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# Determination of nicotine and its metabolites in urine by solid-phase extraction and sample stacking capillary electrophoresis-mass spectrometry

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**Abstract**

The combination of capillary electrophoresis (CE) and mass spectrometry (MS) with solid-phase extraction (SPE) has been used for the identification of nicotine and eight of its metabolites in urine. The recovery of cotinine from cotinine-spiked urine, by C18 SPE, was found to be 98%. Smokers urine (200 ml) was preconcentrated 200-fold via SPE prior to analysis. The sample stacking mode of CE, when compared to capillary zone electrophoresis, was shown to improve peak efficiency by 132-fold. The combination of hydrodynamic and electrokinetic injection was studied with sample stacking/CE/MS. The on-column limits of detection (LOD) of nicotine and cotinine, by this technique, were found to be 0.11 and 2.25 μg/ml, respectively. Hence, LODs of nicotine and cotinine in urine after 200-fold preconcentration were 0.55 and 11.25 ng/ml, respectively.


*Keywords:* Sample stacking; Nicotine, metabolites

## 1. Introduction

Smoking is generally accepted as the major preventable cause of mortality and morbidity in the western world. It is the major cause of cancer mortality in the UK, and may contribute to the causation of many other disease states including atherosclerosis. Thus, smoking cessation may be the most effective strategy for reducing the incidence of cancer, cardiovascular disease and strokes. It appears that although smoking prevalence is declining within the developed world, it is on the increase in most parts of the developing world [1].

The tobacco alkaloid, nicotine, is mainly responsible for its addictive properties [2,3]. Nicotine accounts for approximately 90% of the alkaloid fraction in commercial tobacco, *Nicotiana tabacum*, and is a major component of the particulate phase of tobacco smoke. The presence of nicotine in biological fluids, in humans, is primarily due to exposure to tobacco smoke, the consumption of certain nicotine-containing fruits, such as aubergines, making a negligible contribution [4]. In humans approximately 86% of systemically absorbed nicotine is metabolised to cotinine. The half-life of cotinine is approximately 10-fold greater than that of nicotine [5] and hence cotinine can be

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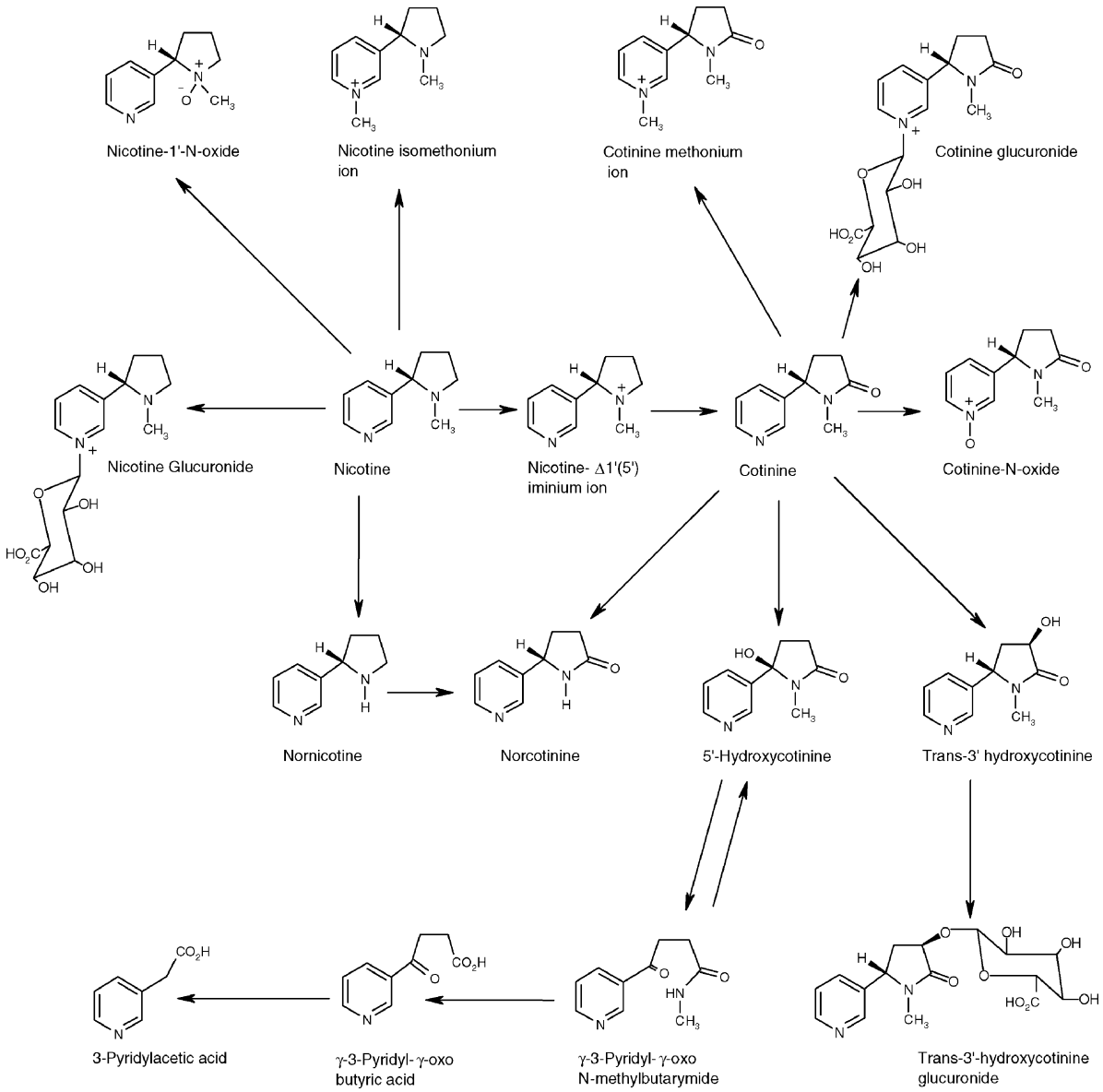


Fig. 1. The major mammalian pathways of nicotine metabolism.

found in greater concentrations than the more rapidly metabolised nicotine [4]. Cotinine has therefore been widely used as the marker of choice for tobacco consumption (Fig. 1).

Currently, the most common analytical techniques employed for the determination of nicotine and its related metabolites in biological fluids are gas chromatography-mass spectrometry (GC-MS) [5,6], high-pressure liquid chromatography (HPLC), radio immunoassay (RIA), enzyme-linked immunosorbent assay (ELISA) [4,7,8] and colorimetric techniques [8]. Of these, GC-MS is the most sensitive and specific method, but is labour-intensive [9]. HPLC methods (including LC-MS) have been used, but require extensive method development due to the low separation efficiencies obtained. Immunoassay techniques are

relatively sensitive but cannot discriminate between several active analytes because of specificity limitations due to cross reactivity with similar molecules [4,8].

In this area of research the application of capillary electrophoresis (CE) is still in its infancy. However, CE offers several potential advantages over GC and HPLC for the analysis of complex mixtures of metabolites; including high separation efficiencies, extremely small injection volumes, short analysis times, rapid method development and low reagent costs [10]. CE may be used to perform highly efficient separations of a wide range of sample types, and the number of theoretical plates in CE may reach several hundred thousand [11].

The main limitation of CE is its lack of sensitivity [11–18]. Detection limits in CE are generally 10–100 times higher than in HPLC [13,14]. The relatively higher limit of detection observed in CE comes from its restricted injection volume (where typical injection volumes are in the nanolitre range) and the short path length for on-capillary detection (when coupled to a UV detector) [10,12]. Thus, a major area of interest in CE is to improve its low concentration sensitivity without sacrificing resolution.

Several techniques have been shown to yield on-column sample concentration and to lower detection limits in CE. These include isotachophoresis [10], sample stacking [15], and filed amplified injection [17]. The most practical way to concentrate a sample is in fact the on-line sample stacking approach. The sample stacking phenomenon was first introduced by Tiselius; and can be achieved through the manipulation of sample and buffer solutions with injection procedures common to any CE instrumentation (Fig. 2).

Palmer et al. [4] recently reported the separation and detection of standards of nicotine and 10 of its metabolites using CE/MS. To date, there are no reported studies, to our knowledge, on the use of CE for the determination of nicotine and its metabolites in clinical samples. Its use has been reported, however, for the quantitative determination of both nicotine in tobacco [19] and tobacco alkaloids (including nictotine) in single plant cells [20] The limits of detection (LOD) reported were, for nicotine in tobacco 286 fg (using electrochemical detection) [19] and for nicotine in plant cells 0.013 pmol (i.e. 2.1 pg) (using ultraviolet detection) [20].

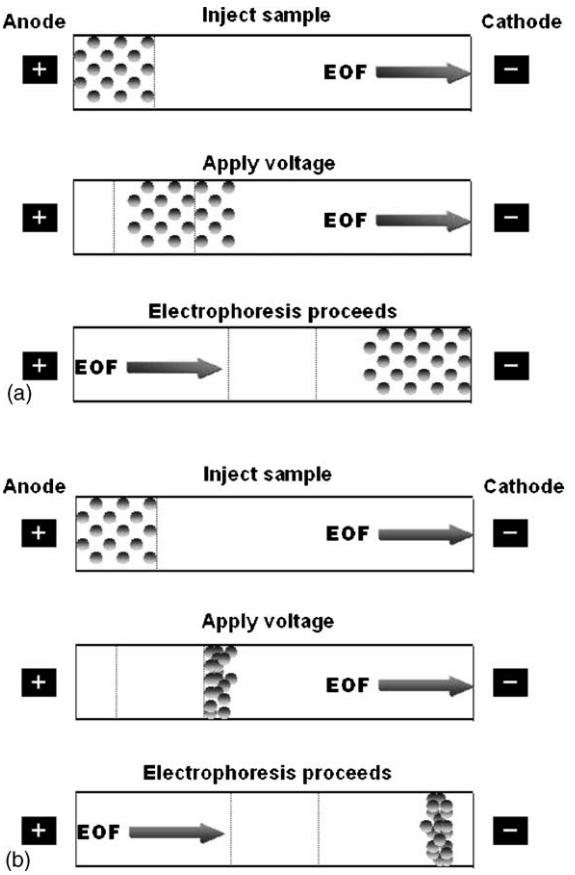


Fig. 2. A comparison of (a) capillary zone electrophoresis and (b) sample stacking. In sample stacking the use of an injection solvent of lower ionic strength than the run buffer results in solute ions forming a narrow stacked zone on application of a voltage. This improves both sensitivity and peak shape.

By combining CE with mass spectrometry (MS) selective identification and also structural elucidation, can be achieved. In this study, we demonstrate the use of solid-phase extraction (SPE) combined with sample stacking CE/MS for the determination of nicotine and its metabolites in urine.

## 2. Experimental

### *2.1. Samples and sample preparation*

Standards of nicotine and its major metabolites were kindly donated by Dr. Peyton Jacob of the Department

of Medicine and Drug Dependence Research Centre, San Francisco General Hospital Medical Centre. These were prepared in buffer for CE experiments or mobile phase for HPLC or one-tenth of the run buffer for sample stacking experiments.

Urine was obtained from five male smokers, labelled A–E, and stored at −20 °C in tissue culture flasks. Two samples, labelled, $C_1$ and $C_2$ were collected from the same subject, but on different days. Prior to extraction urine samples were thawed overnight at 4 °C thoroughly mixed and filtered with a 0.45 μm Supor Acrodisc filter.

### 2.2. Solid-phase extraction

Solid-phase extraction was carried out using a 1 g C18 Isolute cartridge (Jones Chromatography Ltd.). For conditioning purposes 6 ml of methanol followed by 6 ml of deionised water (Milli-Q) were passed through the SPE cartridge sequentially. Then, 100 ml of sample was introduced into the cartridge followed by 6 ml of deionised water. The sample was then eluted with 6 ml of methanol. The eluted product was dried under nitrogen gas. The dried product was reconstituted with 0.5 ml buffer (200-fold preconcentration for CE/MS) or 1 ml mobile phase (100-fold preconcentration for recovery experiment). The reconstituted product was then subjected to ultrafiltration (via a Sorval Tc centrifuge and Vivaspin Concentrator 3000 MW) at 3950 rpm for 20 min.

### 2.3. Buffer preparation

A suitable quantity of ammonium formate (Sigma–Aldrich) was dissolved in the solvent mixture (acetonitrile/deionised water (Milli-Q)); buffer pHs were adjusted with formic acid or HCl (Sigma–Aldrich), as appropriate, and were freshly prepared. Buffers were degassed prior to analysis using a Branson 1210 ultrasonic bath (Branson Ultrasonics, Danbury, CT, USA) and filtered through a 0.2 μm syringe filter (Supor Acrodisk). The buffers prepared were 25 mM ammonium formate (10% acetonitrile, 90 deionised water), which was adjusted to pH 2.5 with formic acid, for CZE; and 10 mM ammonium formate (75% acetonitrile, 25% deionised water) adjusted to pH 2.5 with HCl, for sample stacking experiments.

### 2.4. Recovery experiments

Non-smoker's urine (100 ml) was spiked with cotinine (at 1.25 and 0.625 μg/ml) and subsequently extracted using the SPE method described above. These samples were analysed by LC-MS using a VG Quattro I (Manchester, UK) triple quadrupole mass spectrometer operating in electrospray ionisation (ESI) and selected ion monitoring (SIM) mode.

The HPLC conditions were as follows: column Phenomenex Luna 5 μl C18 column (250 mm × 4.6 mm), mobile phase 15% acetonitrile, 5% methanol and 80% buffer in deionised water (10 mM ammonium acetate, pH adjusted to 4.8 with HCl); flow rate 1 ml/min. A 50:1 split post column was employed with a flow of 20 μl/min being introduced into the mass spectrometer. The injection volume was 10 μl.

### 2.5. CE separation

CE was carried out in a 100 cm, 75 μm i.d. × 365 μm o.d., untreated fused silica capillary (Composite Metal Services Ltd., Hallow, UK). The CE system used (Crystal CE System, Prince Technologies, Lauerlabs, Emmen, The Netherlands) utilizes programmable injection with pressure and voltage. Separations were achieved using +30 kV (a field strength of 350 V/cm). Injections were performed either hydrodynamically (25 mbar/0.2 min) or electrokinetically (30 kV/0.2 min) or hydrodynamically + electrokinetically (25 mbar + 30 kV/0.2 min). Initial conditioning of the capillary took place with 1 M NaOH (5 min) followed by the buffer system (7 min). The capillary was conditioned prior to each run with 1 M NaOH (2000 mbar/1 min) followed by buffer system (2000 mbar/1.5 min). The buffer system was replenished after each run cycle.

### 2.6. CE/MS

A VG Quattro I triple quadrupole mass spectrometer was used throughout. To date, electrospray ionzation (ESI) is the most common technique for coupling CE to MS. In these experiments, CE/ESI/MS coupling was acheived using the co-axial sheath-flow interface developed in-house and previously described by Palmer et al. [4]. The sheath liquid comprised either; 1:1 MeCN/$H_2O$ + 0.1% formic acid, or run

buffer and was delivered by a Harvard Model 11 syringe pump (Harvard Apparatus, Edenbridge UK) at a flow rate of 3–5 μl/min.

Positive ions were generated through the application of 3.5 kV to the probe tip, with a source cone voltage of 25 V. Desolvation was aided by nebulising gas (∼40 l/h) and bath gas (∼225 l/h). Capillaries employed for CZE/MS were 50 μm i.d. × 375 μm o.d. and 100 cm in length.

Data was either acquired by selected ion recording of the analyte protonated molecules employing a 1 Da window with a dwell time of 0.08 s or in full scan mode from 70 to 200 Da in 0.5 s.

## 3. Results and discussion

Table 1 summarises the data obtained from the LC/MS recovery experiments. Percentage recoveries were calculated from a linear calibration plot of eight points, recorded in duplicate. The $R^2$ value for the calibration data was 0.9993. The 1.25 and 0.625 μg/ml cotinine-spiked urine samples were analysed in triplicate and an overall mean percentage recovery of 98% was obtained.

Fig. 3(a) shows non-optimised CE/MS mass electropherograms obtained from the analysis of a standard mixture of the major nicotine metabolites and Fig. 3(b) the corresponding optimised separation for comparison. We have found that several factors are important in obtaining reproducable high quality electropherograms when using the co-axial CE/MS electrospray interface (Table 2).

In order to obtain good signal to noise in CE/MS and hence good detection limits a stable electrospray is required. We were able to improve the stability of the electrospray over our earlier work [4] by: (a) withdrawing the fused silica capillary into the stainless steel sheath capillary until it was level with the sheath capillary and (b) replacing the previously used sheath liquid, i.e. 1:1 acetonitrile/deionised water + 0.1% formic acid, with the buffer system. By withdrawing the fused silica capillary into the stainless steel capillary, the length of the fused silica that can be surrounded by the sheath capillary is maximised. Hence, the maximum electrical contact between the sheath capillary and the fused silica capillary is obtained. The use of the run buffer as sheath liquid improved beam stability presumably due to the homogeneous nature of the sheath liquid and the buffer reducing mixing effects.

A second major issue in obtaining reproducible high quality CE/MS data using the co-axial arrangement is drying out of the electrophoresis capillary. One possible approach to solving this is the application of a supplementary pressure to the head of the column [21]. In our earlier work [4], a supplementary pressure of 50 mbar was in fact employed but used in conjunction with sample stacking to improve sensitivity and peak shape. If we examine Fig. 3a a CZE separation where a supplementary pressure of 50 mbar is used without sample stacking. It can be seen this has a markedly detrimental effect on separation efficiency. This is presumably since the flow profile now has the characteristic parabolic shape of a pressure driven system rather than the plug flow profile of an electrically driven system. Careful arrangement of the relative height of the buffer reservoir and the CE/MS probe allowed us to reduce the supplementary pressure to 20 mbar. Best results were obtained when the buffer reservoir was approximately 1 cm above the CE/MS probe. Combining all of the slight modifications to our previous methodology with sample stacking gives stable and reproducible high quality electropherograms (Fig. 3b).

Sample injection in CE is generally performed by hydrodynamic or electrokinetic injection. During hydrodynamic injection (HE) the sample vial is pressurised, forcing the sample into the capillary. Thus,

Table 1
Percentage recovery of cotinine from a 1 g C18 SPE cartridge ($n = 3$)

| | Unknown concentration (μg/ml) | R.S.D. | Recovery (%) |
|---|---|---|---|
| 0.625 μg/ml cotinine 100 times preconcentration (Isolute 1 g C18) | 63.86 | 0.07 | 102.17 |
| 1.25 μg/ml cotinine 100 times preconcentration (Isolute 1 g C18) | 118.34 | 0.07 | 94.67 |
| Mean | | | 98.42 |

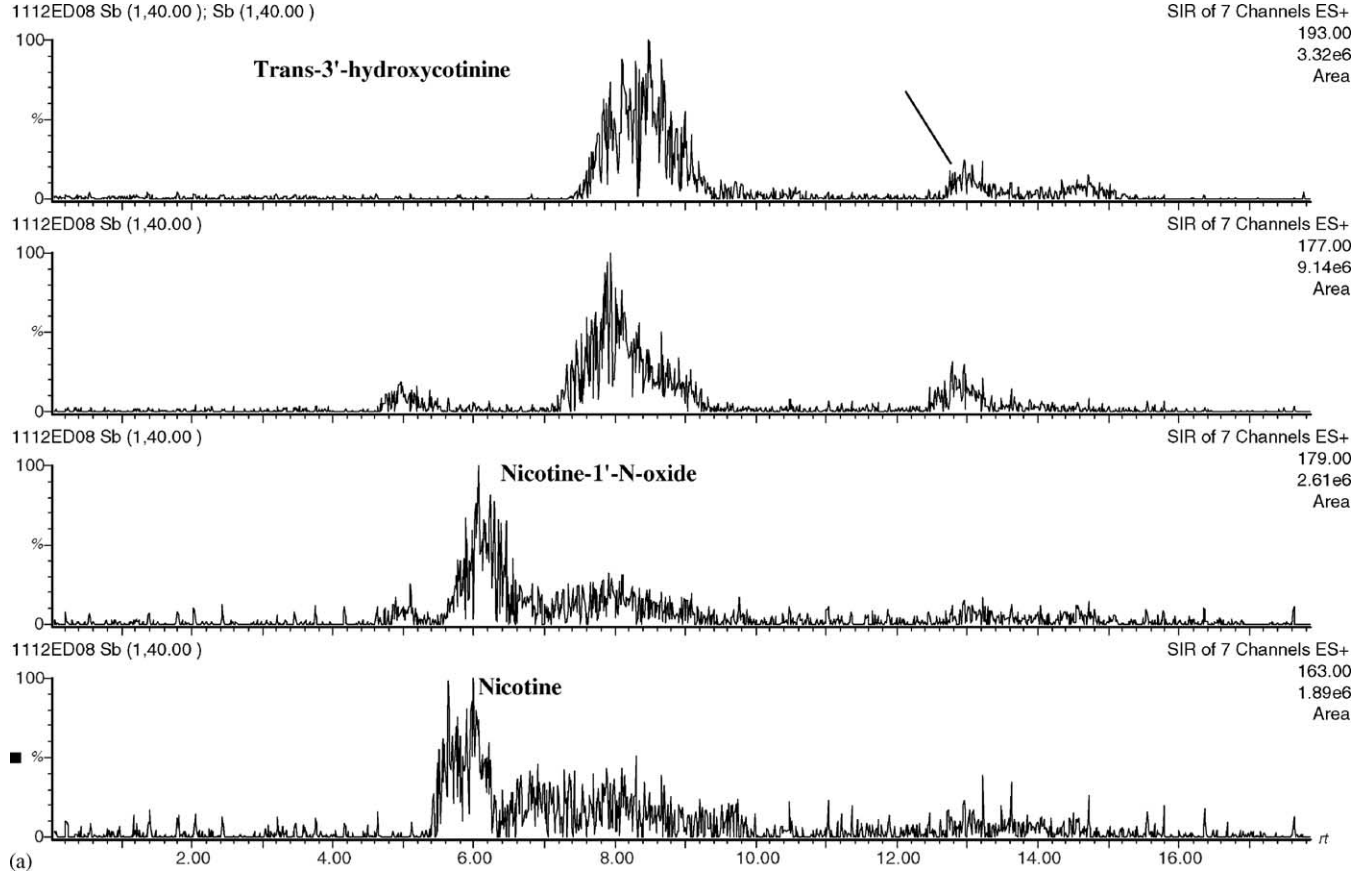


Fig. 3. (a) CZE/MS SIR mass electropherograms of a smoker's urine (sample $C_2$). A 25 mM ammonium formate (10% acetonitrile, 90% deionised water) adjusted to pH 2.5 with formic acid, sheath flow 1:1 MeCN/$H_2O$ + 0.1% formic acid (6 μl/min) + 50 mbar supplmentary pressure. Note the very poor peak shape. (b) Optimised sample stacking CE/MS/SIR mass electropherograms of nicotine and eight of its metabolites. Sample prepared in 10 mM ammonium formate (75% acetonitrile, 25% deionised water) adjusted to pH 2.5 with HCl, run buffer 25 mM ammonium formate (10% acetonitrile, 90% deionised water) adjusted to pH 2.5 with formic acid, sheath flow run buffer (6 μl/min) + 20 mbar supplmentary pressure.

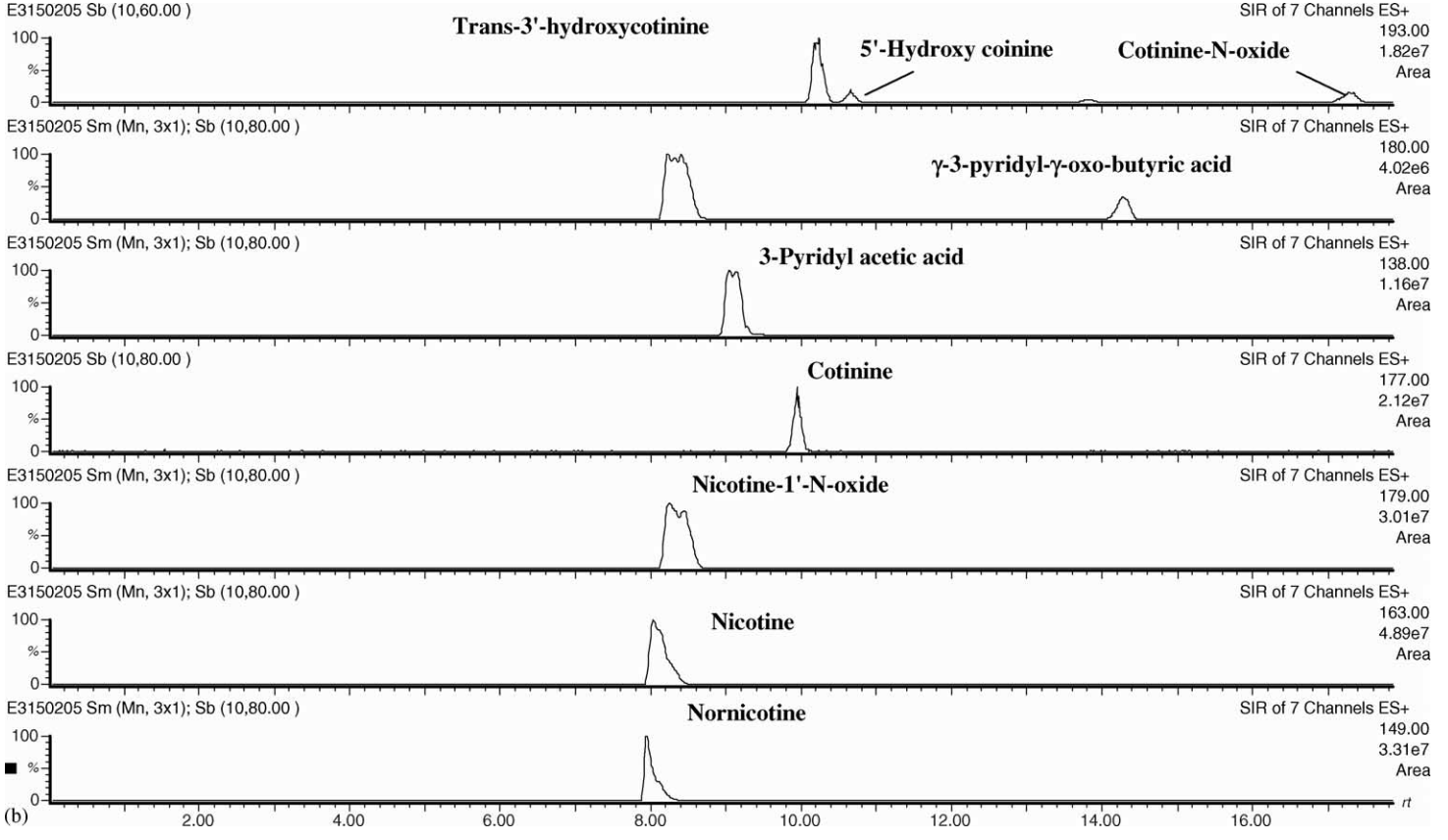


Fig. 3. (*Continued*).

Table 2
A study of the reproducibility of the developed sample stacking CE/MS separation method

| | Mean resolution | Resolution S.D. | Resolution R.S.D. (%) |
|---|---|---|---|
| Sample stacking | | | |
| Sample A (cot-5HC) | 2.59 | $1.17 \times 10^{-1}$ | 3.15 |
| Sample B (cot-5HC) | 1.41 | $1.44 \times 10^{-2}$ | 4.52 |
| Sample $C_1$ (cot-5HC) | 2.21 | $9.78 \times 10^{-2}$ | 1.02 |
| Sample D (cot-5HC) | 2.16 | $7.70 \times 10^{-2}$ | 3.57 |
| Sample E (cot-5HC) | 2.18 | $8.39 \times 10^{-2}$ | 3.85 |
| CZE | | | |
| Sample $C_2$ (cot-5HC) | 2.36 | $6.10 \times 10^{-2}$ | 2.57 |
| Sample stacking (cot-3HC) | | | |
| Hydrodynamic injection | 0.75 | $1.89 \times 10^{-2}$ | 2.51 |
| Electrokinetic injection | 1.26 | $9.98 \times 10^{-3}$ | 0.80 |
| Hydrodynamic + electrokinetic injection | 0.77 | $2.42 \times 10^{-2}$ | 3.15 |

The resolution, $R$, between two adjacent peaks (cotinine and 5′-hydroxycotinine for A–E and cotinine and *trans*-3′-hydroxycotinine for the three injection methods) was calculated as follows: $R = 2(t_2 - t_1)/(w_1 + w_2)$; where $t_1$ and $t_2$, and $w_1$ and $w_2$ are the retention times and peak widths of adjacent peaks, respectively. For smokers urine samples A–E, $n = 3$; $n = 5$ for CZE; $n = 5$ for injection methods; where $n$ is the number of repeat analyses.

the volume of sample injected is dependent on the magnitude and duration of the pressure applied, the capillary dimensions, and the sample viscosity. Electrokinetic injection is performed by placing the capillary and the anode into the source inlet vial and applying a voltage over a period of time. Thus, ionic solutes are injected as a result of their electrophoretic mobilities, with neutrals being pushed through the capillary as a result of the electro-osmotic flow.

Electrokinetic injection provides for sensitivity enhancements in comparison to hydrodynamic injection, since the volume of sample solution that can be introduced does not limit the injected amount of the sample. However, sampling bias is a major drawback of electrokinetic injection, since larger quantities of higher mobility solutes are injected than those of lower mobility. An example of sampling bias was observed in this work. When electrokinetic injection was employed,

Table 3
Sample injection data calculated for CE-sample stacking/MS

| | Injected quantity (ng) | Injected volume (nl) | Sample plug length (mm) | Mean migration time (min) | $t_m$ R.S.D. (%) |
|---|---|---|---|---|---|
| Hydrodynamic + electrokinetic | | | | | |
| Nicotine | 0.83 | 132.04 | 29.89 | 10.27 | 1.52 |
| Cotinine | 0.74 | 118.15 | 26.74 | 12.24 | 2.08 |
| Thiourea | 0.51 | 18.6 | 82.17 | 24.58 | 4.04 |
| Uracil | 0.51 | 18.48 | 81.65 | 24.41 | 3.70 |
| Hydrodynamic | | | | | |
| Nicotine | 0.29 | 45.98 | 10.40 | 9.59 | 3.77 |
| Cotinine | 0.29 | 45.98 | 10.40 | 11.2 | 3.83 |
| Thiourea | 0.29 | 45.98 | 10.40 | 22.2 | 3.05 |
| Uracil | 0.29 | 45.98 | 10.40 | 22.1 | 2.71 |

The viscosity of the buffer system was calculated as 0.51 cP. $Q_{inj} = (V_i/1 \times 10^6) \times c \times 1000$; $L_p = (\Delta P \times t \times d^2)/(53.3 \times \eta \times L) + (v \times t \times (V_{inj}/V_{sep})$; $V_i = (r \times 1 \times 10^6)^2 \times (L_p \times 1 \times 10^3) \times \pi \times 1 \times 10^{12}$; where $Q_{inj}$ is the injected quantity; $V_{inj}$ the injected volume; $c$ the concentration in μg/ml; $L_p$ the sample plug length; $\Delta P$ the pressure across the capillary; $t$ the injection time; $d$ the capillary inner diameter; $\eta$ the viscosity of the sample solution; $L$ the total length of the capillary; $v$ the velocity of the analyte in mm/min; $V_{inj}$ the injection voltage; and $V_{sep}$ the separation voltage.

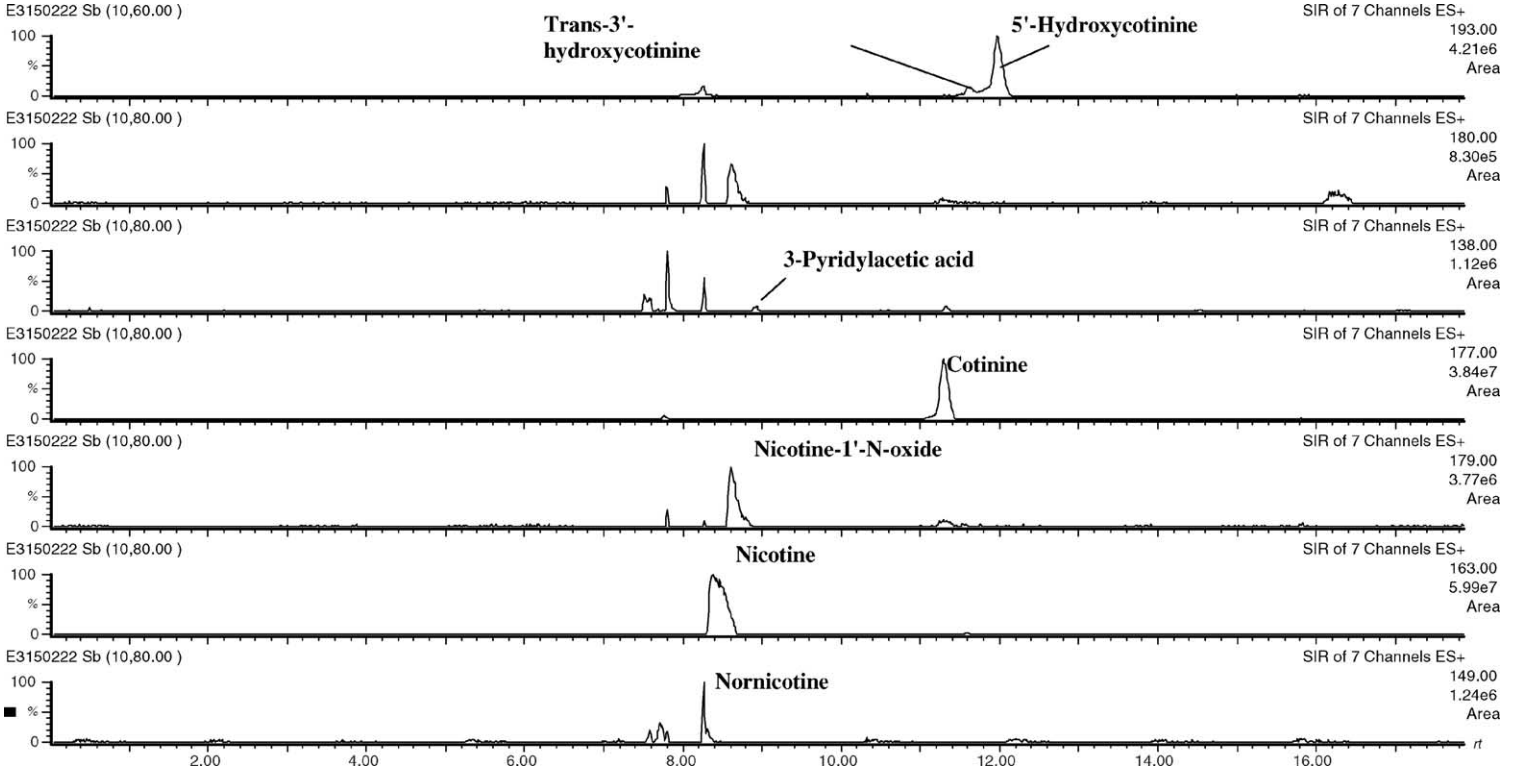


Fig. 4. SIR mass electropherograms of a smoker's urine (sample E); all conditions as Fig. 3b.

γ-3-pyridyl-γ-oxo-butyric acid and cotinine-*N*-oxide were not detected presumably since they were not injected in an adequate amount to yield a sufficient response from the mass spectrometer for detection. In order to eliminate this effect the combination of electrokinetic and hydrodynamic injection was employed. The injected quantities of nicotine and cotinine for the HE method were 0.83 and 0.74 ng, respectively. Of this 0.29 ng of both nicotine and cotinine was contributed by the hydrodynamic injection element (Table 3) and hence electrokinetic injection contributes 0.54 and 0.45 ng of both nicotine and cotinine, respectively. That is 65% of the nicotine HE load and 61% of the cotinine HE load.

Sample stacking was employed in conjunction with the combined injection procedure in order to achieve the best possible sensitivity. The limits of detection, by CE-sample stacking/MS, of nicotine and cotinine, respectively, for the three injection methods were found to be: (1) 0.11 and 2.25 μg/ml for the HE injection method; (2) 2.86 and 6.25 μg/ml for hydrodynamic injection; and (3) 0.18 and 3.27 μg/ml for electrokinetic injection. Thus, it is clear to see that the HE injection method yields the lowest limits of detection, followed by electrokinetic, and finally hydrodynamic injection. The lowest detection limits yielded by the HE injection method can be explained by its ability to introduce the greatest quantity of sample to the capillary (Table 3). It should also be noted that the higher electrophoretic mobility of nicotine results in greater quantities of this solute being introduced to the capillary than cotinine. This, accompanied with the higher ionising potential of nicotine ensured its lower limit of detection. Comparison of the limit of detection obtained for nicotine by HE injection of 0.11 μg/ml, i.e. 14.5 pg on-column (Table 3) with the reported literature values of, for nicotine in tobacco 286 fg [19] (using electrochemical detection) and for nicotine in plant cells 0.013 pmol (i.e. 2.1 pg) [20] (using ultraviolet detection), shows that the sensitivity of our CE/MS method is comparable with that of CE with ultraviolet detection.

Optimisation of the electrospray together with the application of sample stacking (via HE injection) has greatly improved peak efficiency, baseline stability, and detector response to the analytes (Fig. 3b); and the added selectivity that SIM provides ensures the clear identification of nicotine and its metabolites. The order of elution is comparable to that shown by Palmer et al. [4].

When applying this method to urine samples, sample preconcentration via solid-phase extraction was required (with the 200-fold preconcentration of sample achieved). Data from a smoker's urine (Fig. 4) shows the clear identification of nicotine, cotinine and the other major metabolites of nicotine. Since nicotine has only a half-life of approximately 2 h, the relatively high abundance on the mass electropherogram could be attributed to the recent exposure of the smoker to tobacco smoke.

Samples $C_1$ and D (not shown) exhibited a more extensive metabolism of nicotine and hence had a relatively low abundance of this analyte on both mass electropherograms. The high abundance of 5′-hydroxycotinine with respect to *trans*-3′-hydroxycotinine in all the mass electropherograms, with the exception of sample $C_2$, suggests that the major pathway for cotinine metabolism is via 5′-hydroxycotinine. This is further confirmed by the presence of 3-pyridyl acetic acid. However, it is possible that *trans*-3′-hydroxycotinine could mainly exist in the glucuronide form. More work in this area (with respect to patient studies) is required.

The inter-urine sample migration times of the metabolites appeared to differ slightly. The probable cause being, the presence of urobilins and urobilinogens in urine. The presence of this compound in urine may be brought about by the derivation of bilirubin from senescent red blood cells and from other haem-containing proteins, such as cytochromes. Bilirubin is poorly water-soluble and so undergoes glucuronidation to form bilirubin diglucuronide, which is excreted into the bile. The glucronide residues are released in the terminal ileum by intestinal bacterial hydrolases. Free bilirubin is then reduced to colourless urobilinogens, which are oxidised to coloured products known as urobilins. Urobilins are mainly excreted in the faeces, but small proportions of urobilins and urobilinogens are excreted in the urine. Urobilins give urine its yellow pigment. Since ultrafiltration of the urine sample only filters proteins of 3000 MW and over, urobilins and urobilinogens (584.65 MW) are still present in the filtered urine. When preconcentrating urine by 200-fold the concentration of urobilins increase and hence the viscosity increases. Thus, an increase in viscosity will increase

migration times, but not the order of elution providing the fused silica capillary is long enough for effective separation.

## 4. Conclusions

The 100-fold preconcentrations of 1.25 μg/ml and 625 ng/ml of cotinine, in urine, showed a mean percentage recovery of 98% from a 1 g C18 SPE cartridge. Hence, the 200-fold preconcentration of sample could be achieved through drying with nitrogen gas and reconstituting with buffer.

A combination of hydrodynamic and electrokinetic injection was used for sample stacking/MS due to the lower detection limit, and the reasonable number of theoretical plates achieved. Thus, sample stacking mode of CE, when compared to CZE was shown to improve peak efficiency by a mean of 131.6-fold.

LODs of nicotine and cotinine, by CE-sample stacking/MS (via HE injection), were found to be 0.11 and 2.25 μg/ml, respectively. Thus, the LODs of nicotine and cotinine after 200-fold preconcentration would be 0.55 and 11.25 ng/ml, respectively.

The added selectivity that SIM provided ensured the clear identification of nicotine and its metabolites.

The same pattern of elution was observed for nicotine and its metabolites from smoker's urine. However, the inter-urine sample migration times of the metabolites appeared to differ slightly and were attributed to the presence of urobilinogens and urobilins. The extraction of both tetrapyrroles by acetaldehyde and petroleum ether, from urine, is currently under investigation.

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