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Electrophoresis, isotachophoresis, isoelectric focusing

This issue of TrAC highlights electrophoresis, isotachophoresis and isoelectric focusing, all of which separate charged species under the influence of an electric field. Like chromatographic systems they can be used for the separation of both high and low molecular weight compounds. They do, however, possess a number of advantages; particularly significant is their requirement for only minimal sample pretreatment.

Recent years have seen several important advances, and these are covered in this issue of TrAC. High resolution 2-dimensional electrophoresis has made the analysis of complex protein mixtures a practical possibility, rendering it a potent research tool in many areas of biological and biomedical research. Isoelectric focusing now has not only remarkable resolving power, but also a greatly increased protein loading capacity on the preparative scale. Advances in isotachophoresis equipment and detection systems, combined with microprocessors for instrument handling and data processing have contributed to the great flexibility, reproducibility, accuracy and extremely low running costs of this technique.

In the following articles the growing versatility of electrophoresis, isotachophoresis and isoelectric focusing will be amply demonstrated.

Peter T. Shepherd

New directions in isotachophoresis

The great flexibility, reproducibility, accuracy and extremely low running costs of isotachophoresis make it an attractive alternative to HPLC for a number of applications. Recent developments in isotachophoresis equipment, detection systems and the advent of the microprocessor have enhanced the technique's capabilities.

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The development of analytical isotachophoresis (ITP) in narrow-bore tubes began in 1964 at the Eindhoven University of Technology with the work of A. J. P. Martin and F. M. Everaerts. Both anions and cations (as well as amphoteric species) can be separated by ITP, and the system chosen depends on the nature of the sample constituents to be separated (separands). A schematic diagram of an ITP apparatus is shown in Fig. 1. For the separation of ions, the separation compartment and the anode compartment are filled with the ‘leading electrolyte’ (L). This electrolyte consists of an anion with a high effective mobility (velocity per unit of field strength) and a counter ion with low effective mobility, and buffering capacity. The cathode compartment is filled with the ‘terminating electrolyte’ (T), which contains an anion with a low effective mobility. The counter ion is of minor importance since it will not enter the separation compartment. The sample is introduced into the injection compartment at the boundary between the leading electrolyte and the terminating electrolyte (Fig. 2). If the sample load is properly chosen with respect to the separation capacity of the separation compartment, all separands X₁ fitting into the mobility frame mₑ₁ < L > mₑ₁, X₁ > mₑ₁, T will finally migrate with constant velocity.
Fig. 1. (left) Schematic diagram of an ITP apparatus. a = Pt-electrode, b = terminating electrolyte, c = drain, d = septum, e = UV-detector, f = conductimeter, g = septum, h = semi-permeable membrane, i = Pt-electrode, p and q are leads to a current-stabilized power supply. The separation compartment is a PTFE capillary (I.D. = 0.2 mm, O.D. = 0.35 mm, length is about 20 cm).

Fig. 2. (above) Principle of isotachophoresis. After introduction of the separands A and B between the leading electrolyte L and the terminating electrolyte T (Fig. 2 I), the concentrations are adjusted to the conditions of the leading electrolyte during the moving boundary phase (Fig. 2 II). After reaching the steady-state (Fig. 2 III), the zones can be detected isotachophoretically.

Fig. 3. Isotachophoretic analysis of 50 ml of a French wine (Muscadet de Sèvre et Maine) at pH 3.0 with 0.01 M chloride/β-alanine as leading electrolyte (L) and acetate as terminator (T). (a) Trace of conductivity detector. (b) The computer-converted isotachophoretogram which has the properties of a chromatogram, and is treated as such. 1 = sulphate, 2 = sulphite, 3 = phosphate, 4 = malonate, 5 = tartrate, 6 = citrate, 7 = malate, 8 = lactate, 9 = gluconate, 10 = succinate and 11 = dehydroascorbate.
velocity during the steady-state period. The concentrations of the separand zones are adapted to the conditions of the leading electrolyte according to the Kohlrausch Law. Therefore, the length of the zones provides quantitative information, while the electrical conductivity provides qualitative information. Other zone characteristics are temperature, electric-field strength, pH, UV-light absorption, fluorescence and radioactivity. The strength of this separation method, compared with its chromatographic equivalent (displacement chromatography), is its limited diffusion, due to the adaptation of all concentrations of the zones in the separation compartment. Injecting a sample thus causes a concentration or dilution step (Fig. 2).

A characteristic isotachopherogram is shown in Fig. 3a. This isotachopherogram shows the steady state in the analysis of a wine sample. It was analysed at low pH for organic and inorganic acids and an a.c. conductivity detector was used. In standard isotachophoretic equipment minimal detectable amounts are of the order of 100 pmol. Specially adapted equipment and/or sample pretreatment can lower the minimal detectable concentration to c. 1 µM.

On-line and off-line combinations of ITP-MS, ITP-HPLC and ITP-HPLC-MS are currently under investigation by groups in Vienna, Bratislava and Eindhoven. On-line ITP-HPLC looks particularly promising and the results obtained are comparable with the experiments on disc-electrophoresis, performed in 1964 by Ornstein and Davis.

Further lowering of the minimum detectable amount has been achieved by decreasing the inner diameter of the separation compartment to 0.1 mm (Verheggen), by using volume-coupling (Verheggen, Shimadzu) and by two-dimensional column-coupling (Verheggen, Kaniyanski, Eriksson). These developments have enabled the minimum detectable amount to be decreased by a factor of c. 100, compared with commercially available standard isotachophoretic equipment.

### Detection systems

Although accurate, the thermal detector provides insufficient resolution and almost all information collected with a thermometric detector can be obtained using the high-resolution conductometric (potential gradient) detector. A comprehensive comparison of possible ITP detectors can be found in Refs 2 and 3.

With the conductivity (potential gradient) detector, the adjoining zones can be resolved in zone volumes as small as 3 nl, which under standard operational conditions is equivalent to 30 pmols of analyte. The nature of the universal detector signal in ITP, however, makes signal processing by commercially available equipment (chromatographic peak integrators) impossible. The amplitude of the signal provides only qualitative information, whereas the time axis contains both qualitative (sequence of zones) and quantitative (length of zones) information. The differential of the signal is widely used for measuring zone-lengths manually and attempts at automation have not thus far been successful. The only signal processor for ITP currently available (type I-ElB Shimadzu) is, in fact, a modified integrator for chromatography and makes use of the differential of the isotachopherogram for the detection of the zone transitions. Failure to detect a zone transition obscures the quantitative results of other zones, whereas the qualitative accuracy is determined by the stability of the universal detector.

Reijenga has introduced a signal processing method for ITP which converts the linear trace of the isotachopherogram to a signal with chromatographic properties (Fig. 3b) which is then treated as such. The amplitude of the converted signal provides quantitative information. Thus, a great deal of software and hardware developed for chromatography can be used for ITP.

A computer programme for the conversion of ITP signals, written in BASIC, can be used on any
microprocessor with an 8 bit ADC and c. 10 kbyte of RAM. With this programme it is possible to resolve zones, e.g. in trace analysis, which approach the theoretical minimum detectable volume in the detector probe used. Quantitative accuracy in ITP, with a well-defined leading electrolyte transport number, is determined only by the stability of the driving current and the accuracy with which the zonelengths are measured. The method described takes both these effects into account, as the microprocessor also measures the driving current with an absolute accuracy of 0.1%. It has been found sufficient to measure the qualitative information with a resolution of 0.5%. 200 stepheight intervals are available with the microprocessor, which means that, in principle, one can qualitatively identify 199 separands between the leading electrolyte and the terminating electrolyte.

The use of specific detectors, such as UV-absorption or fluorescence detectors, has provided useful additional information in isotachophoresis, especially since at the steady-state the separand zone is mixed only with the counter ion (Fig. 2 III). The concentration is adjusted to the concentration of the leading electrolyte, which makes it necessary to use detector cell volumes less than 10 nl. The introduction of dual-wavelength detection, making use of such a measuring cell\textsuperscript{14} with computerized signal processing, has recently been introduced by the Eindhoven laboratory\textsuperscript{16}. Multiple wavelength detection is possible, especially if optical fibers are used. Scanning detectors are not yet available for ITP because the scan must be completed within 0.1 s to allow the resolution of short zones. Moreover, such a detector would need a more complicated data-system such as that used for GC-MS. At the present time it is possible to choose two wavelengths from 206, 254, 280 and 340 nm with the plasma lamp/filter combinations currently available. Making use of the UV-absorption (or absorbance) ratios, the method has been extremely useful for identification and quantification of steady-state zones, even where these were short.

The detection unit developed for dual-wavelength UV-absorption detection has made it possible to apply fluorescence detection (see Fig. 4). An even more specific detection method uses radioactivity, as introduced by Kaniansky\textsuperscript{12}.

**Recent developments in instrumentation**

Column coupling (Fig. 5), nowadays equipped with a microprocessor for handling the system, for controlling various operations and for stabilizing the electric driving current, enhances the versatility of isotachophoresis without requiring more complex equipment. Column coupling makes use of two PTFE-tubes with different internal diameters. In the pre-separation tube, which has the larger internal diameter, a high pre-separation current is permitted. At a well defined distance from a conductivity detector – a 'tell-tale detector' – the final separation compartment is coupled to the pre-separation capillary
in the bifurcation block. The zones of interest can easily be selected from the sample train, migrating isotachophoretically in the pre-separation compartment via the tell-tale detector. The smaller internal diameter of the final separation compartment permits a higher current density during detection by means of the high resolution detectors described earlier. This system possesses several advantages over conventional isotachophoretic equipment:

- A higher sample load can be handled in the same analysis time.
- Higher concentration ratios of separands are permitted.
- Different operational systems can be applied in the two separation compartments (multidimensional isotachophoresis).
- Various electrophoretic separation principles can be combined, e.g. isotachophoresis followed by zone-electrophoresis.

**Conclusions**

Capillary isotachophoresis makes it possible to analyse both low and high molecular-weight charged substances with a minimum of sample pretreatment. A survey of recent ITP literature (Fig. 6) indicates that there is a considerable overlap in applications with HPLC. Modern developments in isotachophoretic equipment and detection systems, combined with the use of microprocessors for equipment handling and signal processing make this analytical separation technique attractive because of its flexibility, reproducibility, accuracy and its extremely low running costs.

**References**


_Fig. 6. A survey of recent literature on ITP, divided into papers on instrumentation (I), theory (T), patents (P), applications (A) and reviews (R). The field of ITP-applications is broad and shows an overlap with HPLC. C.A. = Chemical Abstracts._