Quantitative estimation of heterocyclic aromatic amines by ion-exchange chromatography and electrochemical detection

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Abstract

Conditions for the quantitative determination of three heterocyclic aromatic amines (HAAs), produced by heat processing of protein-rich food products, were established for a HPLC–electrochemical detection system. The separation of 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ) and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQ$_x$) was achieved on an ion-exchange column with acetonitrile–80 mM phosphate buffer (30:70) mobile phase at pH 5.6. The figures of merit were calculated. Reproducibility gave a relative standard deviation of 1.6–2.4% when measured by peak area. Detection limits (signal-to-noise ratio 3) ranged from 35 pg for MeIQ$_x$ to 70 pg for MeIQ. The method was applied to the determination of HAAs in a commercial beef extract sample.

1. Introduction

Heterocyclic aromatic amines (HAAs) are formed by condensation of amino acids, sugar and creatinine by a Maillard-type reaction [1]. These products, which can be found at low parts per billion (w/w) range in cooked meat and fish products, possess a high mutagenic activity in the Ames test [2–6]. They induce tumors in the liver, lung, breast, small and large intestine and other sites in rodents [7] and are considered as possible human carcinogens.

Daily human exposure to HAAs varies greatly and is dependent on diet habits and cooking practices [8]. These findings suggest the need to develop analytical techniques able to determine low levels present in meats cooked under industrial conditions as well as in traditional cooking practices. Separation and detection of these compounds require fast, selective and sensitive analytical techniques. Sophisticated and expensive techniques, such as HPLC–MS [9,10], GC–MS [11–13] or enzyme-linked immunosorbent assay (ELISA) [14], have been proposed. Restricted analyses are obtained with techniques such as HPLC with UV and/or fluorescence detection [15,16]. However, electrochemical detection (ED) achieves higher selectivity and sensitivity than classical UV detection and studies using this mode of detection have been recently described [17–20]. Separations of HAAs are commonly achieved on a reversed-phase (C$_{18}$) column. However, in the acidic conditions needed for the separation of these compounds,

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amino groups are protonated and interact with the free silanols of the stationary phase. Moreover, in the purification scheme described by Gross and Grüter [15,16], HAAs are successfully purified by adsorption on a propylsulfonyl silica gel.

This work aimed to develop a separation procedure on an ion-exchange column. Conditions were established for the determination of three HAAs identified in commercial beef extracts: 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-f]-quinoline (MeIQ) and 2-amino-3,8-dimethyl-imidazo[4,5-f]quinoxaline (MeIQ$_x$) using ion-exchange HPLC with ED. The method was applied to the analysis of these three HAAs in commercial beef extracts.

2. Experimental

2.1. Chemicals

IQ, MeIQ and MeIQ$_x$, whose structures are presented in Fig. 1. were purchased from Toronto Research Chemicals (Toronto, Canada). Stock solutions (1 mg ml^{-1}) were prepared in methanol and used for further dilutions. Extrelut extraction cartridges and refill material were from Merck (Darmstadt, Germany). Bond-Elut propylsulfonyl silica gel (PRS; 500 mg) and C$_{18}$ (100 mg) cartridges were

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

purchased from Analytichem International (ICT, Basle, Switzerland). Acetonitrile (gradient grade), disodium hydrogenphosphate (analytical-reagent grade) and phosphoric acid (analytical-reagent grade) were from Merck. All the solutions were passed through a 0.45-μm filter before being used in the HPLC system. The pH was fixed in the mixture acetonitrile–buffer solution.

2.2. Extraction scheme

Extraction of the HAAs was carried out using the protocol described by Gross and Grüter [15,16]. Practically, 2 g of beef extract were homogenized in 12 ml of 1 M NaOH. The solution was adsorbed to diatomaceous earth (Extrelut cartridge). The Extrelut cartridges were conditioned with 20 ml dichloromethane. The HAAs were eluted with 35 ml dichloromethane and adsorbed to the coupled PRS cartridge. This cartridge was then connected to a peristaltic pump and rinsed at a flow-rate of 1–2 ml min^{-1} with 6 ml 0.1 M HCl and 15 ml of methanol–0.1 M HCl (4:6), followed by 2 ml water. The PRS cartridge, which contained the polar IQ-type compounds, was then coupled to a Bond-Elut C$_{18}$ cartridge. The HAAs were transferred to the C$_{18}$ cartridges by washing the PRS cartridge with 20 ml of 0.5 M ammonium acetate buffer (pH 8.0). The C$_{18}$ cartridge was rinsed with 1 ml water. The solutes were eluted with 0.8 ml methanol–concentrated ammonia solution (9:1). The solvent was evaporated under nitrogen. Solutes were then dissolved with 100 μl methanol before injection. The volume injected in the HPLC system was twofold the loop volume. Solvent evaporation was minimized by conservation of solutions at 4°C and were injected as fast as possible.

2.3. Instruments

HPLC was carried out with a Kontron (Milan, Italy) Model 420 pump. The sample was introduced via a Rheodyne (Cotati, CA, USA) 7125 injector equipped with a loop of 20 μl. A Spherisorb SCX 5U column (5 μm; 250 × 4.6
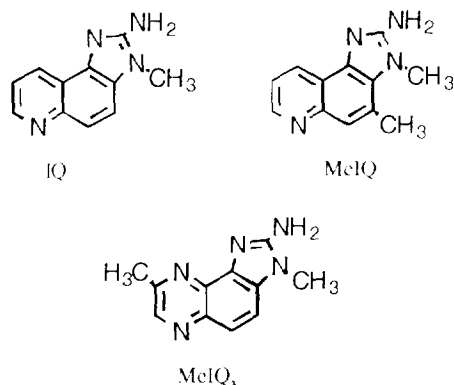
mm) (Alltech, Deerfield, IL, USA) and a Supel-guard LC-8DB precolumn (Supelco, Gland, Switzerland) were used at room temperature. The detection was achieved successively on a UV detector, Kontron Model 432 at a wavelength of 263 nm, and an amperometric detector, BAS (Lafayette, IN, USA) Model LC-4B, equipped with a working electrode (glassy carbon electrode), a reference electrode (Ag/AgCl/KCl 3 M) and with an auxiliary electrode. A data processor Softron (Kontron) was used. Acetonitrile–phosphate buffer (30:70, v/v) at different pH values and ionic strengths was used as a mobile phase at a flow-rate of 1.0 ml min^{-1}.

2.4. Calibration

Calibrations were carried out in quadruplicate with 2-g aliquots of beef extract, which were spiked with 100 μl standard solution of the mixture of the three HAAs (0.25, 0.5, 0.75 or 1 μg ml^{-1}) after extraction. For each compound, linear regression analysis was carried out. Recovery of extraction was determined by spiking beef extract sample before extraction. Concentrations were determined from the calibration curve. Extraction efficiency was expressed as the ratio of amount measured and amount added in the sample.

3. Results and discussion

3.1. Optimization of the chromatographic conditions

Separations on an ion-exchange stationary phase were dependent on the ionic strength of the mobile phase and on the ionization state of the different amines. Mobile phase (acetonitrile–phosphate buffer) was tested for different concentrations of salt. The concentration in Na$_2$HPO$_4$ varied from 10^{-2} to 10^{-1} M. The pH value was fixed at 6.0 by phosphoric acid solution. The capacity factors (k') obtained for each compounds are indicated in Fig. 2. An increase in the ionic strength produces a decrease of k'
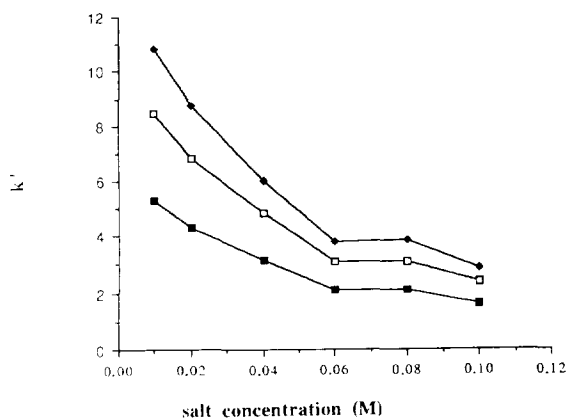

Fig. 2. Effect of concentration in Na$_2$HPO$_4$ on the separation of heterocyclic amines. Mobile phase: acetonitrile–phosphate buffer pH 6.0 (30:70). Working potential: + 1050 mV vs. Ag/AgCl. □ = IQ; ◆ = MeIQ; ■ = MeIQ$_x$.

due to the competitive interaction of solutes and salt with the ionic sites. A compromise must be found between the analysis time and the resolution of the different peaks. The best separation was obtained at a salt concentration of 80 mM.

Separation is also influenced by the pH value of the mobile phase. The capacity factors for the three compounds obtained at different pH values varied from 4.0 to 7.0 and are presented in Fig. 3. The mobile phase was acetonitrile–80 mM Na$_2$HPO$_4$ (30:70, v/v). An increase in the pH
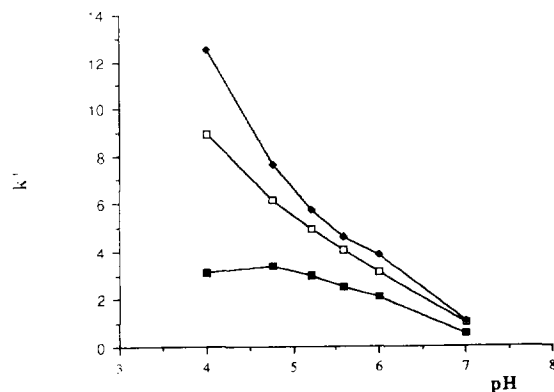

Fig. 3. Effect of mobile phase pH on the separation of heterocyclic amines. Mobile phase: acetonitrile–80 mM phosphate buffer (30:70). Working potential: + 1050 mV vs. Ag/AgCl. □ = IQ; ◆ = MeIQ; ■ = MeIQ$_x$.

value induces a decrease of k' related to a progressive reduction of amine protonation.

It must be stressed that no gradient in the composition of the mobile phase can be applied with ED. Even very small modifications in the composition carry away a shift of the baseline. It can be concluded that the separation of the three HAAs must be realised with a mobile phase of acetonitrile–80 mM aqueous Na_2HPO_4 (30:70, v/v). At pH 5.6, all the compounds can be separated as can be seen in Fig. 4, which shows the chromatogram obtained with a standard solution under the conditions described.

The optimal potential was obtained from the hydrodynamic voltammogram at the separation conditions previously established. The results obtained by variation of potential from +700 to +1100 mV are presented in Fig. 5. The highest surface area with the lowest background noise were obtained at a working potential of +1050 mV which corresponds to a maximum response for the three compounds and is located in the beginning of the plate to limit interferences.

From the voltammogram, it can be observed that the two quinoline derivatives present the same curve aspect whereas the quinoxaline derivative present a reduced tendency to oxidation.
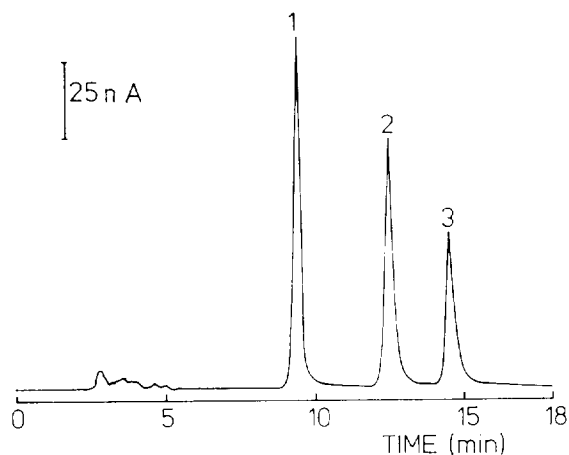

Fig. 5. Hydrodynamic voltammogram. Mobile phase: acetonitrile–80 mM phosphate buffer pH 5.6 (30:70). $\square$ = IQ; $\blacklozenge$ = MeIQ; $\blacksquare$ = MeIQ$_x$.

ED can be used for the characterization of these three compounds in a matrix.

3.2. Linearity and limits of detection

Linearity and limits of detection were determined with standard solution diluted in methanol. Peak area was used as the response in the optimal conditions.

Calibrations were carried out with concentrations in the range 10^{-3}–1 μg ml^{-1}; beef extract containing only trace amounts of HAAs (no higher concentration than 20 ng g^{-1} was studied).

Six replicate determinations of 20 ng (1 μg ml^{-1} solution) of each analyte in methanol were carried out to determine the precision of the method. Relative standard deviations (R.S.D.) obtained were in the range 1.66–2.42%. The detection limits for the heterocyclic amines,

Fig. 4. Chromatogram of standard solution. Mobile phase: acetonitrile–80 mM phosphate buffer pH 5.6 (30:70). Working potential: +1050 mV vs. Ag/AgCl. Peaks: 1 = MeIQ$_x$; 2 = IQ; 3 = MeIQ.
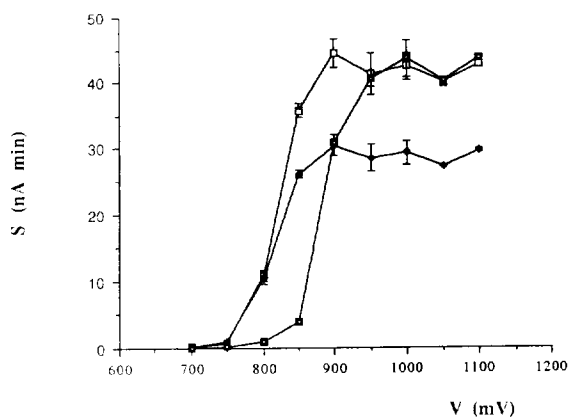
Table 1
Figures of merit

Analyte	Range of linearity (ng)	Limits of detection (pg)	Precision (R.S.D., %)
IQ	1.0–20	37	2.20
MeIQ	2.5–20	70	2.42
MeIQ$_x$	2.5–20	35	1.66

Table 2
Analysis of beef extract

Analyte	Recovery (%)	Precision (R.S.D., %)	Results (ng/g)	Precision (R.S.D., %)
IQ	36.14	12.27	9.59	29.48
MeIQ	50.72	11.24	N.D.	
MeIQ$_x$	27.75	19.90	35.91	18.92

N.D. = Not detected.

based on a signal-to-noise ratio of 3 ranged from 35 to 70 pg (Table 1).

Using these experimental conditions, the detection limit was strongly improved in comparison with the results described in the literature [18–20]; this may be attributed to the pH of the solution, and thus the ionization state of analytes, which plays an important role on the electrochemical reaction [20].

3.3. Application

After optimization of experimental conditions, commercial beef extract was analyzed. Results presented in Table 2 were obtained from four successive extractions. Peak identification was confirmed by UV detection. The percentage recovery is lower than that presented in the literature [15,16,20], which may result from the clean-up procedure; therefore further investigations are needed to clarify this discrepancy.

Chromatograms of the sample and the spiked sample are presented in Fig. 6. No interferences were observed with impurities of the extract and the different compounds are easily quantified. MeIQ could not be detected in the sample (Fig. 6A). Such observation was reported previously for the beef extract [1].

Fig. 6. Chromatogram of a beef extract. Mobile phase: acetonitrile–80 mM phosphate buffer pH 5.6 (30:70). Working potential: +1050 mV vs. Ag/AgCl. (A) Extract. (B) extract spiked before the clean-up (100 ng of IQ and MeIQ). Peaks: 1 = MeIQ$_x$; 2 = IQ; 3 = MeIQ.

4. Conclusions

Quantification of mutagenic heterocyclic amines is essential for the determination of risks to human health. In this study, conditions have been established for the separation and the determination by HPLC–ED with an ion-exchange column of three heterocyclic amines. The applicability, linearity and sensitivity of the method have been studied. The method has been applied to the quantification of IQ, MeIQ and
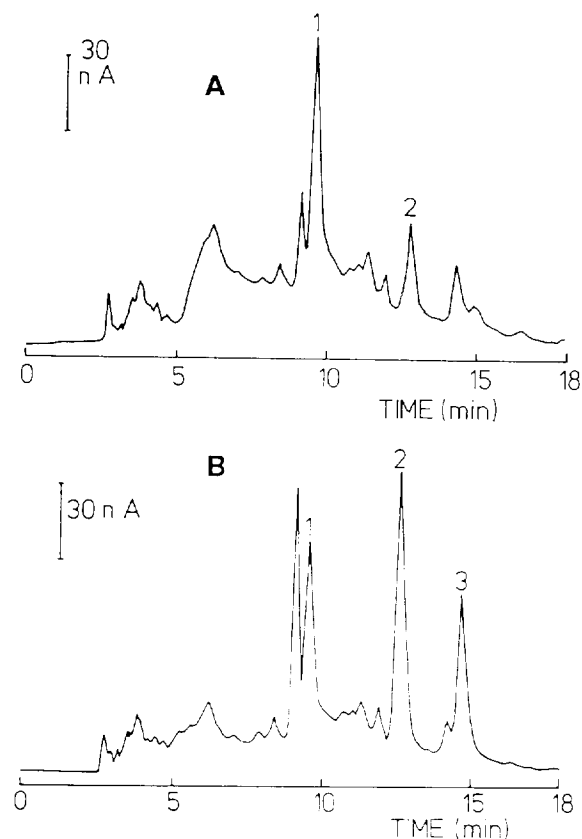
MeIQ$_x$ in commercial beef extract. Research is currently in progress to extend the method to other heterocyclic amines present in beef extract.

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