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Blue Chitin columns for the extraction of heterocyclic amines from cooked meat

Joakim Bång[a], Haruo Nukaya[b], Kerstin Skog[a,*]

[a]*Department of Applied Nutrition and Food Chemistry, Center for Chemistry and Chemical Engineering, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden*

[b]*University of Shizuoka, Shizuoka, Japan*

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Abstract

Mutagenic/carcinogenic heterocyclic amines (HAs) are formed at low levels (ng/g) during heat processing of protein-rich food such as meat and fish. The complex matrix requires effective extraction and purification methods. Blue Chitin columns were used for the extraction of HAs from fried chicken fillets and the samples were analysed with LC–MS. Several HAs were identified at levels ranging from 0.04 to 0.10 ng/g. The use of Blue Chitin columns provides a simple and fast method for the extraction of HAs from meat samples.

Keywords: Meat; Food analysis; Blue Chitin columns; Blue Rayon columns; Stationary phases, LC; Amines, heterocyclic; Aminodimethylfluro[3,2-*e*]imidazo[4,5-*b*]pyridine

1. Introduction

Mutagenic/carcinogenic heterocyclic amines (HAs) are formed during heat processing of protein-rich food such as meat and fish [1–3]. To date, around 20 HAs have been identified in heat-treated foods. The molecular structures of some of them are shown in Fig. 1. HAs have been found to be carcinogenic in long-term animal studies in mice, rats and non-human primates [4]. Furthermore, DNA adducts have been detected in rodents and in humans after consumption of HAs at low doses [5]. Several epidemiological studies have shown a correlation between the intake of fried, broiled or roasted meat, and the development of cancer, while other studies have found no reliable correlation. It has also been suggested that HAs in cooked foods play a role in the aetiology of human cancer [6,7]. There are also indications of a possible relation between HAs and human cancer, where studies have shown a correlation between intake of heavily burnt meat and increased incidences of colorectal, pancreatic, urothelial, and gastric cancer [8].

The daily human intake of HAs varies greatly, depending on dietary habits and cooking practices [9,10]. For estimates of the daily intake of HAs and for reliable risk assessments, it is important to determine the levels of HAs in cooked foods. The amount of HAs is highly dependent of the type of meat and cooking conditions [2,3,11]. As HAs are

*Corresponding author. Tel.: +46-46-222-8319; fax: +46-46-222-4532.

E-mail address: kerstin.skog@inl.lth.se (K. Skog).

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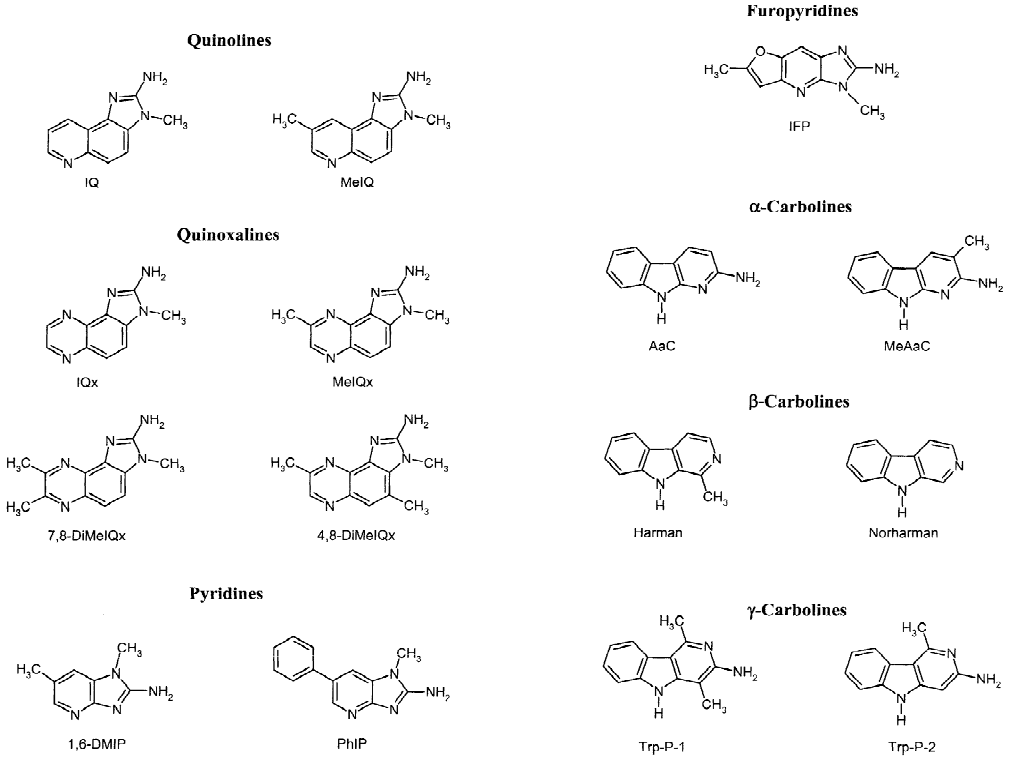

Fig. 1. Chemical structures of the studied heterocyclic amines.

present at low levels in a complex sample matrix, there is a need for an effective purification method and a sensitive and selective analytical method. Reversed-phase high-performance liquid chromatography (HPLC) is often employed for the analysis of HAs, and the different substances have generally hitherto been identified with UV photodiode array detectors. The comparison of UV absorbance spectra for a compound with library spectra of reference compounds, efficiently prevents most false peak identifications. This is crucial because co-eluting interfering compounds are common in chromatograms from heat-processed foods. Fluorescence detectors have sometimes been used as a complement to the UV photodiode array detector. During recent years, several studies have been performed using mass spectrometry (MS), which offers high sensitivity and selectivity, and allows identification of several HAs at very low levels [12–14]. Selected ion monitoring (SIM) can be used to monitor only one or a few masses at a time, thereby increasing the sensitivity and reducing the interference. The possibility of fragmenting the HA ions (MS–MS technique) increases the sensitivity even further [15,16].

Several methods have been used for the extraction and purification of HAs, for example liquid–liquid extraction [17–19] and liquid–liquid extraction on a solid support of diatomaceous earth, followed by solid-phase extraction, a method developed by Gross and Grüter [20]. There are also reports on the use of Blue Cotton for the extraction of HAs from foods [21–23], urine [24,25] and faeces [26]. Blue Cotton is cotton bearing covalently bound copper phthalocyanine trisulfonate, which belongs to a group of blue pigments widely used as dyes [24]. Most of the HAs have three fused aromatic rings in their structures (see Fig. 1), which give them a high affinity to form covalent bonds with phthalocyanine derivatives, yielding a 1:1 complex. Complex formation takes place in water, and the bonds can most effectively be broken by methanol containing a small amount of ammonia. In a similar way, Blue Rayon, i.e., rayon bearing covalently bound copper phthalocyanine trisulfonate has been used for the extraction

of HAs from river water [27–31] and meat extract [32]. In this type of experiment, pieces of Blue Cotton or Blue Rayon are either inserted into the sample solution or packed into a column [33–35]. Synthetic chitin, a poly-*N*-acetylglucosamine, is another material that can be used for the extraction of HAs in a similar way. Chitin can be covalently bound to copper phthalocyanine trisulfonate to a significantly greater extent than cotton and rayon, and this property makes Blue Chitin suitable for packing in a short, narrow column, as shown by Hayatsu et al. [28]. The aim of the present study was to develop a method based on Blue Rayon or Blue Chitin for the extraction of HAs from cooked foods.

2. Experimental

2.1. Chemicals

Acetic acid (suprapur grade), acetonitrile (gradient grade) and methanol (analytical-reagent grade) were purchased from VWR International (Stockholm, Sweden). Water was passed through a Milli-Q water purification system (Millipore, Bedford, MA, USA). The following HAs were used as reference compounds: DMIP (2-amino-1,6-dimethylimidazo[4,5-*b*] pyridine), PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine), IQ (2-amino-3-methylimidazo[4,5-*f*]quinoline), MeIQ (2-amino-3,4-dimethylimidazo [4,5-*f*]quinoline), IQx (2-amino-3-methylimidazo [4,5-*f*]quinoxaline, MeIQx (2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline), 4,8-DiMeIQx (2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline), 7,-8-DiMeIQx (2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline), AαC (2-amino-9*H*-pyrido[2,3-*b*]indole), MeAαC (2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole), 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), and were purchased from Toronto Research Chemicals (Toronto, Canada), while harman (1-methyl-9H-pyrido[3,4-*b*]indole) and norharman (9H-pyrido[3,4-*b*]indole) were purchased from Aldrich (Steinhem, Germany). The chemical purity of the synthetic references was higher than 99%, according to the manufacturers. A mixture of the different HAs (about 2 ng/μl) dissolved in methanol was used as a spiking mixture. For the preliminary study using Blue Rayon, a standard solution of seven synthetic mutagens (IQ, IQx, MeIQ, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, PhIP) in MeOH was prepared (20 ng/μl of each mutagen). The HAs, IQ, IQx, MeIQ, MeIQx, 4,8-DiMeIQX, 7,8-DiMeIQx and PhIP are denoted polar HAs, while harman, norharman, Trp-P-1, Trp-P-2, AαC and MeAαC are denoted less polar, as suggested by Gross and Grüter [20]. In addition, IFP (2-amino-1,6-dimethylfuro[3,2-*e*]-imidazo[4,5-*b*]-pyridine), kindly provided by Mark G. Knize, Lawrence Livermore National Labs., CA, USA, was used as a reference compound. Materials for solid-phase extraction, diatomaceous earth (Isolute), was obtained from Sorbent (Västra Frölunda, Sweden) and the Blue Rayon and the Blue Chitin columns (120 mg) from Funakoshi, Tokyo, Japan.

2.2. Liquid chromatography

The HPLC system included a pump (Spectra-Physics P2000), an autosampler (Spectra-Physics AS3000), a UV detector (Spectra-Physics UV2000), all from Thermo Finnigan (San José, CA, USA), and a Zorbax SB-C_8 StableBond Analytical (150×4.6 mm I.D., 5 μm) HPLC column from Agilent Technologies (Palo Alto, CA, USA). The chromatographic conditions were: solvent A: water (pH adjusted to 3.5 with acetic acid)–acetonitrile (95:5, v/v) and solvent B: water (pH adjusted to 3.5 with acetic acid)–acetonitrile (5:95, v/v). The flow-rate was 1 ml/min and the injection volume was 5 μl. The best separation was obtained by using two gradients, one for polar HAs, and one for less polar HAs. For the polar HAs, the gradient program started at 5% B, increased to 10% B for 10 min and to 70% B for the next 10 min. For the less polar HAs, the gradient program started at 10% B and increased to 60% B for 20 min. IFP was analysed separately, using a gradient program that started at 5% B, increased to 15% B in 8 min and then to 60% B for the next 7 min. The UV absorbance was monitored at 263 nm, and was used only to optimise the settings for the MS detector.

2.3. Mass spectrometry

The HPLC system was coupled to an LCQ$_{DECA}$

ion-trap mass spectrometer with Xcalibur software from Thermo Finnigan, using electrospray ionization. The heated capillary temperature was set at 325 °C. Nitrogen was used as sheath gas and auxiliary gas, with settings of 60 and 10 units, respectively. The MS detector was operated in the positive mode, with an electrospray voltage of 5 kV, a capillary voltage of −7 V, and a tube lens voltage of −20 V. Analysis were achieved in the selected ion monitoring (SIM) mode, with a setting of 3 microscans at a maximum ion time of 200 ms. The SIM windows were adjusted according to the UV chromatogram, to cover one or two peaks, with a mass window of ±0.5 (m/z) for each HA. IFP quantification was performed using the molar extinction coefficient corresponding to PhIP since a synthetic standard is not available [36].

2.4. Extraction of HAs

The first set of experiments (method B) was performed with the spiking mixture and during the search for optimal conditions, only polar amines were analysed in single determinations. Briefly, Blue Chitin columns were conditioned with water, a mixture of MeOH and NH_4OH (25%), and with the solvent in which the sample was dissolved. The sample was applied to the column, which was then washed with water. Finally, the HAs were eluted from the column with MeOH or MeOH–NH_4OH. In addition, the effluent from the washing step was collected and analysed for HAs. Several solvents were tested for dissolving the sample: EtAc (B1), water (B2), 1 *M* acetic acid (HAc) (B3) and 1 *M* sodium hydroxide (NaOH) (B4). The elution solvent was optimised by changing both the total volume and the ratio between MeOH and NH_4OH. For the method yielding the highest recoveries (B4), extractions were made 4–6 times.

When the extraction method had been established for pure references, we attempted to analyse a freeze–dried sample of fried chicken samples (chicken fillet fried at 175 °C for 15 min per side). However, it was necessary to add a pre-purification step, and two different methods were tested: centrifugation (method C) based on an extraction method by Bjeldanes et al. [40] and extraction with diatomaceous earth (method D) based on the method developed by Gross and Grüter [20]. The meat sample (0.5–3 g) was homogenised (Polytron homogeniser) in either HCl (C1) or NaOH (C2, D1 and D2). In method C, the homogenised meat sample was centrifuged and decanted through a filter three times. The pH of the combined HCl filtrate (method C1) was adjusted to 9 with NaOH and the solution was centrifuged to remove precipitate. The sample was then applied to the preconditioned Blue Chitin column. In method D, the homogenised meat sample was mixed with diatomaceous earth, and packed into an empty cartridge, which was eluted with EtAc. The eluate was either applied directly to the Blue Chitin column (D1), or evaporated to dryness, dissolved in NaOH and then applied to the column (D2). Single or double experiments were performed except for the final method (D2), which was repeated 5–9 times.

After evaluation of the results of the various experiments, the method with the highest recoveries (D2) was selected as the ''final method''. A freeze–dried meat sample was homogenised in 10 ml 1 *M* NaOH, then 100 μl spiking mixture was added. Diatomaceous earth (about 60 ml) was added and the dry powder was packed into a 70-ml cartridge. The HAs were extracted with EtAc and 50 ml eluate was collected. The eluate was evaporated to dryness under reduced pressure, and dissolved in 10 ml 1 *M* NaOH. A Blue Chitin column was preconditioned with 20 ml water, 30 ml MeOH–NH_4OH (9:1, v/v), 30 ml water, and finally with 30 ml 1 *M* NaOH. The sample solution was passed through the Blue Chitin column, which was then washed with 50 ml water. The HAs were eluted with 100 ml MeOH–NH_4OH (9:1, v/v). The eluate was collected, evaporated to dryness under reduced pressure, and dissolved in 100 μl MeOH, and then analysed using LC–MS. The extraction and analysis were repeated at different days for estimation of the standard deviation. The limit of detection for a standard solution of HAs was estimated to be 20–200 pg.

3. Results and discussion

The main purpose of this study was to find an extraction method for HAs using Blue Rayon or Blue Chitin. To our knowledge, this is the first paper presenting results on the extraction of HAs from meat samples using Blue Chitin columns in combina-

tion with LC–MS analysis. As no, or only low levels of HAs were found in the eluent after application of the sample, or in the effluent from the washing step, we conclude that HAs are adsorbed onto Blue Rayon and Blue Chitin.

3.1. Blue Rayon experiments

In a preliminary study (method A), Blue Rayon columns were prepared by packing 0.50 g Blue Rayon into a cartridge (bed size 50×5 mm). The sample was then applied to the top of the Blue Rayon column: experiments I and II, 10 μl of the standard solution mixed with 10 ml water (AI) or 10 ml EtAc (AII), experiments III and IV, Trp-P-1 (3.4 ng) and Trp-P-2 (3.7 ng) dissolved in 10 ml water (AIII) or 10 ml EtAc (AIV). The liquid eluted from the bottom of column was collected. The column was washed with 50 ml distilled water (AI and AIII) or EtAc (AII and AIV) and the effluent was collected. The HAs were then eluted with 50 ml MeOH–NH_4OH (50:1, v/v). The different eluates were evaporated to dryness under reduced pressure, re-dissolved in MeOH and analysed. Most of the mutagens were bound to the Blue Rayon and less than 2% of IQ, MeIQx and PhIP were found in the liquid eluting from the column after sample application. When the HAs were mixed with EtAc, the recovery of IQ, IQx, MeIQ, MeIQx, PhIP, Trp-P-1 and Trp-P-2 was 82–110%, and for 4,8-DiMeIQx and 7,8-DiMeIQx it was ~65%. On the other hand, when mixed with water, the recoveries were markedly lower, 27–72%, except for Trp-P-1 and Trp-P-2 (98 and 83%). Thus, it is better to dissolve the samples in EtAc than in water. Previous experiments with Blue Rayon showed the following recoveries for pure reference compounds: IQ, 81%; MeIQx, 78%; PhIP, 75%; and Trp-P-1, 94% [37], which are in agreement with our results. The data indicated that Blue Rayon columns could be used for the extraction of HAs. However, we found it difficult to pack Blue Rayon columns in a standardised way. This led us to try pre-packed Blue Chitin columns, as such columns have been used previously for the extraction of HAs from beef extract and river water [28].

3.2. Blue Chitin experiments

The results of the recovery experiments performed with the spiking mixture are shown in Table 1. Comparing the data from methods B1, B2 and B3, it is clear that the recovery of polar HAs is below 50%, except for IQ and MeIQ. For the other polar HAs dissolved in HCl, the recovery was poor, only

Table 1
Recovery (%) of HAs in spiking mixture or spiked meat samples

	Spiking mixture				Spiked meat samples			
Method/experiment:	B1 (n=1)	B2 (n=1)	B3 (n=1)	B4 (n=4–6)	C1 (n=2)	C2 (n=1)	D1 (n=1)	D2 (n=5–9)
Sample dissolved in/homogenisation solvent:	EtAc	Water	HCl	NaOH	HCl	NaOH	NaOH	NaOH
Solvent applied to Blue Chitin column:	EtAc	Water	HCl	NaOH	NaOH	NaOH	EtAc	NaOH
DMIP	–	–	–	23±4	–	–	–	14±4
PhIP	39	44	6	74±8	27	25	22	53±7
IQ	91	72	39	95±5	27	32	30	76±4
MeIQ	87	65	60	97±5	29	35	32	82±4
IQx	19	45	2	72±4	31	27	41	51±6
MeIQx	38	51	10	58±11	24	33	36	52±4
4,8-DiMeIQx	18	27	6	30±10	20	22	25	28±6
7,8-DiMeIQx	29	39	14	43±12	23	24	25	39±6
Harman	–	–	–	90±8	22	19	–	70±8
Norharman	–	–	–	80±16	30	22	–	63±10
Trp-P-1	–	–	–	95±5	14	24	–	72±6
Trp-P-2	–	–	–	94±4	10	18	–	72±9
AαC	–	–	–	79±14	12	14	–	57±8
MeAαC	–	–	–	70±21	7	13	–	45±14

– Not analysed.

2–14%. The highest recoveries, 70–97% for most of the HAs (both polar and less polar), were obtained when the HAs were dissolved in 1 *M* NaOH (method B4). From this experiment, we concluded that 1 *M* NaOH was the best choice of solvent when applying a sample to the Blue Chitin column. However, some of the HAs showed markedly lower recoveries: DMIP 23%, 4,8-DiMeIQx 30% and 7,8-DiMeIQx 43%. It should be noted that data on the recovery of DMIP and the less polar amines were only obtained with one method (B4). Our results are in agreement with results from other studies showing recoveries for pure reference compounds of 85–100% for IQ, MeIQx, PhIP and Trp-P-2 using Blue Chitin [28]. Using Blue Cotton, the recoveries of IQ, MeIQx, PhIP, Trp-P-1, Trp-P-2, AαC and MeAαC were 57–98% [24,37,38]. The data from our experiments can also be compared with data from studies made with other extraction methods, showing recoveries of 70–86% for IQ, MeIQ, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx and PhIP, and 58–83% for Trp-P-1, Trp-P-2 and AαC [39].

Experiments were also performed to see whether the Blue Chitin columns could be used more than once. The columns were washed with water and MeOH–NH_4OH (9:1, v/v), and used for extraction as described for method B4. No HAs were detected in a blank sample except for traces of 4,8-DiMeIQx, 7,8-DiMeIQx, Trp-P-1 and Trp-P-2. The recovery of HAs (spiking mixture), was about the same as for new columns.

Several combinations of solvents and extraction volumes were tested to find optimal conditions for the extraction of HAs from spiked meat samples. The recoveries of some meat samples are shown in Table 1. Both centrifugation methods (methods C1 and C2) gave low recoveries, 10–30%, with no major difference between the two methods. For the diatomaceous earth methods, the recoveries for method D1 were also low, 20–40% for the polar amines (less polar not tested), but method D2 gave markedly improved results, with recoveries of 50–80% for most of the HAs. We are not aware of any other study presenting data on recoveries of HAs from meat samples after Blue Chitin extraction, but when using Blue Cotton, the recoveries of IQ, MeIQ, MeIQx, 4,8-DiMeIQx and PhIP ranged between 40 and 75% [23,35]. In our study, some of the HAs showed low recoveries: 4,8-DiMeIQx 28%, 7,8-DiMeIQx, 38%, and DMIP 14%. In other studies, DMIP has been extracted using two different solid-phase extraction methods, and the recoveries have been low, 6 and 31% [41]. Overall, the extraction recoveries may vary considerably due to the complexity of the sample matrix and, for example, the commonly used method developed by Gross and Gruter [20] shows recoveries of 33–85% for IQ, MeIQ, MeIQx, 4,8-DiMeIQx and PhIP [20,35,41].

Apart from the choice of sample and elution solvents, the extraction recovery may be affected by the sterical possibility of the HAs to form a complex with copper phtalocyanine trisulfonate. In the case of the quinoxalines (IQx, MeIQx, DiMeIQx), the recovery decreased when the numbers of methyl groups increased. This is probably because the increased number of methyl groups makes complex formation difficult. However, this is not seen for the other classes of HAs, and for the β-carbolines (harman, norharman), the recovery increased when a methyl group was added. The reason for this is unclear. The low recovery for DMIP may be due to its molecular structure; it has only two fused rings (see Fig. 1) which may result in less effective complex formation.

A sample of fried chicken was analysed with method C1. Fig. 2 shows chromatograms of the LC–MS analysis of the fried chicken sample. The peaks of the detected HAs are shaded black. Two samples of fried hamburgers were analysed with method D2, one fast-food burger and one restaurant made hamburger from a restaurant. Table 2 shows the amounts of HAs detected in the different samples. The concentrations are expressed as ng/g of fried meat and corrected for incomplete recovery. PhIP and 4,8-DiMeIQx were detected in all the samples, MeIQx, harman and norharman in the fried chicken and in the restaurant made hamburger. Interestingly, IFP, Trp-P-2 and AαC were detected in the fried chicken sample, IQ and AαC in the restaurant made hamburger and Trp-P-1 and Trp-P-2 in the fast-food hamburger. The reports on these HAs in cooked foods are few in the literature. Generally, the amounts of HAs are low compared to other studies (see review by Skog et al. [3]). There is a great variation of the amounts, probably depending on the cooking conditions. There are other studies

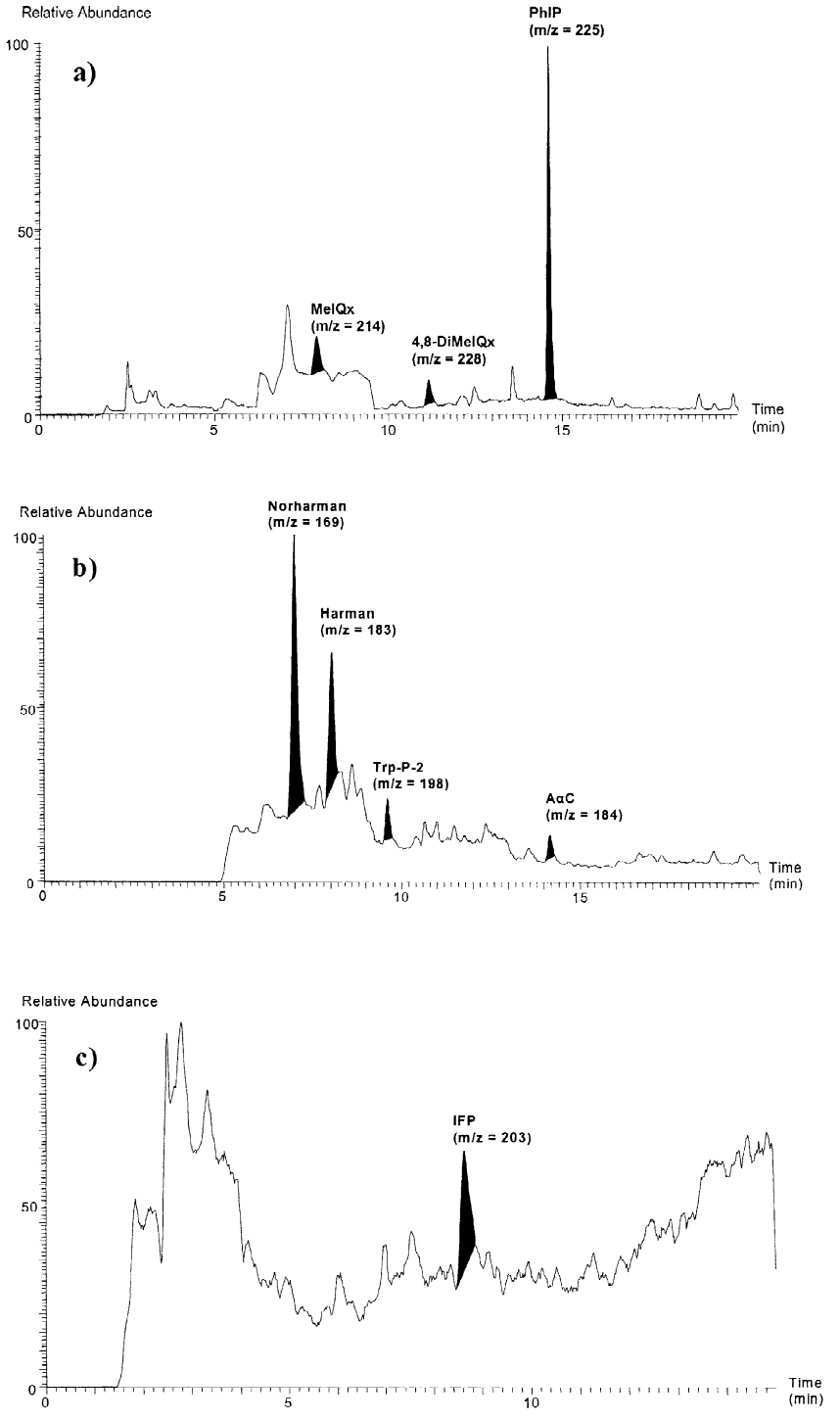

Fig. 2. LC–MS chromatogram in the SIM mode of an unspiked sample of freeze–dried fried chicken. Peaks corresponding to HAs are shaded black; (a) MeIQx, 4,8-DiMeIQx, and PhIP; (b) norharman, harman, Trp-P-2, and AαC; and (c) IFP. Chromatographic conditions are given in the Experimental section.

Table 2
Amounts of HAs in cooked chicken and hamburgers (ng/g cooked product)

	Chicken home-made, extraction method C1 (n=1)	Hamburger fast-food, extraction method D2 (n=1)	Hamburger restaurant made, extraction method D2 (n=2)
DMIP	nd	nd	nd
IFP	0.04	–	–
PhIP	0.4	0.02	0.1
IQ	nd	nd	0.01
MeIQ	nd	nd	nd
IQx	nd	nd	nd
MeIQx	0.1	nd	0.03
4,8-DiMeIQx	0.07	0.05	0.04
7,8-DiMeIQx	nd	0.02	nd
Harman	0.1	nd	0.1
Norharman	0.2	nd	0.08
Trp-P-1	nd	0.01	nd
Trp-P-2	0.3	0.03	nd
AαC	0.08	nd	0.02
MeAαC	nd	nd	nd

– Not analysed, nd=not detected.

that show these levels for ground beef (hamburgers) fried at low temperatures (150 °C) [11]. There are also some reports on markedly higher amounts (6.5–7.0 ng/g) of IFP in cooked chicken [42,43].

4. Conclusions

As shown in the present study, a simple and fast method for the extraction of HAs from meat samples has been developed using Blue Chitin columns. The extraction recoveries of most of the HAs tested were between 50 and 80% in cooked meat samples. Work is now in progress to modify the method to improve the recoveries, and to use Blue Chitin columns for the extraction of HAs from urine samples.

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