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Quantitative analysis of mutagenic heterocyclic aromatic amines in cooked meat using liquid chromatography–atmospheric pressure chemical ionisation tandem mass spectrometry

Philippe A. Guy, Eric Gremaud, Janique Richoz, Robert J. Turesky*

Department of Quality and Safety Assurance, Nestlé Research Center, Nestec Ltd., Vers-chez-les-Blanc, 1000 Lausanne 26, Switzerland

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Abstract

Five mutagenic heterocyclic aromatic amines (HAAs) were quantified from meat extracts, and grilled and pan fried bacon samples using stable isotopically labeled internal standards. These compounds were isolated from the matrices by a tandem solid-phase extraction procedure, followed by separation on reversed-phase liquid chromatography (HPLC) and quantified by atmospheric pressure chemical ionization tandem mass spectrometry (APCIMS–MS). Tandem mass spectrometry (MS–MS) acquisition was done in selected reaction monitoring (SRM) mode to provide a high degree of sensitivity and selectivity for accurate quantification of HAAs. The detection and quantification limits of these HAAs approached 0.015 and 0.045 μg/kg (part-per-billion), respectively, with only 4 g of meat. The HAA levels ranged widely from 0.045 to 45.500 μg/kg, and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) was the predominant HAA found in these samples. The amount of HAAs formed was highly dependent upon the type of meat and method of preparation. An intralaboratory comparison of the extraction procedure showed that estimates of these HAAs obtained by three different individuals at HAA levels below 2 μg/kg were within 5% with coefficients of variation below 19%, indicating the robustness of the analytical method. Moreover, because all of these HAAs from this class of molecules undergo facile cleavage at the *N*-methylimidazole moiety under collision-induced dissociation (CID) conditions, MS–MS analysis in the constant neutral loss mode of $[M+H]^+$-15 enabled the identification of two other HAAs, 2-amino-3-methylimidazo[4,5-*f*]quinoxaline (IQx) and 2-amino-1,7,9-trimethylimidazo[4,5-*g*]quinoxaline (7,9-DiMeI*g*Qx), which have rarely been reported in cooked meats.

Keywords: Solid-phase extraction; Inter–laboratory; Tandem mass spectrometry; Mutagens; Amines, heterocyclic aromatic

1. Introduction

Heterocyclic aromatic amines (HAAs) may be formed in tobacco, meats, poultry, and fish prepared under typical household cooking practises [1]. The formation of HAAs involves the reactions of hexose, amino acids and creatine under high-temperature conditions to give rise to several structurally similar HAA molecules [2]. These HAAs are potent bacterial mutagens and induce tumors at multiple sites in rodents, including the colon and mammary gland, under long-term feeding studies [3,4]. Human tissues metabolically activate HAAs to genotoxins, and therefore HAAs are suspected human carcinogens

*Corresponding author. Tel.: +41-21-785-8833; fax: +41-21-785-8553.

E-mail address: robert.turesky@rdls.nestle.com (R.J. Turesky)

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[5]. There is a need to develop robust, reliable analytical methods to measure the amounts of HAAs in the human diet. Because of the relatively low amounts of HAAs formed in food matrices, it has been a challenge to develop rapid analytical methods that unequivocally identify HAAs in these complex matrices at the low μg/kg level. A number of methods have been reported, which rely on ultraviolet and fluorescence spectroscopy for detection of HAAs [6–10]. However, these methods may provide ambiguous results due to co-eluting peaks when detection approaches the low μg/kg level. Furthermore, these techniques do not readily enable the introduction of reliable internal standards to account for extraction efficiency. More recently, several studies have used LC–MS techniques to measure HAAs in several foods [11–16], although many of these studies did not use stable isotopically labeled internal standards, which is essential for accurate quantification. The goal of our study was to characterise and quantify five major HAAs (Fig. 1) formed in different types of meat and bacon samples using stable deuterated internal standards by LC–MS techniques. We have improved the detection and quantitation limits performed in LC-APCIMS–MS by using a 2.1-mm internal diameter (I.D.) HPLC column instead of the conventional 4.6-mm I.D. column commonly used in such analyses. Because of this high degree of sensitivity and selectivity, we were able to show all the characteristic fragment ions of these HAAs in product ion acquisition mode in the complex food matrices at the low μg/kg (parts per billion) level, which permitted their unambiguous identification. Moreover, the MS–MS analysis in the

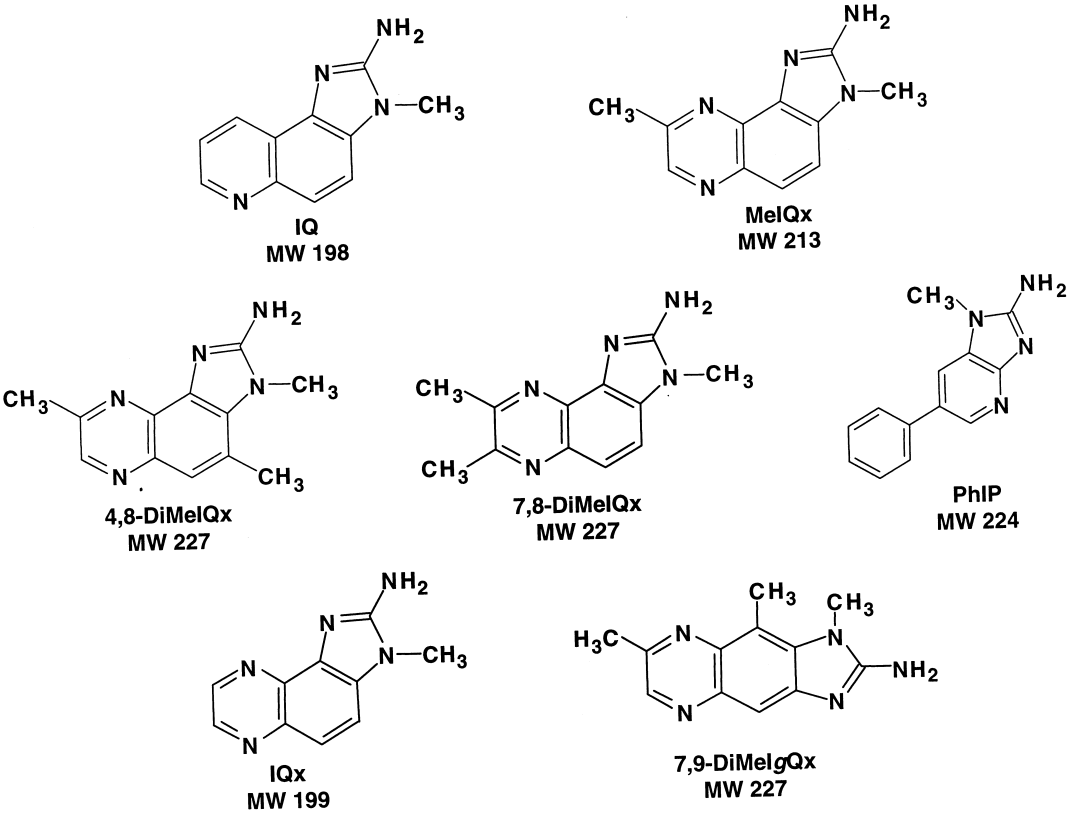

Fig. 1. Chemical structures of the major heterocyclic aromatic amines found in cooked meats.

constant neutral loss acquisition mode enabled us to screen and identify other HAAs which have rarely been reported in cooked meat samples.

2. Experimental

2.1. Chemicals

2-Amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3-methylimidazo[4,5-*f*]quinoxaline (IQx), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-[2-^{14}C]-3,8-dimethylimidazo[4,5-*f*]-quinoxaline (10 mCi/mmol) ([2-^{14}C]MeIQx), 2-amino-3-[$^{2}H_3$]-8-dimethylimidazo[4,5-*f*]quinoxaline (d_3-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) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) were purchased from Toronto Research Chemicals (Ontario, Canada). 2-Amino-3-[$^{2}H_3$]-methylimidazo[4,5-*f*]quinoline (d_3-IQ), 2-amino-3-[$^{2}H_3$]-4,8-trimethylimidazo[4,5-*f*]quinoxaline (d_3-4,8-DiMeIQx), 2-amino-3-[$^{2}H_3$]-7,8-trimethylimidazo[4,5-*f*]quinoxaline (d_3-7,8-DiMeIQx) were synthesized as described by Turesky et al. [17]. 2-Amino-1-methyl-6-[$^{2}H_5$]-phenylimidazo[4,5-*b*]pyridine (d_5-PhIP) was a kind gift from Drs. J. Felton and M. Knize (Lawrence Livermore National Laboratory, Livermore, CA, USA). 2-Amino-1,7,9-trimethylimidazo[4,5-*g*]quinoxaline (7,9-DiMeI*g*Qx) was a kind gift of Drs. A. Tada and K. Wakabayashi (National Cancer Center Research Institute, Tokyo, Japan). All other chemical reagents were analytical or HPLC grade. The isotopic purity of d_3-MeIQx was 96.5%, and the isotopic purity of all other deuterated internal standards exceeded 98.5%.

PRS (500 mg) and Isolute C_{18} (500 mg and 100 mg) cartridges used for the extraction procedure were purchased from ICT (Postfach, Basel, Switzerland).

2.2. Cooking and preparation of meat

Food-grade meat extract samples were obtained from different markets and were received as powder and paste forms. Bacon samples were obtained from local supermarkets in Lausanne. The bacon was cooked on a grill or a pan-fried surface under identical temperature (230°C) and cooking time (1.5 min). The bacon was well done but organoleptically acceptable. The cooked bacon samples were then thoroughly homogenized with a Büchi B-400 mixer (Büchi, Flatwill, Switzerland) and stored at −20°C prior to analysis.

2.3. Extraction of HAAs from meat

The extraction was done by the method developed by Gross [6]. An aliquot of sample (4 g) was homogenised with 1 N sodium hydroxide (15 ml) and 16 μl of a mix of internal standards (d_3-IQ, d_3-MeIQx, d_3-4,8-DiMeIQx, d_3-7,8-DiMeIQx, d_5-PhIP, at 500 ng/ml) was added to achieve a final internal standard concentration of 2 μg/kg. Extrelut-20 (18 g) was added to the homogenate and mixed to obtain a dry, homogeneous mixture. The material was then applied to an Extrelut-20 reservoir (40 ml) connected in series with a 500-mg PRS cartridge equilibrated with dichloromethane. The resin was washed under gravity with dichloromethane until 40 ml of solvent were recovered. The PRS cartridge was removed and rinsed with additional dichloromethane (6 ml) to remove lipids and then dried by water aspiration. The PRS cartridge was then equilibrated with 0.1 N hydrochloric acid (6 ml) and the apolar HAAs eluted with methanol–1 N hydrochloric acid (6:4; 10 ml), followed by water (4 ml) at a flow-rate of 1–2 ml/min. These two fractions were pooled and diluted with water (20 ml) and 25% ammonia (1 ml). The solution was then applied to a C_{18} cartridge (500 mg) that had been prewashed successively with methanol, 1 N hydrochloric acid and water. The apolar HAAs were eluted with methanol–ammonia (9:1; 0.8 ml) directly into HPLC vials and evaporated under nitrogen to dryness.

The PRS cartridge, which still contained the polar HAAs, was then connected in series to a 100 mg C_{18} cartridge which had been prewashed with methanol, 1 N HCl and water. The PRS/C_{18} cartridges were washed with 1 *M* ammonium acetate, pH 8 (20 ml), to elute the polar HAAs onto the C_{18} cartridge. The PRS cartridge was discarded, and then the C_{18} cartridge was washed with water (4 ml) and dried

completely by aspiration. The polar HAAs were eluted with methanol–ammonia (9:1; 0.8 ml) and concentrated to dryness as described above. The dried extracts were dissolved in methanol–water (1:1; 100 μl) and aliquots (20 μl) of both polar and apolar fractions were analysed by LC-APCIMS–MS.

2.4. Liquid chromatography

Separation of the HAAs was done by HPLC with a Hewlett-Packard 1100 HPLC system equipped with a Vydac C_{18} column (2.1 mm I.D.×250 mm length, particle size 5 μm) at a flow-rate of 0.2 ml/min. A binary mobile phase solvent was used. Solvent A contained 25 m*M* ammonium acetate and 5% acetonitrile (pH 8.5); and solvent B contained acetonitrile. A step-wise linear gradient was employed to optimize the separation of HAAs. The solvent conditions started at 10% B, increasing to 16% B in 1 min, then to 20% B at 14 min, increasing from 20% to 100% B at 16 min and remaining at 100% B for 4 min at a flow-rate of 0.23 ml/min. The UV wavelength was set at 265 nm.

2.5. Mass spectrometry

Analyses were performed on a Finnigan TSQ-7000 mass spectrometer equipped with the API 2 interface in atmospheric pressure chemical ionization (APCI) positive mode with the corona discharge set at 5 μA. The heated capillary and the vaporizer temperatures were set at 250 and 500°C, respectively. Nitrogen was used as sheath gas with a pressure set at 20 p.s.i. Instrument tuning was done by infusion of a MeIQx solution (1 ng/ml) at a flow-rate of 5 μl/min via a mixing tee where 0.2 ml/min of 20% solvent B was flushing through the HPLC. In order to avoid contamination of the MS interface, a Rheodyne valve diverter (Omnilab Biosystems, Mettmenstetten, Switzerland) was used for the first 5 min of the gradient. Product ion mass spectra of HAAs were recorded in the mass range of 50-M+10 with a scan time of 2 s. Quantitative analyses were achieved in MS–MS mode for each HAA and its corresponding internal standard, using argon as collision gas at a pressure of 2.2 mTorr. The cleavages of the $N–CH_3$ and $N–CD_3$ bonds were monitored in selected reaction monitoring mode (Table 1). In-source collision was set at 13 eV, whereas the second quadrupole was set at a collision energy of 48 eV. Calibration curves were generated from 10 to 30 000 pg of HAAs containing 3200 pg of internal standards. Peak integration was done automatically with the LCQuan software program (Finnigan, San Jose, CA) with baseline recognition; however, manual integration was performed when high chemical noise precluded reliable integration of the analyte when present at trace levels. HPLC-APCIMS–MS was also performed by scanning the third quadrupole in the mass range of m/z 100-M+5, with the same in-source collision energy while the collision energy of the second quadrupole was set at 58 eV for all HAAs except for PhIP which was set at 65 eV. The MS–MS neutral loss experiments were performed by monitoring the loss of $[M+H]^+$-15 in the low collision energies as described above while scanning the first quadrupole from m/z 180 to 250 with a scan time of 2 s.

2.6. Statistical analyses

Statistical analyses comparing the estimates of HAAs in intralaboratory comparisons were done by a one-way ANOVA using a program by GraphPad PRISM™ (San Diego, CA, USA). Post-tests were not performed.

Table 1
LC-APCIMS–MS analysis conditions of HAAs

HPLC retention time (min)	HAA	MS–MS transition reaction
11.1	d_3-MeIQx	217.1⇒199.1
	MeIQx	214.1⇒199.1
11.5	d_3-IQ	202.1⇒184.1
	IQ	199.1⇒184.1
13.1	d_3-7,8-DiMeIQx	231.1⇒213.1
	7,8-DiMeIQx	228.1⇒213.1
14.5	d_3-4,8-DiMeIQx	231.1⇒213.1
	4,8-DiMeIQx	228.1⇒213.1
20.1	d_5-PhIP	230.1⇒215.1
	PhIP	225.1⇒210.1

3. Results and discussion

3.1. MS characteristics

Product ion mass spectra of the respective protonated molecules of MeIQx, d_3-MeIQx, and [2-^{14}C]MeIQx under low and higher collision energies are shown in Fig. 2. Under low collision energy conditions, one major fragment ion at m/z 199 was found for both MeIQx and d_3-MeIQx, whereas m/z 201 was observed for [2-^{14}C]MeIQx. Under higher collision energies, several major fragment ions were observed at m/z 77, 104, 131, 171 and 199 for

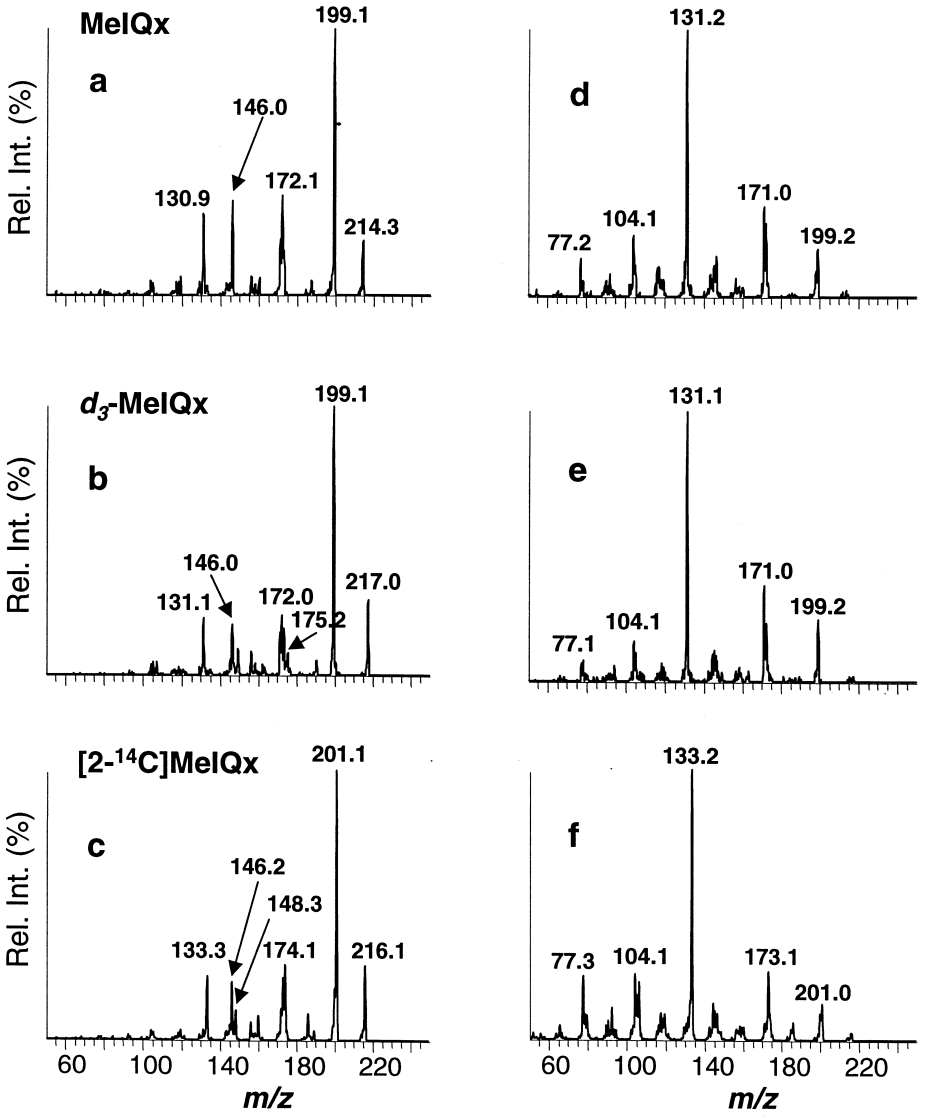

Fig. 2. Product ion mass spectra of protonated (a,d) MeIQx (m/z 214), (b,e) d_3-MeIQx (m/z 217) and (c–f) [2-^{14}C]MeIQx (m/z 216) obtained with a pressure gas of 2.2 mTorr, an in-source collision set at 13 eV and a collision energy in the second quadrupole set at (a–c) 48 eV and (d–f) 63 eV.

MeIQx and d_3-MeIQx, while predominant fragment ions were observed at m/z 77, 104, 133, 173 and 201 for [2-^{14}C]MeIQx. Since MeIQx and d_3-MeIQx give similar fragment ions, the most facile cleavage is at the *N*-methylimidazole moiety to produce the $[M+H]^{+\cdot}-CH_3^{\cdot}$ and $[M+H]^{+\cdot}-CD_3^{\cdot}$ radical cation species (i.e., m/z 199 for both compounds), and most of the other principal fragments are presumably derived at least in part from this charged radical. The product ion mass spectra of [2-^{14}C]MeIQx obtained under the same higher collision energy conditions closely paralleled the fragmentation pattern of MeIQx and d_3-MeIQx, except that the ions were increased by 2 mass units. Therefore, the ^{14}C-radiolabeled atom in the imidazole ring remains intact. Under higher collision energy conditions (Q_2), the base peak is detected at m/z 131 for both product ion mass spectra of MeIQx and d_3-MeIQx, while m/z 133 is the base peak in the product ion mass spectrum of [2-^{14}C]MeIQx. Different parent ion scan experiments of the m/z 131 fragment ion of MeIQx in Q_3 were done at varying in-source collision voltages, which showed that this ion came from the fragmentation pathway of ions at m/z 172 and 199 (data not shown). The ion observed at m/z 131 is also the principal fragment in the product ion mass spectrum of the methylated homologue 7,8-DiMeIQx under similar high collision energy conditions. In contrast to the product ion mass spectrum of 7,8-DiMeIQx, the principal fragment in the mass spectrum of the isomeric 4,8-DiMeIQx derivative was observed at m/z 145, indicating that the 4-methyl group remains intact in this molecule. These data imply that the pyrazine moiety has been lost from these HAAs under higher collision energy conditions with retention of the charge on the benzoimidazole-2-yl-amine moiety of MeIQx and its homologues. Similar MS–MS experiments were performed for IQ, MeIQx, 7,8-DiMeIQx and 4,8-DiMeIQx with higher in-source collision energy conditions and by selecting $[M+H]^{+\cdot}-CH_3^{\cdot}$ radical cation species in the first quadrupole (results not shown). Table 2 summarizes the different fragment ions observed from the product ion mass spectra of these four HAAs under low and higher in-source collision energies. From these results, we have proposed a plausible fragmentation pathway of MeIQx (Fig. 3).

3.2. Calibration graphs

Calibration curves were calculated for each compound. A nine-level calibration curve of each HAA in water with a fixed amount of internal standard (3200 pg injected on column) was performed, quantifying the area ratios of the transition reactions of either $[M+H]^+ \rightarrow [M+H-15]^{+\cdot}$ or $[M+H-18]^{+\cdot}$, against their respective amount ratios. As an example, the calibration curve of MeIQx displayed good linearity ($y=1.0554x+0.0616$) from 10 to 30 000 pg injected ($r^2=0.9997$). The intercept does not go through the origin, and the slope is not equal to 1. These deviations may be explained by the minor contribution of unlabeled MeIQx (3.5%) present in this deuterated standard. To determine the impact of matrix effects on quantitation of HAAs under these MS–MS conditions, calibration curves with triplicate measurements were established in fortified meat-based bouillon samples at three spiking levels (0.1, 0.3 and 0.6 μg/kg) (data not shown). This matrix-based calibration curve also displayed good linearity for all the HAAs screened. As an example, MeIQx was calculated at $y=1.0643x+0.1716$ ($r^2=0.9789$). The slightly higher intercept value of the matrix-based calibration curve is attributed to a small amount of endogenous MeIQx present in the sample.

3.3. Performance of the method

The limit of detection (LOD) from a meat-based bouillon sample for IQ (which was at a non-detectable level in this sample) was calculated at 15 ng/kg (part-per-trillion), corresponding to three times the standard deviation of the observed noise and the limit of quantitation (LOQ) was calculated at 45 ng/kg, corresponding to 10 times the standard deviation of the noise of the same blank sample [18]. The recovery of HAAs was determined by fortifying a series ($n=4$) of non-contaminated meat-based bouillon at 0.2, 0.5 and 1.0 μg/kg level of each HAA added at the beginning of the extraction procedure, whereas 2 μg/kg of each internal standard were added immediately prior the LC-APCI-MS–MS analysis. The mean recovery for the HAAs was estimated at 80% with a standard deviation of less than 10%.

Table 2
MS–MS analysis of four HAAs in the product ion monitoring mode by selecting the $(M+H)^+$ and the $(M+H)^{+\cdot}-CH_3^{\cdot}$ ions in the first quadrupole for each HAA with low and high in-source collision energy (13 and 48 eV), respectively, and a collision energy in Q_2 set at 35 and 25 eV, respectively (gas pressure 2.5 mTorr)

HAA	Low in-source collision		Higher in-source collision	
	Ion selected (Q_1)	Fragment ions[a] (Q_3)	Ion selected (Q_1)	Fragment ions[a] (Q_3)
IQ	199	184 (100%) 157 (23%) 131 (10%)	184	183 (20%) 157 (100%) 156 (56%) 130 (35%)
MeIQx	214	199 (100%) 172 (33%) 146 (31%) 131 (20%)[b]	199	198 (29%) 172 (91%) 171 (49%) 131 (100%)
7,8-DiMeIQx	228	213 (100%) 187 (32%) 172 (26%) 160 (23%) 146 (17%) 131 (36%)	213	212 (22%) 172 (34%) 171 (19%) 131 (100%)[b]
4,8-DiMeIQx	228	213 (100%) 212 (89%) 187 (18%) 186 (13%) 160 (35%) 145 (16%)	213	212 (100%) 185 (56%) 171 (20%) 145 (62%)

[a] Expressed in percentage of relative intensities.
[b] Further higher in-source collision followed by MS–MS of this ion resulted in fragments found at m/z 77 and 104.

3.4. Analysis of meat-based products

Both apolar and polar fractions obtained from the clean-up procedure were analysed and quantified by HPLC-APCIMS–MS. In some meat samples, PhIP, the most lipophilic HAA, partitioned between both fractions, while all other HAAs were recovered in the polar fraction. The sensitivity of HAA detection in a meat-based bouillon sample by LC-APCIMS–MS is shown in Fig. 4. The amounts of HAAs in this sample approached the lower limit of detection (45 ng/kg). A UV chromatogram obtained from a meat extract sample contaminated with high levels of HAAs was added to the figure for comparison. Multiple, unresolved peaks were observed in the UV trace, which precluded unambiguous identification and quantification of HAAs by UV spectroscopy. However, the SRM total ion currents of the HAAs are readily visualized. The order of elution of the HAAs is: MeIQx, IQ, 7,8-DiMeIQx, 4,8-DiMeIQx and PhIP, respectively. Both 7,8-DiMeIQx and 4,8-DiMeIQx and their deuterated standards are baseline separated (rt 13.0 and 14.4 min, respectively) under these gradient conditions, leading to facile quantification of these two isomers. This example highlights the importance of the HPLC–MS coupling system to perform a preliminary separation of the compounds which have identical masses. In this sample, we clearly identified IQ, MeIQx, 4,8- and 7,8-DiMeIQx while PhIP was not detected. A peak eluting with the transition ion of PhIP (225→210) was detected (rt 22.9 min) but did not correspond to the correct rt 22.0 min, further demonstrating the need for internal standards. Stable isotopically labeled internal standards are essential for the unambiguous detection of these analytes, particularly, since the retention times

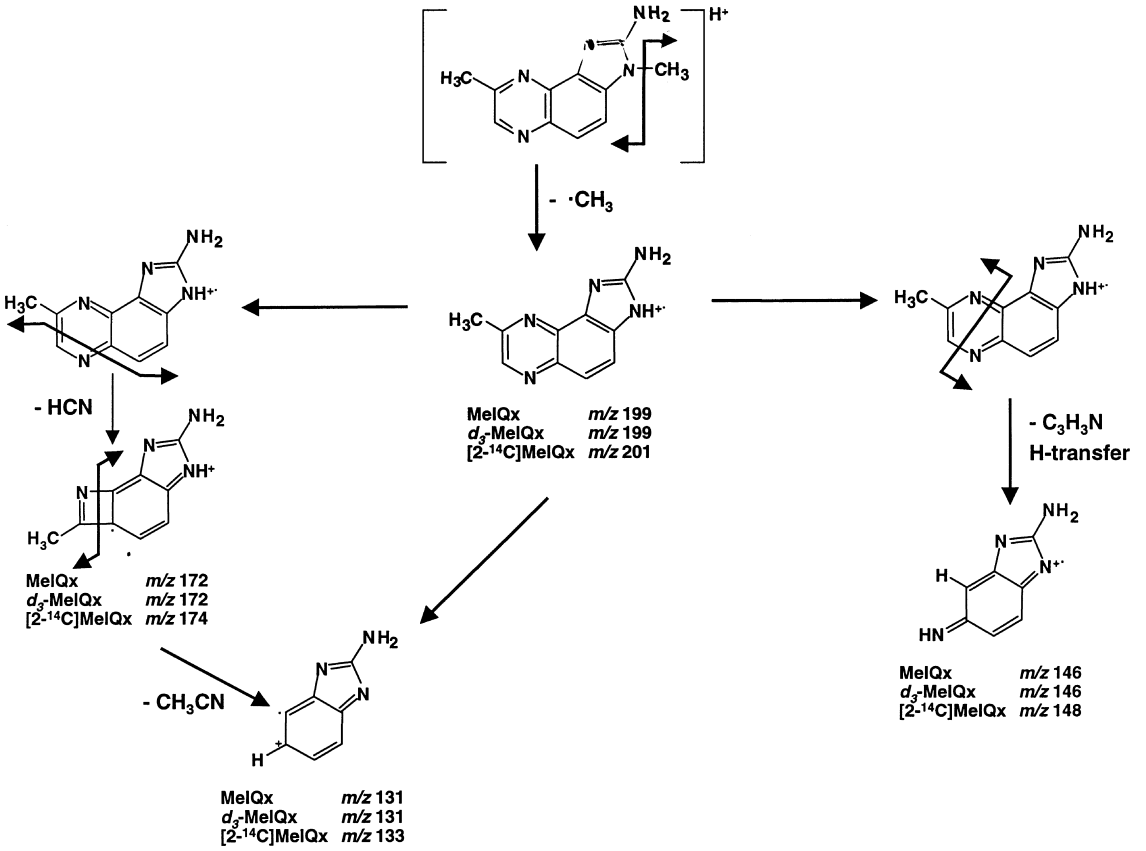

Fig. 3. Postulated fragmentation pathway of the major product ions observed from MeIQx.

of these HAAs may shift by as much as 1.9 min depending upon the food matrix. Quantification of HAAs has already been described using UV/fluorescence detection [6–10]; however, the sensitivity and limits of detection are about two orders of magnitude higher than LC-APCIMS–MS reported here and highly dependent upon the food matrix [19].

The reproducibility of the extraction procedure was assessed by means of an intralaboratory comparison amongst three individuals using a second meat-based bouillon sample. Each analyst prepared the sample in quadruplicate and the LC-APCIMS–MS analyses were performed in duplicate for each case. Table 3 summarizes the amount of HAAs found for each analyst. The estimated values of each HAA were within 5% with coefficient of variation calculated at less than 19% for an amount of 7,8-DiMeIQx estimated at 60 ng/kg, which was the HAA found in this meat sample at the lowest concentrations. Statistical analysis of the means of each HAA analyzed by the three different investigators using a one-way ANOVA revealed no significant difference in estimates of the respective HAAs between the three analysts ($P>0.07$ for 4,8-DiMeIQx; $P>0.27$ for all other HAAs).

The amounts of HAAs found in several meat products are summarized in Table 4. The HAA levels ranged widely from less than 45 to as much as 45 500 ng/kg. Collectively, 7,8-DiMeIQx was found in lowest amounts, consistent with previous reports [12,20], while MeIQx was the predominant HAA found in these meat samples. The divergent values for the grilled bacon samples shows the importance that the method of cooking may have on HAA formation. The bacon sample which was cooked on a smooth heating surface contained 5-fold higher HAA

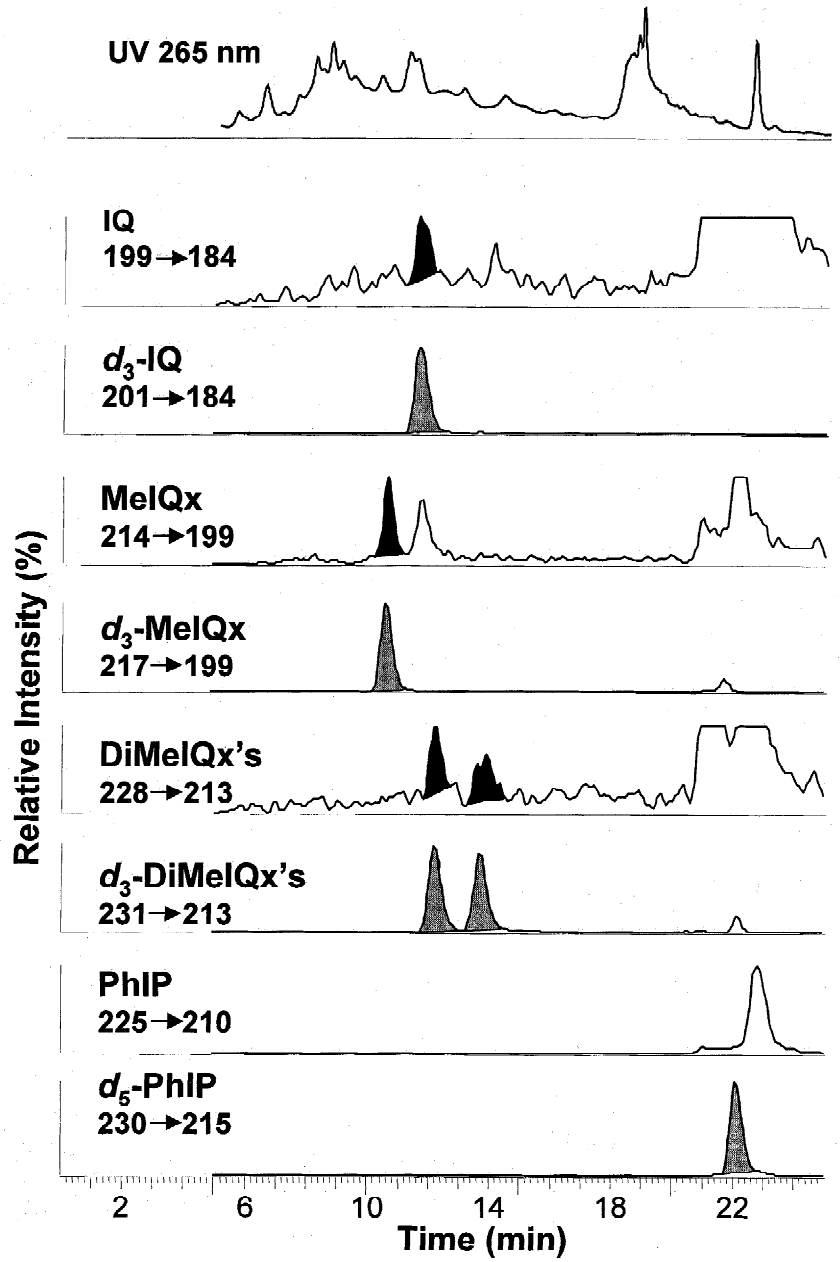

Fig. 4. LC-APCIMS–MS analysis of a meat-based bouillon sample containing low amount of total HAAs (<1 part per billion) monitored in SRM acquisition mode with a gas pressure of 2.2 mTorr, an in-source collision and a collision energy set at 13 and 48 eV, respectively. The UV trace reported on top has been recorded with a wavelength of 265 nm and corresponds to a meat extract sample contaminated with high levels of total HAAs (>10 μg/kg).

levels than the bacon sample cooked on a grill surface, even though time of cooking and the temperature of the heating surfaces were the same for both preparations. The lower amounts of HAAs detected in the grilled bacon sample may be due to their release with the fat drippings. Indeed, it has

Table 3
Intralaboratory comparison of HAA estimates in meat-based bouillon by three different analysts[a]

	IQ	C.V.	MeIQx	C.V.	7,8-DiMeIQx	C.V.	4,8-DiMeIQx	C.V.	PhIP[b]	C.V.
Analyst A	140	12	1845	5	60	15	450	6	640	16
Analyst B	140	8	1800	7	60	15	440	5	600	2
Analyst C	135	14	1880	4	60	14	465	4	600	8
SD	5%		2%		4%		4%		4%	

[a] Four independent extractions were done and analysed in duplicate. The amount of HAAs calculated are expressed as the mean in ng/kg and the coefficient of variation has been calculated in percentage.
[b] PhIP has been calculated from both the polar and apolar phases.

been reported that the fat drippings and grill residue scrapings, which are often used as a base for gravies and sauces, contained levels of HAAs at concentrations from 10- to 100-fold higher than in cooked meats [11].

3.5. Screening for unknown HAAs

For many molecules present in these complex meat extract mixtures, the loss of $[M+H]^+-15$ may be a common fragmentation process and may not be a very selective monitor. However, this tandem solid-phase extraction procedure is specific for the HAA class of molecules and thus may enhance the selectivity for detecting this group of compounds. Because of the close structural similarities in this class of HAAs and common cleavage of the methyl group from the *N*-methylimidazole moiety with tandem mass spectrometry, the constant neutral loss acquisition mode of $[M+H]^+-15$ was monitored for several samples (extraction and clean-up were performed without internal standards) to screen for other possible HAAs. Fig. 5 shows the total ion current of a meat extract in the constant neutral loss acquisition mode and the precursor ion mass spectra of the major peaks detected (Fig. 5, peaks (a)–(f)). Using this acquisition mode, protonated molecular species were detected at m/z 214, 199 and 228 at 10.9, 11.3, and 13.9 min, respectively, and correspond to the expected retention times of MeIQx, IQ, and 4,8-DiMeIQx. Neither 7,8-DiMeIQx (expected rt 13.0 min) nor PhIP (expected rt 19.4 min) were detected, probably because they were under the limit of detection with this acquisition mode. In addition,

Table 4
Amount of HAAs found in different food-based products[a]

HAAs		Meat extract paste 1	Meat extract powder 2	Grilled bacon 3[b]	Grilled bacon 4[c]	Meat extract powder 5	Meat extract paste 6	Meat extract paste 7
MeIQx	Amount (ng/kg)	920	2640	8140	1610	45 510	1180	1580
	C.V. (%)	9	1	5	11	4	3	7
IQ	Amount (ng/kg)	350	110	530	420	4760	130	160
	C.V. (%)	12	1	4	19	16	12	15
7,8-DiMeIQx	Amount (ng/kg)	<45	<45	410	50	650	<45	<45
	C.V. (%)	31	11	5	12	13	22	1
4,8-DiMeIQx	Amount (ng/kg)	180	410	4510	940	13 650	300	370
	C.V. (%)	12	10	1	17	19	3	11
PhIP	Amount (ng/kg)	90	350	28 400	4970	5970	190	220
	C.V. (%)	15	1	3	14	14	8	18
Total HAAs[d]		1540	3510	41 990	7990	70 540	1800	2330

[a] Two independent extractions were done and analysed in duplicate. The amounts of HAAs are expressed as the mean in ng/kg and the coefficient of variation has been calculated in percentage.
[b] Pan frying bacon.
[c] Grilled bacon.
[d] Summation of the HAAs above 45 ng/kg.

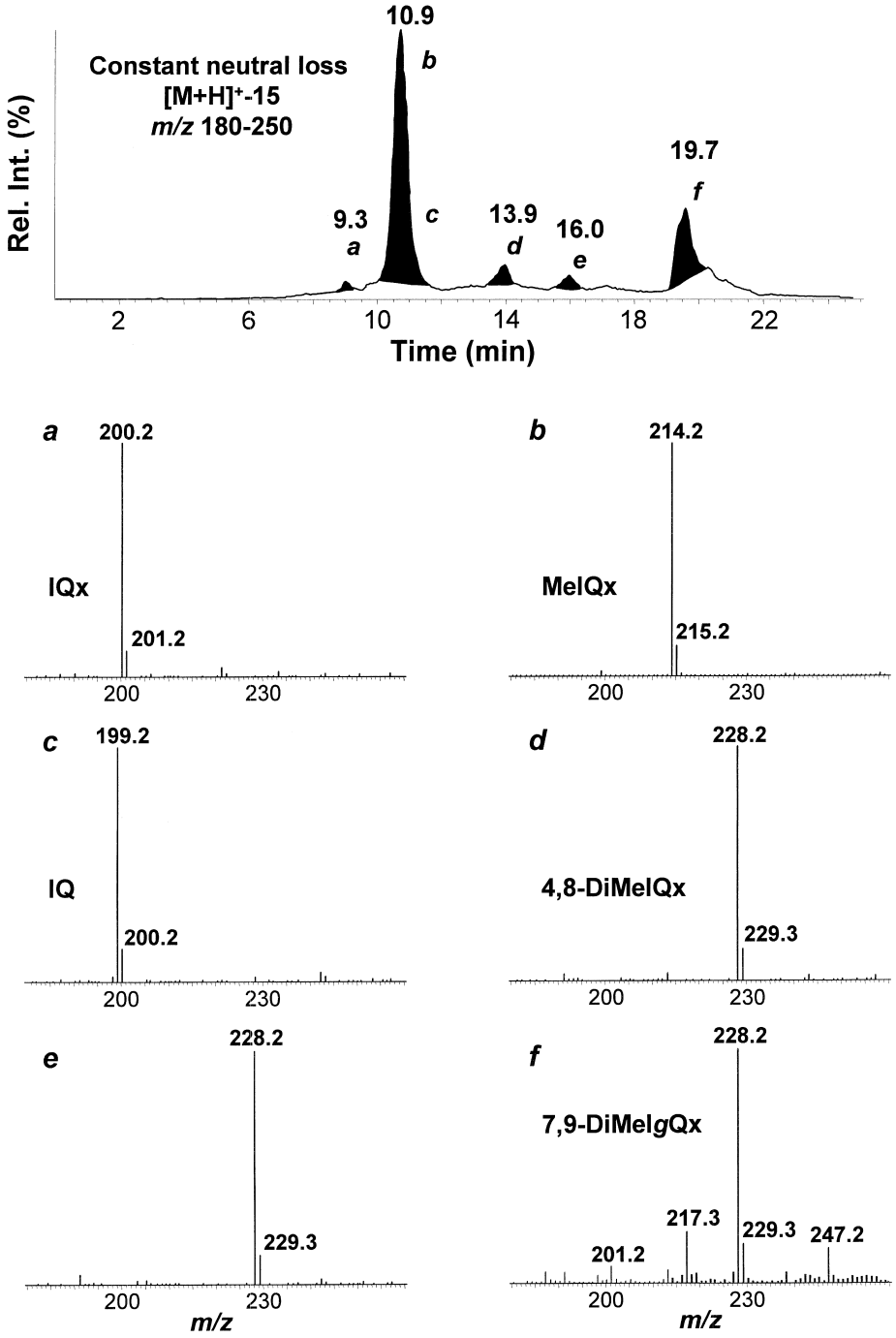

Fig. 5. LC-APCIMS–MS analysis of a meat extract sample contaminated with high levels of total HAAs (>10 μg/kg) monitored in the constant neutral loss acquisition mode of 15 by scanning the first quadrupole in a m/z 180–250 range with a gas pressure of 2.2 mTorr, an in-source collision and a collision energy set at 13 and 48 eV, respectively. (a)–(f) represents the mass spectra of the major peaks obtained.

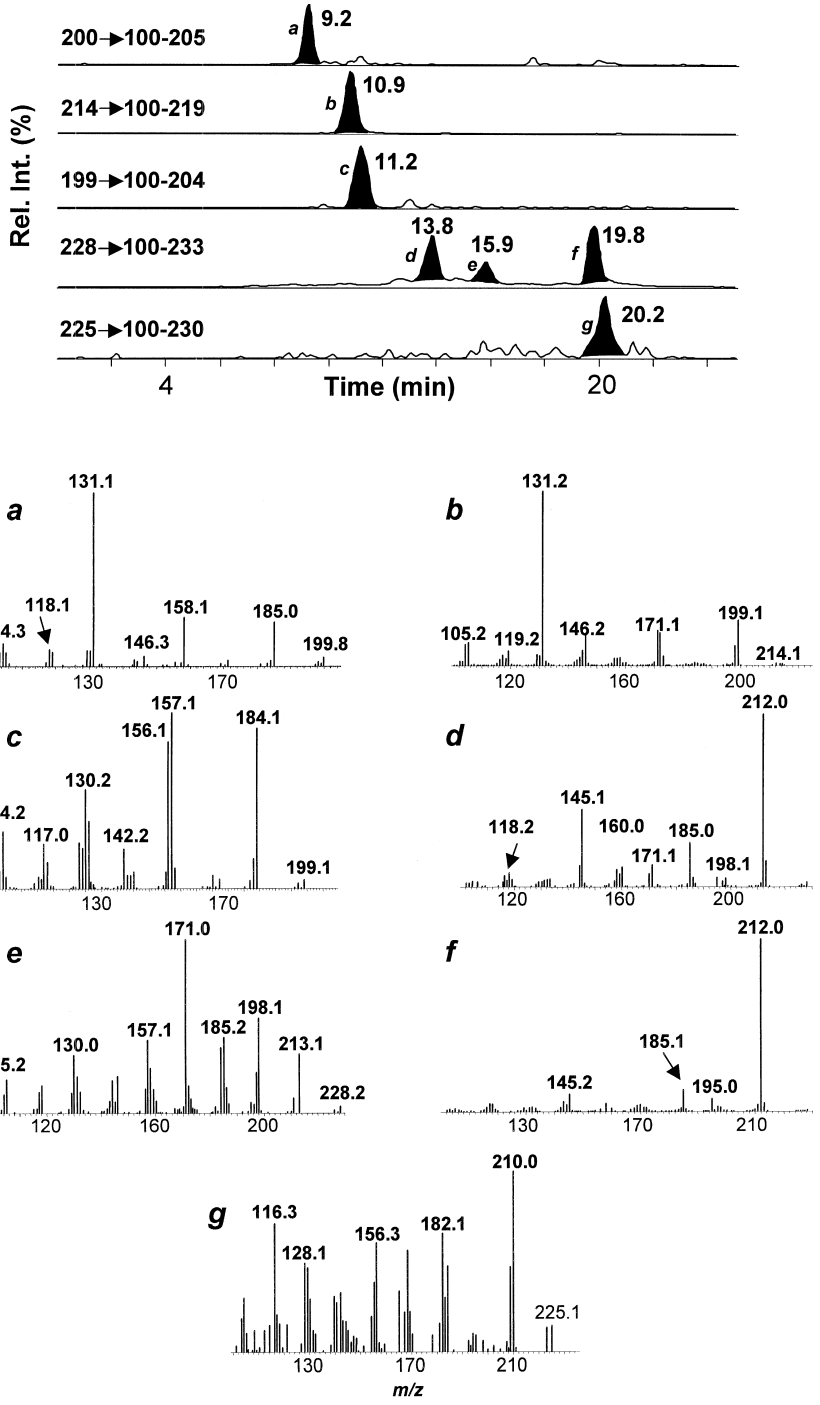

Fig. 6. LC-APCIMS–MS of a meat extract sample contaminated with high levels of total HAAs (>10 μg/kg) analysed in the product ion acquisition mode by selecting m/z 199, 200, 214, 225 and 228 in the first quadrupole with a gas pressure of 2.2 mTorr, an in-source collision and a collision energy set at 13 and 63 eV, respectively. Their respective mass spectra are depicted in (a)–(g) and correspond to: (a) IQx, (b) MeIQx, (c) IQ, (d) 4,8-DiMeIQx, (e) uncharacterized, (f) 7,9-DiMeI*g*Qx, and (g) PhIP.

peaks were also observed at 9.3, 16.0 and 19.7 min, which suggest that several other HAAs containing an *N*-methylimidazole moiety may be present in this meat extract sample. The peak at retention time 9.3 min displays a precursor ion at *m/z* 200, which is 1 mass unit greater than the protonated molecule of IQ. This chemical may be the quinoxaline analogue of IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoxaline (IQx) [20]. Peaks (e) and (f), which display precursor ions at *m/z* 228 may be isomers of DiMeIQx such as 5,8-DiMeIQx and/or 2-amino-1,7,9-trimethylimidazo[4,5-*g*]quinoxaline (7,9-DiMeI*g*Qx) (MW 227 Da) [21]. In order to better characterize these peaks, we repeated the LC-APCIMS–MS analysis of this meat sample in the product ion acquisition mode after selection of *m/z* 199, 200, 214, 225 and 228 in the first quadrupole and scanning the third one from *m/z* 100 to M+5. Fig. 6 depicts the total ion current observed of each product ion monitored and their corresponding mass spectra. The product ion spectrum of peak (b) (rt 10.9 min) of Fig. 6 is in excellent agreement to that of synthetic MeIQx, while peaks (c), (d) and (g) are in excellent agreement to IQ, 4,8-DiMeIQx (7,8-DiMeIQx is under the limit of detection), and PhIP (which is readily detectable using this acquisition mode). The product ion spectrum of the precursor ion at *m/z* 200 (Fig. 6a) shows an intense fragment ion at *m/z* 131, indicating a common fragment ion with quinoxaline-derived HAAs such as MeIQx and 7,8-DiMeIQx and further suggest that this peak may be IQx. The identity of this peak as IQx was corroborated by comparison of the product ion spectrum to that of synthetic IQx and are in excellent agreement (data not shown). Moreover, synthetic IQx was found to elute at the identical retention time of peak a. Based upon the peak area integrated, approximately 3500 ng/kg of IQx was found in this meat extract sample. Similar analyses were performed with 7,9-DiMeI*g*Qx, confirming the presence of this other rarely found HAA [21]. The amount of 7,9-DiMeI*g*Qx was estimated at 22 550 ng/kg in this meat extract. Tada and Wakabayashi have detected this novel HAA in different cooked meats at levels up to 4200 ng/kg (personal communication). The identity of peak (e) (rt 15.9 min, Fig. 6) remains unknown, major fragments were observed at *m/z* 213, 198, and 171 and are indicative of loss of two methyl groups and HCN, which are fragment ions in common to these other HAAs.

4. Conclusions

The use of HPLC-APCIMS–MS permits accurate quantitation and detection of HAAs, which are suspected human carcinogens, at trace levels in daily food products. Our intralaboratory comparison demonstrates that the tandem solid-phase extraction procedure used to isolate HAAs is highly reproducible. The utilisation of LC-APCIMS–MS analysis is very sensitive, with detection and quantification limits of HAAs at 15 and 45 ng/kg, respectively. Moreover, the product ion mass spectra of the protonated molecules allowed us to unambiguously confirm the identities of these HAAs. We have also shown that the constant neutral loss scanning acquisition mode can be used to screen for other HAAs of comparable chemical structure which undergo similar fragmentations. This feature enabled us to detect IQx and 7,9-DiMeI*g*Qx in meat extracts and revealed the possible presence of other structurally related HAAs, whose chemical identities' remain to be elucidated.

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