Isotachophoretic analyses of compounds in complex matrixes : allergenic extracts and aluminium in biological fluids and bone

ISOTACHOPHORETIC ANALYSES OF COMPOUNDS IN COMPLEX MATRICES: ALLERGENIC EXTRACTS AND ALUMINIUM IN BIOLOGICAL FLUIDS AND BONE

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ABSTRACT

The aim of this publication is to show the great versatility of capillary isotachophoresis: (glyco)proteins versus small cations like aluminium.
For the separation of allergenic extracts the UV-patterns obtained, were highly reproducibile, which makes quality control and characterisation of these extracts possible. The determination of aluminium in bone (0.57 g) is possible, when a preconcentration step is used. No disturbing matrix were observed.

INTRODUCTION

In isotachophoresis [1,2,3] separation is obtained by differential displacement of a limited amount of separands by a suitable constituent, the terminator. In order to obtain a 'steady-state' some stringent requirements have to be met. These comprise the application of a discrete amount of sample at the interface of two different electrolytes: the leading and the terminating electrolyte. In its most simple form both the leading electrolyte and the terminating electrolyte contain only one ionic constituent of the same charge sign as the separands and a counter constituent to preserve electroneutrality. The effective mobility of the leading constituent should be higher than that of any of the separands, whereas the terminating constituent should have an effective mobility smaller than that of any of the separands. As soon as the steady state is reached all separands are arranged in contiguous zones, generally in order of their respective effective mobilities. Provided that the electric current density is constant, all zones will migrate at equal and constant velocity without further changes.

According to the Kohlrausch [4] regulation function concept and the moving boundary phenomenon, the concentration within each zone is strictly regulated and the zone boundaries have selfrestoring capabilities against e.g. convective disturbances.

Within a zone the separand concentration is constant and measurement of the zone-length, therefore, provides quantitative information. The determination of the concentration of the separand zones by thermometric, conductimetric, potential gradient, UV-absorption, fluorescence or radiometric means serves qualitative information.

In principal all kind of ionic separands: anions, cations, peptides and proteins [1], can be analysed even in complex matrices. In this paper two totally different examples will be discussed:

- the analysis of allergenic extracts;
- the analysis of aluminium ions (Al) in serum, bone and dialysis fluids.

The first example is chosen because mixtures of (glyco)proteins (e.g. allergenic extracts) can reproducibly be analysed with isotachophoresis [5]. Both for a more detailed characterisation of the various allergenic biomolecules and for the quality control and standardisation of allergenic extracts in physiological salt solutions studies are carried out in our laboratory. The second example is chosen because in established methods (atomic-absorption and -emission spectrometry), the matrix effects disturb the analytical result of aluminium. Also in this case, isotachophoresis offers interesting possibilities to analyse routinely biological fluids and tissue extracts.

EXPERIMENTAL

Equipment

For the profiling of allergenic extracts and the determination of aluminium the equipment built and developed by Everaerts et al. [1] was used. The separation compartment consisted of a PTFE tube (ca. 250 x 0.2 mm I.D.). The direct constant driving current was taken from a Brandenburg (Thornton Health U.K.) high-voltage power supply. The separated zones were detected by measuring the conductivity and UV-absorption at 280 nm.

Operational systems

The operational system used for profiling of the allergenic extracts is a high-pH anionic system (Table IA). In order to avoid interferences from carbonate, both the leading and terminating electrolyte were prepared under flushing nitrogen. The leading electrolyte was made by dissolving 115 mg of tris(hydroxymethyl)aminomethane (TRIS) in 50 ml of a 0.01 Molar HCl solution with 0.2% hydroxyethylcellulose (HEC). A 2% stock solution of HEC was treated with a Type V mixed-bed ion exchanger (Merck, Darmstadt, FRG). This was stored in a refrigerator after addition of HCl to a concentration of 0.01N. For the terminating electrolyte 22 mg of β-alanine and ca. 60 mg of $Ba(OH)_2$ were dissolved in 50 ml of water. The $Ba(OH)_2$ was used both to increase pH and to precipitate carbonate. The terminating electrolyte was filtered over a 0.45 µm luer-type disposable filter (Millipore, Bedford, USA).

The operational system used for the determination of aluminium is a cation system (Table IB). This system was developed for separating heavy metals, but proved also to be applicable for the determination of aluminium. α-Hydroxyisobutyric acid (α-HIBA) was used as a complexing agent and acetic acid was added to obtain the buffering capacity. The leading electrolyte was made by adjusting a 0.02 molar NaOH solution with HIBA until pH=5.2 and adding acetic acid until pH=4.2. To improve zonetransitions 0.05% PVA (polyvinyl alcohol) was added. The water used was taken from the Milli-Q water purification system (Millipore, Bedford, USA). The terminating electrolyte was a 0.005 molar acetic acid solution.

TABLE I Operational systems used for profiling of allergenic extracts (A) and the determination of aluminium (B).

		Leading electrolyte	Terminating electrolyte
System A	Anion	Chloride	β-alanine
	Concentration	0.01 M.	Ca. 0.01 M.
	pH	8.2	9 - 10
	Counterion	TRIS	$Ba(OH)_2$
	Additive	0,2% HEC	-
System B	Anion	Sodium	H^+
	Concentration	0.02 M.	Ca. 0.005 M.
	pH	4.2*	ca. 2.3
	Counterion	Acetate, α-HIBA	Acetate
	Additives	0.05% PVA	-

*- α-HIBA was added to a 0.02 M NaOH solution until pH=5.2 and the acetate was adjusted until pH=4.2. The abbreviations are explained in the text. All chemicals were of analytical grade quality, with exception of PVA (Mowiol 8-8B, Hoechst, Frankfurt, FRG) and HEC (CAT 5568, Polyscience, Warrington USA)

RESULTS

Allergenic extracts

In Fig. 1 an isotachophoretic analysis of an extract of Dactylus glomerata pollens is shown. 10 mg of dry frozen pollen extract (obtained from HAL

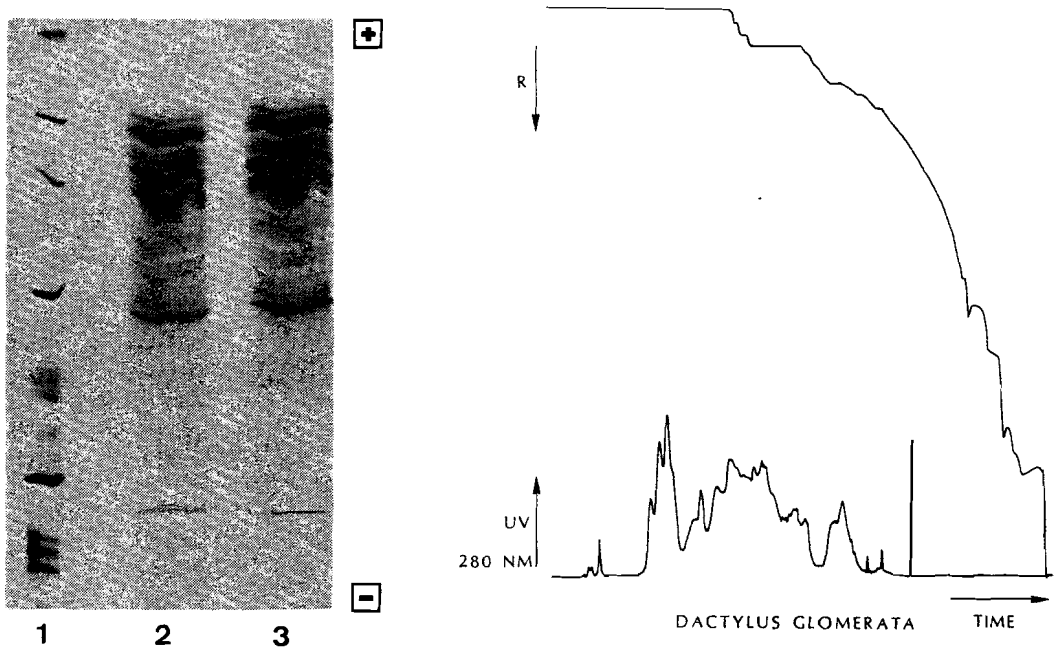

Fig. 1 IEF (lefthand side) and ITP analyses of an extract from pollens of a grass (Dactylus glomerata), which often causes allergic phenomena ("hay-fever"). For the ITP analysis both the conductivity trace (R) and the UV trace are shown. Experimental conditions are given in text. The IEF analysis was performed on agarose (10 mg/ml and 0.5 mm thickness). The pH gradient was ca. pH= 3,5 - 9 and had a length of 104 mm. 1= pH marking proteins IEF G85034; 2= the allergen 30 mg/ml; injected was 25 µl= Dg; 3= the isotonic allergen solution 10 mg/ml dialysed against water; injected was 25 µl=DgD. The authors thank G.T. Hoek (HAL, Haarlem, The Netherlands) for the IEF analyses.

Allergen Laboratories, Haarlem, The Netherlands) were dissolved in 1 ml deionised water. From this solution 1 µl was analysed (operational system, Table 1A) together with 1 µl ampholyte mixture Bio-lyte 3/10, 250 times diluted (Bio-Rad Laboratories, Richmond, USA).

These compounds serve as spacers and carriers for the (glyco)proteins [1, pp 330]. Both the universal conductimetric trace (R) and the specific UV trace (280 nm) were recorded. While the UV trace can be used for "finger-printing", the conductimetric trace is mainly used to correct for deviations in zonelength, due to the injection procedure. This correction is carried out with an Apple IIe microcomputer.

In the study of profiling commercially available allergenic material extracts from the "Haarlems Allergenen Laboratorium" (HAL, Haarlem, The Netherlands) have been used. The allergenic extracts contain isotonic phosphate buffered-saline (PBS) solution. This solution is stabilised with phenol and ε-amino caproic acid. To lower the ionic strength of the samples the solutions were dialysed against double-deionised water with a dialysing membrane (cut off ca 10,000 Dalton). Sample amounts of 200 µl were dialysed in a tube of 5 mm I.D. bent into a U-form and fixed by a clamp. In a 4 liter vessel ten samples could be dialysed simultaneously, whilst the dialysis liquid was stirred permanently.

To determine the time needed for dialysis, each hour an isotachophoretic separation was carried out with a aliquot from the solutions under dialysis. Because all ionic solutes are transported during the separation, the total time for analysis (i.e. the total time needed for the terminator to reach the detector) gives an indication of the ionic strength of the solution under dialysis.

Fig. 2 shows that 4 hours were sufficient for reproducible analyses. It was verified that during this time no degradation of the allergenic (glyco)-proteins occurred (Data not shown).

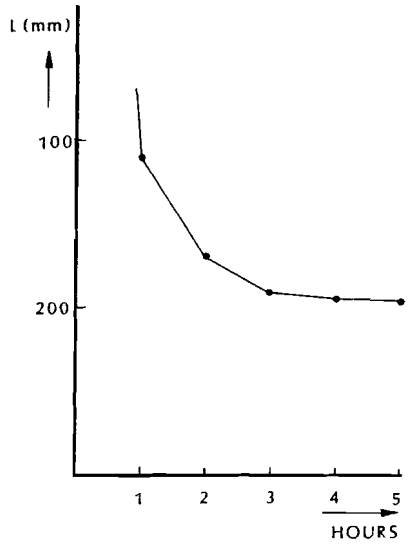

Fig. 2 The determination of the time needed for dialysis in order to reduce low molar weight compounds present in commercial allergenic extracts. This figure shows that after 4 hours, sufficiently low levels were reached. L= the total zonelengths between leading electrolyte and terminating electrolyte.

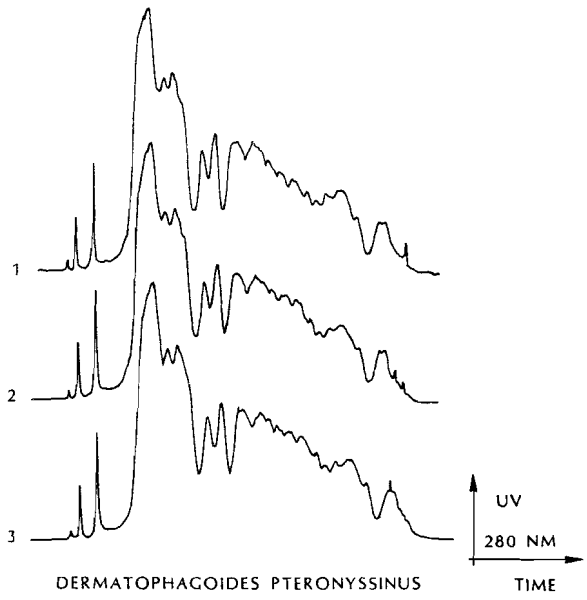

Fig. 3 Analyses of extracts from the house dust mite (Dermatophagoides pteronyssinus). The upper profile shows an extract prepared at +60°C. The trace no. 2 (middle) shows the profile obtained with an extract prepared at -70°C. Profile 3 is a run after duplicating the procedure of no. 2, showing the reproducibility of this method.

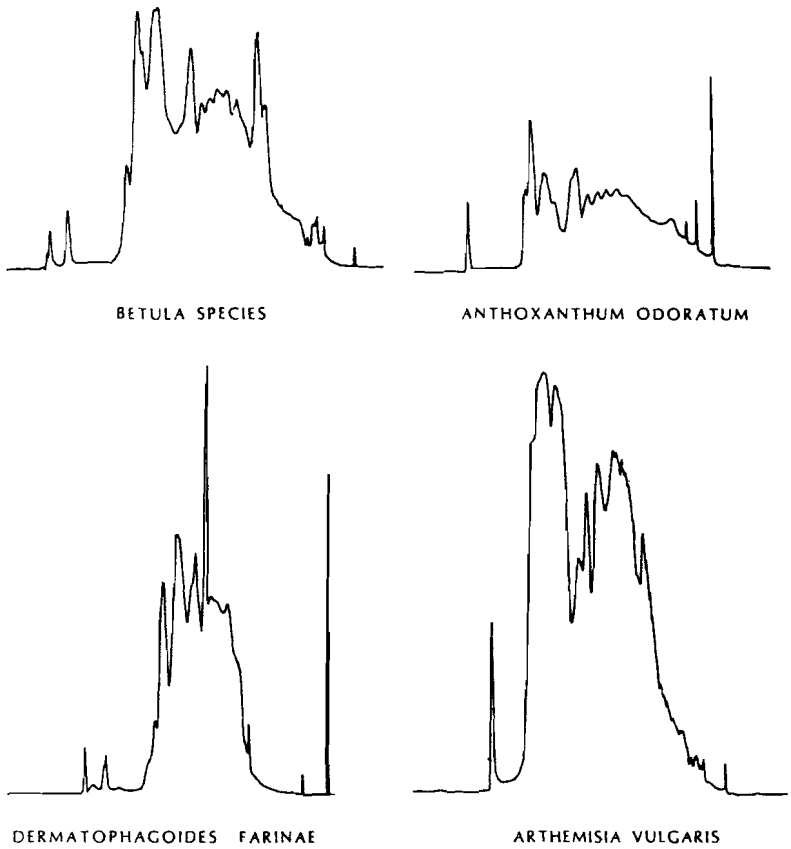

Fig. 4 UV profiles of four different allergenic extracts. The source material obtained from a tree (Birche = Betula), from two grasses (Anthoxanthum and Arthemisia) and from a mite often encountered in house dust (D. pharinae).

Three individual isotachophoretic UV profiles of extracts from the house dust mite (Dermatophagoides pteronyssinus) are shown in Fig. 3. The house dust mite is the major allergenic constituent in house dust, causing allergic symptoms. The upper profile (no. 1) represents an extract which was prepared by extracting a house dust mite culture at +60°C. Trace no. 2 shows the profile of an extract prepared at -70°C.

Profile no. 3 shows a duplicate run with the same extract after a duplicate dialysis procedure as in profile no. 2, in order to illustrate the reproducibility of the technique.

From the profiles shown in Fig. 3 it can be concluded that as far as the isotachophoretic patterns are concerned no differences seem to exist between house dust mite extracts prepared at +60°C and at -70°C.
Fig. 4 shows a number of analyses with extracts from allergens, which frequently cause allergic rhinitis, conjunctivitis and/or asthma.
birche pollens (Betula sp) and the two grasses Anthoxanthemum and Arthemisia show characteristic profiles. The mite Dermatophagoides pharinae, which is also often seen in housedust, gives a pattern which is completely different from that observed in fig. 3, where the mite Dermatophagoides pteronyssinus was analysed.

Determination of aluminium in biological samples

The aluminium sample pretreatment was performed with a cation exchange resin Chelex-100 (Bio-Rad Labs, Richmond, Ca USA), which has a high affinity for aluminium and low for sodium. The procedure was as follows: The samples were mineralised with 1N nitric acid and dried under vacuum at 60°C. The residue was dissolved in double-deionised water. This solution was poored on the Chelex-100 in the acid form. After rinsing with 0,01M nitric acid in order to remove the excess of Na^+ and Ca^{++} the aluminium was eluted with 1N nitric acid. The eluent was evaporated to dryness under vacuum at 60°C the residue is dissolved in 0.005 Molar acetic acid applied as terminator and injected. The whole pretreatment period lasted about 30 min.

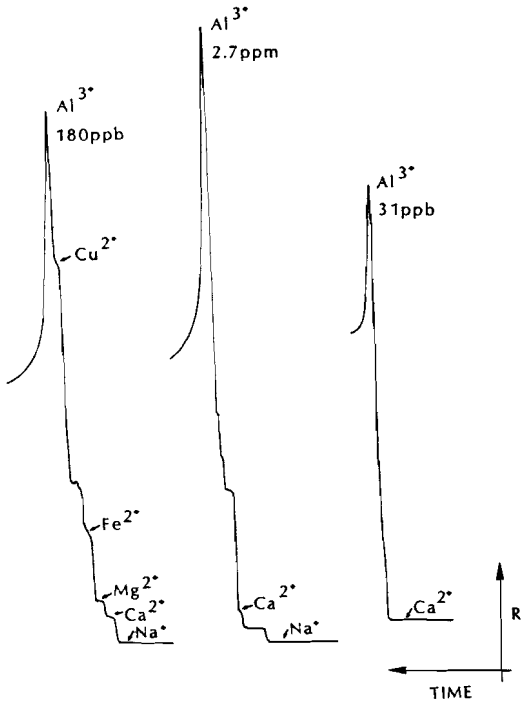

Fig. 5 Isotachophoretic analysis of Al^{3+} in human serum (left hand side), human bone (middle) and fluid (right hand side).

In Fig. 5 the analyses obtained with three different biological samples are shown. The left-hand figure represents the isotachophoretic analyses of serum from a kidney-patient, who had undergone blood dialysis. It can be seen that a number of metal ions are present. Apart from aluminium, all the ionic species shown are also present in normal blood serum. In this sample the aluminium concentration was 180 ppb (6.7 µM).

The profile in the middle of Fig. 5 shows the analysis of a bone extract from a kidney-patient who was dialysed regularly. The bone was taken from the patient after his death. Again here it is evident that aluminium is present in significant amounts. This is not the case in normal human bone.
The right-hand profile of fig. 5 shows the "blank" dialysis fluid from an artificial kidney apparatus. It is evident that a considerable amount of aluminium is present in this fluid. This latter finding, of course, explains the occurrence of aluminium in serum and bone of dialysed patients.

CONCLUSIONS

This study confirms that the isotachophoresis technique is very suitable for the routine profiling of relatively complex (glyco)-protein mixtures. This seems especially valuable in the case of the characterisation of allergenic extracts. This approach enables the direct on-line analysis of the profiling data with the aid of a computersystem, thus making this way of analysis attractive for routine determinations in quality control studies. Studies concerning the latter point are in progress in our laboratory.

To our knowledge, isotachophoretic determinations of aluminium in human bone have not been reported before. We have shown here that it is possible to measure very low levels of aluminium. Although other techniques might be more sensitive, it should be pointed out here that with the isotachophoretic system much less problems concerning disturbing effects of matrices are observed. Therefore, for a rapid routine analysis not only of Aluminium but also of a number of other metals, isotachophoresis seems to be a promising method. Further studies to optimise this system are in investigation.

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