Talanta 82 (2010) 1797–1801

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

On-line concentration and determination of tobacco-specific N-nitrosamines by cation-selective exhaustive injection–sweeping–micellar electrokinetic chromatography

Youyou Yang[a], Honggang Nie[a], Chenchen Li[a], Yu Bai[a], Ning Li[b], Jie Liao[b], Huwei Liu[a,*]

[a] Beijing National Laboratory for Molecular Sciences, Key Laboratory of Bioorganic Chemistry and Molecular Engineering of Ministry of Education, Institute of Analytical Chemistry, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, China
[b] Medical Experiment & Analysis Center, General Hospital of Chinese PLA, Beijing 100853, China

ARTICLE INFO

Article history:
Received 14 April 2010
Received in revised form 23 July 2010
Accepted 28 July 2010
Available online 6 August 2010

Keywords:
Cation selective exhaustive injection
Micellar electrokinetic chromatography
On-line concentration
Sweeping
Tobacco-specific N-nitrosamine

ABSTRACT

In this paper, a micellar electrokinetic chromatography (MEKC) method combined with cation-selective exhaustive injection (CSEI) and sweeping was developed to separate and concentrate four tobacco-specific N-nitrosamines (TSNAs) including N′-nitrosoanabasine (NAB), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol (iso-NNAL). Experimental parameters affecting separation efficiency and enhancement factors were investigated in detail. Under the optimum MEKC condition, NAB, NNK, NNAL and iso-NNAL were baseline separated with high separation efficiencies and good peak shapes. Furthermore, with the preconcentration by CSEI–sweeping–MEKC, the sensitivity enhancement factors for NAB, NNK, NNAL and iso-NNAL in terms of peak areas ranged from 6.0×10^3 to 1.5×10^4, and the detection limits (LOD, S/N = 3) of four TSNAs were in the range of 0.004–0.016 μg/mL. In addition, this method had fairly good repeatability, and the RSDs of retention time and peak area were less than 1% and 5%, respectively. Finally, this method showed promising capabilities in the application of detecting and analyzing TSNAs in human urine samples.

1. Introduction

Tobacco-specific N-nitrosamines (TSNAs) are among the most abundant carcinogenic components in tobacco and tobacco smoke [1,2]. Until now, seven TSNAs have been identified: N′-nitrosonornicotine (NNN), N′-nitrosoanatabine (NAT), N′-nitrosoanabasine (NAB), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol (iso-NNAL), and 4-(methylnitrosamino)-4-(3-pyridyl)butanoic acid (iso-NNAC) [3]. Among them, NNK, NNN, NNAL are the most carcinogenic while NAB is the weak carcinogen, and they are considered to be the leading cause of lung cancer and related diseases [1]. Therefore, it is very important to develop various methods of detection and analysis of TSNAs in order to help evaluate public health risks easily [4].

During the past few years, liquid chromatography (LC), gas chromatography (GC), coupled with mass spectrometry (MS) [5–11] were widely used to analyze TSNAs in tobacco products, tobacco smoke and biological samples. In our previous work, capillary electrophoresis (CE)–UV and CE–MS were also developed to determine TSNAs [12]. However, because of the intrinsic shortcomings, the minute sample amount injected as well as the limited optical path length in CE–UV and the introduction of sheath liquid in CE–MS, the LODs of 0.1 μg/mL could be only achieved even by CE–MS. Therefore, development of concentration techniques is essential for extending CE's applications in this area.

Nowadays, several on-line concentration techniques for CE have been developed, such as isotachophoresis (ITP), sample stacking and sweeping [13]. As the most general and widely used preconcentration methods in MEKC, sample stacking and sweeping could greatly improve the detection sensitivity for both neutral and charged analytes [14,15]. Sample stacking is based on the difference in the electrophoretic migrating velocity of ionic analytes between two buffer solutions with different conductivities [15], while sweeping is based on the picking and accumulating by the micelles that penetrated the sample zone, and the concentration ability is highly determined by the affinity between analytes and micelles [16,17]. Recently, anion-selective exhaustive injection (ASEI)/CSEI–sweeping–MEKC have been established [18–22]. Combining the advantages of both sample stacking and sweeping, it could achieve very high sample enrichment efficiency.

* Corresponding author. Tel.: +86 10 62754976; fax: +86 10 62751708.
E-mail address: hwliu@pku.edu.cn (H. Liu).

0039-9140/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2010.07.075

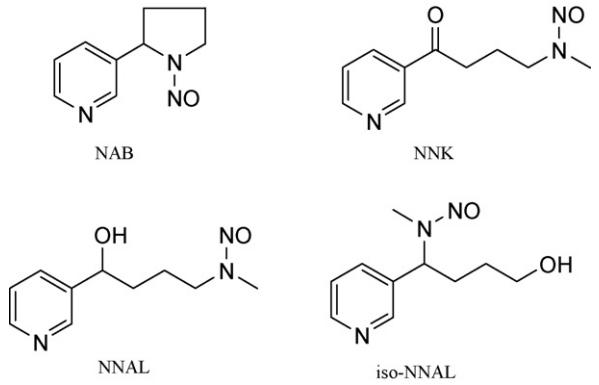

Fig. 1. Chemical structures of the four TSNAs studied.

In this study, four TSNAs (NAB, NNK, NNAL and iso-NNAL, see Fig. 1 for their structures) were first baseline separated by MEKC and also effectively concentrated by CSEI–sweeping–MEKC with SDS as the anion surfactant. All factors influencing enhancement efficiency of CSEI–sweeping–MEKC were investigated in detail. Under the optimum conditions, this method could provide 10^4-fold improvement in detection sensitivity in terms of peak height. Finally, with an appropriate SPE method, this method showed great potential in detecting TSNAs in human urine samples.

2. Experimental

2.1. Apparatus

All experiments were performed on an Agilent 3D CE system equipped with air-cooling and a diode array detector (Agilent Technologies, Palo Alto, CA, USA). Detection wavelength was set at 240 nm, and an untreated fused-silica capillary column (Xinnuo Inc., Hebei, China) of 48.5 cm × 50 μm ID (40 cm effective length) was used for the separation. Each new capillary was preconditioned sequentially with 1 M NaOH (15 min), pure water (20 min). Between consecutive analyses, the capillary flushed with 0.1 M NaOH (2 min), pure water (3 min) and micellar BGE (3 min) in normal MEKC mode or nonmicellar BGE (3 min) in CSEI–sweeping–MEKC. Conductance was measured using a Kangyi DDS-11A conductivity meter (Shanghai, China). All pH values were measured with a Lei-Ci PHS-3 pH meter (Shanghai, China).

2.2. Materials

Standards of NAB, NNK, NNAL, and iso-NNAL (>98%) were obtained from Toronto Research Chemicals (North York, Canada). SDS was purchased from Fluka (Buchs, Switzerland). Analytical-reagent-grade sodium dihydrogen phosphate and phosphoric acid were purchased from Beijing Reagent Company, China. Acetonitrile (chromatographic grade) was purchased from Dima Technology Inc., USA. Purified water was obtained from Hangzhou Wahaha Group Co. (Zhejiang, China). All the solutions were prepared with pure water and filtered with 0.45 μm membrane before use. Stock standard solutions of analytes (100 μg/mL) were prepared in pure water and refrigerated at 4 °C. Prior to the analysis, the standards were diluted with pure water at a desired concentration. The pH of BGE was adjusted with phosphoric acid. The blank human urine samples were obtained through donation.

2.3. Urine sample preparation

Urine sample preparation procedure included the use of Cleanert ODS-SPE cartridges obtained from Agela (Beijing, China) and SCX SPE cartridges (Agilent, USA). In the first stage, after preconditioning of a Cleanert ODS-SPE cartridge with 1 mL methanol and 1 mL water, the urine sample (500 μL) spiked with NAB, NNK, NNAL and iso-NNAL (each 10 μg/mL, 2 μL) was loaded. After washing with 1 mL water and 1 mL methanol/water (10:90, v/v), the eluents were obtained with 1 mL methanol for the next step. In the second stage, an SCX SPE cartridge was firstly preconditioned with 1 mL methanol and 1 mL 2% aqueous formic acid (v/v), and then the former eluents were loaded. After the sample's loading and washing with 1 mL water and 1 mL methanol, the TSNAs were eluted with 1 mL methanol/ammonium hydroxide (90/10 (v/v)). Finally, the extracts were evaporated to dryness under nitrogen flow and dissolved in purified water (100 μL) before CE analysis.

2.4. Electrophoretic conditions

For normal MEKC, the running buffer consisted of 80 mM phosphate buffer, containing 75 mM SDS and 10% (v/v) ACN at pH 2.5. The sample was hydrodynamically injected at 50 mbar for 1 s, and the applied analysis voltage was −25 kV.

For CSEI–sweeping–MEKC, the capillary was first conditioned with non-micellar BGE (80 mM phosphate solution containing 10% (v/v) ACN contained at pH 2.5), followed by the introduction of an HCB plug (109 mM phosphoric acid, 13.3 mm) and then a plug of water (1.3 mm). The sample in pure water with low conductivity was electrokinetically injected at −10 kV for 300 s. After injection, both ends of the capillary were placed in micellar BGE (80 mM phosphate buffer containing 75 mM SDS and 10% (v/v) ACN at pH 2.5) and a voltage of −25 kV was applied. Then the micelles from the cathodic vials would enter the capillary to sweep the stacked analytes into a narrower band. Finally, the preconcentration and the separation were performed.

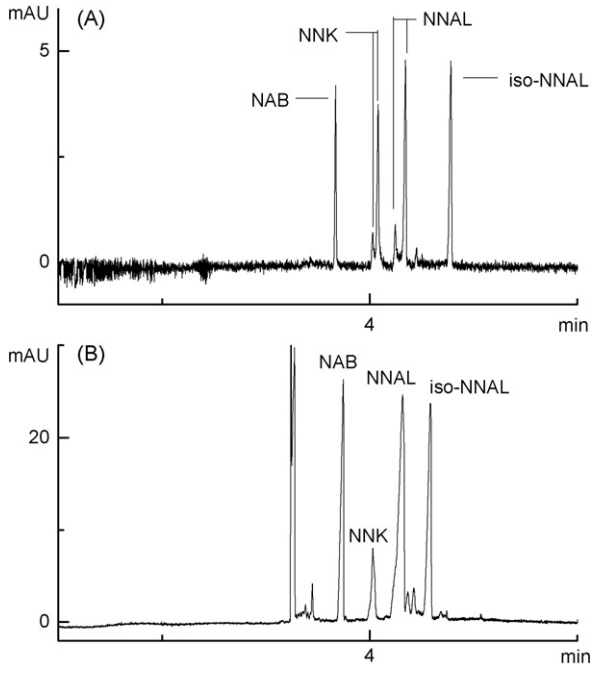

Fig. 2. (A) Separation of NAB, NNK, NNAL and iso-NNAL by normal MEKC. Conditions: running buffer, 80 mM phosphate solution with 75 mM SDS and 10% (v/v) ACN at pH 2.5; sample concentration, 100 μg/mL in pure water; hydrodynamic injection, 1.3 mm; Separation voltage, −25 kV; (B) Typical electropherogram of the four analytes by CSEI–sweeping–MEKC. Conditions: nonmicellar BGE, 80 mM phosphate solution containing 10% (v/v) ACN at pH 2.5; HCB, 109 mM phosphoric acid, 13.3 mm; water plug, 1.3 mm; micellar BGE, 80 mM phosphate solution (pH 2.5) containing 75 mM SDS and 10% (v/v) ACN; sample concentration, 0.1 μg/mL in pure water; electrokinetic injection, 10 kV for 300 s; separation voltage, −25 kV.

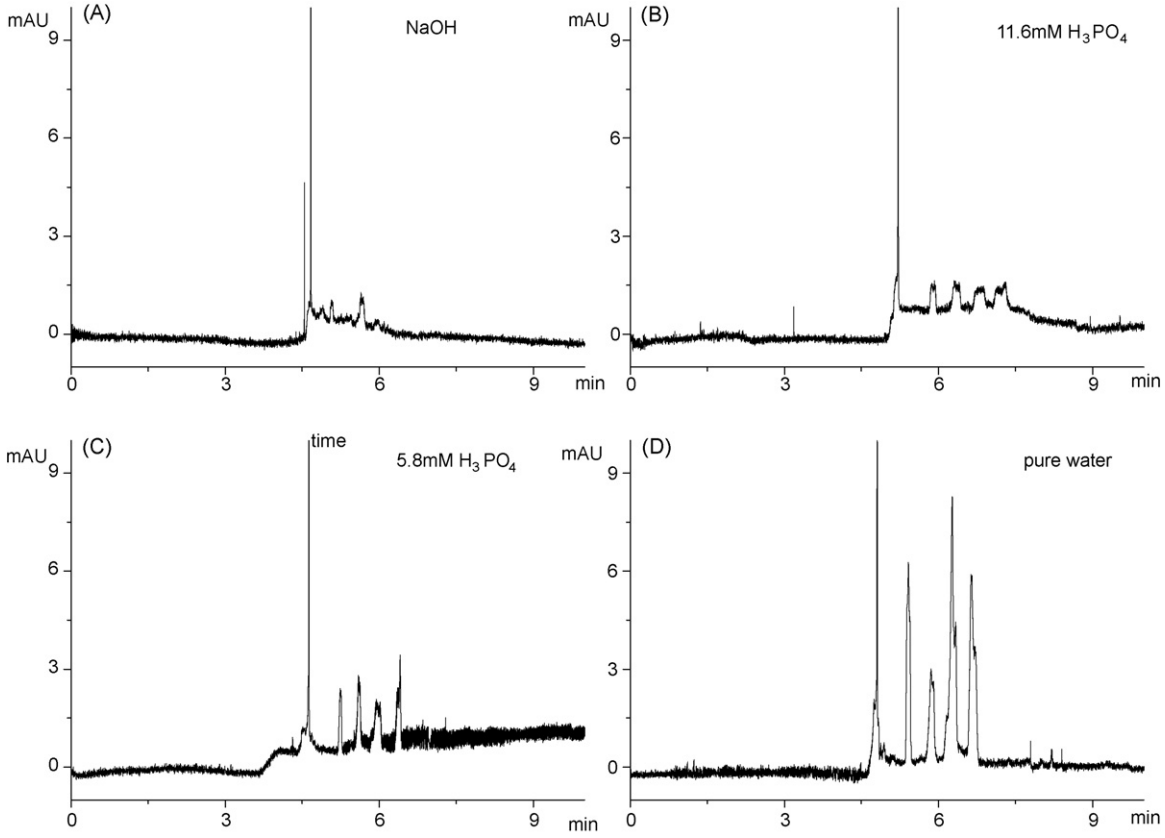

Fig. 3. Influence of sample matrix in CSEI–sweeping–MEKC. (A) 5.8 mM NaOH solution, 0.975 mS; (B) 11.6 mM phosphoric acid solution, 2.130 mS; (C) 5.8 mM phosphoric acid solution, 1.104 mS; (D) pure water, 2.01 μS. Conditions: HCB, 218 mM phosphoric acid, 20 mm; sample concentration, 0.05 μg/mL in pure water. Other conditions were the same as those used in Fig. 2B.

3. Results and discussion

3.1. Optimum separation conditions

Before performing the preconcentration technique, the four TSNAs should be well separated in normal MEKC, so several parameters which would have influence on the separation required to be investigated. In the preconcentration procedure, EOF should be controlled to be as low as possible. Therefore, an acidic phosphate solution was chosen to be the running buffer. Other conditions such as the concentration of phosphate solution (30–100 mM), the concentration of SDS (50–150 mM), pH (2.0–4.0) and the percentage of organic additives (1–12% (v/v)) were also optimized. The effects of the applied voltage and the temperature were considered to obtain the shortest analysis time (data not shown). Fig. 2A represented the separation of 100 μg/mL standard solution of the four TSNAs (in pure water) under the optimized conditions. And the splitting peaks of NNK and NNAL should be due to their (*E*)/(*Z*)-isomers.

3.2. Optimization of CSEI–sweeping–MEKC

To obtain the best separation and the highest enhancement efficiency (Fig. 2B), several key factors were investigated here including the sample matrix, the HCB, the length of water plug and the amount of the sample electrokinetically injected. It should be noted that fresh sample was required in each run since the concentration of sample was decreased after the long time injection (data not shown).

3.2.1. Optimizing the sample matrix

In CSEI–sweeping–MEKC procedure, sample matrix played an important role. Pure water, 5 mM sodium hydroxide solution, 5.8 mM and 11.6 mM phosphoric acids were tested as the sample matrix, respectively (Fig. 3). In electrokinetic injection procedure, both of the electroosmotic mobility and the electrophoretic mobility were significant factors which would affect the sample amount injected. It could be found that when prepared in 5 mM sodium hydroxide solution (Fig. 3A) with conductivity of 0.975 mS, analytes were neutral and rarely concentrated compared with those in phosphoric acid (as shown in Fig. 3B and C). Because the analytes were positively charged in-phosphoric acid, they would be selectively injected. However, samples prepared in phosphoric acid showed less enrichment effect than those prepared in pure water. Sample matrix with lower conductivity would result in higher electric field strength, which could accelerate the cations' mobility and increase the amount of sample introduced [21]. It was clearly observed that with the increase of the electric field strength in the sample matrix from Fig. 3B to D, the enrichment efficiency was improved a lot.

3.2.2. Optimizing the HCB

HCB was also very important in the sample stacking. The introduction of HCB could increase the difference of the electric field strength between the sample zone and the BGE, and would improve the stacking efficiency. Here phosphoric acid was used as the HCB and the effect of different concentrations between 27 mM and 218 mM was tested (Fig. 4A) . It was found that the sensitivity was increased when the concentration of phosphoric acid was changed from 27 to 109 mM. However, with further increase of the concentration, peaks of the four analytes were broadened, since the

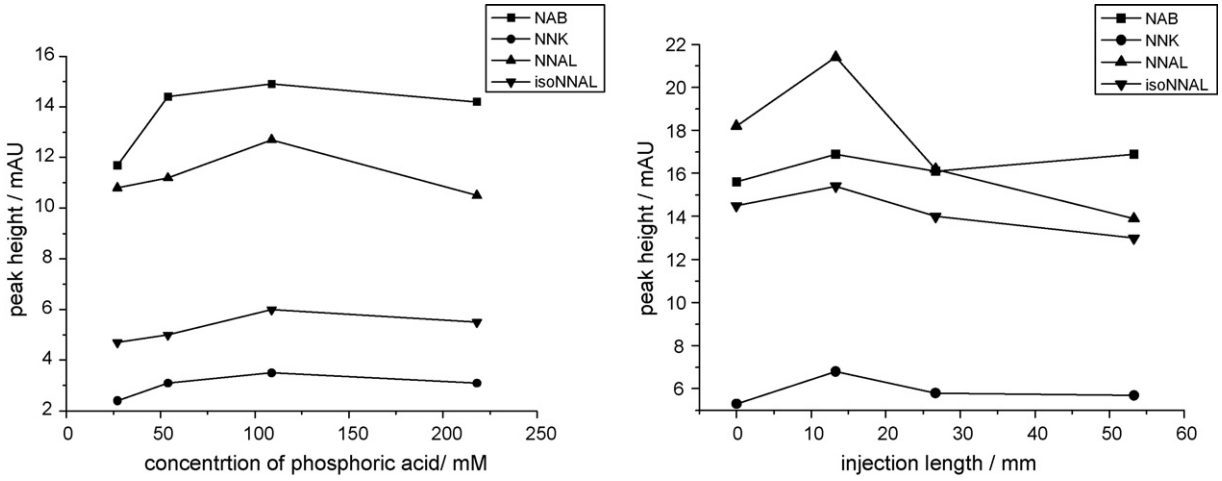

Fig. 4. Effects of the HCB concentration (A) and the HCB length (B) in the CSEI–sweeping–MEKC method. See Fig. 2B for other conditions.

enlarged difference between the Kohlrausch values of the HCB and the non-micellar BGS could lead to the over dilution of the sample zone or destacking at the interface between the HCB and the non-micellar BGS [18]. In addition, the injection length of HCB could also influence the stacking procedure. In this experiment, the length of the HCB from 0 to 53.3 mm was investigated (Fig. 4B), showing that 13.3 mm of HCB could lead to the highest efficiency of concentration.

3.2.3. Optimizing the water plug

Water plug could provide a higher electric field at the tip of the capillary, which would eventually improve the stacking procedure by electrokinetic injection [23]. In this experiment, when a water plug of 1.3 mm was introduced, the peak intensities were a little higher than those without water plug. However, with further increase of the length of the water plug, the peak height was decreased drastically. The possible reason was that the water plug with a large length would give rise to a strong laminar flow which could make the EOF velocities in the sample and buffer zones mismatch [24,25]. Besides, the RSDs of peak areas, peak heights and retention times when absence of water plugs were in the range of 6.9–8.9%, 8.3–10.9% and 0.7–1.0%, respectively, which were much worse than those with a water plug of 1.3 mm (as shown in Table 1).

Table 1
Method validation for the determination of TSNAs by CSEI–sweeping–MEKC and LOD comparison with that by normal TSNAs.

TSNAs	NAB	NNK	NNAL	iso-NNAL
TSNA CSEI–sweeping–MEKC				
Regression equation	$y = Ax + B$			
A	0.31	0.13	0.62	0.38
B	−0.23	−0.51	5.59	3.55
Linear range (ng/mL)	5–500	25–500	5–500	5–500
Correlation coefficient (r^2)	0.9996	0.9980	0.9992	0.9992
RSD (%, $n = 5$)				
Peak height	3.9	2.1	2.4	3.1
Peak area	3.3	4.8	4.0	4.0
Migration time	0.6	0.6	0.6	0.6
LOD (S/N = 3, ng/mL)	4	16	4	4
SEF[a]				
Peak height	6.8×10^3	2.4×10^3	5.4×10^3	5.5×10^3
Peak area	1.4×10^4	0.6×10^4	1.5×10^4	1.0×10^4
Normal MEKC				
LOD (S/N = 3, μg/mL)	5.0	5.0	3.1	3.1

[a] SEF, sensitivity enhancement factor in terms of peak height or peak area = dilution factor × (peak height or peak area obtained with CSEI–sweeping–MEKC/peak height or peak area obtained with normal MEKC injection of 50 mbar 1 s).

3.2.4. Optimizing the injection time

During field-enhanced sample injection procedure, the analyte zone length depended on injection time [18,26]. The longer the injection time, the larger the sample introduced, and the higher the enhancement efficiency. Taking NNAL for an example, Fig. 5 showed that with the increase of the injection time from 100 s to 300 s, the peak height intensity and the peak area was increased all the time since they were directly proportional to the amount of analytes injected. However, when the injection time was increased further, the peak height was not increased any more and the peak width was broadened obviously. This could be explained by the fact that sweeping was only limited by the retention factor k of the analyte in the micelle [18]. And the length of the analyte plug after sweeping was based on the following equation:

$$l_{\text{sweep}} = l_{\text{inj}} \left[\frac{1}{(1+k)} \right] \quad (1)$$

wherein l_{inj} was the original length of the analyte plug injected [17,27]. Therefore, there is a balance between the length of the sample zone and the enhancement efficiency. As a result, a sampling time of 300 s at 10 kV was selected, obtaining the highest enrichment efficiency.

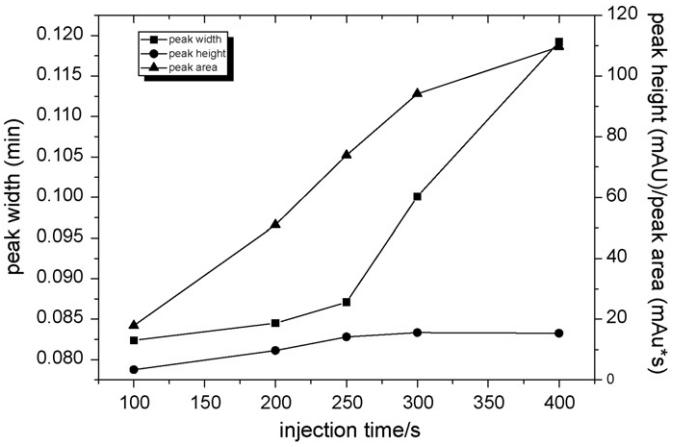

Fig. 5. Effects of the sample injection time in CSEI–sweeping–MEKC for NNAL. Other conditions were the same as those used in Fig. 2B.

3.3. Method validation

Fig. 2B represented a typical electropherogram of the four analytes by the CSEI–sweeping–MEKC method. Under the optimal conditions, NAB, NNK, NNAL and iso-NNAL were effectively concentrated without resolution loss. Compared with normal MEKC, CSEI–sweeping–MEKC could provide 2.4×10^3 to 6.8×10^3-fold enhancements of peak heights, and 6.0×10^3 to 1.5×10^4-fold enhancements of peak areas (as shown in Table 1), and the LODs were 4 ng/mL for NAB, NNAL and iso-NNAL and 16 ng/mL for NNK, which were decreased by three orders of magnitude. In addition, the reproducibility of analysis by CSEI–sweeping–MEKC was satisfactory, and RSD ($n = 5$) was 0.6% for migration time while the RSDs for peak height and peak area were 2.1–3.9% and 3.3–4.8%, respectively. As listed in Table 1, the linearity of this method was examined from 5 to 500 ng/mL for NAB, NNAL and iso-NNAL and from 25 to 500 ng/mL for NNK, demonstrating that the linearity over the concentration range was quite satisfactory with correlation coefficients (r^2) higher than 0.9980. Therefore, CSEI–sweeping–MEKC was reliable and could be used to separate and determine NAB, NNK NNAL and iso-NNAL with high enhancement efficiencies.

3.4. Analysis of human urine sample

Complicated matrices in biological samples like urine, blood and cells brought great difficulties to the analysis, especially with the electrokinetic injection. So it was indispensable to develop a thorough sample preparation method, such as SPE. In this study, CSEI–sweeping–MEKC combined with SPE was used to analyze the four TSNAs in urine samples. SPE procedure underwent two steps, including the removal of the salts and non-cationic analytes based on the hydrophobic interaction and the cation-exchange mechanism, respectively. Fig. 6A and B represented the electropherograms of blank samples without SPE and with SPE, respectively, indicating that the sample procedure in this method was feasible and effective. When SPE was used, the average recoveries of NAB, NNK, NNAL and iso-NNAL were 86%, 74%, 93%, and 89%, respectively, showing the acceptable extraction efficiency. Subsequently, the four TSNAs in spiked urine samples were detected and analyzed by CSEI–sweeping–MEKC, as shown in Fig. 6C.

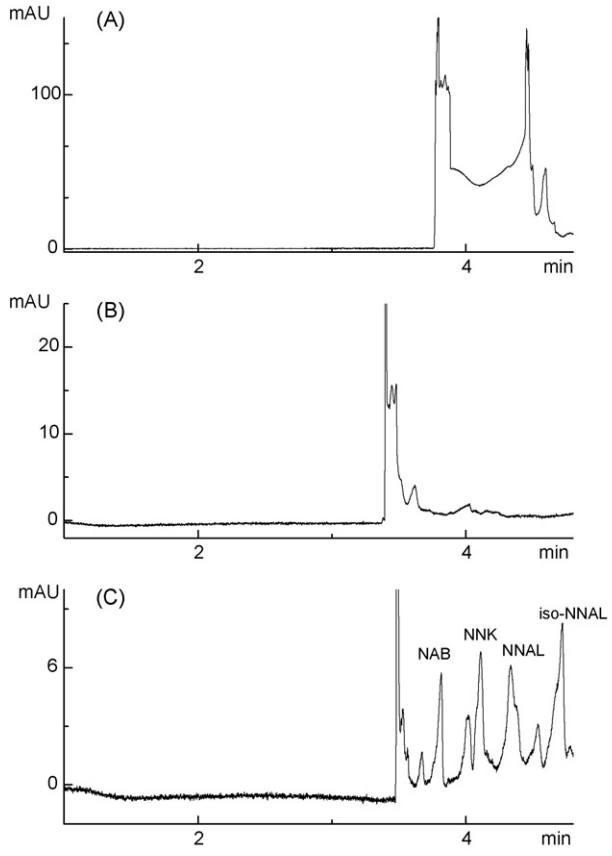

Fig. 6. The electropherograms of (A) blank urine samples without SPE treatment, (B) blank urine samples with SPE treatment, and (C) urine samples spiked with the four TSNAs (each 0.2 μg/mL). Conditions were the same as those used in Fig. 2B.

4. Conclusion

In this study, a highly sensitive CSEI–sweeping–MEKC method was proposed to concentrate the four TSNAs: NAB, NNK, NNAL and iso-NNAL. Compared with GC and LC methods which required the equilibration of the columns, it was less time-consuming. Through the optimization of several key factors such as HCB, water plug, injection time and sample matrix, the detection sensitivity was improved by almost four orders of magnitude in terms of peak area and peak height compared with that by normal MEKC. Furthermore, combined with effective SPE procedure, this method showed a great potential to analyze TSNAs in complicated biological matrices.

Acknowledgements

The authors are grateful to Dr. Nannan Pang and Dr. Zhengxiang Zhang for fruit suggestions regarding the manuscript, and Dr. Dawei Wen for his kindness to provide the SCX SPE cartridges. This study is financially supported by NSFC, Grant No. 90717002 and 20975005. Agilent Technologies for providing HP3DCE system.

References

[1] S.S. Hecht, Chem. Res. Toxicol. 11 (1998) 559–603.
[2] I. Stepanov, S.G. Carmella, S.S. Hecht, G. Duca, J. Agric. Food Chem. 50 (2002) 2793–2797.
[3] K.D. Brunnemann, D. Hoffmann, Crit. Rev. Toxicol. 21 (1991) 235–240.
[4] W.J. Wu, D.L. Ashley, C.H. Watson, Anal. Chem. 75 (2003) 4827–4832.
[5] J. Zhou, R.S. Bai, Y.F. Zhu, Rapid Commun. Mass Spectrom. 21 (2007) 4086–4092.
[6] M. Sleiman, R.L. Maddalena, L.A. Gundel, H. Destaillats, J. Chromatogr. A 1216 (2009) 7899–7905.
[7] D. Kavvadias, G. Scherer, M. Urban, F. Cheung, G. Errington, J. Shepperd, M. McEwan, J. Chromatogr. B 877 (2009) 1185–1192.
[8] C.C. Li, D.W. Wen, J.X. Zhang, Z. Chen, W.H. Cong, Z.H. Rao, H.W. Liu, Anal. Bioanal. Chem. 386 (2006) 1985–1993.
[9] H.L. Lee, C.Y. Wang, S. Lin, D.P.H. Hsieh, Talanta 73 (2007) 76–80.
[10] P. Jacob, C. Havel, D.H. Lee, L. Yu, M.D. Eisner, N.L. Benowitz, Anal. Chem. 80 (2008) 8115–8121.
[11] J.C. Wu, P. Joza, M. Sharifi, W.S. Rickert, J.H. Lauterbach, Anal. Chem. 80 (2008) 1341–1345.
[12] C.C. Li, Z. Chen, D.W. Wen, J.X. Zhang, W.H. Cong, B. Yu, Y.P. Liao, H.W. Liu, Electrophoresis 27 (2006) 2152–2163.
[13] C.H. Lin, T. Kaneta, Electrophoresis 25 (2004) 4058–4073.
[14] J.H. Li, Z.W. Cai, Talanta 77 (2008) 331–339.
[15] J.P. Quirino, S. Terabe, J. Capillary Electrop. 4 (1997) 233–245.
[16] J.P. Quirino, S. Terabe, Anal. Chem. 71 (1999) 1638–1644.
[17] J.P. Quirino, S. Terabe, Science 282 (1998) 465–468.
[18] J.P. Quirino, S. Terabe, Anal. Chem. 72 (2000) 1023–1030.
[19] L.Y. Zhu, C.H. Tu, H.K. Lee, Anal. Chem. 74 (2002) 5820–5825.
[20] Y.H. Lin, J.F. Chiang, M.R. Lee, R.J. Lee, W.K. Ko, S.M. Wu, Electrophoresis 29 (2008) 2340–2347.
[21] H.L. Su, Y.Z. Hsieh, J. Chromatogr. A 1209 (2008) 253–259.
[22] X. Luo, X. Jiang, X. Tu, S. Luo, L. Yan, B. Chen, Electrophoresis 31 (2010) 688–694.
[23] R.L. Chien, Anal. Chem. 63 (1991) 2866–2869.
[24] R.L. Chien, J.C. Helmer, Anal. Chem. 63 (1991) 1354–1361.
[25] S.H. Liu, Q.F. Li, X.G. Chen, Z.D. Hu, Electrophoresis 23 (2002) 3392–3397.
[26] H.Y. Huang, W.C. Lien, I.Y. Huang, Electrophoresis 27 (2006) 3202–3209.
[27] B.C. Giordano, C.I.D. Newman, P.M. Federowicz, G.E. Collins, D.S. Burgi, Anal. Chem. 79 (2007) 6287–6294.