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AUTOMATION OF ANALYTICAL ISOTACHOPHORESIS*

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CONTENTS

1 INTRODUCTION

In isotachophoresis (ITP), sample components separate by forming discrete, consecutive zones with homogeneous concentrations. This system reaches a steady state, in which each sample zone moves with constant velocity, the zone length being proportional to the amount of a sample constituent. The boundaries between adjacent zones are characterized by sudden changes in a number of physical properties which allows quantitation by zone length measurements[1–5] This behavior of electrical transport systems is unique and not duplicated by any other separation technique. Although many publications have praised the high resolution of ITP, other methods, such as high-performance liquid chromatography (HPLC), have been more rapidly explored and introduced in analytical laboratories. Commercially available ITP instruments are very expensive and not easy to operate by a lab technician. In addition to these obstacles, the whole methodology, mainly the choice of a suitable operational system for a specific separation problem, has not yet been compiled in a straightforward way. The superiority of ITP compared to any heterogeneous type of chromatography or any type of field-flow fractionation technique, however, has been fully demonstrated in recent years. Important contributions include: (i) the theoretical description of the isotachophoretic separation process with a model based on migration only[6–8]; (ii) the experimental verification of the separation scheme[9,10]; (iii) the understanding of the zone order in ITP[11]; (iv) computer simulations whose results

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are consistent with the findings in (i)[12] and (iii)[13] and provide further insight into the features of the boundary structure[14], and (v) the development of high-resolution detectors which monitor either universal or specific properties of the separatand pattern.

The automation of the ITP process is necessary for the adoption of this technique as a routine analytical laboratory methodology. It has been clearly established in the past decade that digital electronics has an enormous amount to offer to analytical chemists The advent of versatile and inexpensive microprocessor systems in conjunction with the demonstration of the advantages of ITP can be expected to cause a revolution in the analytical use of ITP. The evolution of an ITP separation as well as the migrating steady-state zone pattern provide the prerequisites for automation. Transient- and steady-state zone distributions can be monitored by a plurality of equidistant detectors along an ITP column (array detector[10,15–17]), or by scanning the separation trough repeatedly with a moving detector[18]. This gives a computer interpretable criterion for the steady state, which is a mandatory condition for automation. Much information of analytical relevance is lost with single detectors placed at the end of the separation space, the method used in all commercial instruments.

Prior to considering possible future developments in the field of automated analytical ITP it is pertinent to reflect on the author's strong links which have generated many of the ideas and predictions formulated in this article. In particular he would like to acknowledge the very significant contributions of E. Schumacher and D. Arn, University of Bern, Switzerland as well as of M. Bier, R. A. Mosher and N. B. Egen, University of Arizona, Tucson, AZ, U.S.A.

This lecture reviews the basic features of automation of analytical ITP. Experimental setups consisting of narrow bore tubes (capillaries) are considered which are self-stabilized against thermal convection. Sample detection in free solution is discussed by listing the detector systems presently used or expected to be of potential use in the near future. The combination of a universal detector measuring the evolution of ITP zone structures with detector systems specific to desired components is proposed as a concept of an automated chemical analyzer based on ITP. In addition possible miniaturization of such an instrument by means of microlithographic techniques is discussed.

2. DETECTION OF ITP ZONES

In principle many of the detection methods developed for HPLC can also be employed for ITP. In addition, unique principles can be used which rely on the constant electric current flow through the separation capillary. ITP zones are therefore characterized by a distinct, constant voltage gradient, resulting in a stepwise electric field change across their boundaries. Conductivity and temperature profiles are of comparable shape. The transition between a pair of sample components can be further marked by a sudden change in a number of other physical properties, such as refractive index, pH and absorbance. A universal detector allows the monitoring of the whole zone structure, the magnitude of the sensor response being a function of the net mobility of the sample constituent[2,3]. The information derived, therefore, has a stepwise character. Methods include the measuring of the potential gra-

TABLE 1

DETECTION SYSTEMS FOR ISOTACHOPHORESIS

Detection system	*Performance*	*Scan*	*Array*
(a) Universal methods			
Potential gradient	Microsensing electrodes in contact with electrolyte		×
Conductivity	Microsensing electrodes in contact with electrolyte		×
Conductivity	Microsensing electrodes without electrolyte contact	×	×?
Temperature	Thermocouple, thermistor		×
Thermal radiation	Heat radiation sensor	×	
UV–VIS absorbance*	Optical micro cell	×	×
Fluorescence quenching**	Optical micro cell	×	×
(b) Optical methods			
Polarimetry	Optical micro cell		×
Schlieren-interferometry	Optical micro cell		×
UV–VIS absorbance	Optical micro cell	×	×
Fluorimetry	Optical micro cell	×	×
(c) Radiometric methods	G M counter	×	
	Scintillation detection	×	×
(d) Electrochemical methods			
Ion-selective electrodes (pH?)	Microsensing electrodes in contact with electrolyte		×
Voltammetry	Microsensing electrodes in contact with electrolyte		×

* If applied with absorbing counter component.
** If applied with fluorescing counter component

dient[2,3,10,15–17,19–23] and the conductivity[2,24] with sensing microelectrodes which are in direct contact with the electrolyte, the determination of the conductivity with sensors outside of the separation column[25] and the detection of the temperature profile with thermocouples or thermistors[2,26] and with heat radiation (IR) sensors[27]. Optical detectors are also considered as being universal if the common counter constituent, but not the sample, can be monitored[2,17,28]. The signal of pH sensors is proportional to the net mobility of the sample components if buffer-free operational systems are employed[1,17,23].

Other methods allowing the visualization of the majority of boundaries include polarimetry, Schlieren-interferometry or ultrasonic imaging. The magnitude of the signals derived therefore are not related to the net mobility of the sample. A non-continuous response is recorded. This is also typical with the use of specific detectors which permit the localization of a desired sample constituent in a complex isotachopherogram. Specific methods include UV–VIS spectrophotometry[2,29], fluorimetry[28], radiometry[30], potentiometry with ion-specific electrodes[2] and voltammetry[31]. Table 1 summarizes the methods of detection currently employed in analytical ITP, together with those of potential use in the near future. Their feasibility for the con-

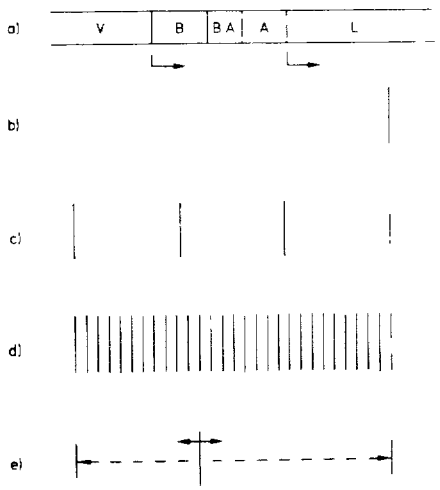

Fig 1 Schematic presentation of detector arrangements along an ITP column. (a) Separation trough with transient zone structure L refers to the leading zone, V to the displacing electrolyte and BA represents a transient mixed zone composed of components A and B (b) Single detector placed at the end of the trough (c) Four detectors along the column (d) Array detector as a plurality of equidistant detectors (e) Single detector adapted for movement (scan detector)

struction of scan and linear array detectors is also included in this list, since location and arrangement of sensors is of crucial importance for a suitable detection and therefore for automation of this analytical technique. A schematic representation of various detector arrangements along an ITP column is given in Fig. 1.

3 MONITORING OF THE SEPARATION PROCESS

The continuous monitoring of the evolving zone structure constitutes the most straightforward approach for the visualization of the separation process. With a plurality of equidistant detectors along the column (array detector as depicted in Fig. 1d) transient- and steady-state zone distributions can be observed. When measuring a general physical property the time development of each zone boundary can be monitored, including the vanishing of mixed zones. An apparatus incorporating a potential gradient array detector as one wall of the isotachophoretic separation trough was constructed at the University of Bern, Switzerland[10,16,17,32]. The sensor array consists of an ordered array of 256 detection electrodes over a length of 10 cm, permitting almost simultaneous measurements of the electric field along the column. Fig. 2 shows the anionic boundary between 2.5 m*M* hydrochloric acid, the leader, and lactic acid as terminator while moving across part of the detection window. The anode is to the right. Data points of 8 scans over 177 detection channels are displayed. They are corrected for irregularities of the crossectional area of the separation trough[33]. The consecutive scans are shifted by an offset in the y-axis for their presentation in one graph. This experiment clearly documents the steady-state behavior of this boundary. Fig. 3 shows corrected data points of scan 11 with maleic acid, tartaric acid and malic acid, 3.5 nmol each, as sample components. The operational system is identical to that of Fig. 2 The presented data are machine treated as dis-

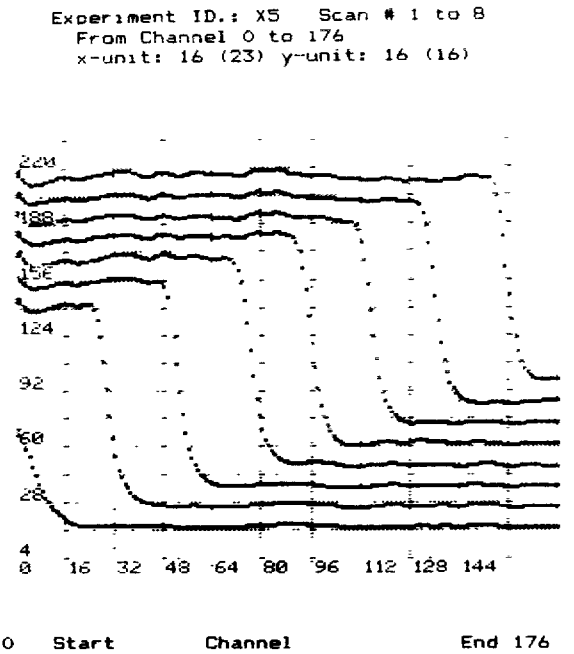

Fig. 2 Evolution of a steady-state anionic boundary as monitored with the potential gradient array detector 2.5 m*M* hydrochloric acid and 10 m*M* sodium lactate were used as anolyte and catholyte respectively Consecutive scans were taken every 46 5 sec under a constant current of 100 μA. Machine treated data points of scans 1 to 8 along a part of the detection window are displayed

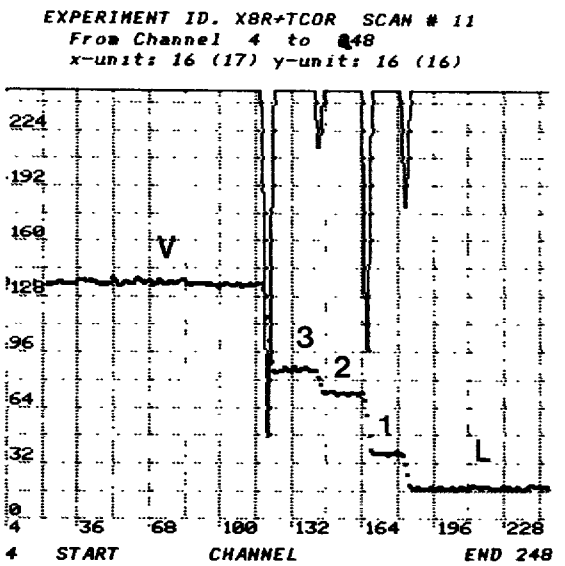

Fig 3 Data of scan 11 representing the electric field profile after 566 sec of current flow across an isotachopherogram with maleic acid (1), tartaric acid (2) and malic acid (3) The leading zone is denoted by L and the displacing electrolyte by V Data points and first derivative from top are machine treated The output protocol provides boundary locations at detector channels 182.46, 165 37, 143 61 and 119 89 Zone heights are 17 44 (L), 36.99 (1), 71 91 (2), 85 76 (3) and 135 34 (V)[33]

cussed by Schumacher *et al.*[33]. Zone lengths are given by the number of detection channels occupied by the zone during the scan. Note that a five-fold decrease of the injected amount of sample still would be detectable with the sensor array. Zone heights are in digital units allocated through the 8 bit analog to digital converter. That the steady-state zone structure was attained within the detection window under the given conditions is shown in ref. 32. An on-line evaluation of data, *i.e.* the comparison of consecutive scans during the experiment is currently under investigation[34,35]. This provides the last step necessary for the completion of the new concept for automated monitoring of the isotachophoretic separation[15].

An alternative procedure represents the use of a single detector adapted for movement along the separation trough (Fig. 1e). By repeatedly scanning and continuously measuring a universal physical property almost instantaneous profiles could be obtained. A necessary requirement is that the scanning speed of the detector has to be much higher than the electrophoretic velocity of the zone pattern. No such detector has been constructed yet Possible candidates include the high-frequency contactless conductivity detection and the heat radiation detection. Data processing in this operational mode is identical to that for the array detector referred to above. It is important to realize that no correction of steady-state zone lengths to equal measuring time is necessary if current density and scanning rate are constant. Every zone is elongated in the laboratory frame proportional to its true length in a moving frame of reference. This is, of course, only correct in a uniform velocity field, which is realized in steady-state ITP. The proportionality is then constant and will drop by calibration from further consideration.

Information about the steady state can also be obtained by comparing the zone patterns from at least three completely different locations of the separation trough (see Fig 1c) Such a multi-sensor procedure for measuring the ITP spectrum at different instants can easily be automated with computer control Simple algorithms allow the evaluation of the digitalized detector responses. This mode of operation has been mentioned in the literature and can be performed using the apparatus with multichannel zone detection[10,16].

Optical multichannel array detectors for the continuous monitoring of specific ITP zones have been proposed[17] but not yet constructed. The application of a linear fiber-optic developed for facsimile transmission[36] could be an interesting approach in this area. Linear diode arrays (LDA) or charge coupled devices (CCD) constitute solid state imaging devices without any mechanical parts and are, together with ultrasonic imaging systems, potentially useful in ITP. The utility of an optical scan detector in electrophoresis has been demonstrated by Hjertén[18]. His quartz tube apparatus, stabilized by rotation about its longitudinal axis, is scanned repeatedly at two UV frequencies. The ratio between the two wavelengths is recorded. By such automatically repeated scans one can follow separation of UV absorbing sample components throughout a run and determine if or when a steady state has been attained.

With just one detector placed at the end of the separation column (see Fig. 1b) it is not certain if the steady state, *i.e.* the number of boundaries (or zones) and/or the constant length of each zone, has been attained at the time of detection. Each experiment must be repeated with different amounts of the sample components followed by a careful comparison of the detected zone structures. This mode of opera-

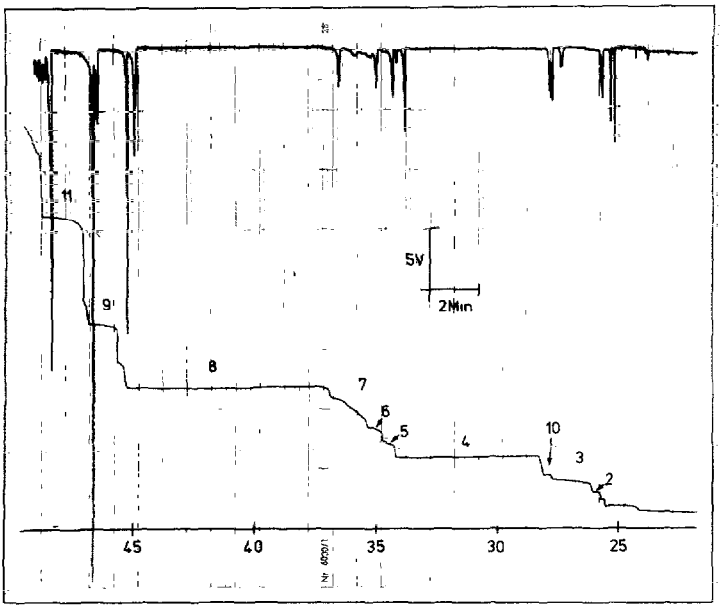

Fig 4 Analysis of 1 5 μl of a Swiss white wine A current of 100 μA was applied Denoted zones: (2) pyruvic acid, (3) phosphoric acid, (4) tartaric acid, (5) citric acid, (6) malic acid, (7) galacturonic acid, (8) lactic and gluconic acid, (9) succinic acid, (10) α-ketoglutaric acid, (11) acetic acid The x-axis scale is the time in min after current application

tion is implemented in all commercial ITP instruments and represents the simplest procedure for the visualization of an electrophoretic zone structure. Its disadvantages are obvious and have been reported extensively. Automation of this mode would not only be inelegant but would not exploit the tremendous capabilities of digital electronics associated with microprocessors.

4 AUTOMATED CHEMICAL ANALYZER BASED ON ISOTACHOPHORESIS

It has been shown that the isotachophoretic separation can be followed with an array detector measuring a general physical property. The ability of an ITP instrument to separate and detect compounds in complex mixtures generally far exceeds that instrument's ability to provide enough information for the identification of those components. Although the magnitude of the universal sensor signal is qualitative information it is often not easy to assign each detected zone to a specific substance. Correlation with standard solutions can be hazardous due to the formation of steady-state mixed zones, produced by constituents which have equal or similar net mobilities in a given operational system. The pherogram presented in Fig. 4 demonstrates the inability of the potential gradient sensor to reliably identify all detected zones. A volume of 1.5 μl of a Swiss white wine was analyzed with hydrochloric acid as leader (pH = 2.60) and hexanoic acid as terminator. To enhance the separation capacity of the given column a concentration cascade[37] of the leading electrolyte was utilized (32.5 μl of 10 mM hydrochloric acid[10]). Detection occurred at channel 250. The denoted zones were determined by the addition of internal standards and by

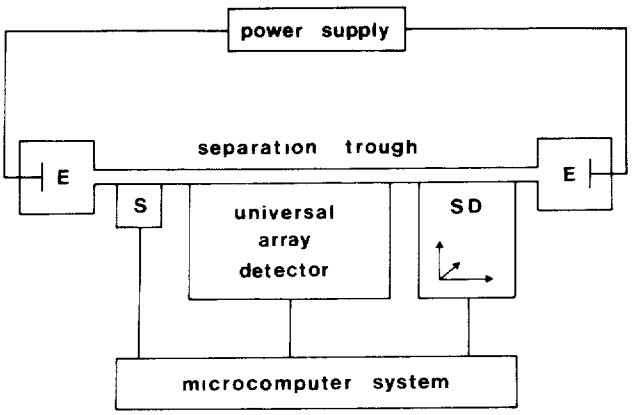

Fig 5 General block diagram of a chemical multicomponent analyzer based on automated analytical isotachophoresis E = electrode compartments, S = sample inlet system, SD = specific detector module

comparison of the zone heights with test samples. Note that in the given operational system lactic acid and gluconic acid were found to have equal net mobilities. It is certainly difficult if not impossible to identify all zones detected in this acid-spectrum. Additional sensors which show specific response of desired components have to be used. They improve considerably the overall performance of ITP. faulty assignments are minimized, unknown components can be identified and further off-line fractionation techniques[38] do not have to be used. The great variety of suitable specific detector systems (see Table 1), together with microprocessor control and chemometrical software, provide flexibility for a wide range of applications. Spectroscopic techniques appear to be the most popular. The construction of simple, interchangeable modules (or module arrays) is required, to allow an easy and quick selection of an appropriate method.

The overall concept of a chemical analyzer based on ITP comprises therefore three important parts, as shown schematically in Fig. 5: (i) a universal detector for the monitoring of the separation process; (ii) a second (or more) detector(s) for the specific detection of selected sample components; and (iii) a microprocessor system for control of the entire apparatus with multiple detection systems, for data storage and data handling as well as for database dictionaries with instructions for the user. Advantages and the necessity of part (i) have been discussed earlier in this manuscript and elsewhere[8,10,16]. It is worthwhile to add some comments pertaining to specific detection systems as well as to the computer system.

UV photometric detection has been employed for a number of years and is included in commercial capillary type apparatus. The combination of this selective detection technique with high-resolution conductance or electric field measurements has been demonstrated as being extremely helpful for the evaluation of complex isotachopherograms[2]. Two sensors placed in series at the end of the separation column were used. New optical microcells for dual-wavelength absorption detection[29] or simultaneous fluorescence (or fluorescence quenching) and absorption detection[28] have been designed and successfully applied to ITP analyses together with computerized signal storage and processing. Data reduction on the basis of signal ratios, fea-

turing greater sensitivity and selectivity has also been developed. Fixed-wavelength detectors have been primarily used. This is a major limitation since only one or two wavelength resolution elements are registered at a time. In HPLC however rapid-scanning UV–VIS photometric detection based on electronic, electromechanical or multichannel devices is already in use[39,40] The gathering of complete absorbance or fluorescence spectra in the order of msec, *i e* the simultaneous detection of a large number of spectral resolution elements, adds an additional dimension of information. The data in digital form can be readily stored, manipulated and presented in any desired format, making it possible to obtain three-dimensional chromatograms within the time needed for a single run. Optical multichannel detectors based on LDA, silicon intensified target (SIT) vidicon or CCD are most promising for the rapid acquisition of spectral data

Radiometric detectors can be used for the analysis of radioactive and radio-labelled ionic compounds. A first approach achieved by Kaniansky *et al* [30] highlights the advantages of this specific detection principle. There is no doubt that this technique will be very valuable in clinical, biomedical and biochemical applications. Little attention has been paid to electrochemical detection so far. The use of electrodes with specific coatings has been discussed by Everaerts *et al.*[2]. Ion-specific sensors based on ChemFET technology[41] (pH?) are yet to be applied for detection in electrophoresis Voltammetry remains another detection technique to be introduced. Electrochemical detection is widely used for HPLC because of its excellent sensitivity for many electroactive compounds The most commonly employed constant potential mode has only modest selectivity. Transient techniques, such as pulse, alternating-current or square-wave voltammetry, however, have been reported to be very useful in liquid chromatography if used with microprocessor-based instrumentation[42]. The simultaneous recording of five or more chromatograms on different time scales with different sensitivities and the recording of complete current–voltage curves at all points in time have demonstrated the advantages and great sensitivity as well as flexibility of these techniques. Rapid potential scanning procedures allow the gathering of entire voltammograms in the order of seconds[42–44]. Three dimensional data presentation is therefore possible which is comparable to that obtained with optical multichannel detectors.

The microcomputer represents the cybernetic center of the apparatus. Since the detailed approach to the steady isotachophoretic state is followed by the computer, an automated decision about the completion of the separation is provided. Separation time and separation location are thereby known. As soon as the criterion for stability is fulfilled, the microcomputer takes several scans over the total component spectrum to ensure a specified precision of zone length. The calculation of zone lengths from the length and decomposition rate of mixed zones represents an alternate way to obtain insight into the steady state zone structure. This transient approach applies nicely for simple systems but fails for complex configurations where mixed zones contain several components. Data from the array detector are processed as described by Schumacher *et al.*[33] and Arn and co-workers[34,35]. Data from specific detectors are digitalized and compared with those of reference components which are stored from calibration runs in the computer library. The computer assigns each detected zone to a specific substance based on the information gathered from both detector systems. It is important to realize that proper analytical self testing and elaborate data analysis is performed unnoticed by the user.

The aforementioned tasks and advantages of a computer system in an automated instrument are not complete without the establishment of a computer database containing methodological instructions for the user. The wide variety of operational systems suitable for ITP analyses should be summarized and stored in the computer. Such a compilation enables the user to find appropriate leading and terminating electrolytes as well as the specific detection system suitable for a specified analysis. This information enables an untrained user to perform successful runs. Similar libraries have been found useful in various areas of analytical chemistry, such as in automated titrimetry.

5. DISCUSSION

Many analytical procedures based on wet chemistry are extremely time-consuming and consequently can only be economically justified if automated. Although various steps in a manual wet chemical method can be mechanized, the objective should be to automate the entire procedure. Automated ITP provides many advantages and is an alternative to automatic titration[15]. An array detector measuring a general physical property provides insight into the evolution of the whole zone spectrum. An ideal apparatus requires more than just the capability of following the separation process together with a computer for data storage and handling. It would also require a second detecting system specific to desired sample components. The combination of two (or more) detector systems would be most suitable in routine analysis if automated with computer control. Such a device would provide the basis for an automated chemical multi-component analyzer. The decision making process of a computer system is based upon a comparative procedure. In automated ITP the computer software would compare. (i) the dynamics data of subsequent scans for the validity of the isotachophoretic state at each boundary and (ii) the universal and specific sample responses with a previously compiled data base. Quantitation through zone length measurements is achieved by comparing the detected distance between two boundaries with those from preregistered calibration runs or by the method of standard addition. The availability of methodological instructions for the user, organized as a computer library, would represent another feature of the analyzer.

The potentialities of new detector systems have been discussed on a broad basis. In particular fast scanning optical and swept-potential electrochemical detectors allowing three dimensional projections of data are proposed as important methods for the more complete identification of ITP zone structures. It will certainly take some time for their introduction in ITP. In the meantime it is hoped that this manuscript stimulates new activities in this field of chemical analysis.

The isotachophoretic sorting process is a focusing method in the sense that it is capable of concentrating a dilute component into a narrow zone. Conversely, a concentrated component in the sample will form a relatively wide zone. In both cases there is no further change of the zone length when a steady state is reached, *i.e.* when the spectrum of contiguous sharp zones whose boundaries migrate with equal velocity is fully established. The width of ITP boundaries is dependent upon experimental parameters, such as the current density and the chemical–electrochemical properties of the components involved. It can be predicted by simple models[14] and is usually in the order of micrometers. Each of these unique features is important for auto-

mation of this electrophoretic mode. They also provide favorable scaling laws, *i.e.* an estimate for suitable miniaturization of the detectors and the separation columns, and would allow instrumentation to be shrunk, possibly to the dimensions of a printed circuit board. Technologies developed for microelectronics, namely microlithography and chemical etching, as well as the advent of versatile and inexpensive microprocessor systems provide the means for the construction of new instruments. The introduction of miniatured gas chromatographs[45] and of microconduit flow injection analysis[46] are examples of the straightforward applicability of such methods. Based on these examples, and the experience obtained with the potential gradient array detector, the design steps of a new, more versatile ITP analyzer are given. Its construction should be feasible in the foreseeable future.

The analysis of wine presented in this paper (Fig. 4) illustrates a field of application for ITP as a multicomponent analytical methodology. The determination of acetic (zone 11), succinic (zone 9) and α-ketoglutaric acid (zone 10) allows the prediction of the quality of the wine because of the disagreeable taste of those components. The ratio of the concentrations of certain acids in a wine further provides a method to conclude its fermentation process. ITP is not only an excellent method to monitor simultaneously the entire acid-spectrum of a wine; similar determinations can be done with other beverages (beer, soda, milk, fruit juice), food extracts, body fluids (serum, saliva, urine, sweat), fermentation solutions in genetic engineering, tissue culture media for hybridoma technology and nutrient solution for the hydroponic industry as well. An automated ITP instrument would certainly be very useful in these and other fields of application.

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7 SUMMARY

A review of progress in the field of computer-aided methods for the monitoring of the isotachophoretic separation process and the steady-state zone structure is presented. Sample detection in free solution and the potential utility of three dimensional projections of data from universal and specific detectors are further discussed in the context of a proposed concept of a multicomponent chemical analyzer based on automated analytical isotachophoresis. The analyzer yet to be designed would benefit from favorable scaling laws, which would permit the utilization of microlithographic techniques for its construction. This would lead to miniaturized instrumentation in capillary electrophoresis.

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