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Determination of heterocyclic amines by capillary electrophoresis with UV-DAD detection using on-line preconcentration

Xiao-Qing Fei, Chen Li, Xiao-Dong Yu *, Hong-Yuan Chen

Key Lab of Analytical Chemistry for Life Science, Ministry of Education, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210093, PR China

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Abstract

This paper proposed a method with capillary zone electrophoresis (CZE) for analysis of eight heterocyclic amines in a commercial meat matrix. The influence of composition, pH and concentration of buffer, and applied voltage were investigated. A 5 mmol/L formic acid–ammonium formate solution at pH 2.20 was chosen as the running electrolyte. Also several solid-phase extraction actions were performed for the clean-up of the samples. With 3 s hydrodynamic injection, detection limits ranging from 0.554 to 1.783 μg/g was obtained. To improve sensitivity, field-amplified sample injection (FASI) was used with the conditions of 3 s hydrodynamic injection of a water plug and 25 s electrokinetic injection of the sample. And methanol–water (1:1 in volume) was applied as the sample solvent. Under above conditions, detection limits ranged from 1.329 to 19.39 ng/g, which were at least 50 times lower than those with hydrodynamic injection.

Keywords: Capillary electrophoresis; Heterocyclic amines; On-line concentration; UV-DAD detection

1. Introduction

Heterocyclic amines (HAs) are a group of compounds that have been found to be mutagens and potential carcinogens [1,2]. They are formed from the protein-rich foods during the thermal treatment at ng/g level [3,4]. People are mainly exposed to them by eating fried meat, beef, fish or inhaling the fumes during the cooking procedures. To evaluate the exposure to HAs, it is necessary to propose accurate and sensitive methods for analysis of them in foods and body fluids. And the trace levels presenting in complex food matrix demand rigorous sample purification steps and effective preconcentration before the final analysis.

Common separation methods for HAs included HPLC–UV-DAD [5–10], HPLC–ED [11–13], HPLC–MS [14–17] and GC–MS [18,19]. Recently, capillary electrophoresis (CE) is found to be a powerful tool to separate charged proteins, peptides, organic and inorganic ions and enantiomers. However, only a few studies have been reported using CE for the determination of HAs [20–26,33]. For routine use, regular detectors as optical detectors were more preferred over the expensive and complicated MS system [23,24,26]. And fluorescence detection was limited to only non-polar heterocyclic amines, though it had much higher sensitivity and selectivity. UV and UV diode-array detection (UV-DAD) were most commonly used [20,21,25,33], for they allowed the detection of all the HAs. But the low sensitivity of the UV-DAD detection may not satisfy the demands of the analysis of HAs (at ng/g level) in real samples.

To increase the sensitivity of UV-DAD detection, the preconcentration methods that can combine with CE are intensively required. The most interesting approaches were the on-line preconcentration methods [23,24,27–31]. Those techniques were mostly electrophoretic-based, which utilize the differences in mobilities and conductivities to preconcentrate the analytes. Among them, normal stacking [28], field-amplified sample injection (FASI) [23,24,29] and sweeping [30] were the most frequently used. In normal stacking the sample in a low-conductivity solvent is firstly injected in hydrodynamic mode, and then the separation voltage is applied. However, the main procedures of FASI are as follows: firstly the sample is dissolved in a solvent of lower conductivity than that of the running electrolyte; then the sample is injected in electrokinetic mode; when applying of the voltage, the electric field strength of the low-conductivity zone is increased, producing an increase of

* Corresponding author. Tel.: +86 25 83592774.
E-mail address: yuxd@nju.edu.cn (X.-D. Yu).

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electrophoretic velocities and a narrower analyte zone. What's more, in FASI a plug of a higher-resistivity solvent (such as water) is often introduced in front of the sample zone for a further enhancement of sensitivity [31]. The main difference between normal stacking and FASI is that in normal stacking the stacking process occurs as the separation voltage is applied, while in FASI the focusing process occurs during the electrokinetic injection of the sample. Sweeping is a preconcentration method applied in micellar electrokinetic chromatography (MEKC). In sweeping, the sample is dissolved in the buffer without micelles. When the separation voltage is applied, the micellar phase in the running electrolyte enters into the sample zone. The concentration occurs when the analytes interact with micelles, so concentration effect lies on the interactions between the analytes and the micellar phase. The stronger interactions between may bring out a higher concentration factor which may be in the range of 80–5000.

The present paper utilized capillary electrophoresis with UV-DAD detection for the separation of eight heterocyclic amines. We investigated how the different parameters affected the separation and the sensitivity, which included the running buffer composition, pH and concentration of the running electrolyte, applied voltage and the sample solvent. To improve the detection limits, on-line preconcentration based on FASI was used. Further, this method was applied to determine these amines in a commercial meat extract.

2. Experimental

2.1. Chemicals

The heterocyclic aromatic amines: 2-amino-1-methylimidazo[4,5-*f*]quinoline (iso-IQ), 2-amino-3, 4-dimethy-3H-limidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3, 8-trimethylimidazo [4,5-*f*]quinoxaline (MeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-9H-pyrido[2,3-b] indole(AαC), 2-amino-3-methyl-9H-pyrido[2,3-b] indole (MeAαC) were purchased from Toronto Research Chemicals (Toronto, Canada). 1-Methyl-9*H*-pyrido[3,4-*b*]indole (Harman, Har) and 9*H*-pyrido[3,4-*b*]indole (Norharman, Nhar) were from Acros Organics(Geel, Belgium). Individual stock standard solutions between 10 and 250 μmol/L in methanol–water (1:1 in volume) were prepared and used for further dilution. Reagents used for the preparation of the running electrolytes were of analytical grade: ammonium formate, formic acid (85%, w/w), methanol, hydrochloric acid, glycin, potassium chloride, sodium dihydrogen phosphate and disodium hydrogen phosphate. The formic acid–ammonium formate running electrolyte was prepared as follows: a 100 mmol/L ammonium formate stock solution was prepared by dissolving ammonium formate with doubly distilled water. The running electrolyte was freshly prepared by diluting the ammonium formate stock solution to the desired concentration, and then adjusting to the desired pH with formic acid solution which had the same concentration as the ammonium formate. All solutions were prepared with doubly distilled water and filtered through a 0.22 μm cellulose membrane filter before use.

Refill material Kieselgur was provided by Sigma–Aldrich Laborchemikalien. GmbH (Seelze, Germany). Kieselgur extraction cartridges (6 mL) were provided by Sipore Co., Ltd. (Dalian, China). Aichrom C18 (500 mg) and strong cation-exchange (SCX) (500 mg) cartridges were provided by Bafang Century Technology Ltd. (Beijing, China). The cartridges were preconditioned with dichloromethane (7 mL), 0.1 mol/L HCl (5 mL), water (10 mL) and methanol (5 mL) for SCX and methanol (5 mL), water (5 mL) for C18. A commercial meat extract was obtained from a local market.

2.2. Instruments

Capillary electrophoresis was performed with Agilent Technology (Waldbronn, Germany) CE^{3D} instrumentation equipped with a 50 μm i.d. × 50 cm (41.5 cm effective length) uncoated fused-silica capillary (Yongnian Optical Fiber Factory, Hebei, China). Detection of all the samples was performed by means of a diode-array detector positioned at the cathodic end of the capillary.

2.3. Electrophoresis procedure

Running electrolyte solutions were prepared with doubly distilled water. Temperature was set to 25 °C and voltage applied varied between 9 and 21 kV Before first run, each new capillary was rinsed with 1 mol/L NaOH, doubly distilled water and the running buffer for 30, 10 and 10 min, respectively, then pre-equilibrated with the running buffer at the separation voltage for 10 min. Migration time and other electrophoretic parameters were determined for three sequential sample injections. After each run, the capillary was rinsed with 1 mol/L NaOH, doubly distilled water and the running buffer, each for 2 min. UV-DAD detection was performed at 214 nm.

The sample injection was operated in hydrodynamic mode, electrokinetic mode and FASI mode. In hydrodynamic mode, 50 mbar was applied for 3 s. In electrokinetic mode 10 kV was applied for 25 s. And in FASI mode, a plug of water was injected in hydrodynamic mode with 50 mbar for 3 s, then the sample zone was injected in electrokinetic mode with 10 kV applied for 25 s.

2.4. Clean-up procedure

Sample preparation and clean-up were carried out according to the method proposed by Gross with some modifications [8,23,32]. A 2–3 g meat sample, spiked with amines (100 ng/g), was dissolved in 12 mL 1 mol/L NaOH and homogenized with sonication for 3 h [8]. The alkaline solution was mixed with refilled material Kieselgur (15 g) and then used to fill empty Kieselgur extraction cartridges. The analytes adsorbed on the Kieselgur extraction cartridges were extracted using 75 mL dichloromethane. Extractants were then passed through a SCX (500 mg) cartridge, which was preconditioned with 7 mL dichloromethane, 5 mL 0.1 mol/L HCl, 10 mL water and 5 mL methanol. The SCX cartridge was rinsed with 15 mL methanol–water (4:6 in volume) and 2 mL of water, and amines

were eluted from the SCX cartridge with 20 mL 0.5 mol/L ammonium acetate (pH 8.5). And then extractants were passed through a C18 (500 mg) cartridge. The C18 one was rinsed with 5 mL of water and HAs were eluted with 0.8 mL of methanol–ammonia (9:1 in volume). The solvent was evaporated under a soft stream of nitrogen at room temperature. Finally, the extract was redissolved with 0.1 mL (100 μL) methanol–water (1:1 in volume).

3. Results and discussion

3.1. CE optimization

The effect of the running electrolyte composition on the separation was studied at pH 2.20 using 10 mmol/L formic acid–ammonium formate, 10 mmol/L KCl–HCl, 5 mmol/L phosphate-buffered saline (PBS), 10 mmol/L glycine–HCl and 30% methanol–10 mmol/L NaH_2PO_4 as the running buffer solution. With glycine–HCl, Har and MeAαC co-eluted and the peak shapes were bad. With 30% methanol–NaH_2PO_4, MeAαC and MeIQx co-eluted and the analysis time was larger than the others. Baseline separations were obtained with KCl–HCl, PBS and formic acid–ammonium formate (Fig. 1), but the separation current using PBS was larger than the other two. In view of the high Joule heat that might bring with, we preferred the rest two. Eight amines were separated within 10.60 min with formic acid–ammonium formate solution, and a longer time 11.60 min occurred using KCl–HCl though slightly higher resolutions were achieved. What's more, we got higher responses with the former. So a 10 mmol/L formic acid–ammonium formate solution was chosen for further study.

To study the effect of electrolyte pH on the separation, the concentration of formic acid–ammonium formate was set at 10 mmol/L and pH of electrolyte varied from 2.00 to 2.80. In all the pH conditions, amines were baseline separated and there was no great improvement in resolutions. As can be seen in Fig. 2, resolutions just changed in a small degree. For relative larger responses were obtained using the pH 2.20 solution, it was chosen for the next optimization.

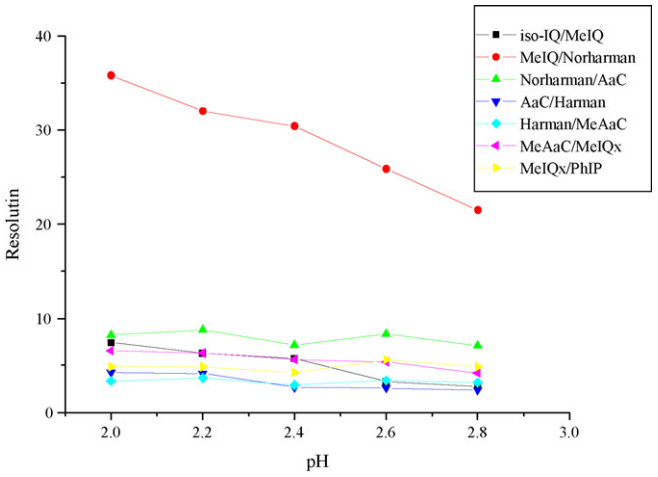

Fig. 2. Effect of different electrolyte pH on resolutions of a 50 μmol/L standard solution. Conditions: capillary, 50 μm i.d. × 50 cm (41.5 cm effective length); capillary temperature, 25 °C; 3 s hydrodynamic injection; applied voltage, 15 kV; λ, 214 nm; the running electrolyte, 10 mmol/L formic acid–ammonium formate.

The concentration of the running electrolyte (pH 2.20) was also optimized, which was varied from 2.5 to 25 mmol/L. There was slight difference in the analysis time and baseline separation was achieved in all the conditions, but the peak shapes of iso-IQ and MeIQ got worse and the baseline became fluctuant as the concentration increased (Fig. 3). And with the 2.5 mmol/L electrolyte, the responses of iso-IQ and MeIQ were small. So the best results were received using the 5 mmol/L electrolyte with better peak shapes and higher responses. As a conclusion, 5 mmol/L formic acid–ammonium formate (pH 2.20) was recommended as the best running electrolyte.

As the voltage applied increased from 9 to 21 kV, there was a decrease in analysis time as the result of the enhanced mobility. At the low values as 9, 12 and 15 kV, the separation time was at least 10 min with relative lower responses. And at higher value as 21 kV, there was a considerable increase in current and background noise, and also band dispersion appeared due to the Jole heat effect. For these reasons, a voltage of 18 kv was chosen

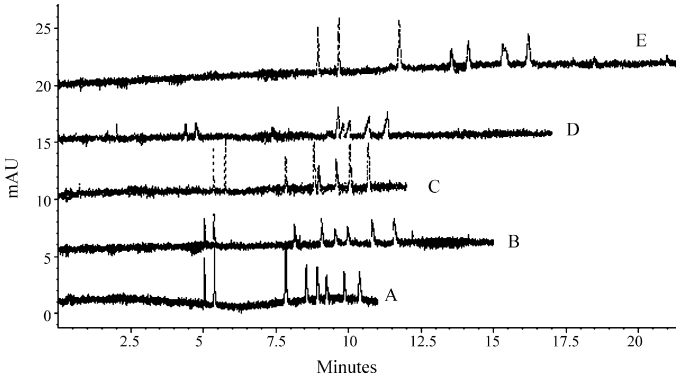

Fig. 1. Electropherograms with different electrolyte composition of a 8 μmol/L standard solution. Conditions: capillary, 50 μm i.d. × 50 cm (41.5 cm effective length); capillary temperature, 25 °C; 3 s hydrodynamic injection; applied voltage, 15 kV; λ, 214 nm. (A) 10 mmol/L formic acid–ammonium formate; (B) 10 mmol/L KCl–HCl; (C) 5 mmol/L PBS; (D) 10 mmol/L glycine–HCl and (E) 30%methanol–10 mmol/L NaH_2PO_4.

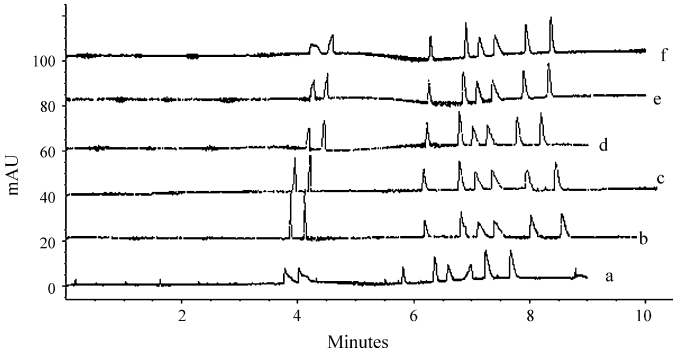

Fig. 3. Electropherograms with different electrolyte concentrations of a 50 μmol/L standard solution. Conditions: capillary, 50 μm i.d. × 50 cm (41.5 cm effective length); capillary temperature, 25 °C; 3 s hydrodynamic injection; applied voltage, 15 kV; λ, 214 nm; the running electrolyte, formic acid–ammonium formate (pH 2.20). (a) 2.5 mmol/L; (b) 5 mmol/L; (c) 10 mmol/L; (d) 15 mmol/L; (e) 20 mmol/L and (f) 25 mmol/L.

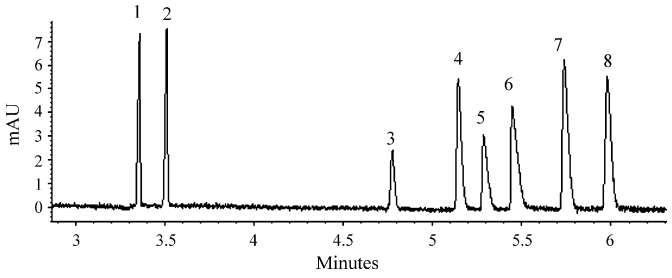

Fig. 4. Electropherograms of a 50 μmol/L standard solution. Conditions: capillary, 50 μm i.d. × 50 cm (41.5 cm effective length); capillary temperature, 25 °C; 3 s hydrodynamic injection; applied voltage, 18 kV; λ, 214 nm; the running electrolyte, 5 mmol/L formic acid–ammonium formate (pH 2.20). Peaks: 1 iso-IQ, 2 MeIQ, 3 Nhar, 4 AaC, 5 Har, 6 MeAaC, 7 MeIQx and 8 PhIP.

Table 1
Calibration data using 3 s hydrodynamic injection

Amines	Linear range (μmol/L)	Linear function[a]	r[b]
Iso-IQ	5.00–50.0	$Y = 0.121x + 0.273$	0.9988
MeIQ	5.00–100	$Y = 0.130x + 0.229$	0.9996
Nhar	25.0–200	$Y = 0.0408x + 0.212$	0.9983
AαC	7.50–100	$Y = 0.106x + 0.0502$	0.9996
Har	10.0–100	$Y = 0.0521x + 0.242$	0.9965
MeAαC	7.50–100	$Y = 0.0743x + 0.272$	0.9987
MeIQx	5.00–100	$Y = 0.107x + 0.356$	0.9979
PhIP	7.50–100	$Y = 0.103x + 0.171$	0.9997

[a] The unit of x is μmol/L.
[b] $n = 7$ except $n = 6$ for iso-IQ.

with resolutions ranging from 1.77 to 25.3 and analysis times no longer than 6.30 min.

3.2. Quality parameters

Under the optimized conditions, eight amines were baseline separated (Fig. 4) and quality parameters were evaluated for 3 s hydrodynamic sample injection (Tables 1 and 2). Detection limits, at 214 nm, based on a 3:1 signal-to-background noise ratio, ranged from 2.28 to 9.51 μmol/L (that is 0.593 to 1.78 μg/g), which did not satisfy the demands of analyzing the trace level amines though acceptable in CE–UV-DAD. As can be seen in Table 2, good precisions were achieved for both migration times and responses (peak heights). Run-to-run precisions were determined by 3 s hydrodynamic injecting in the same work day with five replicates of a 25 μmol/L standard solution. The standard deviations obtained for migration times and responses were lower than 0.389 and 7.47%, respectively. Calibration curves for amines were carried out with standard solutions in the range of 5–250 μmol/L. The correlation coefficients of calibration functions were between 0.9965 and 0.9997 (Table 1). These results showed that the method can be used for quantitative analysis.

Table 2
CE–UV-DAD quality parameters using hydrodynamic injection

Amines	LOD (μmol/L)	LOD (μg/g)	Run-to-run precision, R.S.D. (%), $n = 5$	
			Migration time	Responses
Iso-IQ	2.70	0.596	0.389	4.18
MeIQ	2.85	0.674	0.339	2.88
Nhar	9.51	1.78	0.290	7.47
AαC	5.19	1.06	0.316	4.28
Har	6.87	1.40	0.328	3.95
MeAαC	4.41	0.971	0.335	6.20
MeIQx	2.28	0.542	0.368	3.07
PhIP	4.17	1.05	0.364	3.38

3.3. On-line preconcentration

As detection limits with hydrodynamic injection did not satisfy the demands of analyzing the trace level amines, a preconcentration technique was strongly demanded. In the application of FASI, electrokinetic injection of a sample diluted in a solvent (in our case 1:1 methanol–water) of lower conductivity than that of the running electrolyte. As the application of the voltage, an enhancement of the electric field strength occurs in the lower conductivity zone (the sample zone). As we know, the electrophoretic velocities are in direct proportion to the electric field. So the ions in the sample zone migrate to the front of the zone with significant high electrophoretic velocities until they enter into the running electrolyte zone. Then the ions decelerate in the running electrolyte zone because of the suddenly small electric field strength of the running electrolyte zone. As a result, the sample zone becomes narrower. Thus, a certain preconcentration effect was obtained.

The electrophoretic mobility of the analyte and the electroosmotic flow determine the sample amount injected and the analysis time. In the low pH running buffer (pH 2.20), the activation on the capillary surface was suppressed. So a very low electroosmotic flow was produced and the electrophoretic mobility played the most important part in the migration. In addition, there will be a low degree of protonation of HAs if they are diluted in methanol as in usual studies. A low degree of protonation of the amines may reduce the positive charge the amines carrying, which decrease their electrophoretic mobilities. Sentellas et al. [23] recommended that there was an improvement in the electrophoretic mobility using methanol–water (1:1) or methanol–5 mmol/L formic acid (1:1 in volume). In this study, we compared the experimental results obtained by using three sample solvent: methanol, methanol–water (1:1) and methanol–5 mmol/L formic acid (1:1). In the case of methanol–water (1:1), larger responses and better peak shapes were obtained than the other two, so methanol–water (1:1) was chosen as the sample solvent.

In FASI, a plug of a higher-resistivity solvent (water in our study) is frequently introduced in front of the sample zone for a further enhancement of sensitivity. So the injection times to introduce the plug of water (hydrodynamic mode) and the sample (electrokinetic modes) should be optimized. Hydrodynamic injection (50 mbar) for the water plug during 3 and 15 s, and electrokinetic injection (10 kV) for the sample from 5 to 30 s were tested. We chose a water plug with 3 s hydrodynamic injection (50 mbar) for relative high responses. As we know, the increasing of sample injection time may bring about the increase of the responses, but also an apparent peak broadening. In this case, 25 s of electrokinetic injection of the sample was chosen. Finally,

Table 3
Improved times of responses obtained using different injection modes

Amines	Hydrodynamic injection	Electrokinetic injection[a]	FASI[a]
Iso-IQ	1.00	31.8	42.6
MeIQ	1.00	30.5	35.6
Nhar	1.00	43.6	48.8
AαC	1.00	25.2	23.9
Har	1.00	32.8	34.1
MeAαC	1.00	13.8	16.2
MeIQx	1.00	20.6	22.8
PhIP	1.00	23.0	24.0

[a] The data were obtained with the data of column 2 (responses in hydrodynamic injection) as the standard.

the optimum FASI procedure was as follows: methanol–water (1:1) as sample solvent, 3 s hydrodynamic injection (50 mbar) of water, then 25 s electrokinetic injection (10 kV) of the sample solution.

In Table 3 the responses obtained for amines using different injection procedures were given. A comparison between FASI, the 3 s hydrodynamic injection and 25 s electrokinetic injection (10 kV) of the 25 μmol/L standard solution in methanol–water (1:1) was performed (Table 3). The results showed that the greatest improvement in sensitivity was obtained using FASI. For the slower amines with higher migration times (PhIP, MeIQx and MeAαC), there was less improvement in the signals (about 20 times) than the faster ones (ahout 40 times). This behavior was due to the effect of electrical discrimination with the electrokinetic injection of the sample solution.

Under the above optimum conditions using the FASI, the quality parameters were also evaluated. Detection limits, at 214 nm, based on a 3:1 signal-to-background noise ratio, ranged from 6.01 to 89.7 nmol/L (1.329 to 19.39 ng/g) (Table 4). The results indicate that this method with on-line preconcentration may be used as an alternative to the CE–MS method [23,24,26] with lower cost. Run-to-run precisions were determined as described above with a 1 μmol/L standard solution in methanol–water (1:1). As can be seen in Table 4, the standard deviations obtained for migration times and responses were lower than 1.78 and 11.35%, respectively, which were just a bit higher than those obtained with the method using hydrodynamic injection (Table 1).

Table 4
CE–UV-DAD quality parameters using FASI

Amines	LOD (nmol/L)	LOD (ng/g)	Run-to-run precision, R.S.D. (%), $n=5$	
			Migration time	Responses
Iso-IQ	6.01	1.33	1.08	9.38
MeIQ	30.6	7.25	1.09	5.95
Nhar	18.7	3.51	1.36	3.58
AαC	89.7	18.3	1.50	6.43
Har	19.6	3.99	1.53	4.36
MeAαC	88.1	19.4	1.61	11.1
MeIQx	13.2	3.14	1.69	3.58
PhIP	31.8	7.96	1.78	4.95

Table 5
Results from the spiked extract using FASI

Amines	Recoveries (%)	Run-to-run precision, R.S.D. (%), $n=5$	
		Migration time	Responses
Iso-IQ	35.0	0.358	7.81
MeIQ	51.6	0.372	11.3
Nhar	64.5	0.320	8.24
AαC	78.0	0.296	7.04
Har	49.1	0.322	3.24
MeAαC	64.0	0.293	7.91
MeIQx	54.1	0.319	11.2
PhIP	69.1	0.311	6.10

3.4. Application

Before the method with on-line preconcentration was applied to the determination of the heterocyclic amines in a commercial meat extract, purifications of the real sample were performed according to the method proposed by Gross with some modifications [8,23,32] as described in the Clean-up procedure above. None of the eight amines were determined in the blank extract, but they were quantified in the spiked extract with recoveries from 35.0 to 78.0% (Table 5). Fig. 5 shows the electropherograms of the blank extract and the spiked one. And the standard deviations obtained as described above for migration times and responses were lower than 0.372 and 11.31%, respectively (Table 5).

Wu et al. [33] have used CZE–DAD and separated 13 heterocyclic amines standards in 15 min. The detection limits of these HAs standards were 0.4 ng/μL for IQ, 2.5 ng/μL for Glu-P-2, 1.3 ng/μL for PhIP and 0.5 ng/μL for NH (Nhar) and H (Har). While, only 5 amines (IQ, Glu-P-2, PhIP, Nhar and

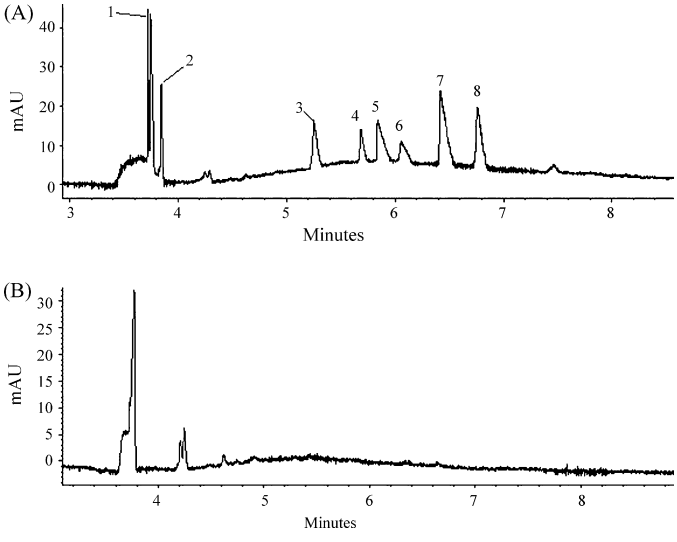

Fig. 5. Electropherograms of meat extract. Conditions: capillary, 50 μm i.d. × 50 cm (41.5 cm effective length); capillary temperature, 25 °C; 3 s hydrodynamic injection (50 mbar) of water, then 25 s electrokinetic injection (10 kV) of the sample; applied voltage, 18 kV; λ, 214 nm; the running electrolyte, 5 mmol/L formic acid–ammonium formate (pH 2.20). (A) spiked extract and (B) blank extract. Peaks: 1 iso-IQ, 2 MeIQ, 3 Nhar, 4 AaC, 5 Har, 6 MeAaC, 7 MeIQx and 8 PhIP.

Har) were confirmed in the food sample extract spiked with 100 ng/g HAs standards. The detection limits obtained in this study without the sample concentration-hydrodynamic injection (0.596–1.78 μg/g, Table 2) were comparable to that reported by Wu et al. [33]. When FASI was used, much lower detection limits, ranged from 1.329 to 19.39 ng/g, were obtained. Furthermore, in this study, all the eight amines could be analyzed successfully in the meat extract spiked with 100 ng/g HAs standards within 7 min.

4. Conclusions

A method for the determination of eight heterocyclic amines was proposed with a perfect separation. The optimum conditions for the determination were: 5 mmol/L formic acid–ammonium formate at pH 2.20 as the running electrolyte, applied voltage 18 kV. Under the optimum conditions, good correlation coefficients and precisions (migration times and responses run-to-run) were achieved. Furthermore, a preconcentration method (FASI) was also performed, which brought out a significant improvement in sensitivity over the normal hydrodynamic injection. Detection limits ranged from 1.329 to 19.39 ng/g with the standard deviations obtained for migration times and responses lower than 1.78 and 11.35%, respectively. Further, the preconcentration method may be used to determine all the eight heterocyclic amines in a meat extract with relative lower cost than the CE–MS systems [23,24].

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