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Application of a digital signal enhancement processor to investigate improvements in sensitivity for capillary electrophoresis and capillary electrochromatography applications

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Abstract

Electroseparation techniques have been very successful in reducing analysis time and improving the resolution of separation. However, a major drawback has been their inherent lack of concentration sensitivity. Several approaches have been adopted to improve sensitivity requiring the use of either expensive detectors, specially adopted flow cells or chemical stacking techniques that are highly dependent on the buffer system and sample matrix. The approach described in this paper employs a signal enhancement processor to significantly improve the signal-to-background noise, without the use of any sample pre-treatment steps or the loss of peak resolution. The reduction in baseline noise also extends the linear dynamic range of measurement. Crown Copyright © 1999 Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Over the last few years there has been growing interest in the use of capillary electroseparation techniques, due to their high separation efficiency and resolution compared to liquid chromatographic methods, and because they can provide rapid analysis with minimal sample and solvent consumption. There are many different modes of capillary electroseparations, with the most recent development being a technique referred to as electrochromatography (CEC) [1–5]—in essence a combination of simple electrophoresis (capillary zone electrophoresis) and high-performance liquid chromatography (HPLC). The various operational modes allow electroseparations to be applied to a very wide range of measurement problems. However, HPLC still dominates as the method of choice for analysing non-volatile components in the majority of laboratories. There are a number of reasons why this remains the case, the principal technical one being the poorer measurement sensitivity of electroseparations.

Although high mass sensitive detection is achievable in capillary electroseparations, the concentration sensitivity is generally poor. This is because the diameter of the capillary necessitates injection of small sample volumes (nanoliters). The need to determine analytes at low concentration, e.g., environmental, biological (including DNA analysis in the ng ml^{-1} range) is commonplace [6]. Improving sensitivity in electroseparations is thus important in

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order to fully utilise the inherent potential of the techniques for these types of applications.

There have been a number of approaches to enhance the measurement sensitivity of capillary electroseparations [7]. Improved detectors [7,8] is one approach, where for example both mass spectrometry and laser-induced fluorescence (LIF) have been used. There is currently much interest in the hyphenation of mass spectrometry and electroseparations, but a reliable commercial interface has yet to be developed, and hence coupling the two techniques together remains an unsolved issue [8]. Fluorescence or LIF detection [7,9] can achieve levels of sensitivity in the sub-pg ml^{-1} range, however, only about 20% of naturally occurring compounds fluoresce: also, in practice current laser technology only allows the use of a limited number of wavelengths for excitation. Therefore, derivatisation is normally required with LIF leading to increased sample preparation time.

A number of approaches have also been adopted to improve the sensitivity of the most commonly used detector in electroseparations (UV–visible absorption), where conventionally the detection limit has been in the $\mu g\ ml^{-1}$ range. These approaches have included both alteration to the detection cell to increase the optical path length (e.g., Z-cells [10] and extended light path capillaries [11]) and mechanisms for increasing the concentration of the sample band within the capillary. The former has proved successful but at the expense of reduced resolution. The second can be further sub-divided into concentration outside of the capillary (solid-phase extraction, etc.), which increases the sample preparation time, and concentration within the capillary using hyphenated techniques such as isoelectric focusing, isotachophoresis, or by on-column stacking (field enhanced pre-concentration) [8,12–16]. These approaches are sample and buffer dependent, difficult to automate, and tend to increase sample preparation time.

Finally it is possible to improve measurement sensitivity by reducing the level and increasing the stability of the background signal from the detectors. This signal emanates from a number of potential sources, including impurities in the buffer and electrical pick-up in the detector wiring circuitry. A route to background reduction and stability is to digitise the data and use mathematical algorithms to smooth out the background noise and amplify the signal. A system for this has been developed by Thomas Swan [17] (the ID/10 signal enhancement processor) which continually gathers data, performs analogue to digital conversion, processes the digital signal and then outputs the data as an analogue signal (and stores the digital signal), with the whole process incurring a delay of only a few seconds. The ID/10 has already been used to achieve lower detection limits in GC and HPLC analysis [17,18] application to capillary electroseparations is equally feasible due to the speed (100 Hz) and resolution of the data collection mechanism.

This paper describes the use of the ID/10 processor to analyse real samples by using capillary electrophoresis techniques. The fast data collection rate and high resolution of the ID/10 processor was used to detect trace impurities in a polymerase chain reaction (PCR) product and a pharmaceutical compound. Improvements in detection are quantified for both capillary gel electrophoresis (CGE) and CEC methods. Signal enhancement from the ID/10 for phenols in tobacco smoke was used to show improvements in the linear dynamic range and data collection for peak area, peak height and retention time.

2. Experimental

2.1. Materials and instrumentation

For CGE analyses, the DNA buffer (89 m*M* Tris, 332 m*M* boric acid, 2 m*M* EDTA pH 7.4) was purchased from Hewlett-Packard, Chemical Analysis Group (Cheadle Heath, Stockport, UK). The buffer component for CEC analysis, disodium hydrogenorthophosphate (analytical reagent 99%) was obtained from BDH (Merck, Poole, UK), orthophosphoric acid from Fisher Scientific UK (Analytical Reagent, Loughborough, UK), and acetonitrile was purchased from Rathburn Chemicals (HPLC S grade, Walkerburn, UK). Buffers were made from 10 m*M* solutions of phosphate salt using ultrapure water (resistivity greater than 18 MΩ cm), with pH adjustment to 2.5 or 3.5 as required. For the

CEC separations, the mobile phase within the capillary consisted of a mix of buffer (pH 2.5 for the measurement of phenols; pH 3.5 for the analysis of the pharmaceutical product) and acetonitrile in the ratio 40:60 (v/v).

A DNA PCR product (304 base pairs: approximately 10 μg ml^{-1} solution of DNA in ultrapure water) was produced in the laboratory by LGCs' Molecular Biology Unit from a DNA constrict Luciferase gene commercially available from Promega Corporation (Southampton, UK); samples (5 mg ml^{-1}) of a weakly basic proprietary product (LY300164) were obtained from Lilly Pharmaceuticals; extracts from tobacco smoke streams were obtained from LGCs' Tobacco Analysis Group. Tobacco extracts and the pharmaceutical compound were prepared for analysis by dissolving in acetonitrile: phenol standards (Aldrich, Gillingham, UK) were prepared from a stock solution (100 mg ml^{-1}) by dilution with acetonitrile.

All buffers, standards and samples were filtered using Pro-Mem 0.45-μm PTFE 25-mm syringe filters (Radleys, hydrophobic solvent resistant), and stored in a refrigerator at 4°C prior to use. Samples, standards and CEC electrolyte mobile phases were sonicated for 5 min at room temperature to remove any air bubbles prior to use.

All electroseparations were performed on a Hewlett-Packard HP^{3D} CE unit (Waldbronn, Germany). CEP-coated capillaries (Hewlett-Packard, Chemical Analysis Group) (48.5 cm×75 μm; inlet-to-detector length of 40 cm) were used for CGE: C_{18} CEC columns were obtained from Hypersil (C_{18} ODS1 33 cm (effective length 25 cm)×50 μm I.D).

2.2. Methods

2.2.1. Capillary gel electrophoresis

The separation of PCR products was performed using a voltage of −16 kV (−330 V cm^{-1}) and UV detection at 260 nm. The column temperature was maintained at 20°C. The samples were introduced into the capillary at the cathodic end by electrokinetic injection (−5 kV, 35 s: approximate on column loading of 1.4 ng). The voltage was then ramped up to the required level over 0.2 min.

2.2.2. Capillary electrochromatography

Separation voltages of 30 kV (909 V cm^{-1}) and 25 kV (758 V cm^{-1}) were employed for the pharmaceutical product and tobacco smoke extract, respectively. The column temperature was maintained at 25°C. Detection was carried out at 240 nm (pharmaceutical compound) or 200 nm (tobacco smoke).

The samples were introduced into the capillary at the anodic end by electrokinetic injection (5 kV, 4 s) or by a combination of voltage and pressure (5 bar at 3 kV, 4 s). The applied voltage was then ramped up to the required level over 0.2 min.

2.3. ID/10 signal enhancement processor

In order to provide an analogue output for connection to the ID/10, a Hewlett-Packard D/A conversion board was fitted to the HP^{3D} CE system. This gave the easiest access to the detector response, with the recognised disadvantage that a significant amount of signal handling had occurred prior to reaching the enhancement processor. It is believed that better performance will be produced by coupling the ID/10 directly to the detector sensor's analogue signal.

The ID/10's analogue output was connected to a second input channel on the HP Chemstation to allow direct comparison between the raw and enhanced signals. In addition a software utility was used to load the data captured in the ID/10 in a digital form directly into the Chemstation: by doing so we were able to exploit some of the additional resolution provided by the ID/10's 24-bit A/D converter by avoiding the limitations imposed by the Chemstation input A/D stage.

The signal enhancement processor (ID/10) provides external control of a number of performance parameters such as the enhancement, sampling, rate and amplification factor.

The enhancement defines the level of algorithms used to smooth the background noise from the detector signal. Sampling rates, from 0.1 to 200 s, can be selected to suit the detector residence time, and amplification of the signal is achieved by a factor of up to 128.

Parameter settings for the three applications studied were: PCR product (enhancement 91, gain 4 and sampling rate 2.0 s); pharmaceutical sample (enhancement 65, gain 64 and sampling rate 6.0 s);

tobacco smoke (enhancement 65, 75 or 80, gain 16 and sampling rate 2.0, 4.0, 6.0 and 12.0 s).

3. Results and discussion

Below are cited examples of case studies used to investigate the most common sensitivity problems encountered with capillary electrophoresis.

3.1. Detection of a PCR product impurity

This shows an application of ID/10's fast data collection rate applied to determine a trace impurity near the peak of interest. Several approaches to the analysis of PCR products by CE [19,20] have been reported. A major limitation, however, has been the lack of detection sensitivity using UV detectors. Fig. 1 shows electropherograms obtained for a typical PCR sample, with and without coupling the detector to the ID/10 processor. An increase in the signal by a factor of 32 was achievable using the ID/10, with the additional resolution of this processor enabling the detection of a 306-base pair PCR product impurity. Further impurities generated from the primers were also observed in the ID/10 electropherogram. The ID/10 enhanced the limit of detection from 1.5 to 0.5 μg ml^{-1} (Fig. 2, electropherogram B).

Figures

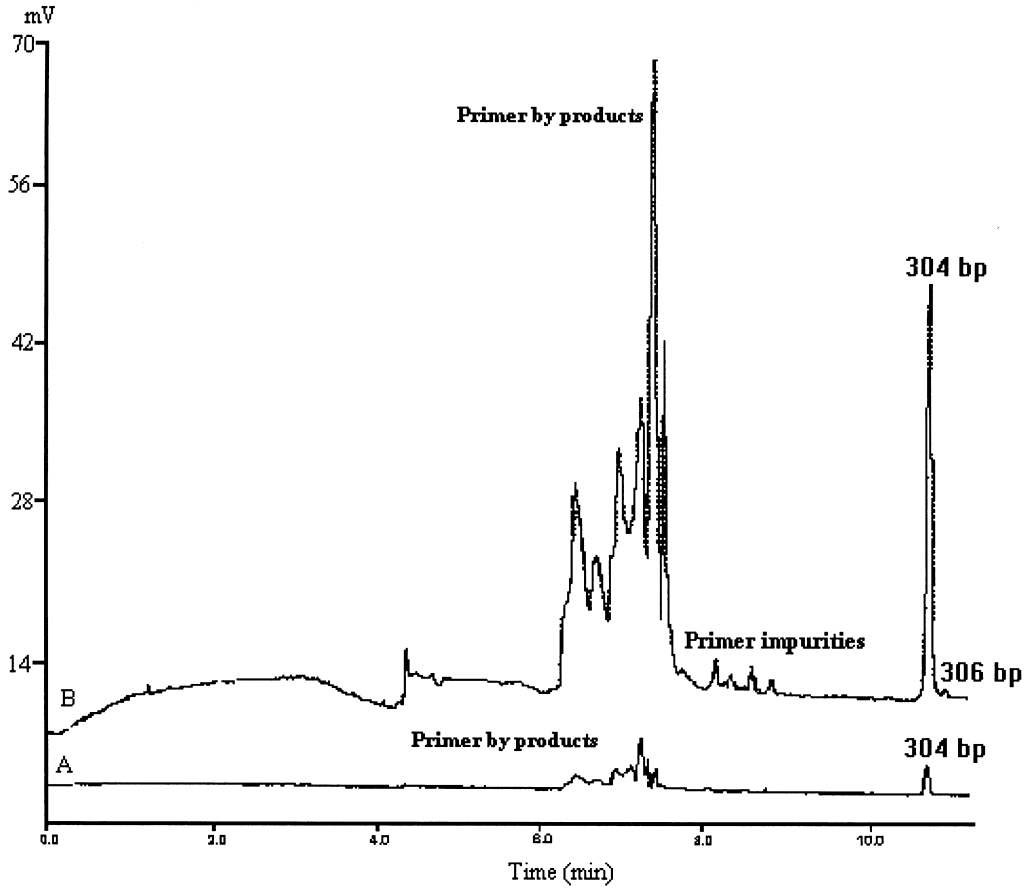

Fig. 1. Electropherograms of a 304-base pair (bp) PCR product (10 μg ml^{-1}) containing a 306-bp PCR product impurity. (A) Data collected on a HP^{3D} Chemstation; (B) data collected using the ID/10 processor.

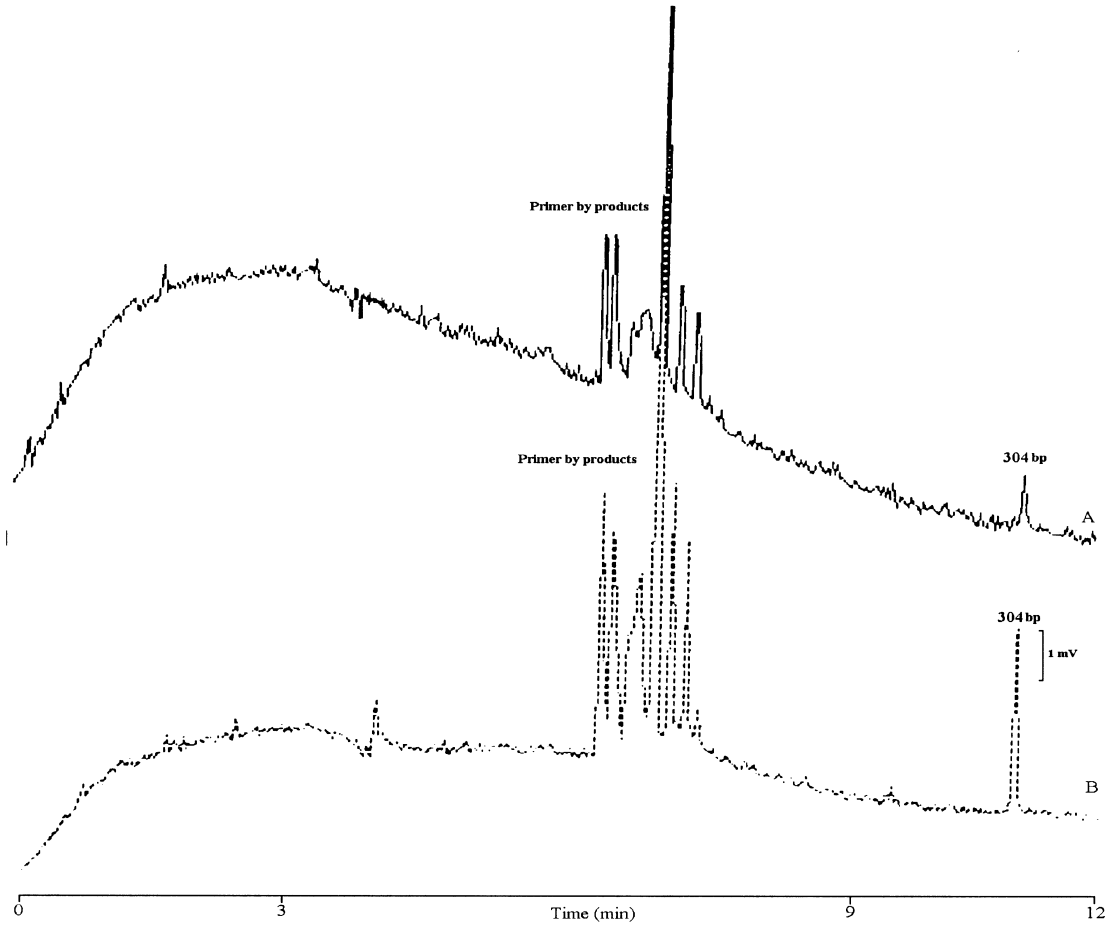

Fig. 2. Comparison of limit of detection (LOD) for a 304-bp PCR product using the ID/10, (A) HP^{3D} Chemstation electropherogram 1.5 mg ml^{-1}; (b) ID/10 electropherogram 0.5 μg ml^{-1} (calculated on a signal-to-noise ratio of 3 the ID/10 provides a LOD of 0.2 μg ml^{-1}).

3.2. Analysis and detection of related impurities of a pharmaceutical preparation

HPLC has been used to characterise this pharmaceutical proprietary product, a similar mobile and stationary phase was used for our CEC work. Table 1 compares the percentage impurity values determined by HPLC with those measured by CEC with and without the use of the ID/10 processor. The ability to detect lower quantities of impurities using the ID/10 led to an increase in the overall levels measured. The improvement in the CEC data achieved by connection to the ID/10 are illustrated in Fig. 3, the limit of detection was enhanced by a factor of 5, from 1.0 to 0.2 μg ml^{-1}.

3.3. Analysis and detection of phenols in tobacco smoke

A CEC method has been developed for the

Table 1
Comparison of total impurity levels for HPLC and CEC

Total impurity levels (%, w/w)	
HPLC	CEC
1.03[a]	0.85[b]
1.03[a]	0.98[c]

[a]Data for $n=6$ HPLC runs.
[b]HP data for $n=6$ CEC runs.
[c]ID/10 data for $n=6$ CEC runs.

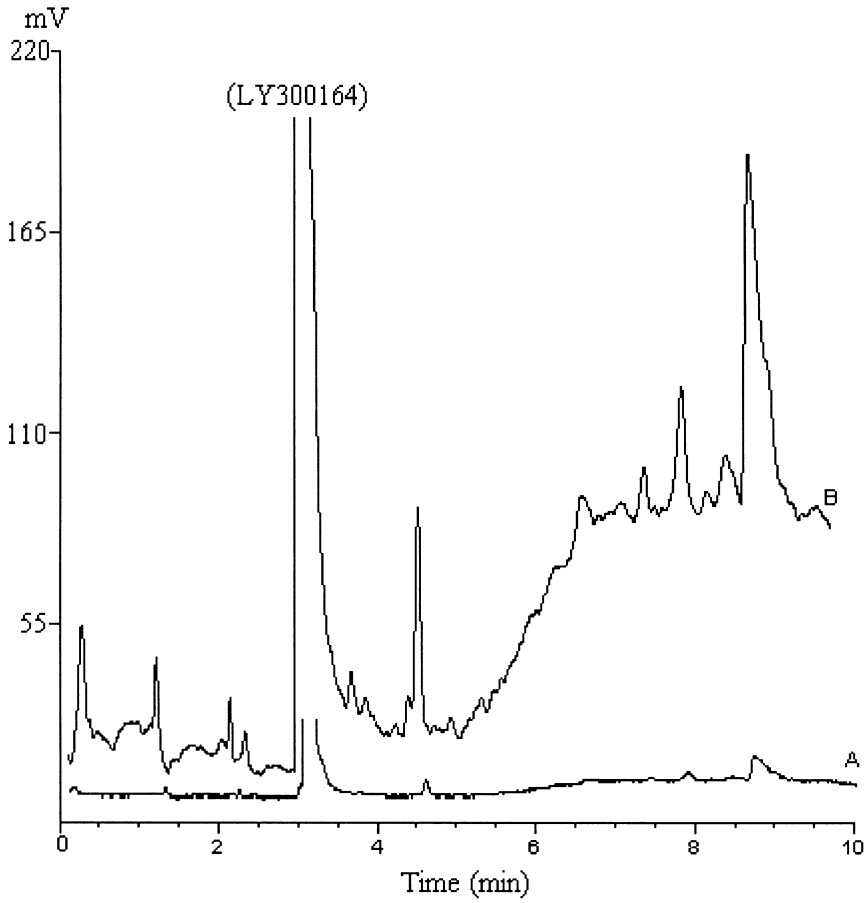

Fig. 3. Determination of related impurities for a pharmaceutical proprietary (LY300164) compound. (A) HP[3]D Chemstation electrochromatogram; (B) ID/10 electrochromatogram. An electrolyte mobile phase acetonitrile–10 m*M* phosphate, pH 3.5 (60:40, v/v) at 30 kV, was employed.

analysis of phenols in tobacco smoke and has been validated using both standard solutions and extracts from filtered cigarette smoke (Fig. 4). A comparison of the data collected with and without the ID/10 shows comparable performance in terms of reproducibility and linearity of response (Table 2). However, the ID/10 was found to extend the linear dynamic range (Table 3) from 50–3.0 to 50–0.25 μg ml^{-1}, and to lower the limit of detection from 1 to 0.2 μg ml^{-1} (Fig. 5, Table 4).

A comparison performed at the 3-μg ml^{-1} level for all six phenol compounds (Table 5) indicates improvements in the R.S.D. for peak height, area, corrected area and retention time by a factor of about 5 using the ID/10. This was attributed to the lowering of the signal-to-noise ratio providing a better baseline for measurement.

4. Conclusions

The ID/10 processor has been shown to significantly improve the sensitivity of the HP[3D] CE instrument to a comparable performance to that of standard HPLC systems. The advantages of electroseparations, namely enhanced selectivity and separation speed, ease of use and method develop-

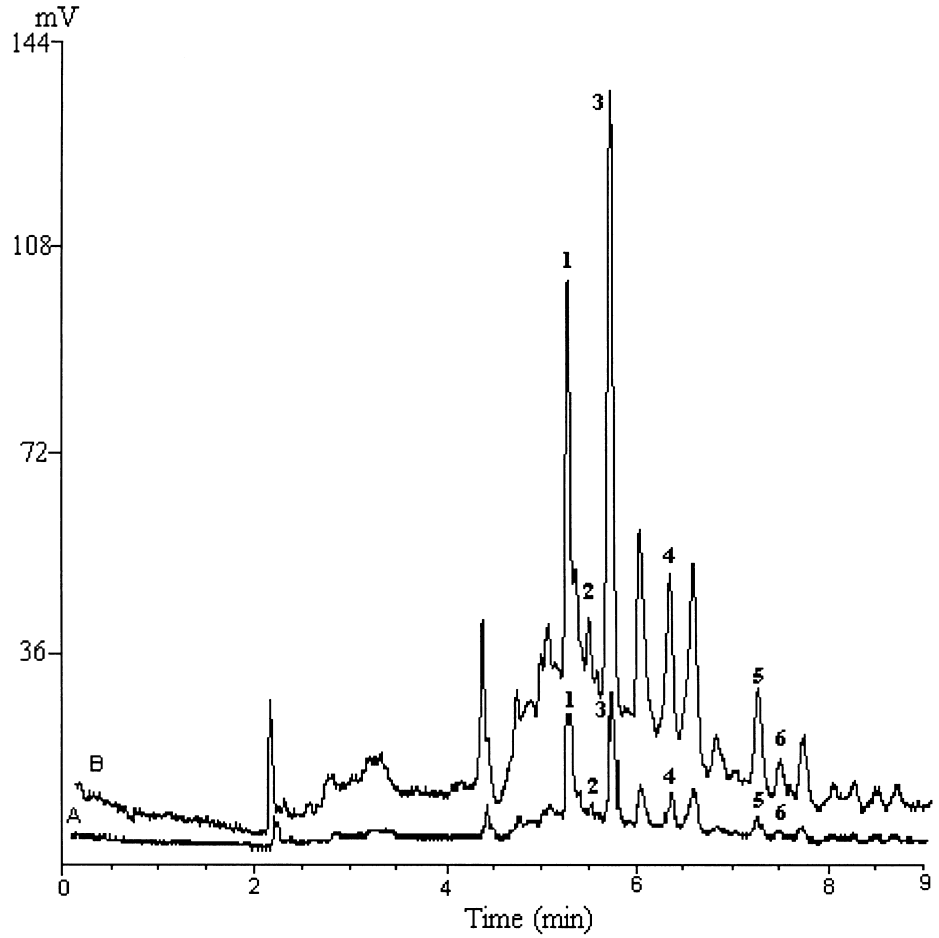

Fig. 4. Electrochromatogram identifying six phenols (1, hydroquinone; 2, resorcinol; 3, catechol; 4, phenol; 5, *m*-cresol and *p*-cresol; 6, *o*-cresol) in a tobacco smoke sample obtained from an laboratory sample of 3.0 μg ml^{-1}, (A) HP^{3D} Chemstation electrochromatogram; (B) ID/10 electrochromatogram.

Table 2
Linear correlation coefficients for phenol determination

	Correlation coefficient (r^2)	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6
Peak height	HP data	0.996	0.999	0.999	0.999	0.999	0.999
	ID/10 data	1.000	0.999	1.000	0.999	0.999	0.999
Peak area	HP data	0.994	0.999	1.000	0.999	0.999[a]	
	ID/10 data	0.998	0.999	0.999	0.999	0.999[a]	
Corrected peak area	HP data	0.994	0.999	0.999	0.999	0.998[a]	
	ID/10 data	0.997	0.999	0.999	0.999	0.999[a]	

Coefficients determined over a concentration range of 50–3 μg ml^{-1} for HP Chemstation data, and 50–0.25 μg ml^{-1} for ID/10 data (n=6).
[a]Total cresols.

Table 3
Comparison of linear dynamic range and limit of detection (LOD) for HP and ID/10 data

	Linear dynamic range (μg ml^{-1})[a]	LOD (μg ml^{-1}) (S/N~3)
HP data	50–3.12	1.00
ID/10 data	50–0.25	0.20

[a]HP data and ID/10 data for n=6 runs.

ment, and lower reagent consumption are all unaffected by coupling to the ID/10. Further advances in sensitivity are believed possible by directly accessing the analogue output of the sensor and also by combining signal enhancement with other detection technologies (e.g., fluorescence detection).

The advances being made in CEC column technology, together with the improved sensitivity of detection, should lead to widespread use of this technique within routine laboratory operations.

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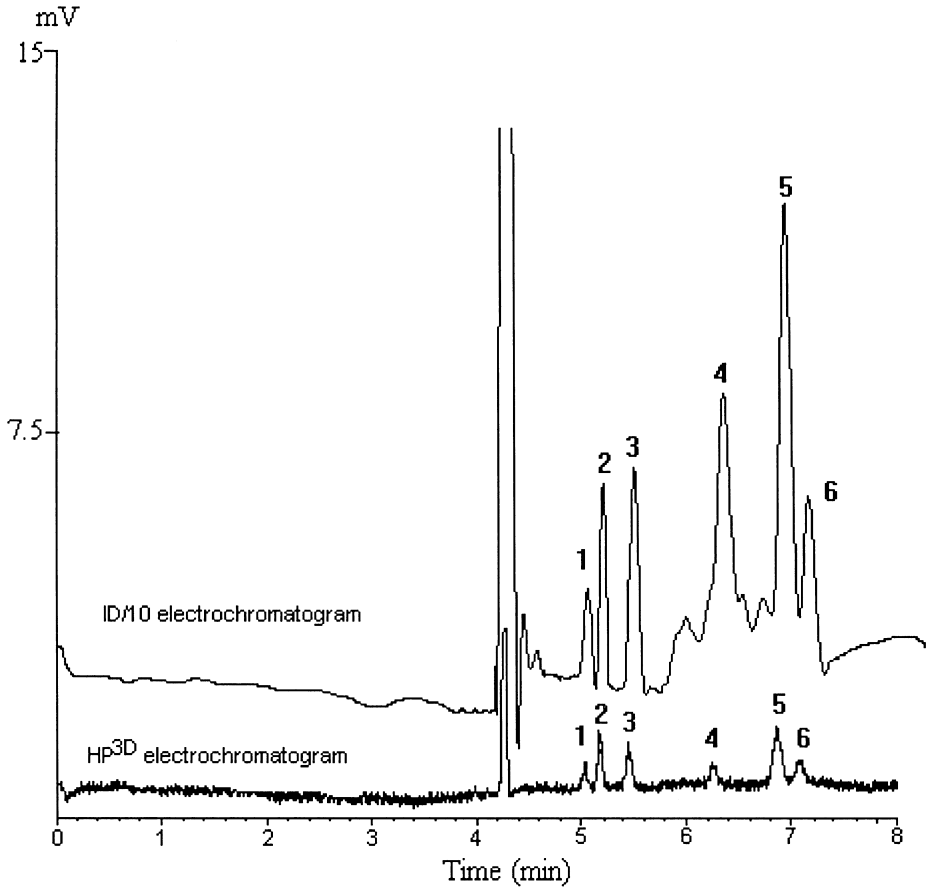

Fig. 5. Electrochromatogram of a six-phenol standard mixture (1, hydroquinone; 2, resorcinol; 3, catechol; 4, phenol; 5, *m*-cresol and *p*-cresol; 6, *o*-cresol). ID/10 electrochromatogram LOD 0.20 μg ml^{-1}, HP^{3D} Chemstation electrochromatogram LOD 1.0 μg ml^{-1}.

Table 4
R.S.D. values at the 0.5- and 0.25-μg ml^{-1} levels for the ID/10 data

	R.S.D. (%)[a]			
	Peak area	Corrected peak area	Peak height	Migration time
0.5 μg ml^{-1}				
Hydroquinone	1.10	1.50	4.01	0.05
Resorcinol	2.34	1.85	5.23	0.05
Catechol	4.55	4.66	3.4	0.00
Phenol	2.92	3.18	7.35	0.09
m-Cresol and *p*-cresol	4.41	4.27	5.81	0.11
o-Cresol	3.32	3.46	6.13	0.19
0.25 μg ml^{-1}				
Hydroquinone	8.68	8.59	11.47	0.06
Resorcinol	3.61	3.52	10.05	0.06
Catechol	12.22	12.19	7.31	0.00
Phenol	3.72	3.92	10.74	0.11
m-Cresol and *p*-cresol	9.29	9.23	9.12	0.13
o-Cresol	6.74	6.74	3.73	0.12

[a]ID/10 data for $n=6$ runs.

Table 5
Comparison of R.S.D. values for phenol peaks at the 3-μg ml^{-1} level for HP and ID/10 data

	R.S.D. (%)[a]			
	Peak area	Corrected peak area	Peak height	Migration time
HP data				
Hydroquinone	4.14	4.06	2.54	0.24
Resorcinol	3.89	3.62	4.62	0.26
Catechol	3.76	3.50	1.86	0.28
Phenol	3.60	3.74	2.56	0.33
m-Cresol and *p*-cresol	2.58	2.56	3.80	0.30
o-Cresol	3.95	3.90	3.84	0.35
ID/10 data				
Hydroquinone	1.98	2.86	1.96	0.10
Resorcinol	1.82	2.67	1.88	0.06
Catechol	0.59	1.53	0.97	0.05
Phenol	1.21	0.79	0.82	0.12
m-Cresol and *p*-cresol	2.01	2.24	1.56	0.15
o-Cresol	2.07	1.71	1.29	0.15

[a]HP data and ID/10 data for $n=6$ runs.

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