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Determination of heterocyclic amines by liquid chromatography–quadrupole time-of-flight mass spectrometry

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Abstract

This paper discusses the applicability of coupling liquid chromatography (LC) to mass spectrometry (MS) using a time-of-flight (TOF) mass analyzer for the analysis of heterocyclic amines (HAs). Accurate mass measurement (<2 mDa) with both MS–MS and in-source CID MS–MS was carried out to confirm the elemental composition of some fragments previously reported. Some isobaric assignments (fragments containing N versus CH_2 and NH_3 versus CD_2H) were distinguished by taking advantage of the resolution provided by the TOF mass analyzer. On the other hand, the LC–MS analysis of HAs in MS acquisition mode was also performed. Quality parameters of the method were established. The linearity range extended over three orders of magnitude, limits of detection were in the pg level and good short-term precision values (R.S.D., 1.2–8.0%) were obtained. The LC–ESI-TOF method was applied to the determination of HAs in a lyophilized meat extract and the results obtained were comparable to those given by MS–MS with triple quadrupole and ion trap instruments.

Keyword: Heterocyclic amines

1. Introduction

In recent years, the time-of-flight (TOF) mass analyzer has re-established itself as a mainstream technique in mass spectrometry (MS) [1–3]. TOF has distinct advantages over scanning instruments. These include the detection of a high percentage of ions, fast acquisition rates, relatively high sensitivity, and large mass range. Recent advances in ionization sources such as electrospray (ESI) and matrix-assisted laser desorption ionization (MALDI), along with the development of high speed electronic and computers, have allowed TOF analyzers to develop rapidly in the last decade. Furthermore, the introduction of an ion-mirror (reflectron) that corrects positional and velocity discrepancies in the acceleration region has permitted an increase in resolution. But while sector instruments provide higher resolution, their use has decreased due to their size and cost and the requirement of specialized technicians. Additionally, high accuracy in mass measurements is another important characteristic provided by TOF mass analyzers for both qualitative and quantitative analysis. These instruments allow confirmation of the elemental composition of the ions when performing fragmentation studies and also provide high selectivity in the determination of the compounds in complex matrices. However, TOF analyzers have an instrumental drift as a consequence of temperature fluctuations, which can cause changes in the applied voltage. Thus, in order to improve mass accuracy, corrections via the mass of an internal standard reference compound (lock mass) is needed. In the last few years, a high number of studies concerning the use of TOF for the analysis of a wide variety of compounds have been published. For instance, high molecular weight compounds such as proteins [4–6] and also small organic molecules [7–11] have been analyzed, which demonstrates the high versatility of the TOF instruments.

Triple quadrupole and ion trap mass spectrometers using tandem mass spectrometry (MS–MS) coupled to liquid chromatography (LC) have proved highly successful in the determination of a family of low molecular weight mutagenic compounds named heterocyclic amines (HAs). To date, more than 20 HAs have been isolated from heated proteinaceous foodstuffs, and some of them are suspected to be human

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carcinogens. Consequently, it is important to develop sensitive and selective analytical methodologies to determine the amounts of HAs present in cooked foods at low ppb level, in order to estimate intakes and risks to human health [12–15]. To our knowledge, the TOF analyzer has not been used until now, although high resolution MS with magnetic sector instruments was employed at the beginning of 1980s for the characterization of mutagenic fractions [16–19]. Low resolution mass analyzers have enabled the analysis of HAs in complex samples such as foods and body fluids, providing high selectivity and sensitivity. Moreover, in the last few years, studies on fragmentation pathways of HAs have been performed [12,20–23], although the ions could only be tentatively assigned due to the low resolution and poor accuracy in mass measurements (>0.1 Da) provided by quadrupole and ion trap analyzers. At present, TOF instruments can solve such problems because they can distinguish between ions that have the same nominal mass but different elemental composition.

In this work, the high mass accuracy measurements provided by the Q-TOF instrument in combination with MS–MS and in-source collision induced dissociation (CID)-MS–MS experiments have been used to establish the fragmentation pathways of HAs. Additionally, the applicability of a LC–ESI-TOF method for the analysis of HAs was studied. Quality parameters were established, and then quantification of HAs in a lyophilized meat extract was carried out to compare the feasibility of the TOF-MS acquisition with respect to the tandem MS mode used previously with other mass analyzers [15].

2. Experimental

2.1. Chemicals

The HAs studied, which are shown in Fig. 1, were 2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine (DMIP), 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3-trideuteromethylimidazo[4,5-*f*]quinoline (D_3-IQ), [2-^{13}C]-2-amino-3-methylimidazo[4,5-*f*]quinoline (2-^{13}C-IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-8-methyl-3-trideuteromethylimidazo[4,5-*f*]quinoxaline (D_3-MeIQx), [2-^{13}C]-2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (2-^{13}C-MeIQx), 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), 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), 2-amino-3,4,7,8-tetramethylimidazo[4,5-*f*]quinoxaline (TriMeIQx), 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC), and 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC), obtained from Toronto Research Chemicals Inc. (Toronto, Ont., Canada), and 1-methyl-9*H*-pyrido[4,3-*b*]indole (Harman) and 9*H*-pyrido[4,3-*b*]indole (Norharman), from Sigma (St. Louis, MO, USA). HAs methanolic stock standard solutions of 80 μg g^{-1} were prepared and used for further dilutions. Diethylamine was purchased from Sigma (St. Louis, MO, USA) and heptylviologen from Aldrich (Milwaukee, WI, USA). These two reagents were dissolved in methanol and water, respectively.

For fragmentation studies, individual standard solutions (10 μg g^{-1}) were used for the infusion of each analyte into the MS system, and they were dissolved in methanol/formic acid. For quantitative analysis, a D_3-IQ methanolic solution as lock mass (0.5 μg g^{-1}) was post-column infused at 1–5 μl min^{-1}.

Standard mixtures of all amines with TriMeIQx as internal standard at different concentration levels were prepared by weight to establish the linearity range and the calibration curves. Standards and samples were passed through a 0.45 μm filter before injection into the LC–MS system.

HPLC grade ethyl acetate and gradient grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Analytical grade ammonia solution (25%) and formic acid (98%) were obtained from Merck (Darmstadt, Germany) and ammonium acetate from Fluka (Buchs, Switzerland). Water was purified in an Elix-Milli Q system (Millipore Co., Bedford, MA, USA). Diatomaceous earth extraction cartridges (Extrelut-20) and refill materials were provided by Merck (Darmstadt, Germany); PRS sodium form (500 mg) and encapped C_{18} (100 and 500 mg) Bond Elut cartridges, coupling pieces and stopcocks were from Varian (Harbor City, USA). Nitrogen (N1) was supplied by Air Liquide (Madrid, Spain); and argon was of 99.995% purity and it was purchased from Carburos Metálicos (Madrid, Spain).

2.2. Instrumentation and MS conditions

A quaternary pump system from Waters (Milford, MA, USA) model Alliance 2690 was used to carry out the separation of HAs by reversed phase liquid chromatography using a Symmetry® C8 column (Waters, Milford, USA), with a particle size of 5 μm, 2.1 mm i.d. and 150 mm in length. Optimum separation was achieved with a binary mobile phase at a flow-rate of 300 μl min^{-1}. Solvent A: acetonitrile; solvent B: 30 mM acetic acid/ammonium acetate buffer at pH 4.5. The gradient elution program was: 0–0.5 min, 5% A, 0.5–15 min, 5–20% A; 15–18 min, 20–60% A; 18–24 min, 60% A; 24–27 min, return to initial conditions; 8 min post-run delay. The sample volume injected was 5 μl.

The LC system was coupled to a quadrupole time-of-flight (Q-TOF-2™) mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray ESI source working in positive mode. Data acquisition was carried out by MassLynx v3.5 software. The infusion of analytes and lock mass solutions was performed by a syringe pump (Harvard Apparatus Inc., Massachusetts, MS, USA). Optimal ionization source working parameters were: capillary voltage, 2.5 kV; cone voltage,

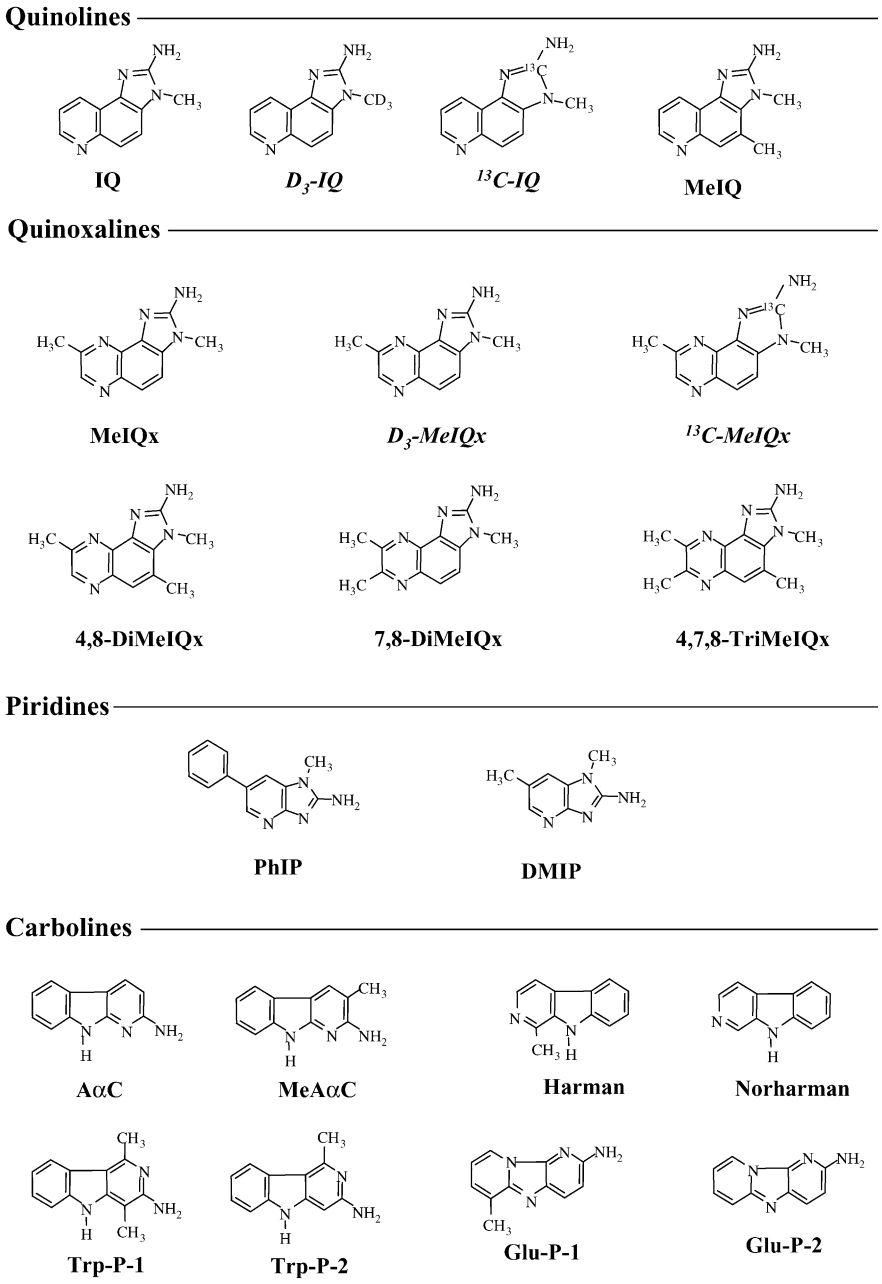

Fig. 1. Structures and abbreviated names of the HAs used in this study.

40 V; source temperature, 135 °C; desolvation temperature, 390 °C; cone gas flow-rate, 50 a.u.; and desolvation gas flow-rate, 300 a.u.

To increase mass accuracy, the Q-TOF instrument was calibrated using six HAs, prepared in methanol–30 mM acetic acid/ammonium acetate pH 4.5, 50:50, at a level of 10 μg g^{-1}. The calibration standard mixture contained DMIP (*m*/*z*: 163.0983), AαC (*m*/*z*: 184.0874), Trp-P-2 (*m*/*z*: 198.1031), MeIQ (*m*/*z*: 213.1140), 7,8-DiMeIQx (*m*/*z*: 228.1249), and TriMeIQx (*m*/*z*: 242.1405). Moreover, in order to extend the calibration mass range, diethylamine (*m*/*z*: 74.0969) and heptylviologen (*m*/*z*: 354.3035) were added to the calibration standard mixture. The infusion of this solution was performed every day for an accurate calibration of the TOF analyzer.

Continuum mode TOF mass spectra were recorded using single MS, MS–MS and in-source CID-MS–MS modes from *m*/*z* 100 to 250 with a duty cycle of 1.0 s. The acquired data were converted to centroid (80% of the top peak area) to implement lock mass adjustment and to generate the accurate mass spectra. D_3-IQ (internal standard reference mass) was post-column infused by means of a tee piece to perform the lock mass correction in the single MS mode acquisition. The relative abundance of the $[M + H]^+$ of D_3-IQ (*m*/*z* 202.1169)

must be ∼10%. A window width of ±0.05 Da was selected to achieve enough selectivity and to decrease the noise (i.e. better limits of detection). For MS–MS mode, the precursor ion (i.e., the protonated molecular ion $[M + H]^+$) was selected in the quadrupole analyzer (*m*/*z* window of 1.0) and fragmented in the hexapole cell by applying collision cell offset voltages between 25 and 35 V, depending on the compound, and using argon as collision gas at a pressure of 15 psi. Product ion spectra were recorded in the TOF analyzer from *m*/*z* 100 to 250. Afterwards, the spectra were lock mass corrected using the *m*/*z* value of the precursor ion. In order to perform in-source CID prior to MS–MS analysis, a cone voltage between 80 and 90 V in the ionization source was used.

2.3. Sample treatment

A lyophilized meat extract containing HAs at a level of 35–60 ng g^{-1} extract was analyzed [24]. To extract the analytes from this sample, a previously described clean-up method [25] was used. Briefly, 1 g beef extract sample was homogenized in 12 ml 1 M NaOH and mixed with diatomaceous earth. The amines were eluted from the extraction column, containing the diatomaceous earth mixture, directly to a propanesulfonic acid (PRS) cartridge using 75 ml ethyl acetate. The cartridge was dried and rinsed with 6 ml 0.01 M HCl, 15 ml MeOH–0.1 M HCl (6:4) and 2 ml of water, which contained the *less-polar* compounds (Trp-P-2, Trp-P-1, AαC, MeAαC and PhIP). After adding 25 ml of water, the combined acidic washing solutions was neutralized with 500 μl of ammonia. The resulting solution was passed through a C_{18} (500 mg) cartridge and the amines retained were eluted, using 1.4 ml of methanol–ammonia solution (9:1, v/v) providing the named *less-polar extract*. The PRS column was then coupled to a C_{18} (100 mg) cartridge, and after that the most polar amines (DMIP, IQ, MeIQ, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, Glu-P-1 and Glu-P-2) were eluted from the cationic exchanger with 20 ml of 0.5 M ammonium acetate solution at pH 8.5. The adsorbed HAs were then eluted from C_{18}, using 0.8 ml of methanol–ammonia solution (9:1, v/v) providing the named *polar extract*. The two extracts were evaporated to dryness under a stream of nitrogen and the analytes were redissolved in 300 μl of a solution containing the internal standard in methanol-ammonium acetate 30 mM at pH 4.5 (1:1, v/v). Finally, the two extracts were separately injected in the LC–MS system.

A Supelco Visiprep and a Visidry SPE vacuum manifold (Supelco, Gland, Switzerland) were used for manipulations with solid-phase extraction cartridges and solvent evaporation, respectively.

3. Results and discussion

Electrospray working parameters were optimized by infusion of HA solutions, and the optimal values achieved are given in Section 2. Due to the high water content (>70%) for the HAs eluted during the first 15 min of the chromatogram, working conditions had to be energetic enough to favor ionic evaporation. For instance, source and desolvation temperatures that provided a maximum response were 135 and 390 °C, respectively. Due to the high efficiency of the ionic evaporation provided by the Z-spray source, post-column addition of acetonitrile was not necessary [15] to increase the response of the analytes.

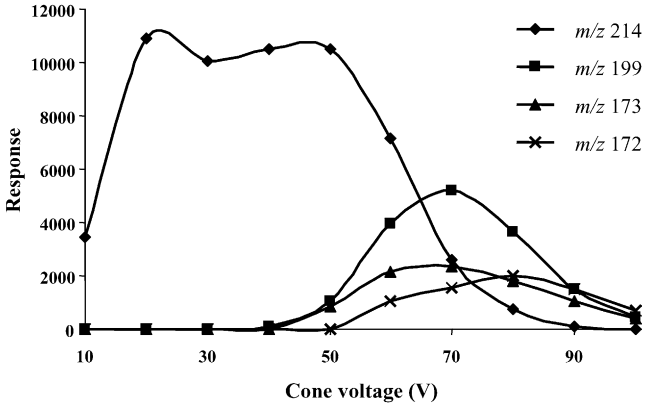

Fig. 2. In-source MS fragmentation curves of MeIQx.

To study in-source fragmentation, cone voltage was varied from 10 to 100 V and curves of ion intensity versus cone voltage were obtained. As an example, Fig. 2 shows the in-source fragmentation curve for $[M + H]^+$ and the three most abundant fragments (*m*/*z* 199, 173, 172) for MeIQx. For all HAs similar behavior was observed and in order to prevent the $[M + H]^+$ fragmentation and to maximize its response a cone voltage of 40 V was selected for further experiments. Under these experimental conditions, single MS spectra of HAs obtained with the Q-TOF instrument agreed with those previously reported using quadrupole and ion trap mass analyzers [15]. For all the analytes, the base peak was the $[M + H]^+$ and no further fragmentation was observed.

The Q-TOF instrument enabled data acquisition with high mass accuracy (<2 mDa) in both V-Optics™ and W-Optics™ configurations. In this last-mentioned case, an additional ion-mirror in the TOF analyzer was activated and the unique flight path of the ions is effectively doubled, producing a two-fold improvement in resolution. As an example, for the $[M + H]^+$ peak of MeIQx, W-mode provided ∼10,800 full width at half maximum (FWHM) height resolution, while V-mode offered ∼5800 FHWM, although with two-fold greater sensitivity. Consequently, the W-mode was chosen to perform the HAs fragmentation studies because of the higher resolution obtained, while for quantitation purposes the V configuration was selected in order to achieve better sensitivity and lower limits of detection.

3.1. Fragmentation pathways of the HAs

The MS–MS spectra of HAs was previously studied using triple quadrupole [12,20,21] and ion trap mass analyzers

[23]. However, due to the low resolution and the poor mass accuracy of these instruments, some ions were explained by different elemental compositions. In this work, the MS–MS spectra of HAs using the Q-TOF instrument were studied to obtain a more accurate assignment. First, argon pressure and collision energies for all analytes were optimized in order to obtain both the maximum signal for product ions and at least ~10% for the parent ion (optimal values are given in Section 2). In these conditions, spectra for all the compounds were obtained and lock mass corrected using the exact mass of the corresponding precursor ion, $[M + H]^+$. Next, the elemental composition of product ions was assigned. In general, the MS–MS spectra of HAs were similar to those obtained previously in an ion trap instrument [23] in terms of fragmentation patterns. For most of these assignments the calculated mass accuracy was <1.9 mDa. Nevertheless, some differences were observed in both relative abundance of ions and fragmentation patterns. This may be due to the fact that multiple collisions occur along the hexapole cell in the Q-TOF while in the ion trap the fragment ions are stabilized just after their generation, avoiding subsequent fragmentations. As an example, Fig. 3 shows the MS–MS spectrum obtained with the Q-TOF instrument for MeIQx, where the assignments with their corresponding mass accuracies are also indicated. Due to the resolution provided by the TOF analyzer, two fragment ions of *m*/*z* 172 can be distinguished and assigned, although the resolution only allowed the *m*/*z* separation at 70% valley, as can be observed in Fig. 3. The fragment at *m*/*z* 172.0860 can be explained by the direct loss of $^{\bullet}CN_2H_2$ as Toribio et al. [23] indicated, while the fragment at *m*/*z* 172.0737 corresponded to the consecutive loss of $^{\bullet}CH_3$ and $^{\bullet}HCN$, which agreed with the MS^3 spectra previously obtained with an ion trap [23]. Similarly, the presence of the ion of *m*/*z* 131.0497 can be explained as the consecutive loss of $^{\bullet}CH_3$ and $^{\bullet}C_3N_2H_4$, by means of the breaking of the pyrazine ring. This fragmentation also occurred for the rest of quinoxalines (Fig. 1).

For the MS–MS spectra of some carbolines (AαC, MeAαC, Trp-P-2, Trp-P-1, Glu-P-2, Glu-P-1), several fragment ions showed higher relative abundances in the Q-TOF instrument than in the ion trap analyzer [23]. As an example, for Trp-P-2 (Fig. 4) the high intensity of the product ion at *m*/*z* 154.0670 can be explained by two simultaneous fragmentation processes, the direct loss of $^{\bullet}CN_2H_4$ and the consecutive loss of $^{\bullet}NH_3$ and $^{\bullet}HCN$ from the ion $[M + H]^+$. Unfortunately, for both cases the elemental composition was the same, so their *m*/*z* values are identical and, consequently, only one peak was observed with a total abundance coming from both contributions. Moreover, adducts at *m*/*z* higher than the precursor ions were only observed in the MS–MS spectra obtained with the ion trap (Fig. 4). As previously explained [23], this is due to ion–molecule reactions inside the ion trap.

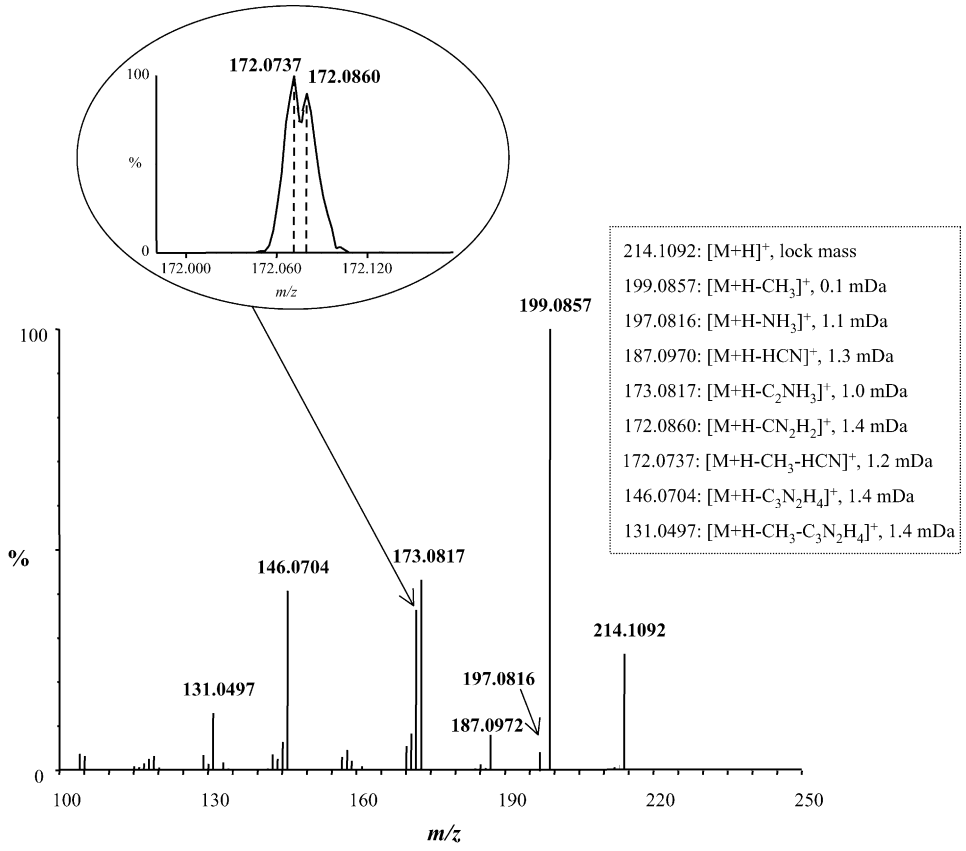

Fig. 3. MS–MS spectra of MeIQx obtained with a Q-TOF instrument, showing the assignment of each fragment with its corresponding mass accuracy.

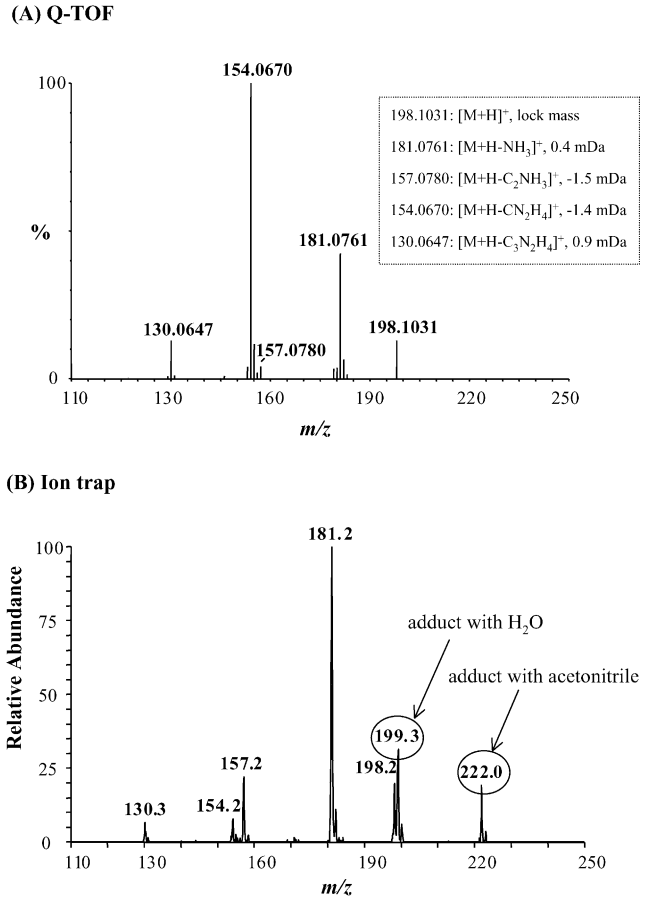

Fig. 4. Comparison of the MS–MS spectra of Trp-P-2 obtained with (A) a Q-TOF and (B) an ion trap instrument.

The MS–MS spectra of IQ and MeIQx showed the loss of NH_3, while, for the deuterated compounds (D_3-IQ and D_3-MeIQx), Toribio et al. [23] have suggested the possible loss of $^{\bullet}CD_2H$ in addition to the loss of NH_3. However, the MS–MS spectra obtained with the Q-TOF instrument showed only one peak with an *m/z* corresponding to the loss of $^{\bullet}CD_2H$ with good accuracy (1.7 mDa, see Table 1). But the loss of NH_3 should not be discarded since it was observed in IQ, MeIQx and ^{13}C labeled compounds although at low relative abundance (∼5%). This low abundance and the high resolution required to separate these ions (∼20,000) prevented their separation in the Q-TOF instrument. These ions could only be differentiated using high resolution MS analyzers such as magnetic sectors or Fourier transform ion cyclotron (FT-ICR) instruments.

In order to study higher order spectra of some of the HAs, in-source CID fragmentation was combined with MS–MS analysis in the Q-TOF instrument. For this purpose, in-source fragmentation curves of IQ and MeIQx and their labeled corresponding compounds were obtained. The collision cell offset voltage was maintained at the minimum value (10 V) in order to prevent fragmentation into the collision cell. The Z-spray source cone voltage was ranged from 10 to 100 V, monitoring in the TOF analyzer the ions formed. As can be observed in Fig. 2, at ∼80 V, several fragment ions of MeIQx were obtained in the ESI source, which agreed with those obtained in the MS–MS spectra using the Q-TOF instrument (Fig. 3). The isolation of these fragments by the quadrupole and their fragmentation in the collision cell allowed us to obtain in-source CID-MS–MS spectra. After lock mass correction of the spectra using the exact mass of the precursor ion, the elemental composition of the fragment ions was assigned. The in-source CID MS–MS spectra are similar to those obtained previously in MS^3 with an ion trap instrument [23], and the mass accuracy obtained was <2.0 mDa. By way of example, Fig. 5 shows the product ions of the ion $[M + H–CH_3]^+$ of IQ, and the assignments with the corresponding mass accuracies are also indicated. Comparing with the MS^3 spectra provided by the ion trap [23], more fragmentation is seen to have occurred with the Q-TOF instrument. For instance, ions at *m/z* 142 and 130 assigned to the losses of $^{\bullet}CN_2H_2$ and $^{\bullet}C_2N_2H_2$, respectively, were more abundant. Moreover, the high mass accuracy obtained with the TOF mass analyzer allowed confirmation of some of the alternatives in the fragmentation pathways previously proposed [23], and Table 1 shows the accuracy in mass measurement for these cases. For

Table 1
Confirmation of the elemental composition of several fragments of HAs, in the MS–MS and in-source CID MS–MS spectra

MS–MS spectra							
HAs	Q-TOF				Ion trap [23]		
	Measured *m/z*	Proposed fragment	Exact mass (Da)	Accuracy (mDa)	Proposed fragment	Exact mass (Da)	Accuracy (mDa)
D_3-IQ	185.0793	$[M + H–CD_2H]^+$	185.0810	1.7	$[M + H–NH_3]^+$	185.0903	11.0
D_3-MeIQx	200.0908	$[M + H–CD_2H]^+$	200.0919	1.1	$[M + H–NH_3]^+$	200.1012	10.4
	In-source CID MS–MS spectra				MS^3 spectra		
	Q-TOF				Ion trap [23]		
	Measured *m/z*	Proposed fragment	Exact mass (Da)	Accuracy (mDa)	Proposed fragment	Exact mass (Da)	Accuracy (mDa)
D_3-IQ	158.0700	$[M + H–CD_2H–HCN]^+$	158.0701	0.1	$[M + H–NH_3–HCN]^+$	158.0794	9.4
	157.0642	$[M + H–CD_2H–DCN]^+$	157.0639	−0.3	$[M + H–NH_3–DCN]^+$	157.0732	9.0
					$[M + H–CD_2H–HCN–H]^+$	157.0623	−1.9
^{13}C-IQ	157.0631	$[M + H–CH_3–^{13}CHN]^+$	157.0640	0.9	$[M + H–CH_3–HCN–H]^+$	157.0595	3.6

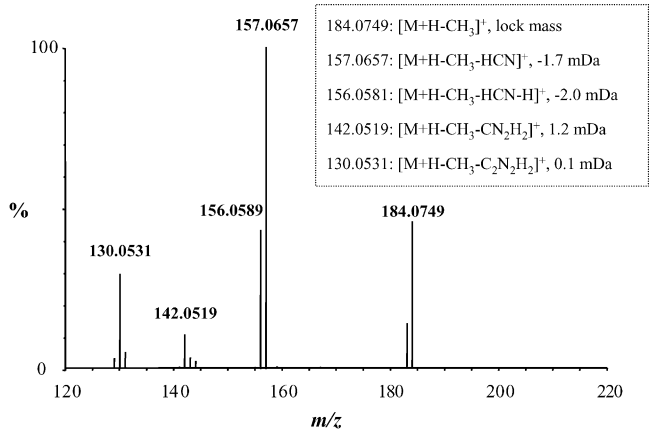

Fig. 5. In-source CID-MS–MS spectra of the ion *m/z* 184, corresponding to the $^{\bullet}CH_3$ loss of the $[M + H]^+$ of IQ, obtained with the Q-TOF instrument.

instance, the in-source CID-MS–MS spectra of the ion of *m/z* 185 $[M + H–CD_2H]^+$ of D_3-IQ gave the product ions at *m/z* 158.0700 and 157.0642, which corresponded to the losses of $^{\bullet}$HCN and $^{\bullet}$DCN (0.1 and −0.3 mDa of mass accuracy, respectively). Likewise, another confirmed fragment of ^{13}C-IQ was the product ion at *m/z* 157.0631, corresponding to $[M + H–CH_3–^{13}CHN]^+$ instead of $[M + H–CH_3–HCN–H]^+$, because of the higher accuracy achieved, 2 ppm (−0.3 mDa), in front of 12 ppm (−1.9 mDa).

3.2. LC–MS analysis of HAs

To attain greater selectivity in an LC–MS method when analyzing complex samples, the use of tandem mass spectrometry is recommended. For HAs, this was demonstrated in a previous study [15], where an evaluation of three LC–MS instruments was performed. Here, the feasibility of an LC–MS(TOF) method that provides selectivity via accurate mass measurements is compared with LC–MS–MS ones with both triple quadrupole and ion trap, that offered selectivity via tandem mass spectrometry.

For the LC–MS(TOF) data acquisition, a lock mass solution of D_3-IQ was continuously post-column infused at 1 μl min^{-1}. The possible existence of ionic suppression induced by the infusion of the lock mass was checked. An HAs standard solution was injected and acquired with and without infusion of the lock mass solution. No decrease in the response of the analyte was observed when this solution was added to the chromatographic eluent, thus demonstrating the absence of ionic suppression.

Once the acquisition had been performed, the LC–MS-(TOF) data were recalculated using the lock mass correction. For quantitation, the *m/z* corresponding to the protonated molecular ion was used with a window width of ±0.05 Da in the V-Optics^{TM} configuration. As an example, Fig. 6 shows the chromatogram of a standard solution of HAs (0.4 μg g^{-1}) acquired at these conditions.

To check methods' performance, quality parameters such as limit of detection (LOD), limit of quantitation (LOQ), lin-

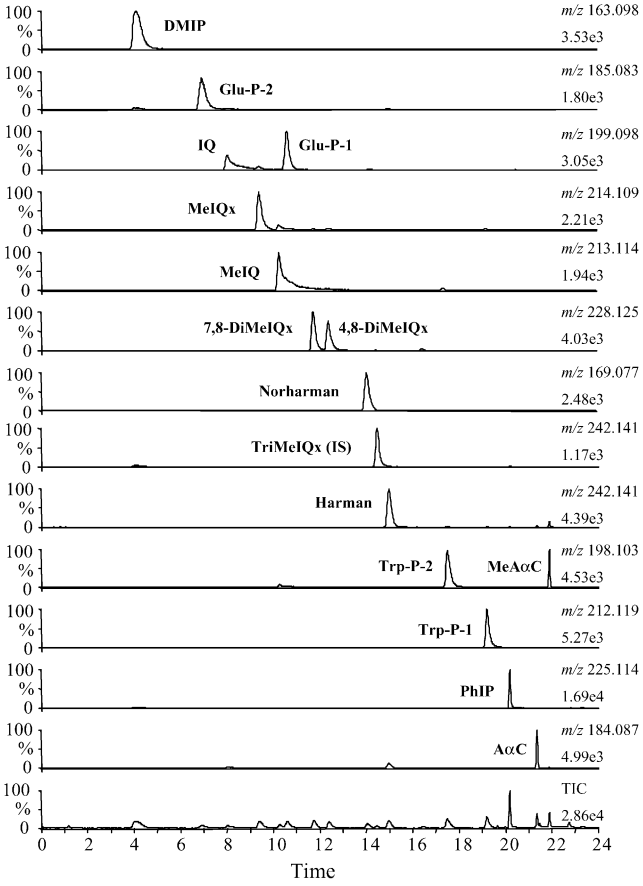

Fig. 6. Extracted ion chromatograms of a standard solution of HAs (0.4 μg g^{-1}) in MS acquisition mode, obtained with a Q-TOF instrument. Working conditions are given in Section 2.

earity range and short-term precision were studied at two concentration levels. LODs were established as the amount of analyte that produces a signal-to-noise ratio of 3:1. They were calculated using standard methanol/buffer solutions at low concentration levels (results in Table 2). They can be compared with those of a previous work [15], showing that LODs with the TOF analyzer are similar to those obtained in SIM mode in a triple quadrupole instrument (∼4 pg) but higher than the LODs in MRM mode (∼1 pg), although with the TOF instrument the high accuracy in mass measurement provides unambiguous identification of the compounds. Moreover, the LODs obtained with the TOF analyzer were similar or slightly lower than those obtained with the ion trap instrument in both full scan (∼20 pg) and product ion scan modes (∼15 pg).

Linearity range was studied from the limit of quantitation (LOQ) to 2.5 μg g^{-1} (LOQ was established as the amount of analyte that produces a signal-to-noise ratio of 10:1). For all analytes, the response was linear up to 1.5 μg g^{-1}, indicating that the linearity range varied over three or four orders of magnitude. It was similar or slightly higher than that obtained using triple quadrupole and ion trap instruments [15]. Calibration curves were established at six concentration

Table 2
Quality parameters

HAs	Standard solution				Meat extract	
	LODs		Repeatability (%R.S.D.) (n = 6)[a]		LODs	
	pg injected	ng g^{-1}	Low concentration	Medium concentration	pg injected	ng g^{-1} extract
DMIP	3	0.7	7.6	7.4	23	1.4
Glu-P-2	16	3.6	6.9	2.4	18	1.1
IQ	5	1.1	7.4	5.6	31	1.9
MeIQx	5	1.1	7.1	5.5	9	0.5
MeIQ	10	2.2	5.8	5.5	31	1.9
Glu-P-1	9	2.0	6.5	6.5	14	0.8
7,8-DiMeIQx	3	0.7	7.8	1.2	7	0.4
4,8-DiMeIQx	4	0.9	7.9	1.6	9	0.5
Norharman	14	3.1	8.0	2.5	30	1.8
Harman	7	1.6	7.1	2.2	19	1.1
Trp-P-2	4	0.9	4.1	2.0	8	0.5
Trp-P-1	2	0.4	7.6	2.3	4	0.2
PhIP	2	0.4	6.1	4.0	10	0.6
AαC	27	6.0	7.3	7.5	49	2.9
MeAαC	5	1.1	7.6	3.9	16	1.0

[a] Low concentration: 0.07 μg g^{-1}; medium concentration: 0.4 μg g^{-1}.

levels using TriMeIQx as internal standard and they were fitted to a lineal function, giving regression coefficients better than 0.999 for all the analytes.

To determine short-term precision, six replicate injections of a HAs standard solution at low (0.07 μg g^{-1}) and medium (0.4 μg g^{-1}) concentration levels were carried out, and external calibration was applied to calculate their concentration. The relative standard deviations (%R.S.D.) were lower than 8% (Table 2), showing that this method provides the values of repeatability required for an accurate analysis of HAs.

3.2.1. Quantitation of HAs in a meat extract by LC–MS(TOF)

The LC–MS method was applied to the determination of HAs in a meat extract, which was used as reference material in a European interlaboratory exercise [26]. In this sample, the noise was high due to the complexity of the matrix; therefore, during analysis, the flow-rate of the lock mass infusion was increased to 5 μl min^{-1} to maintain the relative abundance of the lock mass at a detectable level. To determine the LODs of the method in this sample, a meat extract free of HAs was spiked with very low amounts of analytes (∼3 ng g^{-1} sample) and processed following the clean-up procedure described in Section 2. As shown in Table 2, LODs in meat extract were always higher than those for standard solutions (up to five-fold), due to matrix complexity that affects both noise and ionization efficiency. These values are lower than those obtained in product ion scan (ion trap instrument) and slightly better than those given by SIM (triple quadrupole instrument).

Six individual, fully independent analyses of the meat extract were carried out on three different days. The clean-up procedure is described in Section 2. Quantitation of HAs was performed by the standard addition method, spiking at four concentration levels. As an example, Fig. 7 shows the chromatograms corresponding to the HAs present in the meat extract. The results obtained after the quantitation are given in Table 3. As can be seen, these results are in agreement with those reported by other laboratories which participate

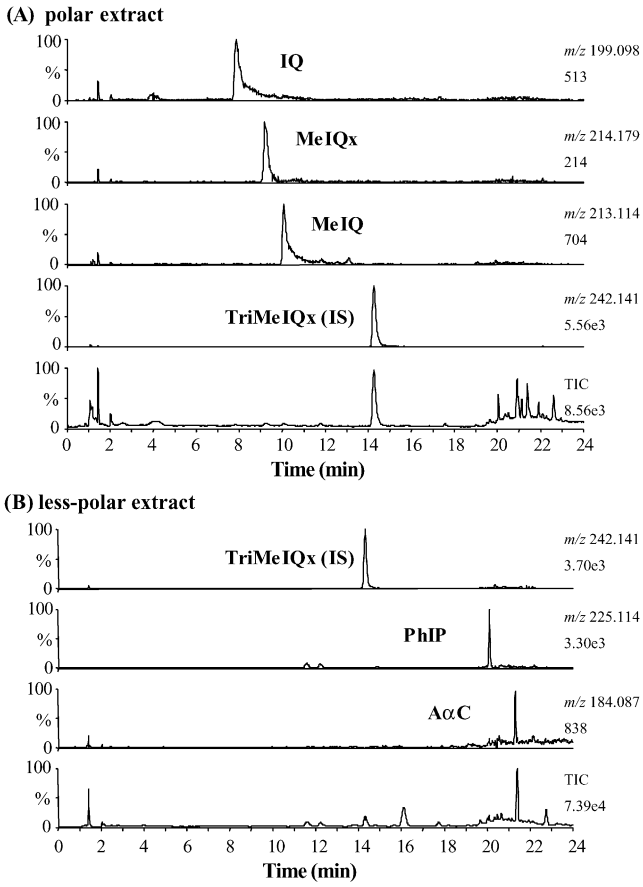

Fig. 7. Extracted ion chromatograms of HAs present in a meat extract in MS acquisition mode, obtained with a Q-TOF instrument. Working conditions are given in Section 2.

Table 3
Results of HAs quantitation in a lyophilized meat extract using LC–MS(TOF) in MS acquisition mode, and comparison with those published previously

HAs	Concentration of HAs (ng g^{-1} meat extract)			
	TOF, MS	Interlaboratory exercise [26]	Triple quadrupole, MRM [15]	Ion trap, MS–MS [15]
IQ	44.9 ± 12.2	39.6 ± 8.9	42.7 ± 3.6	49.4 ± 5.5
MeIQx	45.6 ± 7.3	43.2 ± 11.0	41.5 ± 2.9	46.6 ± 4.7
MeIQ	43.5 ± 9.2	38.1 ± 4.8	42.9 ± 2.7	45.0 ± 5.4
PhIP	32.8 ± 4.5	40.4 ± 4.7	35.2 ± 2.9	40.3 ± 5.3
AαC	39.2 ± 6.7	38.8 ± 8.9	42.8 ± 2.0	37.8 ± 5.1

in the interlaboratory study using different LC–MS methodologies [26]. Moreover, if these values are compared with those obtained using triple quadrupole and ion trap instruments [15], they will be seen as very similar, although with these instruments the use of MS–MS acquisition was necessary in order to achieve higher selectivity and precision values. In conclusion, LC–TOF is a suitable alternative to LC–MS–MS for the identification and quantitation of HAs in complex samples although slightly higher relative standard deviations (∼15–20%) were obtained.

4. Conclusions

Accurate mass measurements were performed in a Q-TOF instrument for qualitative and quantitative analysis of HAs. Most tentative assignments of fragmentation patterns proposed in previous studies [12,23] were confirmed, since the TOF analyzer provided sufficient mass accuracy to enable unambiguous identification. With respect to quantitative analysis in MS mode, good linearity was found over three orders of magnitude. The methodology had low detection limits in both standard solutions and in meat extracts and were similar to data obtained on a triple quadrupole instrument using SIM acquisition mode. Short-term precision at two concentration levels was <8%. The methodology was used for the determination of HAs in a complex sample such as a lyophilized meat extract, obtaining reliable results compared with other mass analyzers. Since both, method development and quantitative procedure, are simpler in TOF instruments than in triple quadrupoles, the use of TOF mass analyzers can be considered a good alternative for the identification and quantitation of HAs in complex samples.

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