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Determination of heterocyclic aromatic amines by micellar electrokinetic chromatography with amperometric detection

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Abstract

A micellar electrokinetic chromatographic system (MEKC) with amperometric detection was utilised to separate eight heterocyclic aromatic amines (HAA), which have been shown to be produced during heat processing of protein rich food. The electrolyte consisted of 15 m*M* borax adjusted to pH 9.1 with phosphoric acid and 5 m*M* cetyltrimethylammonium bromide (CTAB). Baseline separation was obtained within 7 min. Detection limits were at the level of 10 μg/l (3 fg), using a carbon fibre disc electrode at a potential of 0.6 V vs. Ag/AgCl. The method was applied to the quantitative estimation of mutagenic amines in a pan residue extract.

Keywords: Food analysis; Amines; Heterocyclic aromatic amines

1. Introduction

Heterocyclic aromatic amines (HAA) are a group of compounds which have been found to be highly mutagenic [1–3], and are believed to cause cancer in humans [4]. They are formed during the cooking of protein rich food [5,6], and they consist of two or more often three condensed aromatic cycles with one or more nitrogen atoms in the ring system. We are often daily exposed to these substances by eating fried meat or pan residue, or by inhaling the fumes from the cooking procedure [7]. To further establish the effects of HAA it is of vital importance that their occurrence can be monitored by reliable and quantitative methods. The low levels present in complex matrices, ng/g, require sample extraction with subsequent purification procedures and sometimes preconcentration before the final analytical step. Separation methods currently used are immunoaffinity chromatography [8], GC–MS [9–12], HPLC–MS [13–16] and HPLC with various spectrophotometric [17–21] and electrochemical [17,20–25] detectors. Richling and co-workers [15] have recently described a system where the liquid chromatograph is connected via an electrospray ionisation interface to a triple quadrupole mass spectrometer (LC-ESI–MS–MS). The analysis of 10 HAA was achieved in 7 min, due to the highly selective detection mode. Detection limits reported were in the μg/ml region, i.e., 5 μg/ml (25 pg) for MeIQx. The applicability of the system for the analysis of authentic samples has, however, not yet been shown. Further, for routine use, regular detectors have so far been preferred over the more sophisticated and expensive MS-based systems. Fluorescence detection offers impressive sensitivity and selectivity but it is limited to only a few HAA [18,19,21]. UV [17–19] and UV diode-array [20] detection allows the detection of all

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HAA, but are somewhat limited in terms of sensitivity and selectivity. Amperometric detection has been extensively used in combination with HPLC for detection of HAA [17,20–25], mainly because of the high sensitivity obtained but also because of its favourable selectivity properties. Reversed-phase HPLC systems are commonly utilised for the separation step. Recently, Van Dyck et al. have also successfully used an ion-exchange separation system together with this latter type of detector [25].

Capillary electrophoresis (CE) with UV diode-array detection has been utilised for the separation [26–28] and determination of HAA in rain water [27]. CE has the advantage of high separation efficiency and speed, but the low sensitivity of the UV detector restricts the applicability of this method to samples with high concentrations of HAA (>1 mg/l).

In this study a micellar electrokinetic chromatography (MEKC) system has been used together with amperometric detection for the separation and detection of eight aromatic amines. Further, the usefulness of employing this system for monitoring HAA in pan residues from the frying of meat has been investigated.

2. Experimental

2.1. Chemicals

All chemicals and solvents used were of analytical grade. Water was purified with an Elgastat UHQ II (ELGA, UK). The background electrolyte was made as follows: a 15 m*M* Borax solution was adjusted to pH 9.1 with phosphoric acid, and an amount of CTAB corresponding to 5 m*M* was added, giving a final pH value of 9.13. Before use the electrolyte was degassed and filtered.

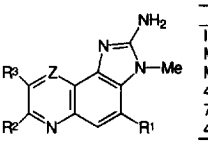
Standard stock solutions (20 mg/l in methanol) were prepared with the following HAA obtained from Toronto Research Chemicals (Toronto, Canada): 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline (7,8-DiMeIQx), 2-amino-3,4,7,8-tetramethyl-imidazo[4,5-*f*]quinoxaline (4,7,8-TriMeIQx), 2- amino-6-methyldipyrido [1,2-*a*:3′,2′-*d*] imidazole hydrochloride (Glu-P-1), 2-aminodipyrido [1,2-*a*:3′,2′-*d*] imidazole hydrochloride (Glu-P-2). The structures of the compounds studied are presented in Fig. 1. Working standard solutions were prepared daily by diluting the stock solutions with a water/operating electrolyte (90:10, v/v).

2.2. Sample preparation

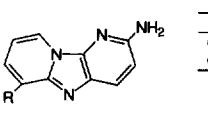
Minced lean pork in 70 g patties was fried at 250°C for 10 min, as described by Berg et al. [29]. After frying each set of patties, the pan was rinsed with 100 ml of water, yielding a pan residue sample. A 20 ml sample of the pan residue, corresponding to 100 g of uncooked pork, was then reduced in volume under vacuum before further purification. The residue was purified according to a method described by Gross and co-workers [19]. The sample was dissolved in a 1 *M* sodium hydroxide solution from which the HAA were extracted into dichloromethane. Further purification was then performed by solid-phase extraction on a propyl sulphonic acid (PRS) cation-exchange column and an octadecyl (C_{18}) reversed-phase column. The sample was finally dissolved in 100 μl of methanol and stored at +4°C until analysis.

2.3. Capillary electrophoresis system

Untreated fused-silica capillaries, 60 cm×20 μm I.D., 150 μm O.D., were obtained from Polymicro Technologies (Phoenix, AZ, USA). Before use, the capillaries were washed with 0.1 *M* HCl for 30 min,



	Z	R¹	R²	R³
IQ	C	H	H	H
MeIQ	C	Me	H	H
MeIQx	N	H	H	Me
4,8-DiMeIQx	N	Me	H	Me
7,8-DiMeIQx	N	H	Me	Me
4,7,8-TriMeIQx	N	Me	Me	Me



	R
Glu-P-1	Me
Glu-P-2	H

Fig. 1. Chemical structures of the heterocyclic aromatic amines.

distilled water for 20 min, and operating electrolyte for 20 min. After every fifth run the capillary was washed with three volumes of 0.1 m*M* HCl and with operating electrolyte.

The input of the high-voltage power supply, ±0–30 kV, (Brandenburg, Thornton Heath, UK), together with the injection end of the capillary, were placed in a Plexiglas box with an interlock on the access door for protection. Rinsing of the capillary was accomplished by applying pressure on the injection buffer. Injections of samples were made hydrodynamically by a laboratory built pressure system, and the injected volume was about 0.3 nl.

Amperometric detection was performed in the end-column mode using a three electrode configuration. The potentiostat was designed according to the circuit scheme presented by Cassidy et al. [30]. Two car batteries, 12 V, were used as the power supply. Control of the detector potential and data acquisition was accomplished with a PC equipped with a PCL-812PG lab card (Advantech, Taiwan), and an in-house developed QBASIC computer program. An ELDS 900 laboratory data system (Chromatography Data Systems, Kungshög, Sweden) was used for integration.

The detector cell has been described in detail before [31]. The working electrode consisted of a 30 mm carbon fibre disc electrode. As reference electrode an Ag/AgCl electrode in 3 *M* KCl gel (BAS, West Lafayette, IN, USA) was used, and the auxiliary electrode was a platinum wire with a diameter of 2 mm (Goodfellow, Cambridge, UK). The working electrode was washed daily with bichromate–sulphuric acid for a few minutes, and then with deionized water. It was positioned directly in front of the capillary outlet at a distance of approximately 5 mm. This was accomplished by adjusting the working electrode with a micropositioner under a microscope. The detector and the injection arrangements were placed in Faraday cages.

3. Results and discussion

Fig. 2 shows an electropherogram of a standard mixture of eight HAA. The electrolyte was a buffer containing 15 m*M* borax (pH 9.1) and 5 m*M* of the cationic surfactant CTAB. According to earlier investigations, the oxidation potential needed to detect the HAA is dependent on the pH, since hydrogen ions are released in the oxidation reaction [24]. To minimise the required oxidation potential, a high pH is favourable. However, at pH 9.1 HAA are uncharged [27], and as a consequence the CE-separation requires the addition of micelles to the electrolyte. The charged micelles serve as a dynamic stationary phase, and separation is governed by differing solubility of the analytes in the micellar phase. The more apolar amines, containing additional methyl groups, increasing their solubility in the micelles, migrate slower than the corresponding amines without methyl groups. If additional nitrogens are present in the aromatic structures, the hydrophobicity decreases, and faster migration times result. CTAB also prevents wall adsorption, since it forms a positively charged layer on the capillary wall, efficiently covering the negative silanol groups. When a similar buffer using the anionic surfactant sodium dodecyl sulphate (SDS) was investigated, peak tailing, gradually increasing with migration time, was observed. This could be due to adsorption to the capillary wall, or adsorption to the electrode during the electrode reaction.

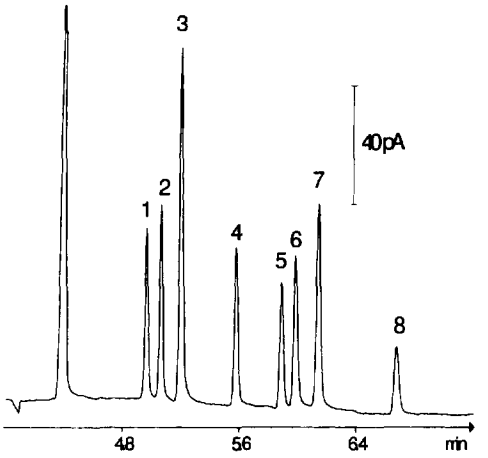


Fig. 2. Electropherogram of 1 mg/l standard solution. Amperometric detection at carbon fiber disc electrode 30 μm, 0.6 V vs Ag/AgCl; buffer: 15 m*M* borax adjusted to pH 9.1 with phosphoric acid, 5 m*M* CTAB; capillary: 60 cm×20 μm I.D.; separation potential: −25 kV; hydrodynamic injection: 0.3 nl. Peaks: 1=Glu-P-2; 2=MeIQx; 3=IQ; 4=7,8-DiMeIQx; 5=4,8-DiMeIQx; 6=MeIQ; 7=Glu-P-1; 8=TriMeIQx.

Hydrodynamic voltammograms for the eight HAA

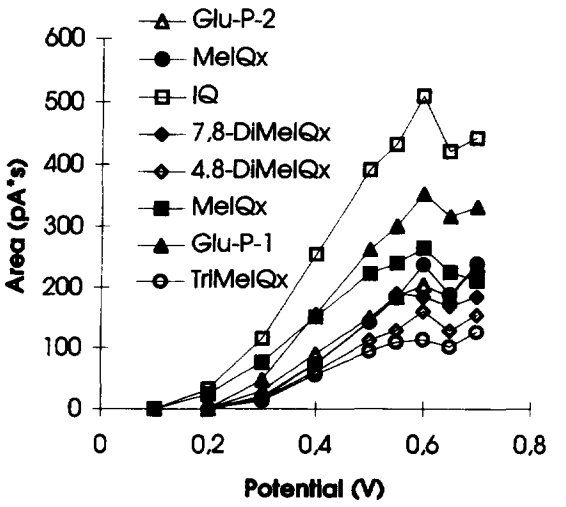


Fig. 3. Hydrodynamic voltammograms for the heterocyclic aromatic amines. Amperometric detection at carbon fiber disc electrode 30 μm; buffer: 15 m*M* borax adjusted to pH 9.1 with phosphoric acid, 5 m*M* CTAB; capillary: 60 cm×20 μm I.D.; separation potential: −25 kV; hydrodynamic injection: 0.3 nl.

are shown in Fig. 3. Above 0.6 V there was no improvement in the detector response. In addition, a considerable increase in background current and noise was observed. For this reason, a potential of 0.6 V vs. Ag/AgCl was used throughout in all the experiments. In contrast, in HPLC there is a problem of finding optimal conditions for both the separation and the amperometric detection of HAA. High oxidation potentials, +0.8–1.1 V, are needed to detect the HAA in the acidic conditions, pH 3–5, required for the separation [24,25]. However, due to the high pH used for the separation in this work, pH 9.1, a lower oxidation potential can be applied, thereby increasing the selectivity and reducing the noise.

Calibration data for the selected amines are presented in Table 1. The calibrations were carried out in the range of 0.02–2.0 μg/ml, using standard solutions. In this range the linearity was good. Repeatability was calculated from eight consecutive injections, and R.S.D. for the peak areas and for the migration times were in the range of 2.2–6.2% and 1.7–2.4%, respectively. These values are expected to improve if an injection system with better precision is used, for instance an autoinjector. R.S.D. values obtained in the sample preparation are generally higher, about 10% [19].

Limits of detection (LOD) values for the amines are also presented in Table 1. The LOD concentration for MeIQx, 7.4 μg/l, is in the same order of magnitude as the LOD values reported for HPLC with amperometric detection, 1.8–27 μg/l [17,20–25]. Mass sensitivity with the proposed MEKC system, on the other hand, is greatly improved, 2.2 fg compared to 35–1300 ng for MeIQx, due to the small volumes injected. To perform a proper injection in CE a few μl of the sample is sufficient, and only a small portion, less than 1 nl, is actually consumed. This may be considered to be an advan-

Table 1
Calibration data

Analyte	Migration time (min)	R.S.D.$^{a}_{time}$ (%)	R.S.D.$^{a}_{area}$ (%)	Detector response (pA/(mg/l))	Concentration range (mg/l)	r^{b}	LOD^{c} (μg/l)
Glu-P-2	4.97	1.7	6.2	57	0.02–2	0.999	8.5
MeIQx	5.07	1.9	3.5	65	0.02–2	0.999	7.4
IQ	5.21	2.0	2.2	120	0.02–2	0.999	4.0
7,8-diMeIQx	5.59	2.1	3.3	51	0.02–2	0.999	9.4
4,8-diMeIQx	5.90	2.1	3.4	41	0.02–2	0.999	12
MeIQ	6.00	2.3	2.3	56	0.02–2	0.999	9.6
Glu-P-1	6.16	2.0	5.4	68	0.02–2	0.999	7.0
TriMeIQx	6.69	2.4	4.5	23	0.02–2	0.999	21

[a] Relative standard deviation calculated from eight injections of 1 mg/l standard solution.
[b] $n=6$.
[c] $S/N=2$.

tage compared to the relatively large sample volumes consumed by HPLC, 10–100 μl. Thus, the main part of the sample still remains intact, and analytical results can be confirmed with alternative methods if desired. With a high mass sensitivity it is also possible to reduce sample quantities, and to develop microscale methods for extraction and purification. Further, improved mass sensitivity is important when very low concentrations of HAA are present in the samples or/and the sample amounts are limited. Monitoring of HAA in indoor air could be an example of such an application in the future, since the concentrations found in fumes are up to two orders of magnitude lower than those present in meat samples [11,32].

Detection limits with amperometric detection on CE were considerably improved compared to those reported with UV diode-array detection, 0.8 mg/l [27]. Because of the low sensitivity obtained with UV, this cannot be considered to be an alternative detection mode for meat related samples.

A pan residue extract was quantitatively analysed using the standard addition method, see Fig. 4. The amounts found are presented in Table 2. The amounts of MeIQx and 4,8-DiMeIQx found in this study correspond quite well with results obtained previously for pan residue obtained from the same type of meat, cooked under comparable conditions [33,34]. Tenfold lower levels are found for some of the other HAA. The presence of IQ, 7,8-DiMeIQx and Glu-P-2 in meat extracts has been reported before [14,19,33–35]. No TriMeIQx could be detected in the extract, which was expected, since it has never been found in any meat related samples. It was included in order to investigate its potential as an internal standard in the future. Even though all peaks in the sample are not fully separated with the MEKC-system, the separation power and the speed have been greatly improved compared to the reported HPLC systems [17,20–25]. Thus, peak identification should be more reliable when using the MEKC system.

One problem with separation of the amines on HPLC is that gradient systems are not compatible with amperometric detection. Consequently, in order to achieve acceptable separation in a reasonable time, two different systems have to be employed [21]. However, with the proposed MEKC system a wide range of HAA with different polarities can be separated in a short time.

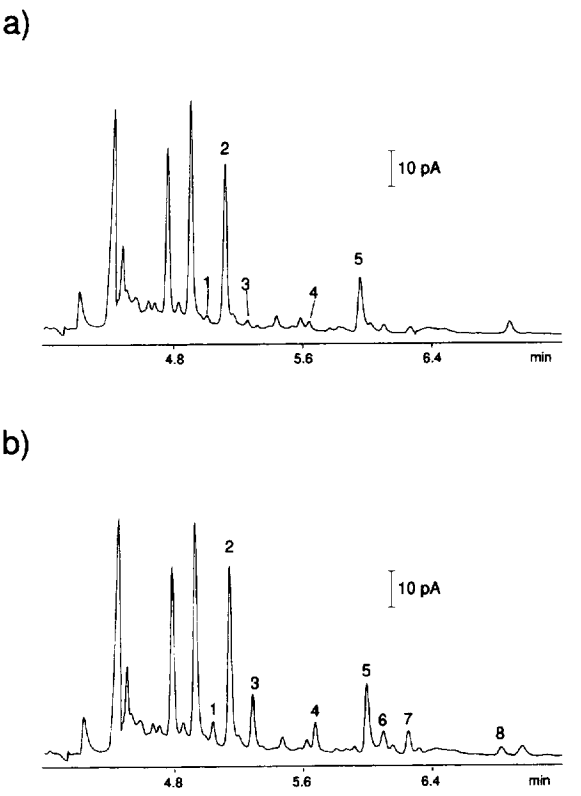


Fig. 4. Electropherograms of pan residue extract. Conditions as in Fig. 2. (a) Extract, (b) extract spiked with 0.1 mg/l of each HAA. Peaks: 1=Glu-P-2; 2=MeIQx; 3=IQ; 4=7,8-DiMeIQx; 5=4,8-DiMeIQx; 6=MeIQ; 7=Glu-P-1; 8=TriMeIQx.

4. Conclusions

Techniques for the determination of HAA in cooked food and cooking fumes are essential to evaluate human exposure to these compounds. Several of the currently applied methods of analysis in this area today utilise insufficiently selective separation and detection techniques. As a consequence, the demands on sample purification become high resulting in complicated sample pre-treatment procedures which, in many cases, might lead to analyte losses and analyte discrimination. A number of different heterocyclic amines are formed in the meat

Table 2
Results from the semi-quantitative analysis of pan residue extract

Analyte	Concentration (mg/l)	Amount related to meat weight (ng/g)[a]
Glu-P-2	0.031	0.038
MeIQx	0.84	1.0
IQ	0.011	0.014
7,8-diMeIQx	0.043	0.054
4,8-diMeIQx	0.41	0.51
MeIQ	not detected	not detected
Glu-P-1	not detected	not detected
TriMeIQx	not detected	not detected

[a] Recovery-corrected values (80%).

cooking procedures, calling for general methods of extraction, sample clean up, separation and detection. In this context the MEKC method described in this paper provides an attractive alternative, since HAA with a wide range of polarities can be rapidly and efficiently separated. Further, the amperometric detection mode employed is capable of detecting all HAA, still providing selectivity and sensitivity. The equipment is inexpensive and the operational costs for routine analysis are low, due to the low consumption of chemicals. However, a disadvantage is that there are few, if any, commercially available amperometric detectors for CE. This has so far limited the universal acceptance and use of this detection technique.

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