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Novel three-dimensional capillary electrophoresis system for complex and trace analysis

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Abstract

A novel triple column capillary electrophoresis system is described. Design specifications facilitate method development and analyses by providing on-line, selective, pre-concentration and clean-up of both high (ml) and low (μl) volumes of specific analytes in two dimensions and separation via an additional third dimension. The system described additionally provides four distinct detection capabilities via both contactless conductivity and UV. The addition of a third dimension to the previously reported "coupled-column" systems, and further modifications made, has allowed for optimal identification, separation, and quantitation of micro-components in complex mixtures. The ability to perform both capillary zone electrophoretic and isotachophoretic separations on-line and in any combination enhances the scope for rapid analytical method development and analysis of complex or trace sample components. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Capillary electrophoresis (CE) is the generic name for a family of electrophoretic techniques. CE has developed rapidly and has generally been accepted as an analytical technique complementary and often superior to other separation methods, e.g., high-performance liquid chromatography (HPLC) and gas chromatography (GC). However, despite the highly efficient separations obtained with standard mixtures, "real world" samples often pose problems associated with the matrix components, limited sample injection volume and consequent lack of concentration sensitivity in conventional CE. Additionally, problems may often be encountered when attempting, for example, to reproducibly quantify micro-constituents (e.g., metabolites in sera) in samples rich with ionic macro-constituents (e.g., high salt concentrations), such as those encountered when dealing with biological and environmental samples.

This paper discusses an attempt to surmount these problems and the design and subsequent development of a novel three-dimensional capillary electrophoresis instrument which overcomes some of the disadvantages of current methodologies is described. The novel three-dimensional CE system facilitates on-line selective, pre-concentration and clean-up of

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specific analytes. In addition, the system allows for separation and quantitation of analytes in more than one dimension while providing dual detection capabilities (UV and contactless conductivity). Moreover, the system is versatile in that more than one mode of CE may be integrated into any one analysis.

2. Theoretical background

2.1. Capillary isotachophoresis

Isotachophoresis (ITP), was first demonstrated by Martin in 1942 (unpublished results) [1]. At that stage, the technique was named "displacement electrophoresis", through analogy with displacement chromatography. Subsequently however, after further independent development by Martin [2] and then Martin and Everaerts [3,4], Verheggen and Everaerts built an ITP instrument in 1968 which formed the basis of commercial production of isotachophoretic equipment [1]. It was not until 1970 however, and after many names being attributed to the technique that the term isotachophoresis was coined [5].

ITP is based upon the separation of ionic sample constituents between two discontinuous electrolytes of differing mobilities. Additionally, this mode of electrophoretic separation is distinguished from others in that there is no background electrolyte to support the electric current and as such no uncoupling of sample zones is observed. Sample introduction is made at the interface (boundary) between an electrolyte with a higher mobility than all sample ions of interest, commonly referred to as the "leading electrolyte" (LE), and an electrolyte of lower mobility, the "terminating electrolyte" (TE). On application of the electric field (set up so that the current level is constant), the initial mixed sample zone separates in order of the individual ion mobilities (in time and thus also along the capillary axis) into a series of contiguous discrete zones, each containing a pure analyte (plus counter-ion) and demarcated by sharp zone boundaries. The potential across each zone is constant but at the zone boundary where the mobility of the ions change instantly, the potential will rise to maintain the same current flow. The net effect of this is that when all the sample ions have separated into their discrete zones they will all travel through the capillary at the same velocity, hence the term isotachophoresis which is derived from the Greek terms "Iso" and "Tacho" meaning constant/equal and velocity, respectively [5].

The step-wise migration of ions under the influence of the applied potential provides an important facet of ITP. Should an ion diffuse out of its own zone, it will experience the electric potential which is driving the zone into which it has diffused. This will cause it to either accelerate or decelerate back into its own zone depending on the magnitude of the potential. This effect produces a self-sharpened zone which serves to maintain sharp zone boundaries.

The "Kohlrausch Regulating Function", which has been well documented elsewhere [1,6,7] also has a profound effect on the output of an ITP experiment. The consequence of this function is that each individual ionic species present will adapt their concentrations to the concentration of the LE and since the concentration of the LE is known it is possible to calculate the concentration of any individual ion species present. Direct quantitative analysis is thus possible, since the amount of a given ion can be directly measured from the step length as concentration is expressed in g ml^{-1} and the bore of the separation capillary is constant. It is for these reasons therefore that ITP is of considerable assistance in trace analysis and can be seen to be an ideal method of sample introduction.

It is interesting to note however, that the exploitation of isotachophoresis in modern analytical laboratories is somewhat sparse and reports of recent utility of coupled column ITP systems as first designed and built by Everaerts et al. [8] which allow on-line selective clean-up, concentration and removal of unwanted macro-constituents even more so [9–16].

It has been with the limitations of current CE technology in mind and the advantages to be gained by utilising capillary isotachophoresis (cITP) in analyses for increased concentration sensitivity, enhanced sample loadability, facilitation of sample clean-up and concentration, that a novel three-dimensional capillary electrophoresis (3D-CE) instrument based upon the coupled-column ITP instrument of Villa Labeco (Spisska Nova Ves, Slovak Republic) has been designed here in the UK by Simpson. Subsequent construction of the system to the design

specifications outlined was carried out by Villa Labeco. The results below contain a detailed description of the 3D-CE system in addition to results from preliminary evaluation experiments.

3. Experimental

3.1. Chemicals

Hydrochloric acid, sodium hydroxide and Tris were purchased from BDH (Poole, UK). Allantoin, allantoic acid, L-histidine, L-glutamic acid and benzoic acid were purchased from Sigma (Poole, UK) and hydroxyethylcellulose (HEC) from Aldrich (Poole, UK).

All solutions, electrolytes and calibration standards were prepared using triple distilled and deionised water from a Millipore Milli-Q system with a resistivity of 18.2 MΩ.

3.2. Instrumentation

The 3D-CE system utilised is described in detail below. Capillaries employed in experiments were as follows: 1st dimension: PTFE 90 mm×800 μm I.D.; 2nd dimension: quartz 200 mm×300 μm I.D.; 3rd dimension quartz: 200 mm×200 μm I.D. Injections of sample and flushing of capillaries between analyses were made with Hamilton micro-syringes and disposable plastic syringes.

3.3. ITP electrolyte systems

Electrolyte systems utilised in all experiments are described in Table 1. All samples were prepared by dissolving in the leading electrolyte.

4. Description of the 3D-CE instrument

The 3D-CE system is shown in the photograph in Fig. 1 and components outlined in the schematic of Fig. 2. The system consists of the following main subunits:

4.1. Three-dimensional CE separation unit

The 3D-CE separation unit is aligned vertically (Fig. 2) with the modules between each dimension tightly connected via knurled connecting screws, and the unit as a whole mounted onto the front panel of the instrument via adjustable screws allowing for adjustments in height, i.e., for when extra components are inserted or dimensions removed. The dimensions of the unit are 265×940×275 mm, with a mass of 25 kg. In its standard configuration, the separation unit consists of the following modules: an injection valve with the terminating electrode compartment; a PTFE preseparation column (first dimension) complete with on-column contactless conductivity sensor, bifurcation block and counter-electrode compartment (140 or 90 mm×800 μm I.D.); a PTFE or quartz separation column (second dimension) with on-column contactless conductivity sensor and adaptor for UV detector optical fibres; bifurcation block

Table 1
ITP electrolyte systems utilised (HEC=hydroxyethylcellulose and LE and TE are the lead and terminating electrolytes, respectively)

	Electrolyte system 1		Electrolyte system 2	
	LE	TE	LE	TE
Solvent	Water	Water	Water	Water
Anion	Chloride	Tris	Chloride	Tris
Concentration	10 m*M*	1 m*M*	10 m*M*	8 m*M*
Counter-ion	Histidine	Glutamic acid	Histidine	Histidine
Concentration	10 m*M*	1 m*M*	10 m*M*	10 m*M*
Additive	HEC 0.15%	–	HEC 0.15%	–
pH	6.0	6.0	8.25	8.5

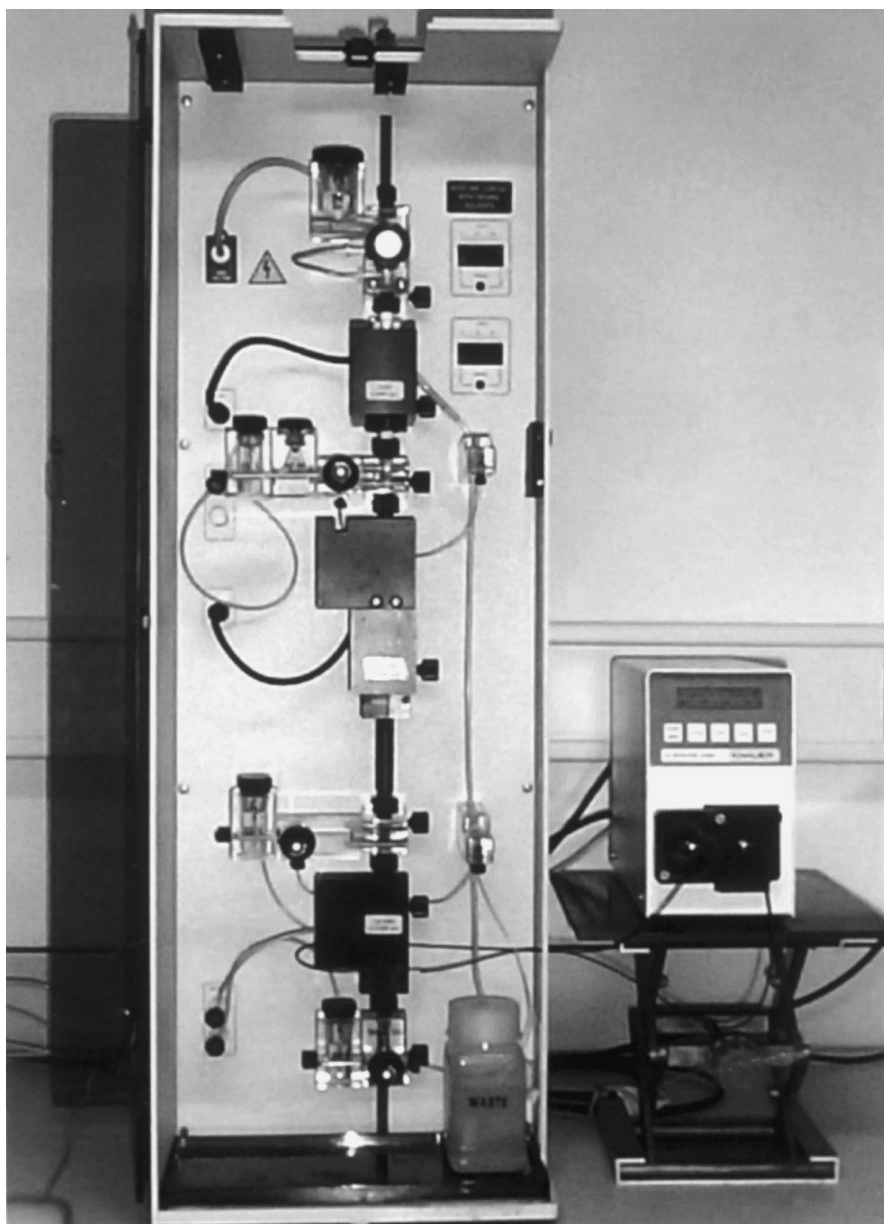

Fig. 1. Photograph of the novel three-dimensional CE instrument.

and counter-electrode compartment (200 mm×300 μm I.D.) and a quartz analytical column (third dimension) with adaptor for UV detector optical fibres and counter-electrode compartment (200 mm× 200 μm I.D.).

4.2. High voltage supply

0–30 000 V variable at set constant current (0–500 μA) with two high-voltage relays allowing switching of electric current between terminating

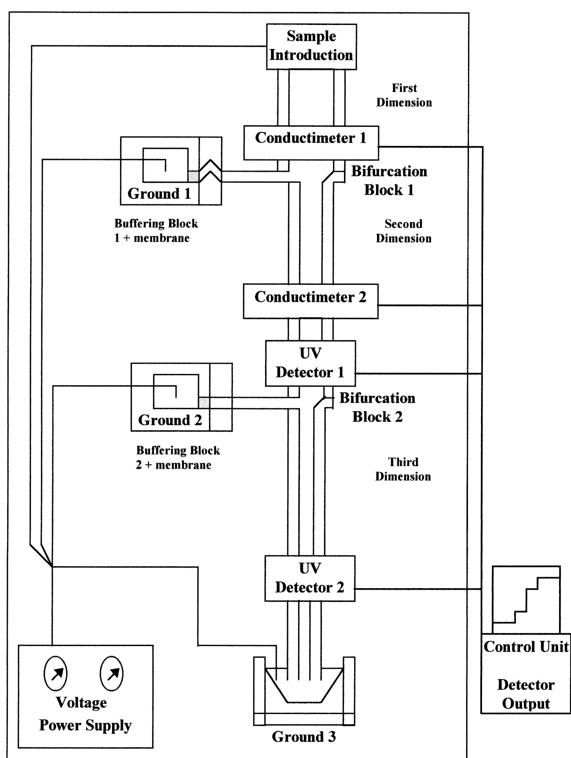

Fig. 2. Schematic of the three-dimensional capillary electrophoresis system components.

electrode and (a) counter electrode for the first dimension and (b) counter electrode for the second dimension, and (c) counter electrode for the third dimension.

4.3. Contactless conductivity detectors

The 3D-CE system has been provided with two contactless conductivity detectors for each of the preseparation and analytical columns, respectively (the details and advantages over conventional conductivity detectors having been previously outlined elsewhere) [17].

4.4. UV absorbance photometric detector

A K-2500 spectrophotometer UV detector (Knauer, Berlin, Germany) was utilised with the instrument and detection is made on-column via an adaptor in either of the second and third dimension capillaries for the optical fibres which transmit and receive the light. The spectrophotometer has a working range between 190 and 740 nm.

4.5. Control unit

The system is controlled via Windows 95 compatible software.

5. Results and discussion

5.1. Initial evaluation of the 3D-CE instrument

After the initial set-up of the analyser, the following parameters were assessed: (1) hydrodynamic flow, (2) injection repeatability, (3) limit of quantitation, (4) linearity and (5) accuracy.

5.2. Hydrodynamic flow

The degree of any hydrodynamic flow in a CE system is an important factor to determine. In order to investigate the degree of hydrodynamic flow in the new 3D-CE system, an experiment was conducted to evaluate the effect of introducing a time interval between injection of sample into the valve (which was subsequently switched onto the capillary) and application of the high voltage. Five injections of the terminating electrolyte were made with two procedures wherein either the application of the high voltage and switching of the valve to the capillary were simultaneous (injection procedure 1) or a 20-s time interval after switching the valve to the capillary was introduced prior to application of the high voltage (injection procedure 2). For this experiment, electrolyte system 1 (Table 1) was utilised and results obtained are shown in Table 2 where the times for the terminating ion to reach the conductivity detector in both the upper and lower dimensions are shown along with related statistics [standard deviation, standard error and relative standard deviation (RSD)]. It was concluded that the injection procedure was important in terms of reproducibility and that a small amount of hydrodynamic flow may occur in the injection valve prior to application of the high voltage, with the best pro-

Table 2
Results and related statistics for investigation into the presence of hydrodynamic flow by assessment of injection repeatability of terminating ion in the first and second dimensions using two injection procedures and electrolyte system 1 (Table 1)[a]

	1st Dimension, migration time (s)		2nd Dimension, migration time (s)	
	Injection procedure 1	Injection procedure 2	Injection procedure 1	Injection procedure 2
INJ 1	233.00	235.40	526.45	545.65
INJ 2	232.25	251.45	506.20	495.50
INJ 3	233.45	234.40	472.10	458.15
INJ 4	235.05	234.40	484.55	603.30
INJ 5	233.95	235.45	512.60	490.30
Mean	233.34	238.22	500.38	4518.58
SD	1.05	7.41	21.87	56.78
S.E.	0.47	3.32	9.78	25.39
RSD (%)	0.45	3.11	4.37	10.94

[a] SD=Standard deviation, S.E.=standard error and RSD=relative standard deviation. Operating conditions: 1st dimension: 250 μA; 2nd dimension: 50 μA.

cedure (i.e., that yielding lowest RSDs) to be the one with no time interval between sample introduction and application of high voltage to the capillary.

In addition, a second investigation to determine hydrodynamic flow in the preseparation and separation capillary (first and second dimension) during analyses was performed. Lead and terminating ion were ''run'' through both dimensions (utilising electrolyte system 1 from Table 1) and this procedure was interrupted midway through each dimension for a period of 50 s. Results obtained indicated there to be no detrimental effect on the run times after taking this 50-s period into account unlike that seen with the introduction of a time interval between sample introduction and application of high voltage to the capillary at the injection valve.

In conclusion, providing the injection procedure is accurately replicated, i.e., the sample valve is switched and high voltage applied to the capillary simultaneously, then results for migration times should be repeatable, even when the high voltage is interrupted mid-run for, e.g., removal of zones to waste via the bifurcation blocks. It is relevant to note that although migration time is often not a qualitative parameter utilised in ITP, it is a necessary and important pre-requisite to have repeatable migration times when calculating timed intervals for zone cutting in sample clean-up and/or preparative experiments.

5.3. Repeatability

The ability of the instrument to yield repeatable results (both qualitative and quantitative) in terms of conductivity (zone length and relative step height) and UV response (peak area) has been investigated. Repeat injections of four different mixture ratios (5:4; 5:1; 1.5:5; and 5:2.5 m*M*) of benzoic and phenylacetic acid (internal standard), respectively, were analysed (using electrolyte system 1 from Table 1) with the conductivity response from the second dimension and UV response from the third dimension recorded. Table 3 contains normalised conductivity zone length and UV peak area:height ratios with the related calculated statistics, e.g., mean, standard deviation, standard error and RSD. From the results shown it can be seen that typical RSDs calculated for the relative step heights (RSHs) of sample ions are between 0.50 and 1.85%, which are far superior to those seen for the normalised conductivity responses (1.38–4.63%) and UV responses (2.72–6.04%). For qualitative purposes this indication of relative step height reproducibility is excellent, facilitating the identification of sample ions especially for sample clean-up/concentration or preparative work.

Additionally, 10 replicate 3 μl and 30 μl injections (i.e., partially and filled loop volumes, respectively) of a solution of benzoic acid (1 m*M* and 0.2 m*M* solutions, respectively) were made,

Table 3
Repeatability of zone lengths and relative step heights for four mixtures of benzoic acid and phenylacetic acid (internal standard) in the second (conductivity) and third (UV) dimensions using electrolyte system 1 (Table 1) with concentration ratios employed being 5:4; 5:1; 1.5:5; and 5:2.5 m*M* for benzoic acid:phenylacetic acid, respectively[a]

Solution	Response	*n*	Mean	SD	S.E.	RSD (%)
A	Zone length	6	1.25	0.022	0.009	2.53
	UV	6	1.26	0.033	0.133	2.77
	RSH, benzoic acid	6	0.73	0.006	0.002	0.79
	RSH, I.S.	6	0.85	0.012	0.005	1.36
B	Zone length	5	5.55	0.274	0.123	1.38
	UV	5	6.04	0.366	0.164	6.04
	RSH, benzoic acid	5	0.70	0.012	0.005	1.85
	RSH, I.S.	5	0.80	0.017	0.008	1.85
C	Zone length	5	0.25	0.011	0.005	4.63
	UV	5	0.26	0.007	0.003	2.72
	RSH, benzoic acid	5	0.68	0.004	0.002	0.50
	RSH, I.S.	5	0.78	0.005	0.002	0.67
D	Zone length	5	2.04	0.091	0.040	4.45
	UV	5	2.07	0.105	0.047	5.18
	RSH, benzoic acid	5	0.70	0.013	0.006	1.82
	RSH, I.S.	5	0.82	0.017	0.008	2.12

[a] Zone length=zone length ratio benzoic acid: internal standard, RSH=relative step height, I.S.=internal standard and UV=peak area:height ratio between benzoic and phenylacetic acid for third dimension. (*n*=Number of injections, SD=standard deviation, S.E.= standard error, and RSD=relative standard deviation). Operating conditions: 1st dimension: 250 μA; 2nd dimension: 50 μA.

these being on different days with fresh sample and leading and terminating electrolytes (electrolyte system 1 from Table 1) on each occasion, and both zone length and RSH response recorded from the first dimension and related statistics (as above) calculated (Table 4). The results from this investigation provide insight into the reproducibility between injections from a filled and partially filled sample loop as well as an indication of inter- and intra-assay variability. Results shown in Table 4 indicate intra-assay variability (in terms of RSD) using two different injection procedures, of 2.53 and 3.31% for zone length response. Inter-assay reproducibility assessed from RSH response was 7.98% (RSD), with the intra-assay variability being 0.18 and 0.75% (RSD). These data indicate that both injection procedures are reproducible and again with the RSH response reproducibility being far superior.

From these results it can be seen the 3D-CE system produces reproducible results in terms of conductivity and UV responses for both inter- and intra-assays. The three-dimensional CE system as such therefore is applicable for methods incorporating sample clean-up and concentration steps.

5.4. Limit of quantitation

The 3D-CE system allows for exploitation of concentration cascades and continuous sampling in addition to volume coupling in two dimensions where the decreased inner diameter of sequential capillaries results in increased detector sensitivity. In preliminary experiments, utilising allantoin (a compound which is difficult to detect due to the lack of strong absorption bands around 260 nm [17]) for which a limit of detection of 5 μ*M* [18] has been previously reported using micellar electrokinetic chromatography (MEKC) methodology, we have so far been able to repeatably detect 200-fold lower concentrations with ease via appropriate choice of leading electrolyte concentration (electrolyte system 2, Table 1) with no concentration cascades or

Table 4
Inter- and intra-assay injection reproducibility results and related statistics for 30 μl and 3 μl injections of benzoic acid (electrolyte system 1)[a]

Injection No.	Day 1		Day 2	
	3 μl injection zone length (s)	3 μl injection RSH (V)	30 μl injection zone length (s)	30 μl injection RSH (V)
1	3.25	0.760	12.70	0.885
2	3.50	0.759	12.05	0.884
3	3.20	0.759	12.15	0.881
4	3.30	0.757	12.45	0.881
5	3.35	0.765	12.15	0.882
6	3.45	0.751	12.55	0.882
7	3.20	0.751	12.95	0.882
8	3.20	0.752	12.30	0.880
9	3.30	0.748	12.15	0.881
10	3.20	0.749	12.80	0.884
Intra-assay variability				
Mean	3.30	0.755	12.43	0.882
SD	0.11	0.006	0.31	0.002
SE	0.03	0.002	0.10	0.001
RSD (%)	3.31	0.748	2.53	0.184
Inter-assay variability (RSH)				
Mean	0.819			
SD	0.065			
SE	0.015			
RSD (%)	7.980			

[a] RSH=Relative step height, SD=standard deviation, S.E.=standard error and RSD=relative standard deviation. Operating conditions: 1st dimension: 350 μA.

continuous sampling techniques. These modifications to the method are currently being investigated in order to further improve the limit of quantitation (LOQ), and so far the only limiting factor which has been experienced in similar quantitative analyses carried out in our laboratory is the significantly higher electric fields incurred and subsequent bubble formation in the system when dealing with extremely diluted electrolytes. It is important to note that since ITP analyses operate with a discontinuous electrolyte system and it is zone lengths and relative step heights which are the quantitative and qualitative responses rather than peak areas, the calculated method of obtaining an LOQ, i.e., the sample concentration calculated to produce a signal ten times the signal-to-noise ratio, cannot be applied (unless the differential of the conductivity signal is utilised). However, the measured LOQ which is the lowest sample concentration that can be precisely and accurately measured is more appropriate.

Preliminary experiments to investigate the increase in sensitivity and resolution achieved resulting from the addition of the third dimension have been carried out. By comparing UV responses recorded from the second and then third dimensions of the same biological sample, any differences in resolution, or number of components separated may be observed. Fig. 3a and b show both the conductivity and differentiated conductivity signals obtained for a urine–water (1:10, v/v) sample in the first and second dimensions, respectively, where an undesired macro-component (not yet characterised) has been removed via the ground-switching technique after detection in the first dimension and Fig. 3c the UV trace for the third dimension analysis of the same sample. From these results it is clear that the addition of a third dimension improves the resolution for sample containing complex matrices. The improvement in resolution and number of components detected after transfer from the second to the third

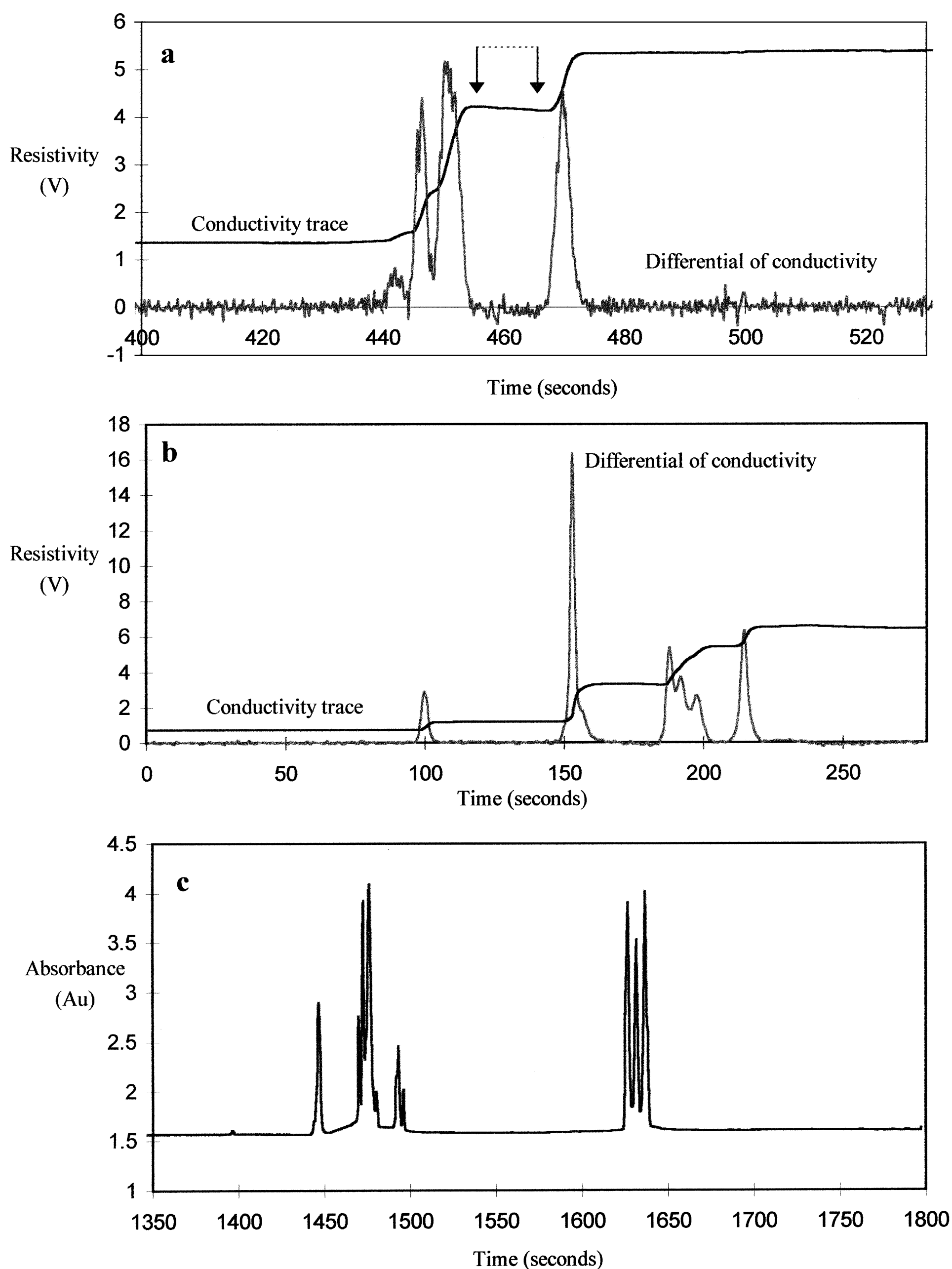

Fig. 3. Conductivity and UV electropherograms for a sample of urine (diluted 1:10 with water) analysed in three dimensions where: (a) first dimension conductivity and differentiated signal and portion enclosed within arrows representing portion of zone removed by ground switching, (b) second dimension conductivity and differentiated signal for zones transferred from first dimension and (c) third dimension UV signal (220 nm) for zones transferred from second dimension. Operating conditions: 1st dimension: 250 μA; 2nd dimension: 50 μA; 3rd dimension: 30 μA.

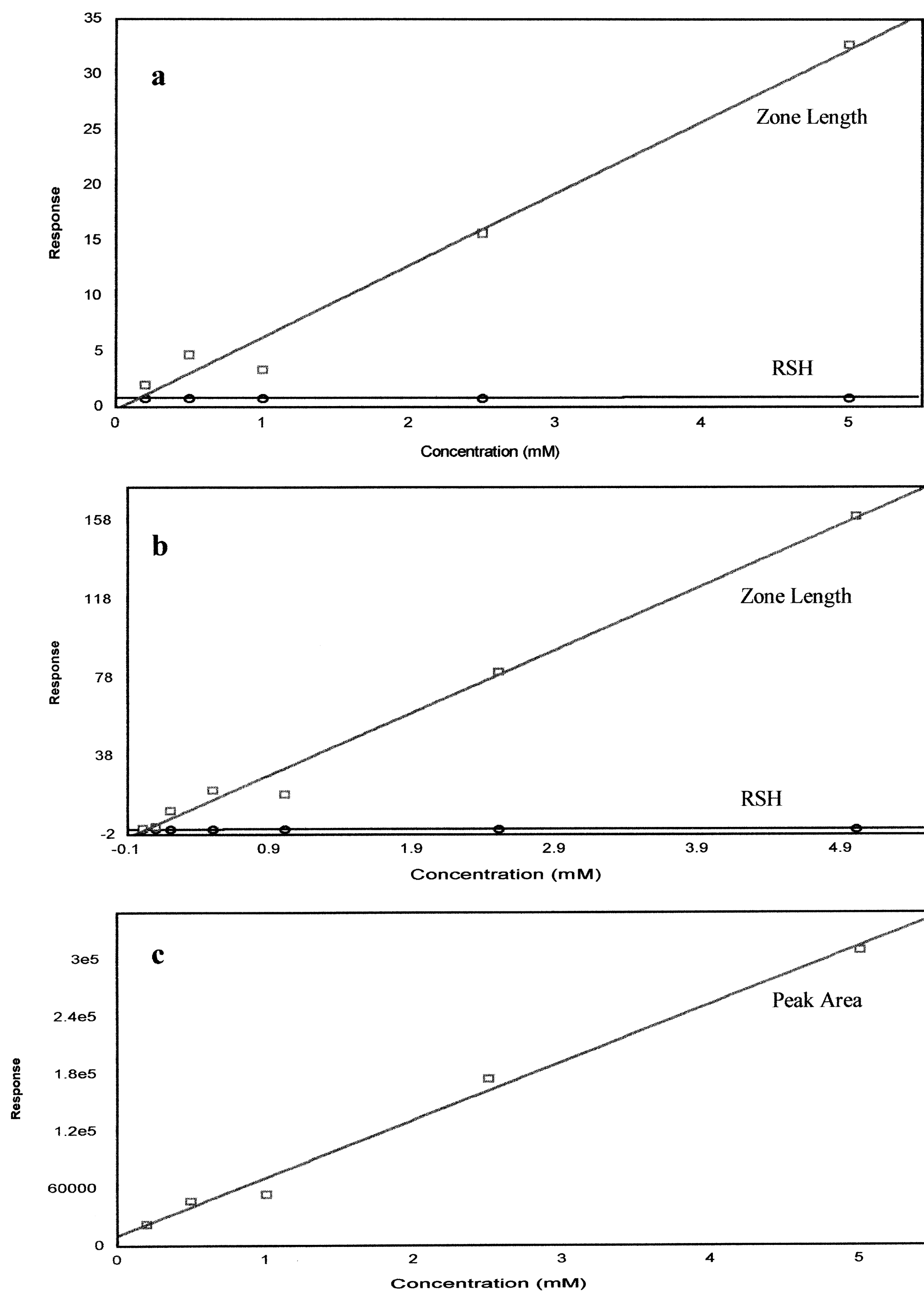

Fig. 4. Graphs showing linearity of detector response for a sample of benzoic acid analysed using electrolyte system 1 (Table 1) and concentration ranges as follows: first dimension: 0.5–5 m*M*; second dimension 0.01–5 m*M*; and third dimension 0.2–5 m*M*. (a) First dimension contactless conductivity detector [responses=relative step height (RSH) and zone length], (b) second dimension contactless conductivity detector [responses=relative step height (RSH) and zone length], and (c) the third dimension UV (response=peak area). Operating conditions: 1st dimension: 350 μA; 2nd dimension: 70 μA; 3rd dimension: 40 μA. Equations for the line in each case are: a: $y=0.15x+0.07$, $R=0.991$; b: $y=0.03x+0.02$, $R=0.995$; and c: $y=0.00002x-0.15$, $R=0.995$.

Table 5
Percentage recoveries of benzoic acid, allantoin and allantoic acid spiked human urine in three dimensions where the 1st and 2nd dimensions utilise contactless conductivity and the 3rd dimension UV detection[a]

Sample	1st Dimension	2nd Dimension	3rd Dimension	*n*
Benzoic acid	97.46	99.39	98.14	3
Allantoin	86.79	98.52	–	5
Allantoic acid	75.84	82.99	–	5

[a] Operating conditions as in Fig. 3 for benzoic acid. Operating conditions for allantoin and allantoic acid samples: 1st dimension: 250 μA; 2nd dimension: 30 μA.

dimension was not observed when continuous capillary diameters were utilised in both dimensions, hence eliminating increased separation volume as the cause of improvement rather than sequential decrease of capillary diameter.

5.5. Linearity of contactless-conductivity response

The calibration curve plotted from the analysis of five standard solutions of benzoic acid are shown in Fig. 4a and b, respectively. Results observed indicated the contactless conductivity detectors within each of the first two dimensions to be responding linearly (i.e., linear correlation coefficients achieved being >0.99) in addition to yielding a highly repeatable relative step height response.

5.6. Accuracy

In order to assess typical accuracy achievable in each of the three dimensions of this system, a known concentration of benzoic acid (2.5 m*M*), allantoic acid and allantoin (both 2.5 m*M*) were spiked in dilute human urine. Blank urines were additionally prepared in order to calculate percentage recoveries of each analyte ion. Results for percentage recoveries are shown in Table 5 where the recoveries are calculated from zone lengths observed at the correct RSH for each sample ion.

6. Conclusions

At present capillary zone electrophoresis (CZE) and MEKC are currently the most widely employed of the various CE techniques. However, these techniques are disadvantaged by factors such as limited (nl) sample loading and sub-millimetre detection path lengths which result in relatively high concentration UV detection limits for most analytes ($>10^{-6}$ *M*). For these reasons, biological samples containing physiologically relevant levels of analyte can often prove difficult to analyse due to the excess of other components present in high concentrations, e.g., chloride ions. In these cases it is necessary to perform various clean-up or concentration techniques or to employ alternative detection techniques [18]. The novel 3D-CE system presented in this paper has been designed to overcome these specific problems, whilst additionally providing more scope and variability in selection of electrophoretic modes for method development purposes over that of previously described coupled-column systems [10–16] as various combinations of cITP, CZE and MEKC may be utilised.

In conclusion, the successful design, construction and evaluation of the 3D-CE system have been described. The novel system has been assessed in preliminary experiments to yield repeatable, accurate and linear responses under the stated method conditions. The system facilitates selective on-line sample clean-up and pre-concentration via cITP in addition to providing the means whereby multi-mode electrophoresis may be achieved with four independent detection capabilities.

The exploitation of this system should be of particular importance for researchers involved with trace analyses in clinical, environmental and pharmaceutical fields.

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