Journal of Chromatography A, 655 (1993) 101–110
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 034

High-performance liquid chromatographic determination of ten heterocyclic aromatic amines with electrochemical detection

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ABSTRACT

Conditions for the HPLC–electrochemical detection determination of ten mutagenic heterocyclic amines that can be produced by heat processing of protein-rich food products were established. The use of reversed-phase chromatography and acetonitrile–ammonium acetate (10:90) at pH 4.0 for the separation of 2-amino-3-methylimidazo[4,5-*f*]quinoline, 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) and 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), and of acetonitrile–ammonium acetate (30:70) at pH 6.0 for the separation of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-3-methyl-9H-pyrido[2,3-*b*]indole, 1-methyl-9H-pyrido[4,3-*b*]indole (harman) and 9H-pyrido[4,3-*b*]indole (norharman) was proposed. The figures of merit were calculated; reproducibility gave a relative standard deviation of 1.6–4.6% when measured by peak area. Detection limits (signal-to-noise ratio 2:1) ranged from 0.2 ng for Trp-P-2 to 3.4 ng for 4,8-DiMeIQx. The method was applied to the determination of some of these compounds in a commercial beef extract. Levels of 5.1 ng g^{-1} MeIQx, 5.8 ng g^{-1} MeIQ and 15.5 ng g^{-1} of Glu-P-1 were found.

INTRODUCTION

Aromatic amines are one of the few classes of chemical compounds for which there is convincing evidence that some members induce cancer in humans [1]. This group of compounds includes several heterocyclic amines of two or generally three condensed aromatic cycles with one or more nitrogen atoms in the ring system; most of these heterocycles belong to two main classes of compounds: aminocarbolines and aminoimidazo azaarenes. These products, which can be found in cooked protein-containing food products in the low parts per billion (w/w) range, have shown mutagenic activity by means of the Ames test, and in some cases have also been found to be carcinogenic [2–6]. Humans are continually exposed to carcinogenic heterocyclic amines in heat-processed foods, and some heterocyclic amines have been detected in urine samples from healthy volunteers eating a normal diet [7].

These findings suggest the need to develop analytical techniques to determine these compounds in various heat-processed foods. To screen for mutagenic and carcinogenic compounds it is necessary to develop a simplified method that can analyse these compounds simultaneously using a fast, selective and sensitive analytical technique. The techniques presently available either require sophisticated and/or expensive equipment, such as enzyme-linked immunosorbent assay (ELISA) [8], HPLC–MS [9,10] or GC–MS [11,12], or are restricted to the determination of a selected group of compounds. HPLC with UV detection has been found appropriate to determine most of these compounds simultaneously [13]. However, HPLC with electrochemical detection (ED) is an option to be considered, since high selectivity and sensitivity

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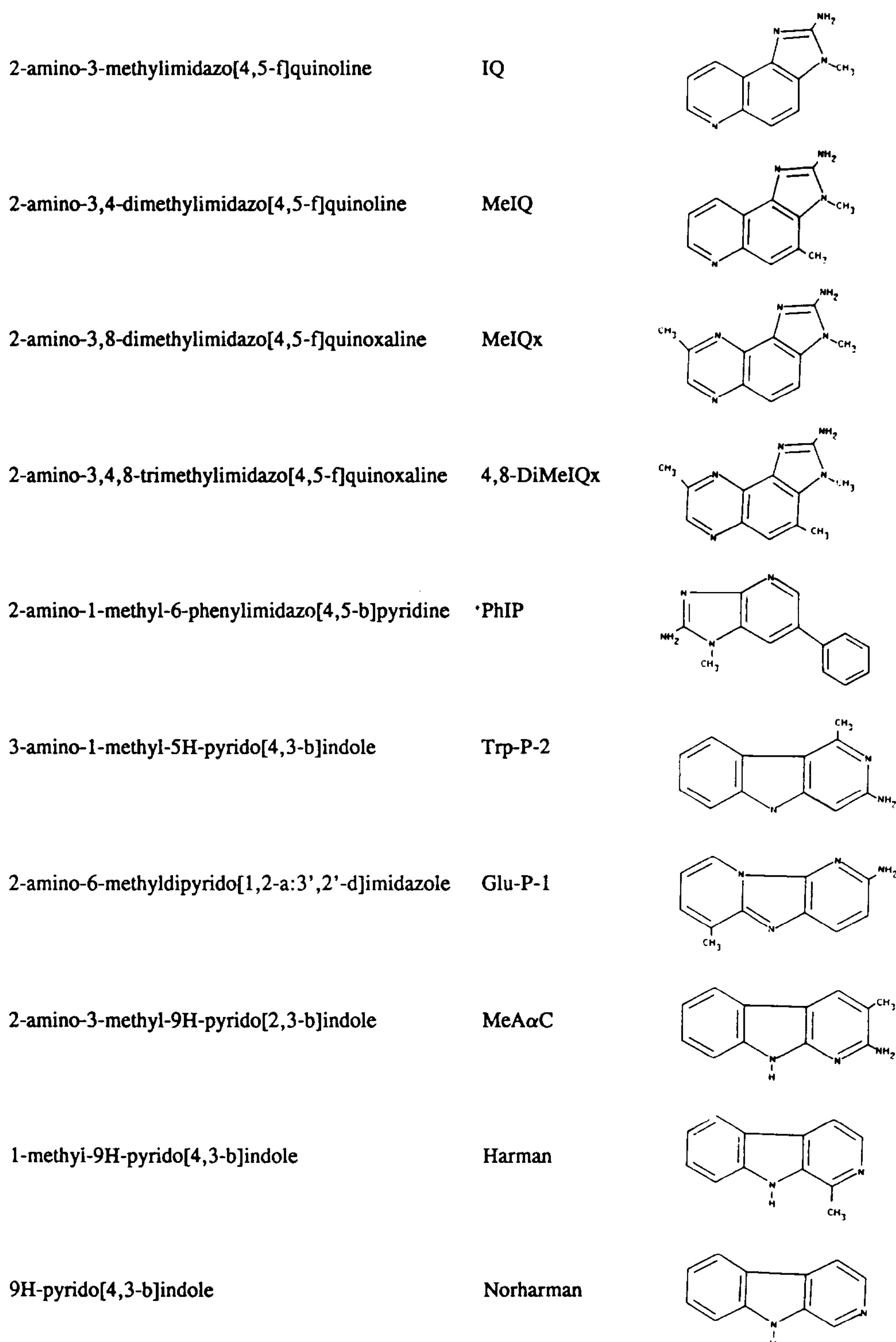

Fig. 1. Chemical structures of the mutagenic heterocyclic amines.

are achieved. Some aminoimidazo azaarenes have been detected using this technique [14–16], and recently both separation and ED of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2 - amino - 6 - methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole (Trp-P-1) and 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole(Trp-P-2) have been described [17], although in all cases the procedures are restricted to the determination of a reduced number of compounds.

In this study conditions are established for the determination of ten heterocyclic amines, selected from those identified in beef extracts [IQ, 2 - amino - 3,4 - dimethylimidazo[4,5-*f*]quinoline (MeIQ), MeIQx and 2 - amino - 3, 4, 8 - trimethylimidazo[4, 5 - *f*]quinoxaline (4, 8 - DiMeIQx)] [13,18–20], and in cooked meat and fish [2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), Glu-P-1, Trp-P-2, 2-amino-3-methyl-9H-pyrido[2,3-*b*]indole (MeAαC), 1-methyl-9H-pyrido[4,3-*b*]indole (harman) and 9H-pyrido[4,3-*b*]indole(norharman)] [11,13,15,16,18–25], using HPLC–ED. The figures of merit have been calculated and the method has been applied to the determination of some of these compounds in a commercial beef extract.

EXPERIMENTAL

Chemicals

The compounds studied are listed in Fig. 1 and were purchased from Toronto Research Chemicals (Toronto, Canada), except harman and norharman, which were from Aldrich (Steinheim, Germany); stock standard solutions of 100 μg ml^{-1} in methanol were prepared and used for further dilutions. 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline (7,8-DiMeIQx) and 2-amino-3,4,7,8-tetramethylimidazo[4,5-*f*]quinoxaline(4,7,8-TriMeIQx) were used as internal standards. Extrelut extraction cartridges (20 ml) were provided by Merck (Darmstadt, Germany). Bond-Elut propylsulphonyl silica gel (PRS; 500 mg) and C_{18} (100 mg) cartridges as well as coupling pieces and stopcocks were from Analytichem International (ICT, Basle, Switzerland). These cartridges were respectively preconditioned with dichloromethane and water–methanol (1 + 3 ml).

Other solvents and chemicals were HPLC or analytical grade, and the water was purified using a Culligan (Barcelona, Spain) system. All the solutions were passed through a 0.45-μm filter before injection into the HPLC system.

Instruments

HPLC was carried out with a Gilson Model 302 pump with an 802 monometric module (Gilson, Villier-le-Bel, France) and a Metrohm wall jet electrochemical detector, Model 656,

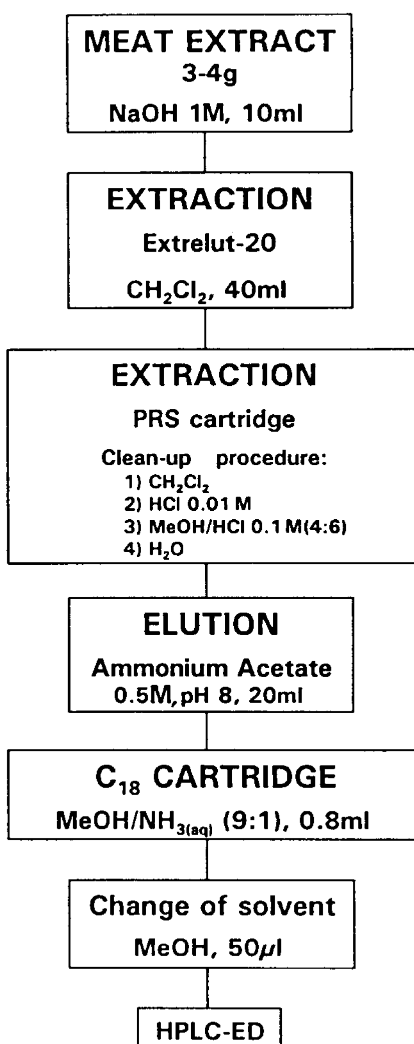

Fig. 2. Clean-up procedure.

TABLE I

CAPACITY FACTORS (k') OF HETEROCYCLIC AMINES AT DIFFERENT ACETONITRILE CONCENTRATIONS

– = Retention time > 50 min; + = not detected. Retention times > 50 min at 10% acetonitrile and pH 6.0 and 6.5.

	pH 3.5				pH 4.0				pH 4.5				pH 5.0				pH 5.5				pH 6.0			pH 6.5		
Acetonitrile (%) Analyte	10	20	25	30	10	20	25	30	10	20	25	30	10	20	25	30	10	20	25	30	20	25	30	20	25	30
IQ	3.17	0.69	0.00	0.00	4.16	0.83	0.00	0.00	5.92	0.94	0.00	0.00	8.59	1.36	0.67	0.00	9.50	1.50	0.82	0.00	1.97	0.89	0.79	1.98	1.04	0.00
Glu-P-1	3.80	0.96	0.00	0.00	5.06	1.44	1.01	0.90	9.20	1.96	1.45	1.01	13.50	3.05	1.87	1.43	–	3.75	2.27	1.45	4.78	1.38	1.64	4.53	2.43	1.53
MeIQx	5.20	0.96	0.00	0.00	6.03	0.97	0.00	0.00	7.54	1.03	0.00	0.00	9.90	1.45	0.76	0.00	10.58	1.42	0.86	0.00	2.06	0.91	0.71	1.89	0.98	0.00
MeIQ	5.02	0.96	0.00	0.00	7.19	1.10	0.00	0.00	10.35	1.45	0.82	0.00	–	2.04	0.97	0.00	–	2.29	1.27	0.87	3.20	1.91	1.00	3.47	1.70	0.99
4,8-DiMeIQx	21.21	1.70	0.97	0.00	13.97	1.75	1.03	0.00	–	1.87	1.09	0.00	–	2.72	1.27	0.86	–	2.75	1.49	0.94	3.63	1.49	1.07	3.47	1.68	0.88
7,8-DiMeIQx	11.21	1.43	0.00	0.00	11.93	1.51	0.84	0.00	–	2.97	0.91	0.00	–	3.73	1.60	0.00	–	2.17	1.94	0.80	2.76	2.18	0.86	4.96	1.38	0.81
TriMeIQx	–	2.18	1.30	0.75	–	2.39	1.39	0.90	–	2.72	1.57	0.87	–	3.65	1.69	1.00	–	4.08	1.85	1.09	4.77	1.94	1.29	4.79	2.15	1.17
Trp-P-2	–	4.14	2.44	1.25	–	4.53	2.61	1.48	–	5.29	2.56	1.52	–	6.68	2.76	1.64	–	6.08	2.90	1.73	7.57	3.08	1.93	7.60	4.10	1.78
PhIP	–	+	+	+	–	+	+	2.36	–	9.64	5.41	3.03	–	15.89	6.89	3.93	–	–	8.23	4.25	13.77	6.89	4.50	–	9.12	4.38
Harman	–	+	+	+	–	+	+	+	–	+	+	+	–	–	+	1.93	–	–	4.40	2.60	–	8.14	4.14	–	11.67	3.19
Norharman	–	+	+	+	–	+	+	+	–	+	+	+	–	–	+	2.50	–	–	6.05	3.60	–	8.78	5.46	–	12.82	5.21
MeAαC	–	10.76	7.52	4.24	–	–	11.29	6.59	–	–	13.91	7.35	–	–	14.23	7.93	–	–	14.27	7.99	–	3.33	8.00	–	14.60	8.17

equipped with a working electrode (Glassy Carbon Electrode, Model 6.0805.010), a reference electrode (Ag/AgCl/KCl 3 *M*) and an auxiliary electrode (Glassy Carbon Electrode, Model 6.0805.010) (Metrohm, Herisau, Switzerland). A data processor, Chromatopac C-R3A (Shimadzu, Kyoto, Japan), was used. The sample was introduced by a Rheodyne 7125 injector (Rheodyne, Cotati, USA) equipped with a loop of 50 μl. A TSK-Gel ODS 80T (5 μm) C_{18} column (25.0 cm × 4.6 mm) (Toso Haas, Stuttgart, Germany) and a Supelguard LC-8-DB precolumn (Supelco, Gland, Switzerland) were used at room temperature. Acetonitrile–50 m*M*

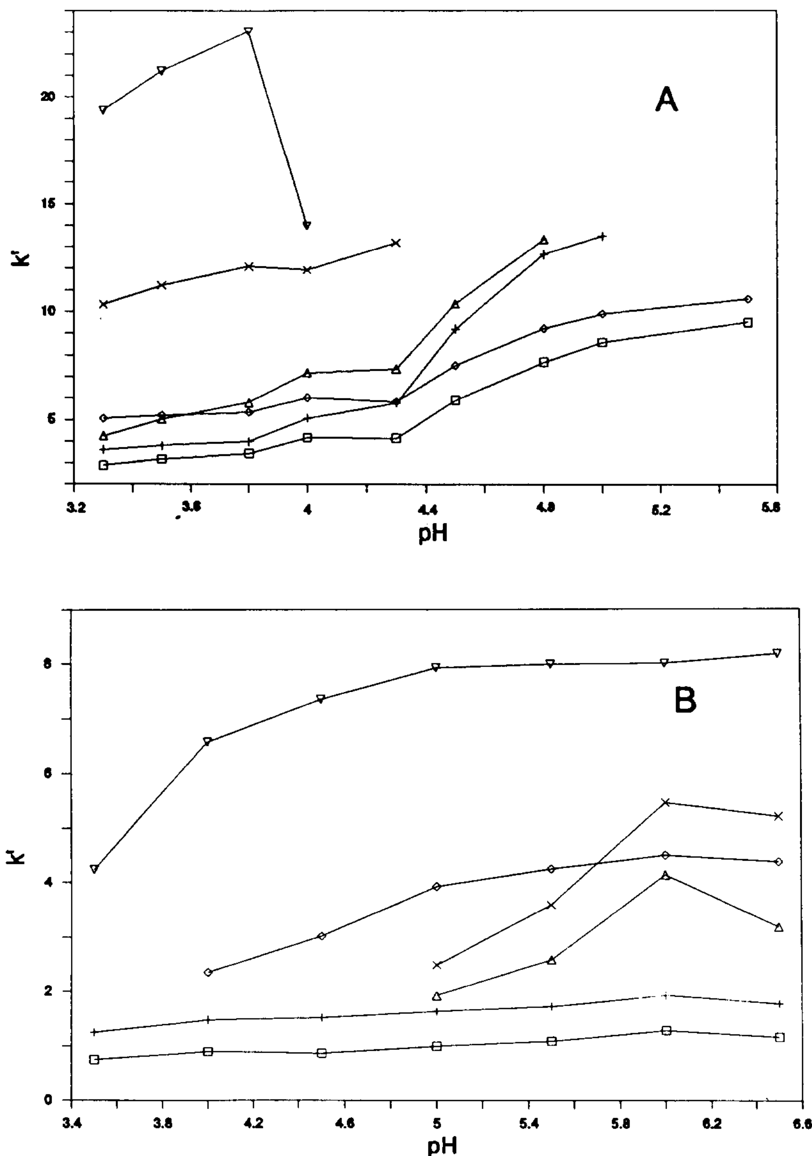

Fig. 3. Effect of mobile phase pH on the separation of heterocyclic amines. (A) Mobile phase: acetonitrile–50 m*M* ammonium acetate (10:90). + = Glu-P-1; □ = IQ; △ = MeIQ; ◇ = MeIQx; ▽ = 4,8-DiMeIQx; × = 7,8-DiMeIQx. (B) Mobile phase: acetonitrile–50 m*M* ammonium acetate (30:70). + = Trp-P-2; △ = PhIP; ▽ = MeAαC; × = harman; ◇ = norharman; □ = 4,7,8-TriMeIQx.

ammonium acetate at different pH values was used as mobile phase at a flow-rate of 1.0 ml/min. A Supelco Visiprep and Visidry SPE vacuum manifold (Supelco, Gland, Switzerland) were used for the clean-up procedure.

Analytical procedure

Sample preparation and clean-up were carried out according to the method proposed by Gross [13], which is summarized in Fig. 2. The method uses a diatomaceous earth cartridge (Extrelut-20) coupled to a propylsulphonic cartridge (PRS). The sample was eluted from the first cartridge and introduced into the second using dichloromethane. Elution was carried out with ammonium acetate pH 8.0, and clean-up using a C_{18} cartridge was carried out to give the final extract (methanol–ammonia). The solvent was evaporated with a stream of nitrogen and the analytes were dissolved with 50 μl of the internal standard (I.S.) in methanol. IQ, MeIQ, MeIQx, Glu-P-1 and 4,8-DiMeIQx were analysed by HPLC using 50 m*M* ammonium acetate (pH 4.0)–acetonitrile (90:10, v/v) as mobile phase, and 7,8-DiMeIQx as internal standard; otherwise Trp-P-2, PhIP, harman, norharman and MeAαC were analysed using 50 m*M* ammonium acetate (pH 6.0)–acetonitrile (70:30, v/v) as mobile phase; the internal standard used in this case was TriMeIQx. The working potential of the electrochemical detector was set at +1000 mV.

The compounds were quantified by the standard addition method. The spiked samples were prepared by addition of accurately measured amounts of each standard (125, 250 and 500 ng) at the beginning of the clean-up process. Recoveries were estimated from the slope of the regression line performed with the added amount *versus* the measured amount.

RESULTS AND DISCUSSION

Optimization of the chromatographic conditions

In order to establish the chromatographic conditions for the separation of the ten aromatic amines, variables such as pH and composition of the mobile phase were studied. Different binary phases of acetonitrile–50 m*M* ammonium acetate at different pH values were tested. The mixtures contained between 10 and 30% acetonitrile and the pH varied from 3.5 to 6.5. Values of capacity factor (k') obtained for the compounds in all the mobile phases are indicated in Table I. The increase in acetonitrile caused a reduction of k' values for the compounds at all the pH values. An increase in the pH produced an enhancement of k' due to a decrease in the amine ionization. In Table I it can be seen that PhIP, harman and

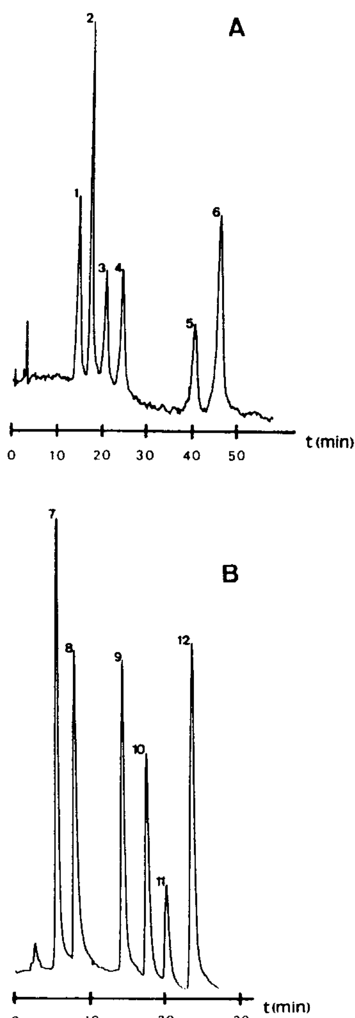

Fig. 4. Chromatogram of standard solution. (A) Mobile phase: acetonitrile–50 m*M* ammonium acetate (pH 4.0) (10:90). Peaks: 1 = IQ; 2 = Glu-P-1; 3 = MeIQx; 4 = MeIQ; 5 = 7,8-DiMeIQx; 6 = 4,8-DiMeIQx. (B) Mobile phase: acetonitrile–50 m*M* ammonium acetate (pH 6.0) (30:70). Peaks: 7 = 4,7,8-TriMeIQx; 8 = Trp-P-2; 9 = PhIP; 10 = harman; 11 = norharman; 12 = MeAαC.

norharman are not detected at pH less than 5, probably because of their low degree of oxidation at this pH, since the electrochemical oxidation of aromatic amines takes place with the loss of hydrogen ions [26].

From the values in Table I, it can be concluded that for the separation of the ten abovementioned compounds using isocratic mode (gradient detection is difficult to perform with this detector) it is necessary to work at two different conditions. Thus the analysis of IQ, MeIQ, MeIQx, Glu-P-1, 4,8-DiMeIQx and 7,8-DiMeIQx can be performed using acetonitrile–50 m*M* ammonium acetate (10:90) (see Fig. 3A, which shows the plots of k' *vs.* pH). At pH 4.0 all the compounds can be separated, as can be

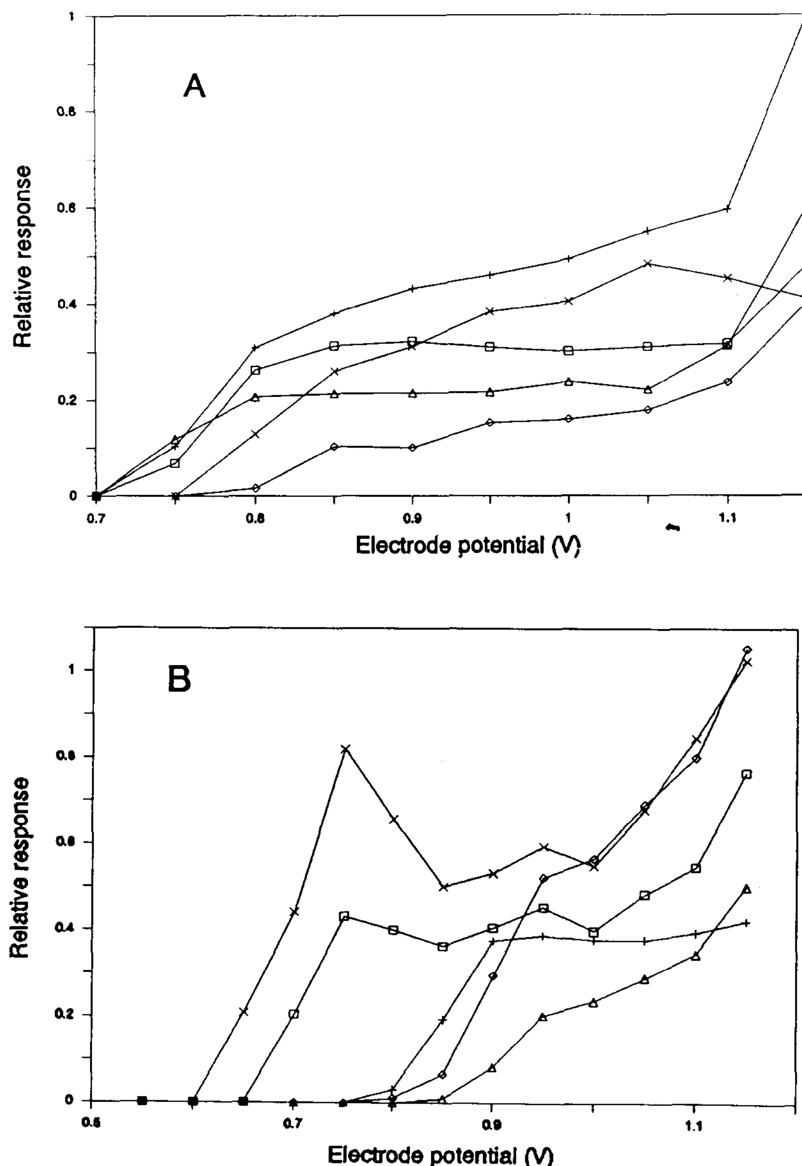

Fig. 5. Hydrodynamic voltammograms. Relative response to Glu-P-1 signal. (A) Mobile phase: acetonitrile–50 m*M* ammonium acetate (pH 4.0) (10:90). + = Glu-P-1; □ = IQ; △ = MeIQ; ◇ = MeIQx; × = 4,8-DiMeIQx. (B) Mobile phase: acetonitrile–50 m*M* ammonium acetate (pH 6.0) (30:70). □ = Trp-P-2; + = PhIP; × = MeAαC; △ = harman; ◇ = norharman.

seen in Fig. 4A, which shows the chromatogram of a standard solution of these compounds at this pH. Mobile phases with 30% acetonitrile are used for the separation of Trp-P-2, PhIP, harman, norharman, MeAαC and TriMeIQx. Their capacity factors at different pH values are given in Fig. 3B, and the separation obtained at pH 6.0 can be seen in Fig. 4B.

The optimum working potential was obtained from the hydrodynamic voltammograms of the compounds at the separation conditions previously established. Fig. 5A and B shows the hydrodynamic voltammograms obtained by raising the potential from +600 to +1150 mV. High responses were obtained for all compounds at +1150 mV; at higher potentials an increase occurred in both the background noise and the residual current, so the working potential chosen to give the best response for all the compounds and low background noise was +1000 mV.

From the voltammograms it can be observed that at electrode potentials lower than +750 mV no detectable response were obtained for any compound except for the aminoindol derivatives Trp-P-2 and MeAαC, which gave high responses at this potential; this behaviour can thus be used for the characterization of these compounds in complex samples.

Quality parameters

Calibrations for heterocyclic amines in methanol were carried out in the optimum separation conditions for each compound, with concentrations in the range 0.1–15 $\mu g\ ml^{-1}$. Peak area was used as the response. The correlation coefficients of calibration functions in the interval of linearity were better than 0.999 for all the heterocyclic amines.

Six replicate determinations of 30 ng (2 $\mu g\ ml^{-1}$ solution) of each heterocyclic amine in methanol were carried out under the optimum conditions to determine the precision of the analysis. Relative standard deviations (R.S.D., %) in the range 1.6–4.6% based on peak area were obtained.

The detection limits for the quinoline and quinoxaline derivatives, based on a signal-to-noise ratio of 2:1 ranged from 0.7 to 3.5 ng, according to their retention times, as can be seen in Table II. The low detection limit for Glu-P-1 may be related to its higher relative response at the working potential. The other compounds analysed with mobile phase acetonitrile–50 m*M* ammonium acetate (30:70) pH 6.0 gave lower detection limits related to their higher responses at this pH. These values are higher than the ones indicated in the literature for IQ, MeIQx, 4,8-DiMeIQx by Takahashi and co-workers [15,16] and Billedeau *et al.* [17]. The differences can be attributed to the pH of the mobile phase; at higher pH values lower detection limits are obtained (about 0.1 ng) but the separation of the compounds is poorer, as can be seen in Table I. Thus, if good separation is needed the use of the mobile phase at pH 4.0 is compulsory, but in this case the detection limits are high. In order to

TABLE II

FIGURES OF MERIT

Analyte	Interval of linearity (ng)	Limits of detection (ng)	Precision (R.S.D., %)
IQ	3.14–156.8	0.74	2.91
Glu-P-1	3.42–171.2	0.51	3.50
MeIQx	4.35–222.4	1.34	3.01
MeIQ	3.36–163.2	1.72	2.58
4,8-DiMeIQx	6.46–161.6	3.37	3.78
Trp-P-2	1.62–161.6	0.19	2.33
PhIP	1.65–164.8	0.26	4.57
Harman	2.61–260.8	0.26	3.95
Norharman	1.60–160.0	0.26	1.68
MeAαC	1.55–155.2	0.74	1.61

improve the limit of detection, an increase in the pH is required, but under these conditions a worse separation is achieved. Values for Glu-P-1 obtained at pH 5.5 are in agreement with those obtained by Billedeau *et al.* [17] for this compound.

Application

The analytical method studied in this paper was mainly developed to determine heterocyclic amines and related compounds in processed-food samples. Estimates of the amounts of IQ, MeIQ, MeIQx and 4,8-DiMeIQx in beef extract have been reported previously [13,15,16,18–20], but procedures that allow the simultaneous analysis of a large number of heterocyclic amines by HPLC–ED have not been reported. So the method was applied to the determination of these compounds in a commercial beef extract. Purification of the sample was performed by the method proposed by Gross [13], as described in the Experimental section. The percentage recovery for each compound is indicated in Table III. These results are in agreement with the data published by Gross [13], and for some compounds our results are slightly higher. Amines such as Glu-P-1 gave low recovery factors, and PhIP, Trp-P-2, MeAαC, harman and norharman were not recovered by this method. The use of different clean-up methods for recovery of these compounds is currently being studied.

Glu-P-1, MeIQ and MeIQx were identified and quantified in the beef extract, and the results are given in Table III. Fig. 6 shows the chromatograms of the sample and the spiked sample

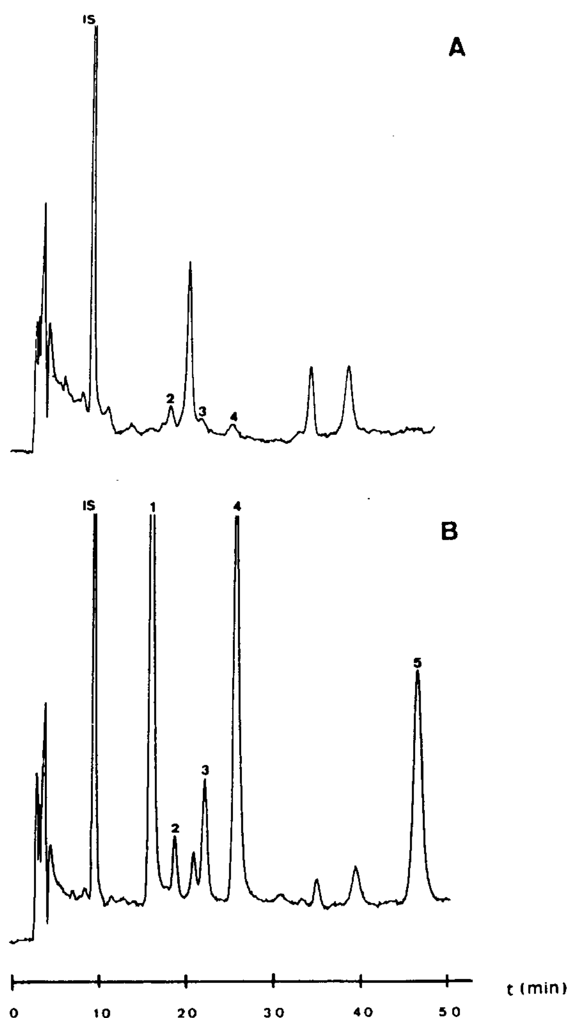

Fig. 6. Chromatograms of a beef extract. Mobile phase: acetonitrile–50 m*M* ammonium acetate (pH 4.0) (10:90). (A) Extract, (B) extract spiked before the clean-up (500 ng of each compound). Peaks: IS = aniline; 1 = IQ; 2 = Glu-P-1; 3 = MeIQx; 4 = MeIQ; 5 = 4,8-DiMeIQx.

and confirms the presence of Glu-P-1, MeIQ and MeIQx. Furthermore, it can be seen that the peak eluted at 21.1 min is not MeIQx, which appears at higher retention times (see spiked sample); likewise, the peaks at 35.0 and 39.0 min were identified. The internal standard used was aniline because of the interfering peak that appeared near to 7,8-DiMeIQx. IQ and 4,8-DiMeIQx, which have been detected in some beef extracts, could not be detected as clear peaks in the sample (Fig. 6). The limits of

TABLE III

ANALYSIS OF A BEEF EXTRACT

ND = Not detected.

Analyte	Recovery (%)	Results (ng/g)
IQ	68.28	ND
Glu-P-1	17.66	15.54
MeIQx	62.93	5.07
MeIQ	77.37	5.77
4,8-DiMeIQx	66.26	ND

detection of these compounds in our system are 0.74 and 3.4 ng, respectively. Thus, if they were present it would be at <0.8 and <3.5 ng g^{-1}, respectively.

CONCLUSIONS

Quantification of mutagenic and carcinogenic heterocyclic amines in cooked foods is essential for estimation of their risk to human beings. In this study conditions have been established for the separation and determination by HPLC–ED of ten heterocyclic amines. The applicability, selectivity, linearity and sensitivity of the method have been studied. The procedure has been applied to the determination of IQ, MeIQx, MeIQ, 4,8-DiMeIQx and Glu-P-1 in a beef extract. Research is currently in progress to optimize the extraction and clean-up methods for the determination, in cooked foods, of the other heterocyclic amines whose chromatographic characteristics and detection limits have been established in this study.

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