*Journal of Chromatography*, 256 (1983) 126–134
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,401

## Note

# Discontinuous fractionation technique for capillary isotachophoresis

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(Received September 27th, 1982)

When a complex analytical problem has to be solved, *e.g.*, the identification of ionic constituents present in a multi-component sample of unknown qualitative composition, capillary isotachophoresis (ITP) is usually not used alone. Several other separation and/or identification techniques are combined with ITP to achieve the desired result (see, *e.g.*, ref. 1). Such a combination of analytical methods stimulated the development of preparative techniques suitable for the micro-scale fractionation of ionic components after their ITP separation.

A continuous fractionation technique suitable for ITP was developed by Arlinger[2], in which the constituents separated by ITP are collected on a cellulose acetate strip. For some analytical methods that follow ITP (*e.g.*, immunoelectrophoresis, zymograms, some types of radioactivity measurements) the use of strips is advantageous[3–5]. However, for other analytical techniques also compatible with ITP[high-performance liquid chromatography (HPLC), gas chromatography (GC), mass spectrometry (MS), GC–MS, analytical ITP] a need to cut out the strips and elute the components of interest is a disadvantage.

A different approach to the isolation of separated constituents was proposed by Kobayashi *et al.*[6]. In this instance the constituents of further analytical interest are withdrawn with a microsyringe and can be immediately introduced into a sampling device of the analyser used for the subsequent analysis. For some techniques (GC, GC–MS), however, the fraction must usually be derivatized before the analysis. On the other hand, the resolution of the withdrawn components is lost, which can be a disadvantage in certain applications (*e.g.*, immunoelectrophoresis and related electrophoretic techniques). The possibilities of the complete fractionation of the mixture separated in one run are not clear from the performance description given[6].

In this laboratory a simple technique for the discontinuous fractionation of the components separated by ITP is currently being used. This paper describes the arrangement of the separation unit adapted for this mode of ITP and considers its performance characteristics for a single fraction trapping and for a complete fractionation of the separated mixture.

0021-9673/83/0000 0000/$03.00 

## EXPERIMENTAL

The separation unit adapted for preparative ITP working in the discontinuous mode is shown schematically in Fig. 1. The only difference from conventional ITP instrumentation[7] is the fractionation valve between the counter-electrode compartment and the detector. To achieve a higher load capacity (higher isolated amounts of material), a fluorinated ethylene–propylene copolymer (FEP) capillary tube of 0.85 mm I.D. is used in this separation unit.

The fractionation valve is shown in detail in Fig. 2. The positions of the plunger of the valve during the separation, trapping phase and for refilling of the trapping channel are also shown.

## RESULTS AND DISCUSSION

### *Determination of the recovery of fractionation*

In general, the discontinuous fractionation technique described in this work can be used for two purposes: (1) a component of further analytical interest is isolated

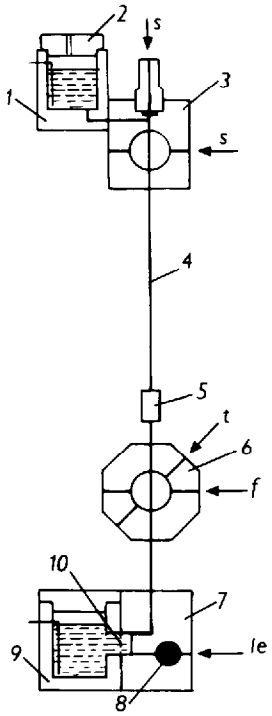


Fig. 1. Separation unit for micropreparative work in the discontinuous mode. 1 = Terminating electrolyte reservoir; 2 = cap of the reservoir; 3 = sampling block; 4 = capillary tube; 5 = conductivity detector; 6 = fractionation valve; 7 = block for the filling of the capillary tube with the solution of the leading electrolyte; 8 = needle valve; 9 = counter-electrode compartment; 10 = membrane; s = positions for the sample introduction (microsyringe or valve); t = trapping position; f = refilling position; 1e = place for the filling of the capillary tube.

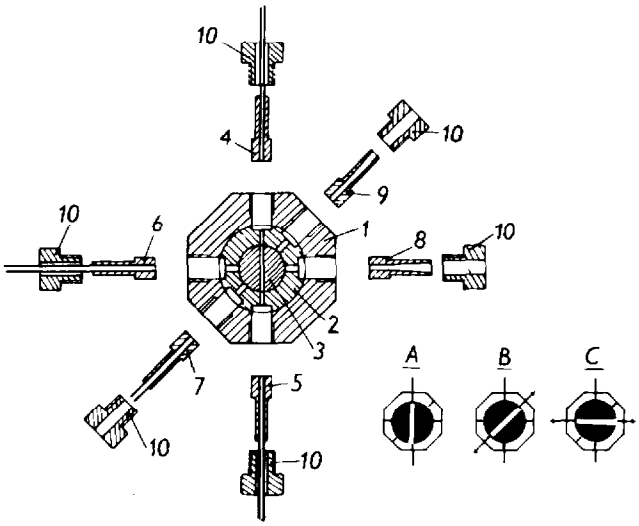


Fig. 2. Fractionation valve for the discontinuous isolation of the separated constituents. 1 = Acrylic housing of the valve; 2 = PTFE static part of the valve; 3 = PTFE plunger with trapping channel; 4, 5, 6, 7 = pieces for clamping the capillary tubes to the body of the valve; 8, 9 = pieces for fixing the syringes used for the washing (7) and refilling (8) of the channel in the plunger (3). A = Position of the plunger during the separation; B = trapping of the desired constituent(s); C = position for the refilling of the channel with the solution of the leading electrolyte.

from the accompanying components representing a more or less complicated matrix (only one fraction is isolated in one run); (2) the complete mixture is separated into a finite number of fractions, which are subjected to further analysis.

In both instances the recovery of the isolation procedure is of a prime analytical importance, especially when the isolation is intended as a step prior to further quantitative evaluation. In the latter instance, moreover, cross-contamination of the fractions must be prevented and the overall reproducibility of the runs used for

TABLE I

OPERATIONAL SYSTEMS

BALA = β-alanine; HIS = histidine; BTP = 1,3-bis[tris(hydroxymethyl)methylamino]propane; HEC = hydroxyethylcellulose; CAPR = caproic acid.

| *Parameter* | *System No.* | | | | |
|---|---|---|---|---|---|
| | *1* | *2* | *3* | *4* | *5* |
| Leading anion | $Cl^-$ | $Cl^-$ | $Cl^-$ | $Cl^-$ | $Cl^-$ |
| Concentration (m*M*) | 10 | 2 | 10 | 2 | 10 |
| Counter ion | HIS | HIS | BALA | BALA | BTP |
| Additive to the leading electrolyte | HEC | HEC | HEC | HEC | HEC |
| Concentration (%) | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| pH of leading electrolyte | 6.0 | 6.0 | 3.2 | 3.2 | 6.0 |
| Terminating anion | CAPR | CAPR | CAPR | CAPR | CAPR |

TABLE II

EVALUATION OF RECOVERY OF MALEIC ACID

| *No.** | *Amount injected*** (ng)* | *Measured zone length*** (mm)* | *No. of parallel determinations* | *Expected zone length**** (mm)* |
|---|---|---|---|---|
| 1 | 25 | 3.5 | 4 | 4.3 |
| 2 | 25 | 4.6 | 3 | 4.3 |
| 3 | 25 | 4.2 | 3 | 4.3 |
| 4 | 25 | 4.4 | 3 | 4.3 |
| 5 | 50 | 8.6 | 3 | 8.5 |
| 6 | 50 | 9.1 | 2 | 8.5 |
| 7 | 50 | 8.7 | 2 | 8.5 |
| 8 | 75 | 11.1 | 2 | 12.5 |
| 9 | 75 | 12.3 | 2 | 12.5 |
| 10 | 75 | 12.0 | 2 | 12.5 |
| 11 | 75 | 13.0 | 2 | 12.5 |
| 12 | 100 | 16.8 | 2 | 18.0 |
| 13 | 100 | 16.7 | 2 | 18.0 |

* Fractionation run.

** One tenth of the amount injected into the micropreparative instrument and trapped by the valve was analysed; zone length as measured on the isotachopherograms obtained by a conductivity detector at the chart speed of 1 mm/sec (the values given are averages for several parallel determinations on the same fraction).

*** The amount representing 100% recovery under the conditions of recovery evaluation.

repeated fraction collections must be acceptable high, otherwise the reliability of conclusions drawn from the analysis of the fractions could be doubtful.

The recovery of the fractionation technique was evaluated for a single constituent trapping. To investigate the importance of some of the factors that contribute to the recovery values, two series of experiments were performed. In the first a mixture (maleic acid, 50 ng/$\mu$l; formic acid, 46 ng/$\mu$l; acetic acid, 60 ng/$\mu$l) was injected in volumes of 5, 10, 15 and 20 $\mu$l. After the separation (system No. 1, Table I) maleic acid was trapped as described above. A 50-$\mu$l volume of doubly distilled water was sufficient to wash out the contents of the trapping channel quantitatively.

To evaluate the recovery, an aliquot of the trapped maleic acid was analysed by ITP (0.3 mm I.D. capillary tube; system No. 2, Table I). The amounts representing 100% recoveries were determined under identical conditions. The results of the determination of the recovery of maleic acid are summarized in Table II.

In the second series of the recovery experiments, hydrophobic cinnamic acid was separated at a low pH (system No. 3, Table I). Recoveries not exceeding 50% were obtained when a 100-$\mu$l volume of water was used for the washing of the trapping channel in the fractionating position. Part of the material was found in the following fraction and the rest of the cinnamic acid migrated out of the valve. Using the same volumes of 10 m*M* Tris in water, recoveries close to 95% were found by ITP and by UV spectrometry (for details, see Fig. 3). Undoubtedly, adsorption of cinnamic acid on the walls of the trapping system (a high surface to volume ratio) explains the low recoveries when water was used.

The above experiments were mainly concerned with a single component isolation. Obviously, accompanying components trapped in the fraction (3-$\mu$l volume of

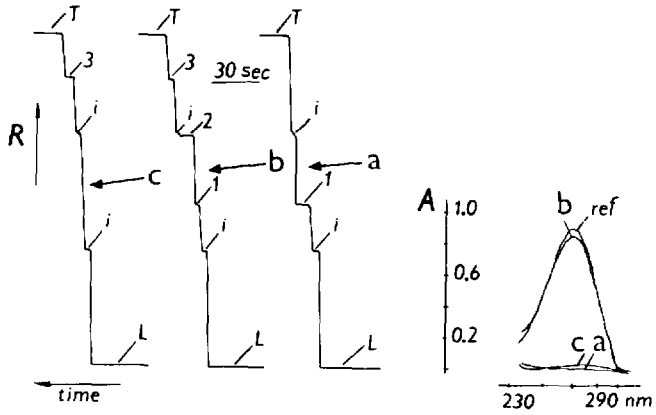


Fig. 3. ITP and UV spectrometric evaluations of the isolation of cinnamic acid. A 20-$\mu$l volume of a mixture (cinnamic acid, 40 ng/$\mu$l; succinic acid, 60 ng/$\mu$l; propionic acid, 35 ng/$\mu$l) was separated at pH 3.2 with subsequent fractionation. No additives were used in the solution of the leading electrolyte in the fractionation runs (the capillary tube was thoroughly washed with an aqueous solution of HEC before the analysis). UV spectra of the fractions were measured in a 100-$\mu$l cell with a 1-cm pathlength. Aliquots of the fractions were then analysed by ITP (0.3 mm I.D. capillary tube; system No. 4, Table I) using a conductivity detector. a,b,c = Identification of the trapped fractions; $A$ = increasing absorbance; $R$ = increasing resistance; ref = amount of cinnamic acid representing 100% recovery. 1 = Succinate; 2 = cinnamate; 3 = propionate; L = chloride; T = capronate.

the trapping channel) must not interfere in the subsequent analytical steps, otherwise further purification is required. In general, the demands on the amount and purity of the isolated material are determined by the analytical methods used after preparative ITP.

UV spectrometry (Fig. 3) is a method compatible with preparative ITP performed on the micro-scale and, in some instances, is applicable to the identification of the isolated constituents. In this respect, however, an on-line technique for the measurement of the UV spectra is a more satisfactory alternative[8,9]. Nevertheless, there are a variety of analytical methods for which an off-line combination of a particular method with ITP is sufficiently flexible. An off-line combination of ITP with mass spectrometry can serve as an example[10].

*Complete fractionation of mixtures*

The complete fractionation of a mixture separated by ITP in one run can also be of analytical interest, *e.g.*, when identification of several unknown components is required.

An analytical scheme describing the identification of ionic constituents when only ITP is used is exemplified as follows:

(1) Analysis in several operational systems is carried out in a conventional ITP instrument.

(2) Separation with a subsequent fractionation of the separated constituents is performed in a suitable operational system.

(3) The fractions are evaluated by analytical ITP in the same operational system in which the fractionation experiments were performed.

(4) The fractions are analysed in the operational systems used in the first stage.

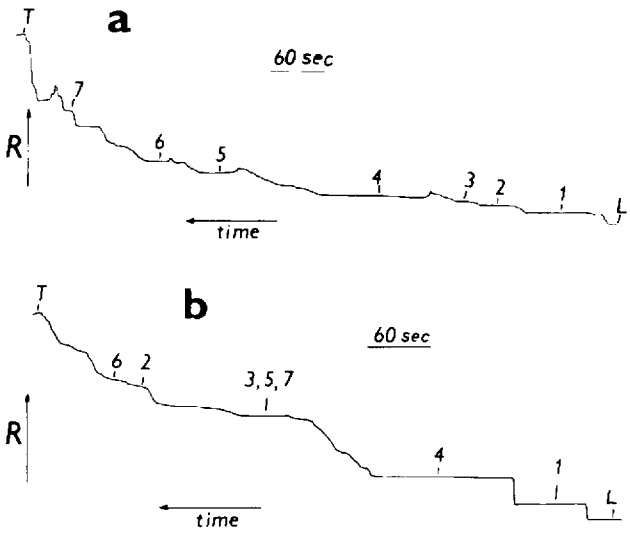


Fig. 4. Isotachopherograms from the analysis of the acids present in cooking liquor after the delignification of wood. An instrument with coupled columns[12] was used (only responses of the conductivity detector in the analytical column are given). (a) Separation performed in system No. 3 (Table I); driving current in the analytical column, 35 $\mu$A; a 1.5-$\mu$l volume of undiluted cooking liquor[11] was injected. (b) Analysis of the same sample as in (a) performed in system No. 1 (Table I); driving current, 45 $\mu$A. $R$ = increasing resistance. 1 = Oxalate; 2 = dichloroacetate; 3 = monochloroacetate; 4 = formate; 5 = hydroxyacetate; 6 = lactate; 7 = acetate; L = chloride; T = capronate.

(5) Isotachopherograms obtained in the first stage are interpreted from the results obtained.

(6) Analyses of the sample and/or the fractions may be carried out in order to increase the certainty of identification.

Identification of the acids present in a cooking liquor after the delignification of wood performed by ITP illustrates the capability of the fractionation technique for this type of application. Isotachopherograms for the separation of the acids present in the cooking liquor are given in Fig. 4. When these isotachopherograms were compared with those obtained for the expected acids[11] (parallel runs and/or spiking), only a few of the acids could be unambigously excluded from the further analysis. A high degree of uncertainty remained for identification of the other acids because of both the qualitative and the quantitative complexity of the sample.

Therefore, in accordance with the above scheme, fractionation of the sample was carried out in system No. 1 (Table I). Five runs with a subsequent fractionation were performed to obtain amounts of material sufficient for further analyses (identical fractions from these runs were collected in the same vessels). Analytical evaluation of the fractions was performed in a conventional ITP instrument[7] and the operational system used for the fractionation was employed.

Analysis of fractions in different operational systems (differentiation of the constituents according to p$K$ values, ionic mobilities, charges) combined with parallel runs in which the expected acids were analysed provided the possibility of identifying some constituents. Finally, the proposed identity of the acids was confirmed in runs

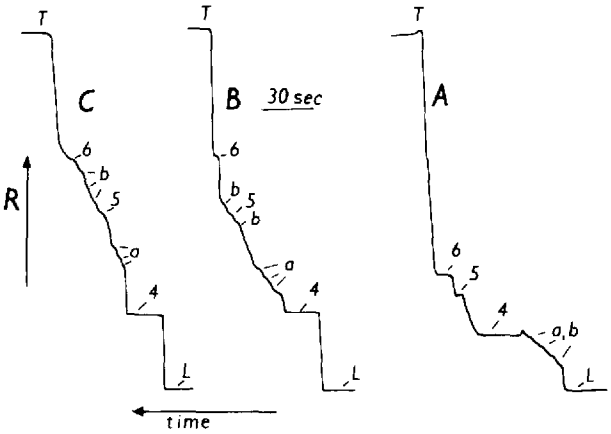


Fig. 5. ITP evaluation of fraction No. 3 in different operational systems. A,B,C = Unidentified constituents. Other symbols as in Fig. 4.

with spiked fractions. In this way we were able to identify the zones on the isotachopherograms in Fig. 4. In spite of the wide range of the acids used in the investigation (40 low-molecular-weight acids are reported to be present in samples of such an origin[11]), a number of the constituents could not be identified. The data obtained, however, may be helpful in a search for other reference compounds.

Isotachopherograms for the analysis of fraction No. 3 given in Fig. 5 indicate that unidentified groups of constituents (a,b) are stronger acids than formic acid (Fig. 5A) and at pH 6.0 behave as divalent anions (Fig. 5B and C; for details, see ref. 13). From these results and from the sample prehistory[11], low-molecular-weight hydroxy and chloro derivatives of dicarboxylic acids represent a group that should be taken into consideration. Similar conclusions could be drawn from the analyses of the remainder of the fractions[11].

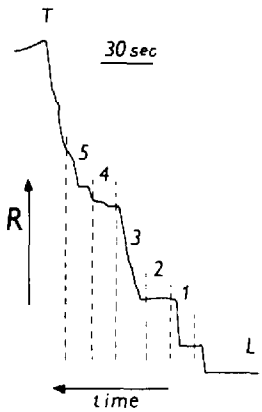


Fig. 6. Isotachopherogram from the micropreparative run on the same sample as in Fig. 4. The instrument shown in Fig. 1 was used (0.85 mm I.D. capillary tube). Vertical dashed lines indicate the boundaries of the collected fractions (1–5 are the numbers of the fractions). The separation was carried out in system No. 1 (Table I); driving current, 250 $\mu$A. Symbols as in Fig. 4.

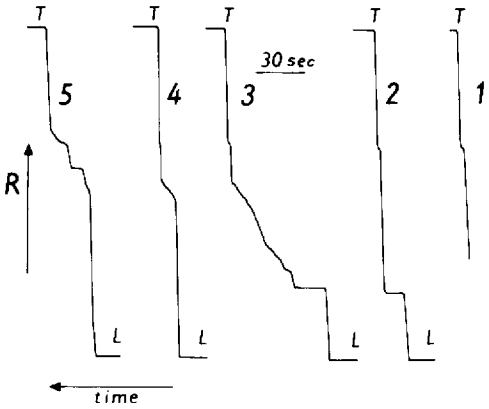


Fig. 7. ITP evaluation of the fractions collected in five repeated runs. Analysis was carried out in a conventional instrument[7] provided with a 0.3 mm I.D. capillary tube. The same volumes of the trapped material were dosed (only for fraction No. 3 was this volume three times higher). Symbols as in Fig. 6.

It is clear from the above that this fractionation technique is working in a discontinuous mode, *i.e.*, the driving current is switched off during the fraction collection and refilling of the trapping channel (positions B and C in Fig. 2). Although for a single-fraction trapping possible disturbances of the zone boundaries outside the trapping channel due mainly to diffusion are of no interest (the experiment is finished after the fraction is trapped), they can be disadvantageous when a complete fractionation of the mixture is carried out.

Experiments with mixtures containing coloured substances showed that switching-off of the driving current leads to a slow zone-mixing effect. This effect was eliminated within 10–15 sec after the current was switched on again. Obviously, the time for which the current is interrupted should be as short as possible (in our work the time needed for a manual manipulation during the fractionation was less than 20 sec). The time interval during which a new part of the separated mixture refilled the trapping channel was 10–15 sec in the above experiments, *i.e.*, close to that needed for resharpening of the zone boundaries. Further, in the experiments with coloured substances no visible convective disturbances were observed when the plunger of the valve was turned into different positions (see Fig. 2).

When several fractionation runs are necessary in order to collect a sufficient amount of the material, a high reproducibility of the fractionation procedure is essential. The isotachopherograms in Figs. 6 and 7 undoubtedly indicate that this characteristic of the fractionation technique used is good.

Column-coupling techniques[12] also used in this work (see Fig. 4) give similar possibilities to a combination of preparative and single column analytical ITP. The need to carry out the pre-separation run in each experiment, however, leads to an undesirable increase in the number of experiments. Moreover, a reversed migration configuration of ionic constituents[1,14,15] for some of the operational systems applied in the pre-separation and analytical columns can lead to increased demands on the load capacity of the analytical column. On the other hand, after the identification phase has been completed and repeated analyses need to be performed, the use of the instrument with coupled columns is by far the most suitable alternative when complex mixtures are involved.

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