Potential of Capillary Electrophoresis (CE) and Chip-CE with Dual Detection (Capacitively-Coupled Contactless Conductivity Detection (C^4D) and Fluorescence Detection) for Monitoring of Nicotine and Cotinine Derivatization

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Capillary electrophoresis (CE) coupled with capacitively-coupled contactless conductivity (C^4D) and fluorescence (FD) detectors and chip-CE for monitoring of nicotine and cotinine derivatization was demonstrated. Separation of the substrates and intermediates could be achieved by CE-C^4D in 7 min (R_s > 2.7) using 45 mM acetic acid (pH 3.0) and this system was applied to detect the intermediate formation. Final fluorescent products could be analyzed by micellar electrokinetic chromatography (MEKC-FD) in 5 mM borate buffer (pH 9.0) containing 10 mM sodium dodecylsulfate (SDS) (%RSD < 3.00%). Transferring of MEKC-FD to chip-CE allowed for shorter analysis time (2.5 min) and decreased sample consumption. The chip-CE-FD shows good detection and quantitation limits (< 7.5 μM) and precision (%RSD < 2.81%) and was employed to determine nicotine and cotinine in artificial urine. This work reveals the potential of CE and chip-CE with dual detectors as simultaneous, convenient and rapid methods for monitoring pyridine derivatization.

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Introduction

Pyridines are heterocyclic aromatic compounds present in many biologically active compounds including nicotine and cotinine (Fig. 1). Nicotine is a major alkaloid in tobacco and is the main known addictive component of tobacco smoke. Nicotine is metabolized to more than 20 different derivatives, among them, cotinine is a primary metabolite (70 – 80%) with a closely related structure to nicotine.1 Nicotine and cotinine are frequently used as biomarkers, which appear in different human biological fluids and matrices, for evaluations of smoke exposure and diagnostic smoking related diseases. Additionally, nicotine is contained in some pharmaceutical formulations used as smoke cessation products. Hence, the determination of tobacco alkaloids, especially nicotine and its oxidized metabolite/product cotinine, is important for both pharmaceutical and food industries in order to regulate commercial nicotine-related products.

Analyses of nicotine and cotinine in different matrices by several methods have been described in the literature, including high performance liquid chromatography (HPLC),2–8 gas chromatography (GC),9–13 capillary electrophoresis (CE),14–19 radioimmunoassays (RIA)20,21 and enzyme-linked immunosorbent assay (ELISA).21–23 Among the above methods, CE is highly attractive due to its advantages in terms of applicability for a wide range of compounds, high separation efficiency, relative simplicity, low solvent consumption and short analysis time. Previously, our group demonstrated the use of CE with UV detection for simultaneous analysis of nicotine, cotinine and related pyridines in pharmaceutical formulations.19 In order to achieve limit of detections (LODs) required for biological samples, a substantial increase in detection sensitivity can be realized by combining CE with fluorescence detection (FD).15 However, due to the lack of natural fluorescence of the target analytes, to apply FD, employment of derivatization reactions is necessary. The procedure from Krishnan et al.24 was employed in a modified form as pre-separation derivatization in the current work. Owing to the complexity of the derivatization reactions, a combination of CE-capacitively-coupled contactless conductivity detection (C^4D)25 and FD was used in this work as a convenient solution for studying the derivatization reactions through separation of the reaction intermediates and products. CE-C^4D also allows for the detection of analyte species in the reaction...
They were protected from light, stored at 2 – 8°C for monitoring of nicotine and cotinine, respectively, in 10 mL water. Commercially available and its potential as a generic chip standard nicotine and cotinine, respectively, in 10 mL water. 

**Reagents and chemicals**

Nicotine, citric acid, sodium sulfate (Na₂SO₄), calcium sulfate (CaSO₄) and magnesium sulfate (MgSO₄) were purchased from Riedel-de Haën (Seelze, Germany). Cotinine was obtained from Sigma-Aldrich (St. Louis, MO). Aniline, creatinine and boric acid were purchased from Sigma (St. Louis, MO). Cyanogen bromide (BrCN) and sodium tetraborate decahydrate were obtained from Aldrich (Wisconsin, USA). All reagents were of analytical grade. Water was treated with a Milli-Q water purification system.

Stock solutions (10 mM) of nicotine and cotinine were prepared separately by dissolving 16.23 and 17.62 mg of standard nicotine and cotinine, respectively, in 10 mL water. They were protected from light, stored at 2 – 8°C and used within 7 days.

**Experimental**

**Reagents and chemicals**

Nicotine, citric acid, sodium sulfate (Na₂SO₄), calcium sulfate (CaSO₄) and magnesium sulfate (MgSO₄) were purchased from Riedel-de Haën (Seelze, Germany). Cotinine was obtained from Sigma-Aldrich (St. Louis, MO). Aniline, creatinine and boric acid were purchased from Sigma (St. Louis, MO). Cyanogen bromide (BrCN) and sodium tetraborate decahydrate were obtained from Aldrich (Wisconsin, USA). All reagents were of analytical grade. Water was treated with a Milli-Q water purification system.

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**Apparatus**

**CE with C/D and FD.** An in-house portable CE setup was employed in this work. An external high voltage supply (Electro Automatic, EA-PS 2016-050) was used as a power source. Fused silica capillaries (Polymer Technologies) of 50 μm i.d. with a total length of 38.5 cm and an effective length of 30.0 cm were employed for all experiments. The derivatized mixtures were hydrodynamically injected at a height of 10 cm for 10 s. The integrated C/D-FD detector was designed as described previously. In brief, it was assembled on an aluminum detector assembly disk with a subminiature version A (SMA) optical fiber connector, which also served as an alignment platform for the capillary, the FD excitation and emission optical fibers, and C/D electrodes. The placement of the two separating capillary holder/C/D tubular electrodes with the stainless-steel tubes, which were also acting as guides for positioning the CE capillary, was precisely aligned using a microscope to allow the excitation light from the 50 μm i.d. optical fiber to pass through the separation capillary. For FD, light emitting diodes (LEDs) were used as light sources with an excitation wavelength of 470 nm (B3B-447-IX, Roithner Lasertechnik, Austria), which was powered with 30 mA using laboratory-made constant current power supplies. Fluorescence emission was monitored by a photomultiplier tube (PMT) (Hamamatsu, Japan) connected to an emission pick-up optical fiber (Polymicro Technologies, 300 μm) positioned in another piece of metal tube. The angle between the CE capillary and the fluorescence emission pick-up fiber was 45°. The optical fiber position relative to the capillary was adjusted under the microscope. A long pass filter (Edmund Optics, UK) was inserted to the entrance of the PMT to block the excitation light and reduce the FD background noises. For C/D, a high-frequency electric field was produced by a function generator (ISO-TECH GPG-8216A) coupled to the excitation electrode of the C/D. A pick-up amplifier equipped with a feedback resistor of 1 MΩ was inserted between the detection electrode of the C/D, which was placed separately. The two independent signals (FD, C/D) were recorded by an eDAQ (New South Wales, Australia) multichannel data acquisition station with chromatographic (eDAQ Chart) software.

**Chip-CE with blue light emitting diode induced fluorescence (LED-IF) detection.** Chip-CE analyses were performed on the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) using DNA chips. The microfluidic chip features a separation channel with a total length of approximately 40 mm,
with 14 mm effective length from the injection point to the detection point. Microfluuidic chips were filled and flushed with buffer by manually pipetting into the buffer reservoir and applying vacuum to the buffer waste reservoir using a homemade device consisting of a 3-mL plastic syringe and a piece of pipette tip that fitted tightly into the reservoirs on top of the chip. Microchips were typically flushed with background electrolyte (BGE) for 5 min and the sample reservoirs were filled with 7 μL of sample, while the buffer reservoirs were filled with 10 μL of buffer. Programmed high voltage control for the 16 independent platinum electrodes of the microchip was achieved by developing a new script via the assay and script developer mode. In brief, filling the injection intersection was achieved by application of 100 V to the sample waste and 1500 V to the buffer. Detection was equipped with blue LED-IF with an excitation wavelength at 470 nm (a bandwidth of 25 nm) and an emission wavelength at 525 nm (a bandwidth of 30 nm).

Procedure
Pre-separation derivatization. The modified König reaction was applied for the derivatization of nicotine and cotinine. Reagents (i.e., BrCN and aniline) used in derivatization steps were freshly prepared prior to CE analysis. BrCN and aniline were separately dissolved in water and 1 M HCl, respectively, to obtain the concentration of 5% w/v. The intermediate derivatives were prepared by mixing 10 μL of 10 mM nicotine/cotinine standard solution with 5 μL of 5% w/v BrCN at room temperature. After 5 min, 5 μL of 5% w/v aniline was added as a coupling reagent to produce fluorescent products. Derivatization mixtures were allowed to react for 5 min and adjusted to 500 μL with water.

Artificial urine was prepared by dissolving 55 mmol NaCl, 67 mmol KCl, 2.6 mmol CaSO₄, 3.2 mmol MgSO₄, 29.6 mmol Na₂SO₄, 19.8 mmol NaH₂PO₄, 310 mmol urea and 9.8 mmol creatinine in water, adjusted to 1.0 M L⁻¹ and was used within 7 days. Standard solutions of nicotine and cotinine were spiked into the artificial urine, and then they were derivatized with the above method without sample pretreatment.

CE optimization. Monitoring of intermediate formation was performed by CE-C4D using capillary zone electrophoresis (CZE) mode. Various types of BGE (e.g. citric acid, histidine-citric acid and acetic acid) at different concentrations (e.g. 5, 45, 520 and 850 mM) and pH (e.g. 2.5 and 3.0) were investigated.

Further optimization for monitoring of fluorescent product formation was carried out by MEKC-dual detectors (C4D and FD). C4D could be utilized for determination of EOF marker or non-fluorescent products, which possibly occurred in the reaction mixtures. MEKC optimization was carried out using phosphate (10 mM NaH₂PO₄ (pH 7.0)) and borate (5 mM sodium tetraborate and 4.5 mM boric acid (pH 9.0)) buffer containing 10 mM SDS. The optimized MEKC conditions were evaluated from resolution (Rₛ), sensitivity (signal-to-noise, S/N) and relative standard deviation (%RSD).

Method validation. The optimal MEKC-FD method was transferred to chip-MEKC coupled with a blue LED-IF detector. Firstly, resolution and separation efficiency was evaluated and then the chip-CE method was validated in terms of precision, recovery, limits of detection (LOD), and quantitation (LOQ). Method precision, calculated from the %RSDs of nᵢ and the peak area, was determined from an injection repeatability (n = 10), intra-day (n = 3), and inter-day (n = 6) assay of the working standard solutions. Recovery of the method was evaluated by spiking nicotine and cotinine into the artificial urine (n = 3). The LOD and LOQ were based on S/N ratios of 3 and 10, respectively.

Results and Discussion
Monitoring of intermediate formation by CE-C4D
Substrate containing pyridine with a hydrogen adjacent to the heterocyclic nitrogen can react with BrCN to produce a derivative of glutaconic aldehyde based on hydrolysis reaction of the substrates. Consequently, derivatization of nicotine and cotinine was performed based on this concept. An initial step for nicotine and cotinine derivatization is the opening of pyridine rings by BrCN and their subsequent cleavage to yield glutaconic aldehyde derivatives, as the intermediates of the modified König reaction (Fig. 1). The substrate consumption and intermediate formation were then monitored by CE-C4D. The optimal CE-C4D condition was obtained by varying types, concentrations and pH of BGE (e.g. citric acid, histidine-citric acid and acetic acid). The low pH (2.5 and 3.0) BGE was selected to enable the substrates in their protonated forms. No peaks were observed in preliminary experiments using 10 mM histidine-520 mM citric acid (pH 2.5) as the BGE. Using 5 mM citric acid (pH 2.5) provided the separation of nicotine and cotinine (Rₛ = 4.5), but intermediate peaks could not be detected due to the small conductivity differences between the BGE and intermediates. Thus, acetic acid was employed as alternative BGE. At high concentrations (580 mM acetic acid, pH 2.5), the substrates could be separated with baseline noises. However, nicotine intermediate (NI) overlapped with cotinine, and cotinine intermediate (CI) could not be found. Lowering the acetic acid concentration to 5 and 45 mM allowed for the separation of all analytes, but a better resolution of 1.05 was achieved with 45 mM acetic acid. The final CE condition with improved resolution (Rₛ > 2.7) was achieved with 45 mM acetic acid at higher pH (pH 3.0), which provided baseline separation of the substrates and intermediates in 7 min (Fig. 2). Table 1 shows electrophoretic mobilities (μₑ) of nicotine, cotinine and their intermediate, which indicate that all analytes were cationic and migrated prior to the EOF. We reasoned that, under these conditions, nicotine and cotinine remained in di- and mono-protonated forms, respectively.

Formation of nicotine and cotinine intermediates was
monitored for 60 min, using the CE-C4D condition in Fig. 2. Intermediate formation was achieved during the first 5 min of the reaction, with %yields of 51 and 31%, respectively, (Fig. 3). We observed that prolonging of the reaction times did not further increase the percent yields. Percent yields were evaluated by firstly defining the reaction ratio of the substrate and intermediate, which was found to be 1:1. The substrate and intermediate concentrations were calculated from the peak area and %yield of the nicotine and cotinine intermediates were determined by taking ratios to the initial peak area of nicotine or cotinine. Thus, the CE-C4D procedure could be employed for monitoring the nicotine and cotinine intermediate formation.

**Monitoring of fluorescent product formation by CE-C4D and FD**

Nicotine and cotinine intermediates were coupled with aniline, which subsequently produced polymethine dye derivatives as fluorescent products (Fig. 1). The fluorescent products could not be detected under the CE conditions in Fig. 2 due to limited water solubility. Thus, MEKC was selected as an alternative to monitor the fluorescent products, using phosphate (10 mM NaH2PO4 (pH 7.0)) and borate (5 mM sodium tetraborate and 4.5 mM boric acid (pH 9.0)) buffer containing 10 mM SDS as the BGE.

Results show that S/N of nicotine (NFP) and cotinine fluorescent products (CFP) were 42 and 12 in borate buffer and 10 and 3 in phosphate buffer, respectively. Therefore, borate

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<table>
<thead>
<tr>
<th>Analyte</th>
<th>Electrophoretic mobility (μe/m² V⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine</td>
<td>4.01 × 10⁻⁸</td>
</tr>
<tr>
<td>Nicotine intermediate</td>
<td>2.35 × 10⁻⁸</td>
</tr>
<tr>
<td>Cotinine</td>
<td>2.61 × 10⁻⁸</td>
</tr>
<tr>
<td>Cotinine intermediate</td>
<td>1.40 × 10⁻⁸</td>
</tr>
<tr>
<td>EOF marker (50% MeOH)</td>
<td>8.82 × 10⁻⁹</td>
</tr>
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a. For CE condition: see Fig. 2.

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**Table 1** Electrophoretic mobilities of nicotine, cotinine and their intermediates under CE-C4D

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### Fig. 3 Monitoring of nicotine and cotinine intermediate formation using CE-C4D. CE-C4D condition: see Fig. 2. Reaction condition: mixing of 200 μM nicotine/cotinine standard solution and 5% BrCN in 1 N HCl at room temperature for 5 - 60 min to produce nicotine/ cotinine intermediate (see the Pre-separation derivatization section).

### Fig. 4 MEKC of nicotine, cotinine and their fluorescent products. Conditions: 5 mM sodium tetraborate, 4.5 mM boric acid (pH 9.0) containing 10 mM SDS; capillary, 38.5 cm total length (8.5 cm to the detector), 50 μm i.d.; hydrodynamic injection for 10 s by height differences of 10 cm; temperature, 25°C; voltage, 15 kV; detection by C4D, 15 kHz and FD, LED with excitation wavelength at 470 nm and emission wavelength at 525 nm. (NFP: nicotine fluorescent product, CFP: cotinine fluorescent product).

### Fig. 5 Chip-CE based MEKC analysis of fluorescent products of: a) nicotine and cotinine standard solutions, b) spiked nicotine and cotinine in artificial urine, c) spiked mixture of nicotine and cotinine in artificial urine. Conditions: 5 mM sodium tetraborate, 4.5 mM boric acid (pH 9.0) containing 10 mM SDS; capillary, 14 nm separation length; temperature, 25°C; injection and separation voltage, 1400 V and blue LED-IF detection with excitation wavelength at 470 nm and emission wavelength at 525 nm.
buffer was superior to phosphate buffer since it provided 4.0 times higher sensitivity (S/N) (Fig. 4). Precision for resolution values and peak areas of fluorescent products shows %RSDs between 1.80 to 3.00% (n = 3). Mobilities of NFP and CFP were $2.81 \times 10^{-4}$ and $2.63 \times 10^{-4}$ m$^2$ V$^{-1}$ s$^{-1}$, respectively.

**Chip-CE with blue LED-IF detection**

The optimal MEKC method used in conventional CE could be successfully transferred to chip-CE with FD (blue LED-IF detection) with minor modifications such as adjustment of the applied voltage. Figures 5a and 5b present electropherograms of separated standard and spiked artificial urine of nicotine and cotinine, and 5c shows the mixture of the drugs in spiked artificial urine. The fluorescent products of nicotine (NFP) and cotinine (CFP) migrated at 2.03 and 2.25 min, respectively with the R, of 1.2. The shorter analysis time in chip format (2.5 min) was proportional to the increased field strength (500 V/cm for CE and 1000 V/cm for chip-CE). Comparing to MEKC-FD (Fig. 4), the chip-CE showed improved resolution, higher separation efficiency, with plate numbers for NFP and CFP of 8000 and 10000, respectively, and lower peak dispersion (Fig. 5).

The chip-ce-blue LED-IF method with pre-separation derivatization was applied to analyze the spiked nicotine and cotinine in artificial urine without sample clean-up procedure. In comparison to blank signals, the NFP and CFP peaks were distinct without matrix interferences (Fig. 5). These promising results could be obtained due to the highly sensitive FD and specific derivatization reaction resulting in low background interference from complex matrices.

LOD, LOQ and precision data of the chip method is presented in Table 2. LODs and LOQs were less than 7.5 µM and %RSDs (n = 6) for the migration times and the peak areas of all the analytes were 1.45 and 2.81%. Percent recoveries of nicotine and cotinine were 96.4 and 89.7%, respectively, with %RSD of < 2.40. These results indicated that chip-CE with FD shows possibility for rapid analysis of nicotine and cotinine in 2.5 min. Analytical performance characteristics support that chip-CE is applicable for the quantitative analysis of nicotine and cotinine in low levels. The analysis of NFP and CFP in the spiked artificial urine samples were 97.9 and 54.3 µM (%RSD of <2.80), respectively.

**Conclusions**

This investigation shows that nicotine and cotinine derivatization, using the modified König reaction, can be monitored by CE combined with C-D and FD detection. Glutaconic aldehyde intermediate formation and substrate consumption can be simultaneously analyzed within 7 min by CE-C-D. The intermediates were then coupled with aniline to produce polymethine dye derivatives (fluorescent products), which could be detected by MEKC-FD. The MEKC-FD condition was transferred to chip-CE without losing any resolution or separation efficiency. Impressively, resolution, peak symmetry and efficiency even improved in chip-format. This is the first report on the monitoring of nicotine and cotinine derivatization by CE with dual detections. The combination of two detection techniques (C-D and FD) offers greater advantages than the individual detector method namely, enhanced convenience and speed for the determination of the diverse components in complex mixtures.

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