driving currents were 150 and 30 μA in the preparative and analytical columns, respectively. For the composition of the electrolyte system, see Table I. L and T = leading and terminating zones, respectively. *A*, *R* and *t* = increasing light absorption, resistance and time, respectively.

of 2 was *ca.* 75 pg (the noise was determined by run-to-run fluctuations of the peak area corresponding to the UV-absorbing impurities originating from the electrolyte solutions and migrating between the same spacing constituents as the analyte). This decrease in the detection limit in comparison with the previously estimated value[15] can be ascribed to the use of discrete spacers instead of a "continuous" spacing mixture. In this way we prevented undesirable dilution of the analyte by a carrier effect due to some of the constituents present in mixtures of synthetic ampholytes.

The choice of the mobile phase for the chromatographic separations was made by considering mainly the following requirements: (i) a compatibility of the mobile phase with the electrolyte solutions in ITP; (ii) a minimum peak volume of asulam at the column outlet; and (iii) reproducible retention time of asulam in the analysis of soil extracts. Of the alternatives tested[26], the best results were achieved with the mobile phase given under Experimental.

Under the chromatographic conditions employed, the detection limit for asulam

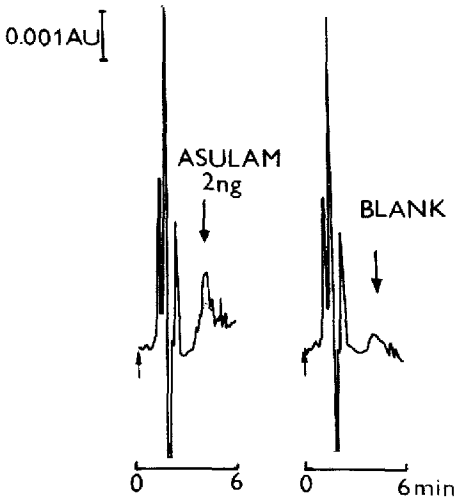

Fig. 2. Chromatograms from the determination of the detection limit of asulam. Asulam was present in a 10^{-2} *M* aqueous solution of sodium sulphate to prevent its losses by adsorption (the blank is a corresponding run with the solution of sodium sulphate). For the composition of the mobile phase and the other working conditions, see Experimental.

was *ca.* 2 ng for a signal-to-noise ratio of 2 (see Fig. 2). This value is considerably higher than that obtained under comparable conditions (the same injection volume, the same detection wavelength and close pH values of the solutions) by ITP. Such a difference may be surprising when a more favourable cell path length of the chromatographic detector is considered (*ca.* 40 times higher than for the ITP detection cell). However, when the dilution of the injected sample during the chromatographic process (*ca.* 15–20 times in this instance) and its concentration during the ITP separation (*ca.* 2000-fold concentration in this instance) are also considered, this difference in the detection limits is obvious. At the same time, these data suggest that here the sequence HPLC–ITP should lead to more favourable analytical results than the opposite order.

Sample clean-up by RP-HPLC with analytical evaluation by ITP

Soil extracts were chosen for this study as they represent matrices of high ionic complexity. Hence the analytical capabilities of the proposed off-line combination of the separation methods could be evaluated for an extreme type of application.

From the characterization of the extraction procedures (see Experimental), it is clear that the methanolic extracts contained very small amounts of the matrix constituents. Chromatograms obtained from their analyses gave the UV profiles shown in Fig. 3. A comparison of the blank run (an extract corresponding to 15 mg of soil sample was injected) with the run in which the extract was spiked with asulam shows that the analyte was eluted on a strongly tailing peak of co-extracted matrix constituents. Further attempts to optimize the chromatographic conditions did not lead to an improvement in the resolution of the analyte from the matrix constituents[26].

An isotachopherogram from the analysis of the same extract as in the blank chromatographic run (Fig. 3, right) shows (Fig. 4) that many of the UV-absorbing

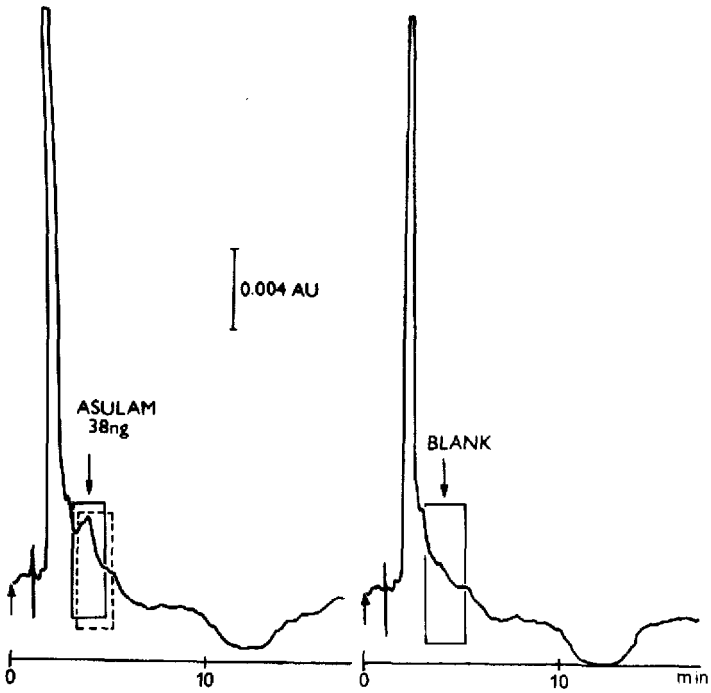

Fig. 3. Chromatograms from the analyses of a methanolic soil extract (right) and the extract spiked with asulam (left). Boxes on the recorder traces mark the collected fractions. For further details, see text.

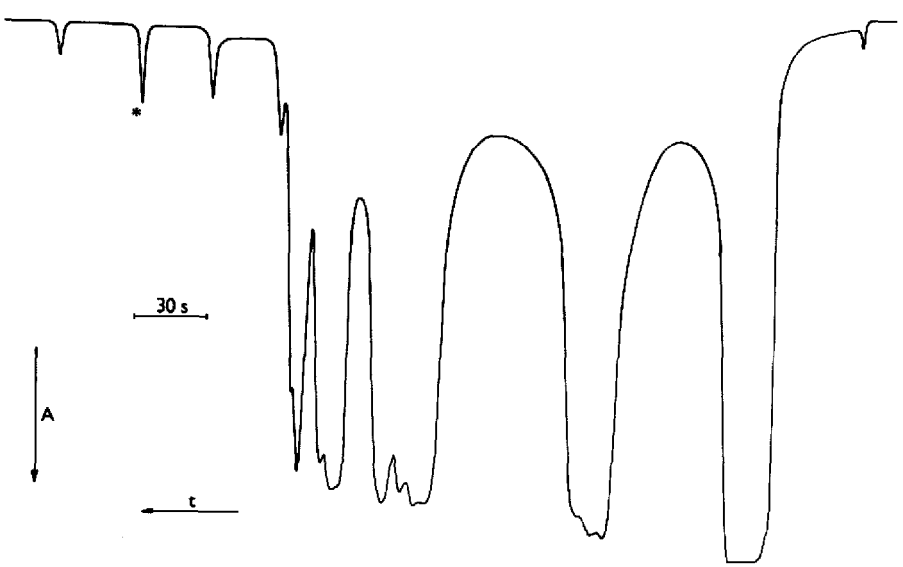

Fig. 4. Isotachopherogram from the analysis of a methanolic soil extract. The same sample as in the blank run in Fig. 3 was taken for the analysis. The same spacing constituents as in Fig. 1 were added to the sample. For the driving currents and the composition of the electrolyte system, see Table I. Symbols as in Fig. 1.

constituents present in this sample are relatively strong acids. Although only a very small part of the matrix constituents was present in the migration position of the analyte (marked with an asterisk in Fig. 4), their contribution to the positive systematic analytical error was equivalent to *ca.* 0.2 ppm of the analyte.

The isotachopherograms in Fig. 5 were obtained from analyses of the corresponding HPLC fractions of the same extract. The isotachopherogram at the top is a complete trace from the photometric detector as obtained from the analysis of the extract containing 0.25 ppm of the analyte (this amount could not be detected in HPLC). To illustrate the contributions of the electrolyte solutions and matrix constituents to the determination of the analyte, only the peaks in its migration position are shown in the box. These peaks clearly show that the impurities from the electrolyte solutions (subtractable from the results of the analyses) represented the main contribution to the positive systematic error in the quantification. Only a 3% contribution of the detected systematic error (equivalent to 0.008 ppm of the analyte) could be ascribed to the matrix constituents. The considerable improvement in this performance parameter in comparison with direct ITP analysis (see above) demon-

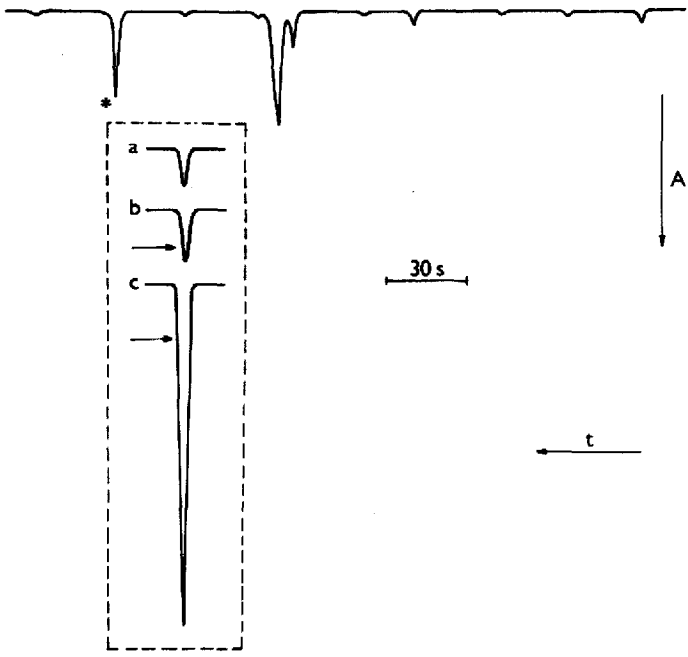

Fig. 5. Isotachopherograms from the analyses of a methanolic soil extract after HPLC clean-up. The same sample as in Figs. 3 and 4 and spiked with asulam at 0.25 ppm was taken for the analysis. The complete upper trace from the photometric detector was obtained in the analysis of fraction of the extract containing asulam (marked with an asterisk). (a) Peak at the migration position of asulam as obtained in a blank run (a mixture of discrete spacers was injected); (b) analysis of a fraction of the unspiked methanolic extract with added discrete spacers (the arrow indicates the peak height corresponding to the impurities in the electrolyte system); (c) as b except that the extract contained asulam (0.25 ppm) (the arrow indicates the peak height that corresponds to the total contribution of the electrolyte solutions and matrix constituents). Isotachopherograms a, b and c were registered with the detector set at a 5-fold higher sensitivity in comparison with the upper trace.

strates the powerful clean-up efficiency of HPLC. As in comparison with the situation shown in Fig. 4 there were almost no UV-absorbing constituents migrating in front of the analyte, this suggest that the separating conditions in both methods were dissimilar (non-correlated).

The sample fractions trapped by HPLC were three times wider relative to the baseline width of the asulam peak to guarantee its quantitative recovery also with fluctuations in the retention times of the separands. However, in this particular instance, it was the widest fraction that could be collected as only a 30-s shift of the start of the fraction collection to shorter retention times (solid-line box on the left-hand chromatogram in Fig. 3) led to a 5-fold increase in the systematic errors of quantitation (a systematic error equivalent to 0.04 ppm of asulam) due to the sample matrix. This is understandable when general requirements concerning multi-dimensional separations in the column-coupling systems are considered[28].

Interactions of pesticides with both inorganic and organic soil constituents are well known[29,30]. Therefore, in such instances more efficient extraction procedures are required. Extraction with borate buffer (see Experimental) belongs to this group of soil extraction procedures. Although in this work there were no reasons to expect problems due to the adsorption of asulam by soil constituents[31], we tested the proposed combination of the separation methods also for such an extreme matrix. From the data characterizing the extraction procedures employed (see Experimental) it is apparent that the borate extract contained a 360-fold excess of soil constituents relative to the methanolic extract. This excess can be ascribed mainly to the extraction of humic acids by the borate buffer solution. Humic acids extracted in this way have pK_a values in the range 1–6 with a maximum occurring close to $pK_a = 4$[32]. Therefore, when these constituents accompany asulam ($pK_a = 4.82$) in the extract, it is apparent that its trace determination by either HPLC or ITP alone would be very tedious.

A typical chromatogram from the analysis of a borate soil extract (pretreated by the SPE procedure as described under Experimental) is given in Fig. 6. From magnified part of the chromatograms at the position of the analyte circled in Fig. 6, it is apparent that its detection in the pretreated borate extract is cumbersome at trace concentrations. The same sample could be analysed by ITP under the working conditions employed only after an appropriate dilution, otherwise high column overloading occurred. In the analysis of the diluted extract, however, we obtained considerably lower systematic errors in the determination of asulam in comparison with the results reported previously[15]. This can be ascribed in part to the improved detection limit (see above) and also to the partial clean-up achieved on the disposable columns. On the other hand, by using HPLC for the isolation of fractions of interest a "clean" sample for the ITP analysis was easily obtained as in this way disturbing inorganic (*ca.* 10^5 excess relative to asulam) and organic constituents (*ca.* 10^6 excess relative to asulam, as estimated from the characterization of the extract) were removed to a substantial extent.

The isotachopherograms in Fig. 7 were obtained from ITP analyses of the borate soil extract after the HPLC clean-up. The complete isotachopherogram from the photometric detector, shown at the top, was obtained in the analysis of a soil sample fortified with the analyte at 12 ppb[a]. The contributions of the electrolyte solutions and

[a] Throughout this article the American billion (10^9) is meant.

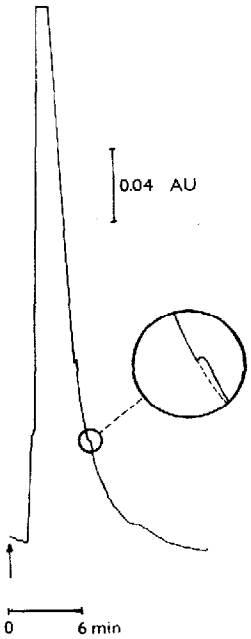

Fig. 6. Chromatogram from the analysis of a borate soil extract spiked with asulam at 0.25 ppm. The injected extract corresponded to 312.5 mg of soil and in the run with the spiked sample it contained 52 ng of asulam. The difference in the chromatograms for the spiked (solid line) and unspiked (dashed line) extracts is shown in the circle. For further details, see text.

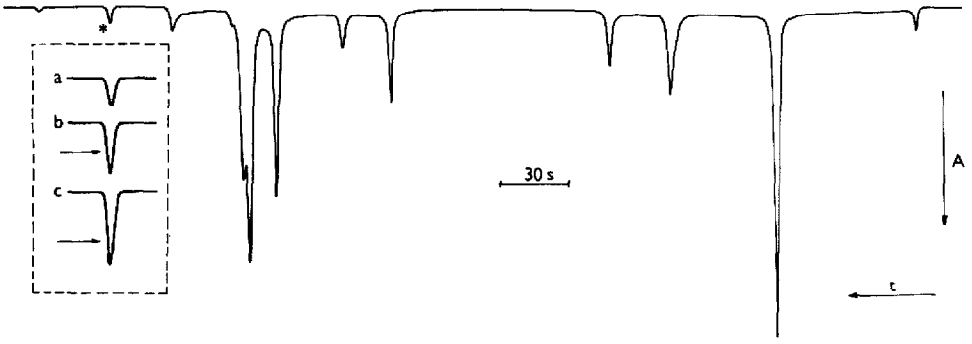

Fig. 7. Isotachopherograms from the analyses of borate soil extracts after HPLC clean-up. The injected parts of the fractions of soil extracts corresponded to 7.8 mg of soil and in the run with the spiked sample 95 pg of asulam was also present. The upper trace was obtained from the photometric detector in the analysis of the fraction containing 12 ppb of asulam (marked with an asterisk). (a) Peak at the migration position of the analyte in a blank run (only a mixture of the discrete spacing constituents was injected); (b) analysis of the fraction from the unspiked extract (the arrow indicates the peak height corresponding to the electrolyte impurities); (c) as b except that the extract was spiked with 12 ppb of asulam (the arrow indicates the peak height corresponding to the sum of comigrating impurities from the extract and electrolyte solution). Isotachopherograms a, b and c were registered at a 5-times higher amplification of the photometric detector in comparison with the upper trace.

the soil matrix to its determination are illustrated in the box. In this instance the matrix constituents migrating at the position of the analyte represented a positive systematic error of 12 ppb. This value is close to that achieved with the methanolic extract and clearly indicates that a very efficient sample clean-up was achieved by HPLC also in this instance. Thus, in this instance (12 ppb of the analyte), a *ca.* 100% systematic error was involved. When the complexity of the matrix is considered, in many instances this is a tolerable bias of the determination.

CONCLUSIONS

The results clearly show that RP-HPLC can be a very efficient sample preparation method in ITP analysis. In our experiments its use was straightforward as the sample clean-up was a key requirement and an inherent sample dilution during the chromatographic separation was compensated for in the final analytical step by the concentrating power of ITP. In this respect, the low detection limit achieved in the ITP analysis owing to the use of selective detection in the spike mode of quantification was also advantageous. However, our previous experience in the use of HPLC for sample preparation in ITP[33] indicated that in the evaluation of the analysis using a high-resolution universal detector the chromatographic dilution of the constituents of interest may require injection volumes as large as several millilitres. Although sample injection devices suitable for such volumes have been developed for ITP[23], the presence of ionogenic constituents in the HPLC mobile phase at concentrations as low as 10^{-3}–10^{-4} *M* may require unrealistic load capacities of the ITP columns. These facts indicate that when this sequence of the separation methods is intended for trace analytical work, the use of selective detectors in the ITP step may be essential.

As in this work a very simple configuration of the chromatographic instrument was satisfactory for the trace analysis experiments with matrices of extreme ionic complexity, it appears that the use of HPLC for sample preparation in ITP is an economic solution in general. In this respect, it should be stressed that by using the ITP equipment with the column-coupling configuration of the separation unit there are additional possibilities for further sample clean-up and/or for concentration of the analyte.

The time needed for a complete analysis (not including the soil extractions) was *ca.* 35 min or less. In comparison with previous procedures[15] this represents at least a 50% reduction in the analysis time. It appears that a short analysis time is a typical feature of the sequence HPLC–ITP because, *e.g.*, removal of inorganic salts from the samples can considerably reduce the time requirements for the ITP analysis.

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