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Journal of Chromatography B, 802 (2004) 107–113
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of heterocyclic aromatic amines in beef extract, cooked meat and rat urine by liquid chromatography with coulometric electrode array detection

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Abstract

This paper describes a method for the determination of heterocyclic aromatic amines (HAs; DMIP, IQ, MeIQ, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, AαC, PhIP) by high-performance liquid chromatography (HPLC) with coulometric electrode array detection. The compounds are separated on reversed phase columns (LiChroCart Superspher 60 RP-select B, 250 mm × 2 mm, 4 μm and LiChrospher 60 RP-select B, 250 mm × 4 mm, 5 μm) using mobile phases consisting of acetonitrile/buffer/distilled water and detected at eight working electrodes at potentials between +190 and +680 mV against modified palladium electrodes. In the context of an EU-interlaboratory exercise, the method was applied to analyse a standardised test solution and—after isolation of the analytes by several clean-up steps—for the analysis of standardised beef extract and grilled meat. Further, the method could be applied for the analysis of HAs in suspensions of bacteria and rat urine without any sample preparation step beyond sample dilution. The data obtained show that HPLC with coulometric electrode array detection gives accurate results.

Keywords: Heterocyclic aromatic amines; Coulometric electrode array detection; Food analysis

1. Introduction

Heterocyclic aromatic amines (HAs) are an interesting class of compounds for food chemists since some of them are potent mutagens and possibly also carcinogens [1,2]. They can be formed by cooking protein-rich foods like meat or fish. The influence of different physical and chemical parameters on the formation of HAs has been investigated in several meat model systems [3–7]. These studies have shown that cooking temperature and cooking time seem to play a more important role than the presence of precursors (e.g. creatine, sugars, free amino acids) or the water content of the food product.

In order to estimate the risk posed by food containing these compounds, it is necessary to complement toxicological data by realistic exposure data which can only be obtained by analysing frequently consumed food products [8–15]. The accurate determination of HAs is, however, a difficult analytical task since traces of these compounds have to be determined in highly complex food matrices. This problem can only be solved by combining both elaborate sample preparation steps with selective separation steps followed by detection methods allowing the quantification of HAs at low levels.

Sample clean-up frequently consists of at least one solid phase extraction step [16]. The determination is usually carried out by chromatographic techniques, e.g. high-performance liquid chromatography (HPLC), in combination with different detection systems [17]. Fluorescence detection [18,19], electrochemical detection [8,20–23] and mass spectrometry [24,25] offer both an increased selectivity and sensitivity compared to UV detection [8,26].

Abbreviations: DMIP, 2-amino-1,6-dimethylimidazo[4,5-b]pyridine; IQ, 2-amino-3-methylimidazo[4,5-f]quinoline; MeIQ, 2-amino-3,4-dimethylimidazo[4,5-f]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline; 4,8-DiMeIQx, 2-amino-3,4,8-trimethyl-imidazo[4,5-f]quinoxaline; 7,8-DiMeIQx, 2-amino-3,7,8-trimethylimidazo[4,5-f]quinoxaline; AαC, 2-amino-9H-pyrido[2,3-b]indole; MeAαC, 2-amino-3-methyl-9H-pyrido[2,3-b]indole; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; Trp-P-1, 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole; Trp-P-2, 3-amino-1-methyl-5H-pyrido[4,3-b]indole

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doi:10.1016/j.jchromb.2003.10.063

Among the electrochemical detectors, amperometric detectors have frequently been used to quantify HAs [8,20,21], whereas coulometric electrode array detection which offers the possibility to detect compounds at various potentials simultaneously has only been applied in a few cases [22,23].

The present paper demonstrates the applicability of HPLC coupled to the coulometric electrode array detector for the analysis of HAs in different sample matrices.

The first part of the paper contains results obtained as part of an European project on heterocyclic aromatic amines. It describes the development of the method using a standard test mixture of polar and less polar HAs and its application for the analysis of a standardised beef extract. In order to evaluate the method, our data were compared with those obtained by other participating laboratories applying different analysis methods. In addition, our analysis method was used to quantify polar HAs in some meat samples purchased in a local fast-food shop.

The second part of the paper demonstrates the versatility of the combination of HPLC separation and coulometric electrode array detection by describing its application for the analysis of HAs in a suspension of *Lactobacillus bulgaricus #291* and rat urine. These analytical problems were posed by the observation that strains of lactobacilli contained in dairy products can attenuate genotoxic and carcinogenic effects of dietary mutagens including HAs [27–30]. It has been suggested that the detoxification process is a result of direct binding of HAs to the cell walls of the bacterial strains [29]. In order to test this hypothesis in vitro it was necessary to determine the decrease of the (free) HA concentration in a saline solution caused by the addition of lactobacilli.

Additional experiments were designed to demonstrate the modification of the genotoxic effect of HAs in vivo. Urine HA-levels of rats fed with a standard mixture of five HAs were compared with the HA-levels present in the urine of rats which had been fed with the HAs standard mixture and lactobacilli. The composition of the standard mixture used in these experiments (PhIP, MeIQx, 4,8-DiMeIQx, AαC and IQ) is regarded as representative for fried beef and chicken [31].

2. Experimental

2.1. Chemicals and reagents

HAs were purchased from Toronto Research Chemicals (Toronto, Canada). According to the producers, the chemical purity of these compounds was higher than 99%. Glacial acetic acid (p.A.) was bought from Fluka (Buchs, Switzerland). Ammonia (32%), dichloromethane, sodium hydroxide, sodium acetate, hydrochloric acid (all of p.A. grade) were obtained from Merck (Darmstadt, Germany). Methanol and acetonitrile, both of HPLC grade, were obtained from Riedel-de Haen (Seelze, Germany).

Diatomaceous earth extraction cartridges (Extrelut NT) and refill material were obtained from Merck. Propylsulfonic acid silica (PRS) columns (500 mg) and octadecyl silica (C_{18}) Bond Elut columns (100 mg) were received from Varian (Harbor City, USA).

Stock solutions of HAs were prepared by dissolving about 5 μg of the substances in 5.0 ml methanol. Various standard solutions of different concentrations were prepared by dilution with mobile phase. Solutions were stored in flasks covered with aluminium foil at 4 °C.

The HA mixture with a composition regarded as representative for the HA content in fried beef (57.00% PhIP, 26.30% MeIQx, 4.70% 4,8-DiMeIQx, 10.83% AαC and 1.17% IQ) had a total HA concentration of 20.0 mg/ml physiological saline [31].

Lactic acid bacteria strains *(L. bulgaricus #291* and *Bifidobacterium longum #BB536*) which are currently used for yoghurt production were obtained from Danisco Cultor GmbH & Co. (Niebuell, Germany). The cells were stored deep frozen at −70 °C as concentrated cultures. Immediately before the experiments, the cultures were defrosted in warm water (+40 °C) and suspended in sterile physiological saline. The viability of the cells was determined by plating [32], MRS agar was used for *L. bulgaricus #291* and reinforced Clostridal agar for *B. longum #BB536* [33]. Bifidobacterium plates were re-incubated in an anaerobic jar (Oxoid Ltd., Basingstoke, Hampshire, UK). To create anaerobic conditions, Anaerocult© A (Merck, Darmstadt, Germany) was used. *L. bulgaricus* plates were incubated under aerobic conditions. All plates were incubated at +37 °C for 5 days in the dark, subsequently the colony numbers were counted manually.

2.2. Samples

2.2.1. Test solution and standardised beef extract

The test solution and the standardised beef extract were received from the co-ordinator of the interlaboratory exercise, M.T. Galceran from the Department of Analytical Chemistry, University of Barcelona. The test solution contained five less polar (Trp-P-1, Trp-P-2, PhIP, AαC and MeAαC) and five polar (DMIP, IQ, MeIQ, MeIQx and 4,8-DiMeIQx) HAs in the range from 0.8 to 2 μg/g in methanol. The beef extract was spiked with the same HAs in the range from 50 to 100 ng/g.

2.2.2. Meat samples

Grilled sausage, knuckle of pork and minced meat were bought in a local fast-food shop. All products were very well-done.

2.3. Sample preparation

2.3.1. Test solution

After adding 4,7,8-TriMeIQx (70.8 ng/100 μl) as internal standard the test solution was diluted 1:7 with mobile phase. An aliquot of 20 μl was injected into the HPLC system.

2.3.2. Standardised beef extract

Clean-up was carried out by slightly modifying the recommended procedure from Toribio et al. [16]. 1 g of the beef extract was dissolved and homogenised in 12 ml of 1 M NaOH using an Ultra Turrax mixer. Then the extract was mixed with 13 g of diatomaceous earth (Extrelut) and transferred to an empty glass column (200 mm × 25 mm i.d.). HAs were eluted from Extrelut with 75 ml dichloromethane and adsorbed onto a PRS column which had been preconditioned with 7 ml of dichloromethane. After having eluted the less polar HAs by washing the column with 6 ml of 0.01 M HCl, 15 ml of MeOH/0.3 M HCl (50:50, v/v) and 2 ml water, polar HAs were eluted with 20 ml of 0.5 M ammonium acetate, pH 8.5, directly in another C_{18} cartridge (100 mg). After washing the column with 5 ml of distilled water the polar HAs were eluted with 0.8 ml of methanol/ammonia (90:10, v/v). The extract was evaporated to dryness under a stream of nitrogen and redissolved in 100 μl of acetonitrile/water (50:50, v/v) containing 438.5 ng of internal standard (7,8-DiMeIQx). Finally, 20 μl of the extract were injected into the HPLC system.

2.3.3. Meat samples

Five grams of the meat samples were dissolved and homogenised with 20 ml of 1 M NaOH. After mixing the extract with 23 g of Extrelut further clean-up was carried out according to Section 2.3.2.

2.3.4. In vitro binding studies

Fifty microlitres of the HA solution being representative for the HA content in fried beef (see Section 2.1) were mixed with 700 μl of physiological saline and 250 μl of *L. bulgaricus #291* suspension (10^8 bacteria/ml or 10^9 bacteria/ml). Samples were incubated at 37 °C for 4 h. After centrifugation, the supernatant fractions were diluted 1:25 with mobile phase. Concentration of (unbound) HAs was measured by injecting 20 μl aliquots into HPLC. The control sample was obtained by mixing 50 μl of the HA solution with 950 μl of physiological saline.

2.3.5. In vivo studies

A scouting experiment was carried out with two male Fischer 344 rats purchased from Harlan-Winkelmann GmbH (Borchen, Germany). Rats were housed under controlled conditions (24 ± 2 °C, 50 ± 5% humidity) and provided with tap water ad libidum.

The rats were treated orally with 100 mg of HAs per kg body weight by administering the corresponding volume of the HA solution described in Section 2.1. Subsequently, the 24 h urine was collected and used as a control. One hundred milligrams of HAs per kg body weight were then administered together with a suspension of 5×10^9 Lactobacilli/ml, either with *L. bulgaricus #291* or with *B. longum #BB536* followed by another collection of urine for 24 h. After sterile filtration, the urine samples were diluted with mobile phase and 20 μl aliquots analysed by HPLC.

2.4. Chromatographic separation and coulometric electrode array detection

The modular liquid chromatograph used consisted of a high pressure gradient pump (model L-6200, Merck), an autosampler (model 2000A, Merck) adapted with a 20 μl sample loop and a column thermostat (CROCO CIL^{TM}, ERC GmbH, Riemerling, Germany). Electrochemical detection was carried out with a coul array electrode system, (ESA, Chelmsford, MA, USA) equipped with two cell blocks consisting of eight working electrodes. Chromatograms were evaluated using the Coul Array Win software.

2.4.1. Test solution

The analytical column was a LiChroCart Superspher 60 RP-select B, 250 × 2 mm I.D., 4 μm (Merck). Elution was carried out isocratically at 22 °C with a flow rate of 0.3 ml/min. The mobile phase consisted of acetonitrile/buffer/distilled water (15:10:75, v/v/v). The buffer was prepared by dissolving 4.9 g of trichloroacetic acid in 30 ml glacial acetic acid and mixing it with 3.7 g sodium acetate dissolved in 70 ml distilled water. The potentials of the eight working electrodes (eight channels) were adjusted to +280, +320, +360, +400, +440, +480, +520 and +560 mV.

2.4.2. Meat extracts and samples from in vitro and in vivo binding studies

The analytical column was a LiChroCart LiChrospher 60 RP-select B, 250 × 4 mm I.D., 5 μm (Merck). Elution was carried out isocratically at 40 °C with a flow rate of 1.2 ml/min. The mobile phase consisted of acetonitrile/buffer/distilled water (12:10:78, v/v/v). The buffer was prepared as described in Section 2.4.1. Separation of the analytes was achieved within 35 min. After each analysis the analytical column was washed with acetonitrile/distilled water (70:30, v/v) for 15 min and then reconditioned for 27 min with mobile phase.

The potentials of the eight working electrodes were adjusted to +190, +260, +330, +400, +470, +540, +610 and +680 mV.

2.5. Standard addition

For analysing HAs in the standardised beef extract and the meat samples the standard addition method was used. Two unspiked and four spiked samples (40, 80, 120 and 160 ng of each of the HAs/g) were analysed as described above.

Peak height ratios were plotted against the amounts of analytes added. Recoveries were determined by dividing the slope of the linear regression lines for the standard addition by the slope of the linear regression lines of HAs standard solutions.

2.6. External calibration

The HPLC system was calibrated by injecting seven standard solutions in the concentration range from 5 to 400 ng/ml

mobile phase. The analysis function was obtained by linear regression of the ratio of peak heights (HA-IS) on standard concentrations.

3. Results and discussion

3.1. Test solution

The participants of the European Project on Heterocyclic Aromatic Amines had to analyse a HA test solution provided by the project co-ordinator M.T. Galceran (see Section 2.2.1). Participants were allowed to use any determination method available to them. The results obtained by the participating laboratories were evaluated with regard to precision and accuracy.

In our laboratory the five polar HAs present in the test solution were quantified by HPLC with coulometric electrode array detection. The less polar amines were excluded because of problems detecting MeAαC and Tr-P-2. Since the solution did not contain any matrix compounds it was not necessary to apply purifying sample preparation steps. However, since the concentration of HAs was in the range from 0.8–2 μg/g and therefore beyond the calibration curve, the solution was diluted 1:7 with mobile phase before injecting into HPLC. Table 1 compares the data obtained by repeated measurement of the test solution with the mean of the means, confidence intervals and coefficients of variation obtained by the participants in the European project [34]. The data show that for DMIP, MeIQ, MeIQx and 4,8-DiMeIQx the mean values obtained by HPLC with coulometric electrode array detection fell within the confidence limits of the mean of the means. Only for IQ a mean value was slightly lower than the means obtained by other participants.

3.2. Standardised beef extract

Participants of the European Project also received a standardised beef extract which should be analysed. For sample preparation, a frequently used procedure had been proposed [16] but could be modified.

Fig. 1 shows a representative electrode array chromatogram of the beef extract. In Table 1, the results obtained in repeated analysis of the beef extract by our method and the data collected from all participants are summarised. When we tried to quantify HAs by HPLC with coulometric electrode array detection, in some cases problems due to matrix interferences occurred. Since due to these interferences some determinations did not lead to reliable values for IQ ($n_1 = 4$) and MeIQx ($n_1 = 2$), less than six values were sent to the project co-ordinator. Since in the evaluation process outliers had been eliminated from the data set also different numbers of determinations were available to calculate the mean of the means, its coefficient of variation and the confidence limits. The recovery determined by the standard addition method was quite different for each compound. The lowest value was found for DMIP (38.5%) and the highest one for MeIQ (78.5%). In spite of our problems for all HAs except MeIQ our method led to mean values which fell within the confidence limits of the mean of the means (see Table 1). The limits of detection ($S/N = 3$) for DMIP, IQ, MeIQ, MeIQx and 4,8-DiMeIQx were 0.8, 1.4, 2.1, 1.5 and 2.5 ng/g, respectively.

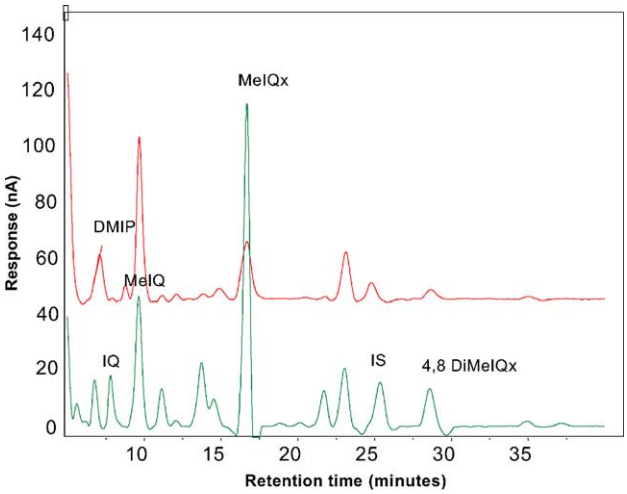

Fig. 1. Electrode array chromatogram of a standardised beef extract (channel 5: +470 mV and channel 6: +540 mV). IS: 7,8-DiMeIQx.

3.3. Meat samples

The analysis method developed was applied to determine polar HAs in some typical Austrian meat products purchased

Table 1
Results of the analysis of polar heterocyclic aromatic amines in the test solution and in the standardised beef extract

HA	Test solution								Beef extract							
	Mean (μg/g)	n_1	CI (μg/g)	CV (%)	Mean of all means (μg/g)	n_2	CI (μg/g)	CV (%)	Mean (ng/g)	n_1	CI (ng/g)	CV (%)	Mean of all means (ng/g)	n_2	CI (ng/g)	CV (%)
DMIP	1.34	6	0.06	4.5	1.36	6	0.23	15.9	81.0	6	15.2	17.9	68.1	4	14.5	13.4
IQ	1.53	6	0.03	2.0	1.78	6	0.18	9.6	65.0	4	6.6	6.4	64.5	5	10.9	13.6
MeIQ	1.38	6	0.05	3.6	1.38	6	0.23	15.9	45.9	6	5.2	10.7	63.6	5	14.4	18.2
MeIQx	1.52	6	0.15	9.2	1.37	6	0.19	13.1	63.4	2	60.2	10.6	64.5	5	19.3	24.1
4,8-DiMeIQx	1.57	6	0.29	17.8	1.44	6	0.15	9.7	77.1	6	15.4	19.1	71.6	4	13.8	12.1

n_1: number of determinations; n_2: number of participants; CI: confidence interval; and CV: variation coefficient.

Table 2
Concentration of polar heterocyclic aromatic amines (ng/g) in some typical Austrian fast-food meat samples

Sample	DMIP (ng/g)	IQ (ng/g)	MeIQ (ng/g)	MeIQx (ng/g)	4,8-DiMeIQx (ng/g)
Grilled sausage	1.5	5.1	ND	ND	ND
Knuckle of pork–crust	NA	3.7	7.4	5.9	2.1
Minced meat	ND	ND	1.6	0.4	ND

NA: not available due to matrix interferences; and ND: not detected (DMIP < 1.0 ng/g; IQ < 1.2 ng/g; MeIQ < 1.4 ng/g; MeIQx < 0.3 ng/g; and 4,8-DiMeIQx < 1.4 ng/g).

in a local fast-food shop (Table 2). In Fig. 2, a representative chromatogram of an extract of the crust of a knuckle of pork is shown. The concentrations of HAs in the analysed food samples are summarised in Table 2. In each of these food products polar HAs were detected. In general, the highest concentrations of HAs were found in the crust of the knuckle of pork. In this extract the quantification of DMIP was not possible due to matrix interferences. In the grilled sausage concentration of IQ was higher than in the crust of the knuckle of pork. In the minced meat extract only low levels of MeIQ and MeIQx were detected.

3.4. Binding of HAs to Lactobacilli

In the context of attempts to arrive at a realistic human exposure assessment, several papers have been published focusing on the binding of HAs to the cell walls of different bacterial strains [27–30]. In order to enlarge the data base to include both polar and less polar HAs, in vitro and in vivo experiments were carried out on the impact of lactobacilli on the distribution of a test mixture consisting of IQ, MeIQx, 4,8-DiMeIQx, PhIP and AαC. Since the analysis method described above was developed for analysing the five polar HAs (DMIP, IQ, MeIQ, MeIQx and 4,8-DiMeIQx) in other matrices (standardised beef extract and meat samples), it was necessary to examine if the analysis method was versatile enough to be easily adapted for this analysis problem.

Table 3
Excreted amounts of HAs found in urine (μg)

	IQ	MeIQx	4,8-DiMeIQx	PhIP	AαC
Applied dose of HAs/rat (μg)					
Rat 1	200	4470	800	9700	1850
Rat 2	210	4840	860	10490	2000
Excreted amount of HAs found in urine (μg)					
Rat 1					
Without LB	31.5	249.0	30.0	98.3	33.8
With LB 536	28.0	191.1	19.6	88.9	23.8
Rat 2					
Without LB	15.4	273.9	30.8	100.1	31.9
With LB 291	10.4	168.0	16.0	79.2	27.2

LB: lactic acid bacteria; LB 536: *Bifidobacterium longum #BB536*; and LB 291: *Lactobacillus bulgaricus #291*.

3.4.1. In vitro binding

In vitro binding studies were carried out by incubating the HA mixture with various numbers of *L. bulgaricus #291*.

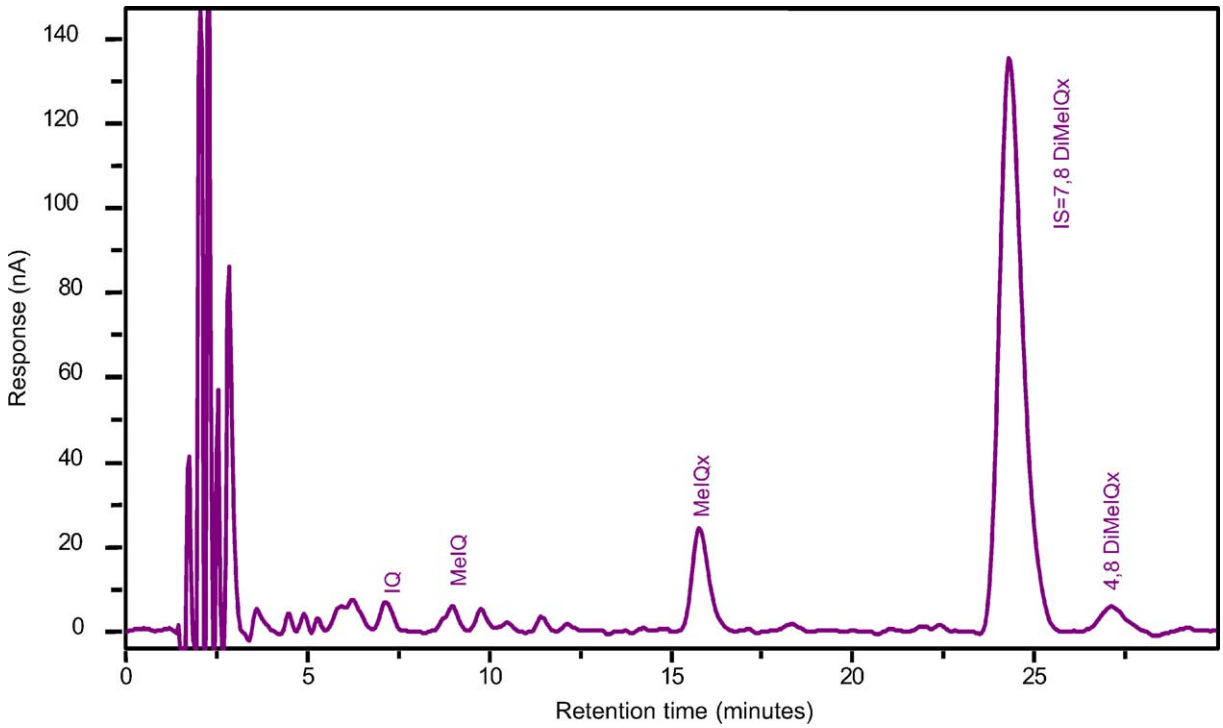

Fig. 2. Chromatogram of an extract of the crust of a knuckle of pork (channel 7: +610 mV). Internal standard (IS): 4,8-DiMeIQx.

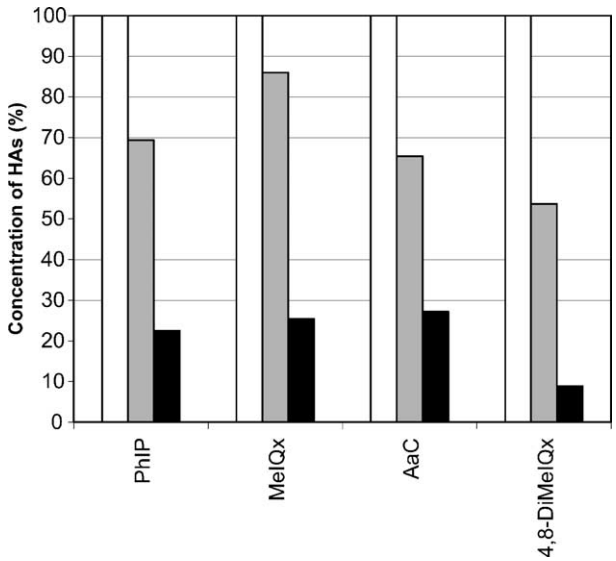

Fig. 3. Influence of the number of lactobacilli/ml on the applied concentration of HAs found in the supernatant fraction. White bar (□): without lactobacilli; grey bar (▨): with 10^8 lactobacilli/ml; and black bar (■): with 10^9 lactobacilli/ml.

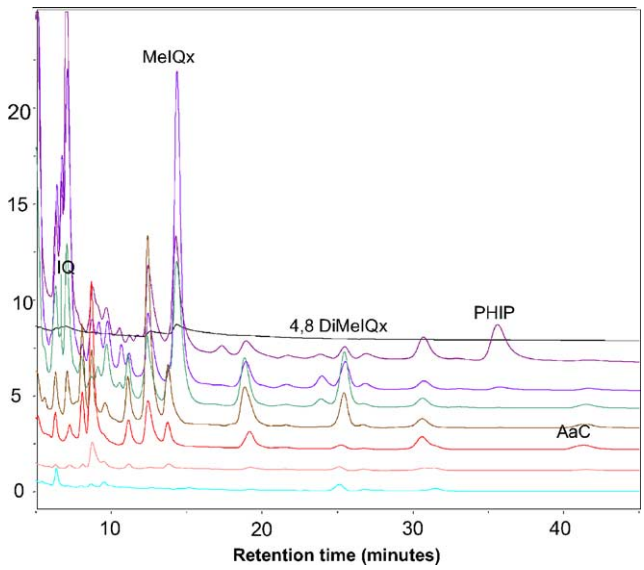

Fig. 4. Chromatogram of rat urine (diluted 1:1 with mobile phase) after feeding with HAs in the presence of bacteria (channel 1–8: +190 to +680 mV, increments: 70 mV).

After removal of the bacterial cells by centrifugation, the supernatant was diluted with mobile phase and the concentration of (unbound) HAs was measured by injection into HPLC. The method originally developed for the analysis of five polar HAs in meat extracts could be applied without any modifications. Incubation with *L. bulgaricus #291* resulted in a remarkable decrease of the concentrations of PhIP, MeIQx, AαC and 4,8-DiMeIQx in the supernatant fraction compared to the control solution (see Fig. 3). It can be seen that the extent of binding increased with the number of bacterial cells added. The concentration of IQ in the supernatant, however, was not significantly decreased by the addition of lactobacilli.

3.4.2. In vivo binding

Up to now, most data on the binding of HAs to lactobacilli derive from in vitro studies. In the present paper, it was investigated if lactobacilli simultaneously administered with HAs have an influence on the excretion of HAs in rats. Fig. 4 shows a representative chromatogram of a 24 h urine (diluted 1:1 with mobile phase) collected after simultaneously feeding a rat with lactobacilli and the HA mixture. The chromatogram demonstrates that the analysis method developed is selective enough to allow the determination of HAs in rat urine. Quantification of HAs was possible without any matrix interferences. Table 3 compares the excreted amounts of each of the HAs when administered without lactobacilli (control) and simultaneously with lactobacilli. The table shows that the simultaneous treatment of male rats with HAs and lactobacilli slightly decreased the excretion of HAs in urine compared to the administration of HAs alone.

4. Conclusion

It could be shown that HPLC with coulometric electrode array detection is applicable to analyse mixtures of polar and less polar amines in different matrices. In some cases, the detection of specific HAs poses problems which can be due to the interference of matrix compounds and/or to some extent to the poisoning of the working electrode surfaces by electrochemical reaction products. Electrode problems can be reduced by cleaning the electrodes electrochemically each day. In the absence of such problems, low concentrations of HAs could be determined.

Since problems in detecting a specific HA have been observed with some food sample matrices but not with others, interference by matrix components seem to be the main cause. The standard addition method can be used to achieve accurate results by eliminating the effects of electrode poisoning and the influence of the matrix on the recovery of the HAs.

Acknowledgements

We thank the European Union for supporting this project (HC Amines, QLK1-1999-01197) and the Fonds zur Förderung der Wissenschaftlichen Forschung (project number 13171-CHE) for the coulometric electrode array system.

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