Aspects of detection and identification in isotachophoresis

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ASPECTS OF DETECTION AND IDENTIFICATION IN ISOTACHOPHORESIS

J. C. REIJENGA, A. A. G. LEMMENS, Th. P. E. M. VERHEGGEN and F. M. EVERAERTS*

Laboratory of Instrumental Analysis, University of Technology, P.O. Box 513, 5600 MB Eindhoven (The Netherlands)

SUMMARY

The detector response in isotachopheresis is usually associated with qualitative parameters such as mobility (universal detection) and molar absorbance (specific detection). A more specific response (valency) is obtained from the a.c. conductivity detector when using coated electrodes. When using UV absorption of the counter ion, a more universal character of the signal is obtained. A number of anionic and cationic operational systems are suggested. In addition, quantitative accuracy and precision are discussed with special reference to detection principles, detector cell design, driving current stability and electroosmotic disturbance.

INTRODUCTION

For identification and structure elucidation purposes in general, techniques such as mass spectrometry, Fourier transform infrared spectrometry and nuclear magnetic resonance are the most powerful techniques. However, they require the sample to be pure or in a well defined matrix but in practice these conditions are usually not fulfilled. Physical separation methods are necessary to isolate the sample constituent from the matrix prior to identification, which has led to the introduction of versatile combinations such as gas chromatography-mass spectrometry.

However, the use of a physical separation method in itself sometimes yields information on the identity of the compound of interest. Gas chromatography gives an indication of boiling point and gel permeation chromatography and polyacrylamide gel electrophoresis give information on molecular size. Here, retention data are used for identification. Sometimes there is no unequivocal relationship between retention and molecular structure.

In capillary isotachopheresis, there is also no unambiguous connection between effective mobility and solute properties such as structure, molecular size or even charge-to-mass ratio. Attempts have been made to obtain linearized relationships for homologous series of compounds, but these are not valid universally¹⁻⁴. Here universal detection, giving information on the effective mobility, was concerned. Specific detection systems have been developed in order to obtain structural information from the signal amplitude.

QUALITATIVE ASPECTS

The replacement of low-resolution thermal detection by high-resolution potential gradient/conductivity detection has greatly improved quantitative resolution. In contrast, the qualitative accuracy was not increased, owing to the limited linearity of the electronics used and the possible occurrence of electrode reactions. Sometimes, electrode reactions will lead to coating of the electrode surface, *e.g.*, Kolbe electrolysis. Experiments with coated electrodes⁵ have revealed that the total a.c. resistance of the detector cell depends on the measuring frequency and that the effect of coating is negligible in the d.c. mode. The above-mentioned frequency dependence was observed especially for multivalent ions. In earlier work⁵ we gave the results of a separation of nitrate and sulphate, detected with a cell coated with 1-aminoanthracene and operated in the d.c. and a.c. modes (Fig. 1). The relative step height of sulphate is clearly frequency dependent. This would offer attractive possibilities for the determination of the valency of unidentified sample components. Another more laborious way is to determine the concentration dependence of the effective mobility, but the results are not satisfactorily unambiguous⁶.

Fig. 1. Isotachopheretic separation with conductivity detection of nitrate (1) and sulphate (2) at pH 6.0. The leading ion was 0.01 *M* chloride with histidine as a counter ion. The terminator was 2[N-morpholino]ethanesulphonic acid (MES). The electrodes of the detector were coated with 1-aminoanthracene. Detection was (a) d.c. and (b) a.c. conductivity⁵.

Specific detectors show responses only to certain zones in the isotachopherogram, depending on the properties of the component concerned. UV absorption detection⁷ is mostly used. The choice of wavelength will determine the specificity: the lower the wavelength, the more components will show absorption. Under certain conditions (operational system, capillary material), even detection at 206 nm is possible⁸. For identification, scanning UV detection⁹, dual wavelength detection^{10,11} and fluorescence emission or quenching^{12,13} give additional information. An improvement in scanning detection, especially in terms of speed, is achieved by the use of a diode array. Even more specific is radiometric detection¹⁴, where only components emitting β -radiation will be detected. Similarly, an energy spectrum thus obtained can be used to identify the nuclide concerned.

Fluorescence quenching makes use of specific properties of the counter ion and can consequently be defined as universal detection. An empirical relationship was

derived between fluorescence quenching and whether a component ion is weak or strong¹³.

As early as 1974, Arlinger and Lundin^{15,16} used UV absorption of the counter ion as a universal detection method in a similar way. The method was said to make use of the pH dependence of the molar absorbance of the counter ion in the pH range used, utilizing the stepwise change of pH between the successive zones. The response is most favourable if the change in molar absorbance of the counter ion ranges over a decade or more. The choice of wavelength here obviously plays an important role.

Another effect that occurs is the stepwise decrease in concentration of the counter ion in the successive zones. This effect will normally not predominate over the pH effect, except when an absorbing substance is added as a strong co-counter ion at low concentration.

For low pH anionic operational systems, quinine (pK_1 4.3) can be used because of its high UV absorption and low effective mobility. Fig. 2 shows an analysis of a standard mixture of anions at pH 3 with quinine as a counter ion. For some of the zones, additional absorption is caused by the component to be separated, *e.g.*, ascorbate or sorbate. The analysis shows good resolution with a detection limit of certainly less than 100 pmol, comparable to conductivity detection.

Fig. 2. Analysis of anions at pH 3 quinine as a counter ion (see Table I), with UV detection at 254 nm. 1 = Phosphate; 2 = salicylate; 3 = tartrate; 4 = citrate; 5 = malate; 6 = lactate; 7 = gluconate; 8 = succinate; 9 = benzoate; 10 = ascorbate; 11 = glutamate; 12 = acetate; 13 = sorbate; 14 = propionate. Amount of sample components 300 pmole each.

Table I lists some examples of operational systems suitable for UV detection of the counter ion at 254 nm. In spite of the difference in construction of the UV slit and the conductivity cell, similar detector cell volumes are achieved. Because of the straightforward construction of the UV slit, the method of universal UV detection deserves more attention than it actually receives.

QUANTITATIVE ASPECTS

Other than in elution techniques, such as chromatography, the detection limit in isotachopheresis is not associated with detector noise and specific amplitude. The

TABLE I
OPERATIONAL SYSTEMS FOR UNIVERSAL UV DETECTION AT 254 nm

System	Leading ion	Counter ion	pH range	Terminator
Anionic	Chloride	Quinine	ca. 3-5.0	Propionate, glutamate
Anionic	Chloride	Creatinine	ca. 4-5.5	Glutamate, MES
Cationic	Potassium	Sulphanilate	ca. 3-5.0	H ⁺
Cationic	Potassium	Barbital	ca. 7-8.5	Tris

minimum detectable amount is determined by the volume of the detector cell and the concentration of a component in its zone during detection. The latter is approximately equal to the leading electrolyte concentration, which is limited in range owing to requirements of solubility and buffering capacity. The detection limit in concentration units also depends on the composition of the sample injected. For a specific matrix, the amount that can be injected will be proportional to the volume of leading electrolyte between the points of injection and detection. In fact, the performance of isotachophoretic equipment can be given by a performance index, defined as the ratio of the leading volume mentioned and the detector cell volume (see Table II). The most favourable values of the performance index are obtained with volume coupling¹⁷ and column coupling¹⁸. Here, flexibility of the configuration is combined with a detector cell volume of *ca.* 3 nl in a 0.2 mm I.D. capillary. A further decrease in this volume will be limited by considerable practical restrictions.

As mentioned, the detector cell volume should be as small as possible. A distinction is necessary between the theoretical and the effective cell volume. For example, a 15- μ m thermocouple measures zone lengths in centimetres, owing to the heat transfer limitation.

TABLE II
PERFORMANCE INDEX OF ISOTACHOPHORETIC EQUIPMENT

See text for further explanation.

Manufacturer	Leading volume (μ l)	Detector		Performance index	Remarks
		Volume (nl)	I.D. (mm)		
LKB, Sweden	102	20	0.5	5100	250 mm capillary
Shimadzu, Japan	98	20	0.5	4900	100 mm pre-separation
THE, NL*	48	20	0.5	2400	—
THE, NL*	12	3	0.2	4000	—
THE, NL*	23	3	0.2	7700	Volume coupling
THE, NL*	76	3	0.2	25,000	Column coupling
Ustav Radio- ekologie, Czechoslovakia	106	7	0.3	15,100	Column coupling

* Laboratory of Instrumental Analysis, University of Technology, Eindhoven, The Netherlands.

Inside the capillary, additional effects take place at the zone boundary to be detected: a radial zone boundary profile, due to radial temperature differences and electroosmosis, and an axial concentration distribution, due to diffusion. The latter is approximately equal to $4mRT/\delta mFE$ where m is the average effective mobility, R the gas constant, T the absolute temperature, δm the difference in effective mobility of the adjacent zones, F the Faraday constant and E the average electric field strength¹⁹. For a relative effective mobility difference of 10% at 10^4 V m^{-1} and room temperature, this diffusion thickness is *ca.* 0.1 mm. This is of the same order of magnitude as the detector cell length. Therefore, it can be argued that a decrease in this length serves no purpose. Consequently, the axial concentration distribution affects the precision of the determination of the zone boundary because of the uncertainty of the exact location of that boundary. In contrast, the radial zone boundary profile will influence the accuracy of zone length measurements, as will be shown. For the construction of the conductivity detector cell, two types have been reported²⁰, those with axially and those with radially mounted electrodes. A theoretical cell volume of $\pi r^2 l$ can be calculated, where r is the internal radius of the capillary and l the length of the cell. However, in both a.c. and d.c. measurements, the field line density distribution will determine the effective cell volume. This is not easily established as it will depend on the specific resistance of the zone, the dielectric constant of the solvent, the temperature and the measuring frequency.

A qualitative representation of the field line density distribution will illustrate which type of cell is to be preferred for the accurate determination of zone transitions and zone lengths (see Fig. 3).

Fig. 3. Schematic representation of the field line density distribution in a conductivity detector cell with (a) axially and (b) radially mounted electrodes during the detection of a zone boundary.

In cell type (a), the field line density increases with increasing distance from the central axis. This is not the case with cell type (b). Fig. 3 shows that a zone boundary with a pronounced profile is not properly detected. When half of the volume between the circular electrodes is filled with zone 2, it is seen that the resistance of the cell is determined by zone 1 for more than 50%. The front of zone 1 (not shown in Fig. 3) will certainly show a less pronounced profile, so that the error in detecting the beginning of zone 1 is less than that at the rear of the zone. The net result will be that zone 1 seems longer than it actually is. The effect mentioned above has been verified experimentally by analysing an anionic sample component under standard operational conditions. With a leading electrolyte of 0.01 *M* chloride buffered at pH 6.0 with histidine, a solution of benzoate was injected, with lactate as an internal standard. The zone length of benzoate was measured with a type (a) con-

TABLE III

ZONE LENGTH OF BENZOATE IN A LEADING ELECTROLYTE OF 0.01 M CHLORIDE/HISTIDINE, pH 6, WITH DIFFERENT TERMINATORS

Analysis at 25 μ A in a 200 $\times$ 0.2 mm I.D. capillary with type (a) detector.

Terminator	Average zone length (sec)	n	Standard deviation (sec)
<i>p</i> -Aminobenzoate	7.9	6	0.12
MES	9.0	5	0.14

ductivity detector cell, using the internal standard to correct for possible injection errors. The results are summarized in Table III, indicating a significant increase in zone length when benzoate was followed by MES as a terminator instead of *p*-aminobenzoate. The benzoate-MES boundary has a more pronounced profile.

The effect amounted to a difference of up to 1 sec at normal current densities in a 0.2 mm I.D. capillary with a type (a) cell. In many instances this effect will explain a slight intercept in standard calibration graphs and should be corrected for in trace analyses.

The use of spacers or a smaller current density during detection will help as the zone boundaries will be straightened. Unfortunately, it will also increase the thickness of the diffusion-controlled boundary. Therefore, an increase in accuracy unfortunately coincides with a decrease in precision.

Another possible source of error in zone length measurements is current instability of the high-voltage supply. During detection, the driving current should be as constant as possible. The stabilization is then lower because of the high voltage. However, this instability can be adjusted by a coulometric device as introduced by Boček²¹. The coulometer drives the stepping motor of the recorder, so that the paper speed is directly proportional to the driving current. Fig. 4 illustrates the principle²². The current is monitored as the potential drop over a series resistor on the earth side. This voltage is amplified and converted to a transistor transistor logic (TTL)-compatible pulse train that drives the stepping motor of the recorder. A pulse counter for monitoring the progress of the analysis or for special functions (recorder on/off) can also be attached. The ultimate accuracy is also determined by the quality of the stepping motor. The resolution of the coulometer (the number of coulombs corre-

Fig. 4. Schematic diagram of coulometric registration in isotachopheresis^{21,22}. VFC = voltage-to-frequency converter. See text for further explanation.

sponding to 1 pulse) is also important. When working at 25 μA in a 0.2 mm I.D. capillary, a resolution of 1 μC is sufficient²². In this way, the instability can be reduced to 0.004% within 15 min at 25 μA . The coulometer also makes it possible to work at a constant voltage or to switch the current during registration.

CONCLUSIONS

The identification of unknown components in isotachopheresis is possible on the basis of the signal amplitude of both universal and specific detectors. More detailed spectral information (UV, fluorescence) will give an indication of possible chromophores. Initial experiments with coated electrodes indicate that the response of the conductivity detector yields information on the valency of the sample components.

UV absorption of the counter ion as a more universal detection technique can often be an attractive alternative to the use of a conductivity detector without loss of resolution. A number of cationic and anionic operational systems have been evaluated. Quantitative errors can be due to diffusion in the zone boundaries (this will affect the precision) or to the radial zone boundary profile, caused by radial temperature and electroosmotic profiles (this will affect the accuracy). Both effects, in addition to practical limitations, will impose a limit on the detection limit of the order of picomoles under practical operational conditions.

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