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Evaluation of different liquid chromatography–electrospray mass spectrometry systems for the analysis of heterocyclic amines☆

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Abstract

Three liquid chromatography–electrospray ionisation (LC–ESI) MS systems are evaluated for the analysis of heterocyclic amines (HAs). The electrospray sources and analysers (ion trap, single quadrupole and triple quadrupole) have been compared in terms of performance and quality parameters. In all cases, a C_8 reversed-phase column and (acetic acid–ammonium acetate 30 mM pH 4.5)-acetonitrile (ACN) as mobile phase were used. Ionisation source parameters, post-column addition and working conditions for each acquisition mode (full scan, product ion scan, selected ion monitoring, and multiple reaction monitoring) were optimised for each instrument. The MS–MS spectra obtained with the ion trap and the triple quadrupole systems were very similar in both fragment ions and relative abundances, except for carbolines that showed adduct formation in the ion trap. Quality parameters were established and good precision (relative standard deviations (R.S.D.) $< 12\%$) and very low limits of detection were obtained, mainly when using the triple quadrupole (<9 pg injected). The content of HAs in a lyophilised beef extract was determined using the three instruments in order to compare their applicability for routine HAs analysis.

Keywords: Mass spectra; Liquid chromatography–mass spectrometry; Amines, heterocyclic aromatic

1. Introduction

In the last few decades, scientists have discovered that humans are exposed to a range of potentially toxic substances including mutagenic and carcinogenic agents. At present, it is well known that one of these groups is made up of heterocyclic aromatic amines (HAs). These compounds belong to a large group of substances present in grilled and fried meat, poultry, and fish [1–3]. They were discovered as potent bacterial mutagens between 1975 and 1977 by Japanese scientists in heated proteinaceous foodstuffs [4]. To date, more than 20 HAs have been isolated as mutagens, and their structure has been elucidated [5]. Moreover, it is known that under long-term feeding studies HAs induce tumours at multiple sites in rodents, including the colon and mammary gland [6,7]. Finally, HAs are suspected to be human carcinogens. So it is important to quantify the amounts of these mutagens present in a variety of cooked foods in order to estimate intakes and risks to human health.

Because of the relatively low amounts of HAs formed in food matrices, the challenge has been to develop rapid analytical methods that unequivocally identify HAs in these complex matrices at the low ppb level. Usually, a purification step is carried out, followed by a separation technique such as liquid chromatography (LC) [8–15], gas chromatography (GC) [16,17], or capillary electrophoresis (CE) [18,19]. Nevertheless, co-extracted compounds from the food sample matrix frequently appear and can interfere in the analysis of HAs. One of the most important aspects of the determination of HAs is the confirmation of the chromatographic peaks using selective techniques, since numerous co-elutions can occur leading to false peak identification. To solve this problem, the coupling of more selective techniques like mass spectrometry that allows the unambiguous identification of the compounds, can be used. In the last few years, several laboratories have worked with atmospheric pressure ionisation sources for the determination of HAs by liquid chromatography–mass spectrometry (LC–MS), using different analysers. Among them, single and triple quadrupole in selected ion monitoring (SIM)

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acquisition mode have been the most frequently used [20–29]. Nevertheless, in the last few years, the selectivity provided by the triple quadrupole analyser working in multiple reaction monitoring (MRM) mode has allowed limits of detection to be decreased [30–32] in complex samples. Ion trap instruments, which provide MS and MS–MS spectra information for unequivocal identification in real samples when working with full scan and product ion scan modes, have also been used [33–36]. However, no comparison of the results obtained with these analysers has been published. Moreover, several chromatographic separation conditions, different LC columns (C_{18}, C_8 and cyano) of various internal diameters (2.1 and 4 mm), and different mobile phase composition have all been used. It thus becomes difficult to compare the results given in the literature as they have been obtained using different MS analysers, ionisation sources, chromatographic conditions, and also sample matrices.

In this work, we have evaluated three LC–MS systems equipped with an electrospray (ESI) as ionisation source and different analysers, API 150EX (single quadrupole), API 3000 (triple quadrupole) and LCQ (ion trap), for the analysis of 16 HAs. In all cases, the same chromatographic conditions have been used. For each instrument ionisation source parameters and working conditions of each acquisition mode have been optimised, and quality parameters have been established. The performance of each LC–MS system has been evaluated by analysing HAs in a lyophilised beef extract.

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-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (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-dimethylimidazo[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, Canada), and 1-methyl-9*H*-pyrido[4,3-*b*]indole (Harman)

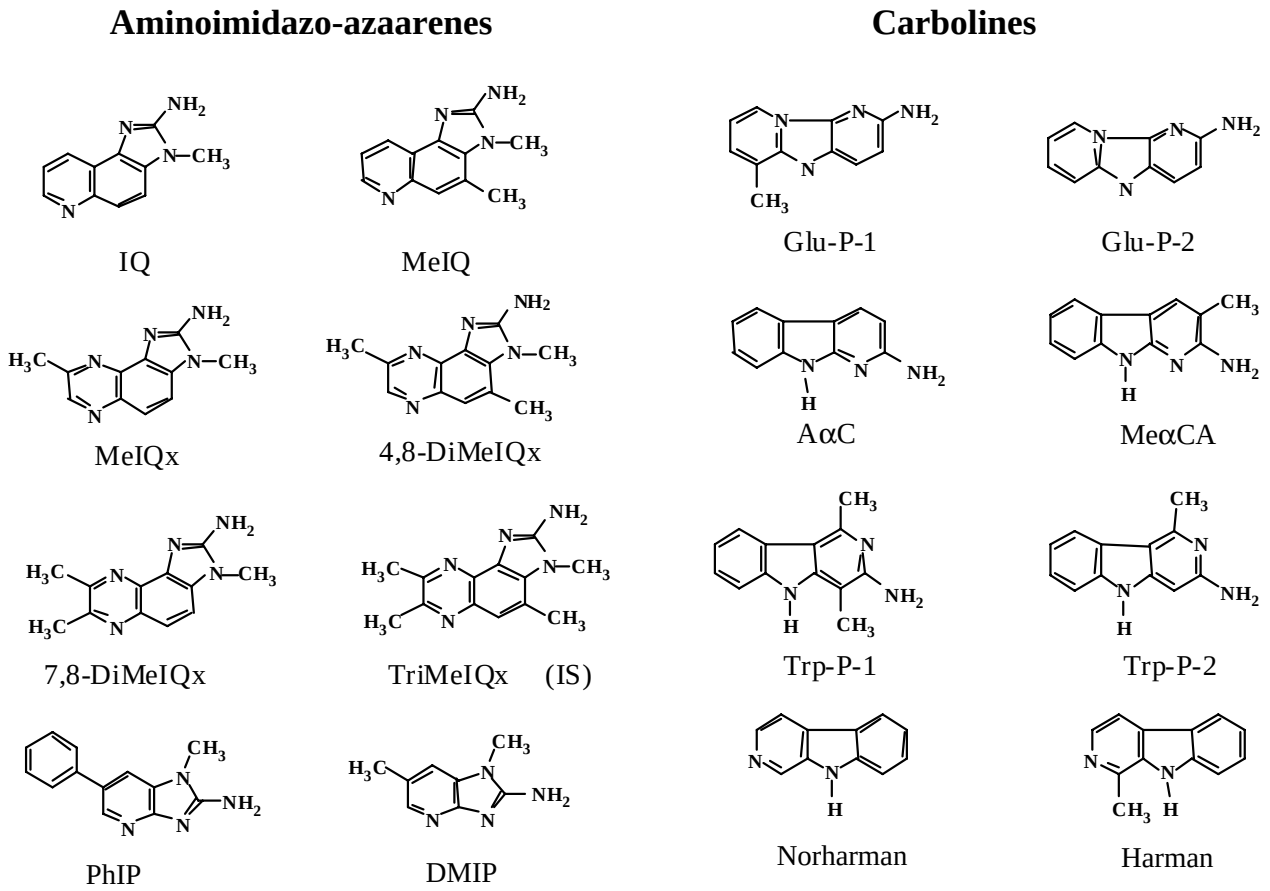

Fig. 1. Structures and abbreviated names of HAs used in this study.

and 9*H*-pyrido[4,3-*b*]indole (Norharman), from Sigma (Missouri, USA).

HPLC-grade ethyl acetate and gradient grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Water was purified in an Elix-Milli Q system (Millipore, Bedford, MA, USA). Analytical grade ammonia solution (25%) and formic acid (98%) were obtained from Merck (Darmstadt, Germany) and ammonium acetate from Fluka (Buchs, Switzerland). Diatomaceous earth extraction cartridges (Extrelut-20) and refill material 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). Helium of high purity and N_2 (N1) were supplied by Air Liquide (Madrid, Spain). HA methanolic stock standard solutions of 80 $\mu g\,g^{-1}$ were prepared and used for further dilutions. Standard mixtures of all amines with TriMeIQx as internal standard at different concentration levels were prepared by weight to establish the range of linearity and for the calibration curves in all systems. Standards and samples were passed through a 0.45 μm filter before injection into the LC–MS system.

2.2. Instrumentation

A quaternary pump system from Waters (Milford, MA, USA) model Alliance 2690 was coupled to a LCQ (Thermo-Finnigan, San Jose, CA, USA) equipped with an electrospray as ionisation source and an ion trap as analyser. Data acquisition was carried out by Xcalibur 1.2 software. Optimal ionisation source working parameters were: spray voltage, 3 kV; sheath gas, 90 arbitrary units (a.u.); auxiliary gas, 60 a.u.; heated capillary temperature, 280 °C; capillary voltage, 31 V; and tube lens offset, 9 V. The data acquisition was performed using: (i) full scan, scanning from *m*/*z* 150 to 250 u in centroid mode, with a maximum injection time of 200 ms, three microscans, and automatic gain control activated; (ii) product ion scan, using as precursor ion the protonated molecular ion $[M+H]^+$ and scanning *m*/*z* from 110 to 250 u; the normalised collision energy (NCE%) applied was between 37 and 45%, the isolation width (IW, *m*/*z*) was 1.5, the activation time (AT) was 30 ms and the activation Q (AQ) was 0.45. When using the product ion scan the chromatogram were segmented into five windows, as shown in Table 1, to reduce the number of precursor ions to analyse in each segment and gain sensitivity.

A binary pump system from Shimadzu (Japan) model SCL-10ADVP was coupled to a PE Sciex API 150 EX (Applied Biosystems, Foster City, CA, USA) equipped with a Turbo Ionspray as ionisation source and a single quadrupole as analyser. Data acquisition was carried out by Analyst 1.1 software. Optimal ionisation source working parameters were: electrospray voltage, 2.5 kV; nebuliser gas, 11 a.u.; curtain gas, 14 a.u.; turbo ionspray gas flow rate, 7 a.u.; turbo ionspray gas temperature, 450 °C; declustering potential, 30 V. Selected ion monitoring was used as data acquisition mode using *m*/*z* corresponding to the protonated molecular ion $[M+H]^+$. The chromatogram was segmented into four windows, and only 3 or 4 *m*/*z* values were monitored simultaneously for each one, as shown in Table 2.

A quaternary pump system from Agilent Technologies (USA) model Series 1100 was coupled to an PE Sciex API 3000 (Applied Biosystems, Foster City, CA, USA) equipped with a Turbo Ionspray as ionisation source and a triple quadrupole as analyser. Data acquisition was carried out by Analyst 1.1 software. As the ionisation source is the same as API 150 EX instrument, the same optimal ionisation source working parameters were obtained. The data acquisition was performed using: (i) selected ion monitoring, at the same conditions as for API 150 EX, and (ii) multiple reaction monitoring, using as precursor ion the protonated molecular ions $[M+H]^+$; the collision cell offset voltage applied was between 35 and 49 V with a collision gas pressure (N_2) of 6 a.u., and the *m*/*z* corresponding to the most abundant product ion was monitored. The chromatogram was also segmented into four windows, and the parameters used are given in Table 2.

2.3. Chromatographic conditions

The chromatographic separation of amines was carried out by reversed-phase liquid chromatography using a Symmetry C_8 column (Waters, Milford, MA, USA), with a particle size of 5 μm, 150 *mm* × 2.1 *mm* I.D. Optimum separation was achieved with a binary mobile phase at a flow rate of 300 μl/min. Solvent A: acetonitrile (ACN); 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.

2.4. Sample treatment

A lyophilised meat extract candidate to laboratory reference material [37] was analysed. It was prepared from a commercial beef extract (Bovril), which was spiked before the lyophilisation with IQ, MeIQ, MeIQx, PhIP and AαC at a level of 35–60 ng/g extract. To extract the analytes from a lyophilised meat extract a previously described clean-up method [11] was used. Briefly, 1 g beef extract sample was homogenised in 12 ml 1 M NaOH and mixed with diatomeaceous earth. The amines were eluted from the extraction column, containing the diatomeaceous earth mixture, directly to a propanesulfonic acid (PRS) cartridge using 75 ml ethyl acetate. It 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. After adding 25 ml of water, the combined acidic washing solution was neutralised with 500 μl of ammonia. It 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)

Table 1
MS–MS parameters used with ion trap LCQ instrument (cf. Section 2)

Segment	Time (min)	Analyte	Precursor ions (*m*/*z*)	NCE (%)	Product ions used for quantification (*m*/*z*)	Production scan range (*m*/*z*)
1	0–10	DMIP	163	41	148	140–170
		Glu-P-2	185	43	158	150–190
		IQ	199	41	184	150–205
2	10–14	MeIQx	214	41	199 173	165–220
		MeIQ	213	40	198	165–220
		Glu-P-1	199	44	184 172	165–210
		7,8-DiMeIQx	228	42	213 187	180–235
		4,8-DiMeIQx	228	41	213 187	180–235
3	14–17	Norharman	169	45	167 142 115	110–175
		TriMeIQx	242	41	227 201	195–250
		Harman	183	44	181 168 115	110–190
4	17–21	Trp-P-2	198	40	222 199 181	175–225
		Trp-P-1	212	40	236 213 195	190–240
		PhIP	225	43	210	200–230
5	21–25	AαC	184	39	208 185 167	165–215
		MeAαC	198	37	222 199 183 181	175–225

Activation $Q = 0.45$; activation *time* = 30 ms; isolation width (*m*/*z*) = 1.5.

Table 2
SIM and MRM parameters used with single (API 150 EX) and triple quadrupole (API 3000) instruments.

Segment	Time (min)	Analyte	SIM (*m*/*z*)	MRM		
				Precursor → product ion (*m*/*z*)	Collision offset voltage (V)	Dwell time[a] (ms)
1	0–9.5	DMIP	163	163 → 148	37	400
		Glu-P-2	185	185 → 158	37	400
		IQ	199	199 → 184	39	400
2	9.5–12	MeIQx	214	214 → 199	38	400
		MeIQ	213	213 → 198	38	400
		Glu-P-1	199	199 → 172	37	400
3	12–17	7,8-DiMeIQx	228	228 → 213	40	300
		4,8-DiMeIQx	228	228 → 213	40	300
		Norharman	169	169 → 115	49	300
		TriMeIQx	242	242 → 227	38	300
		Harman	183	183 → 115	49	300
4	17–25	Trp-P-2	198	198 → 181	35	300
		MeAαC	198	198 → 181	35	300
		Trp-P-1	212	212 → 195	36	300
		PhIP	225	225 → 210	43	300
		AαC	184	184 → 167	38	300

[a] Interchannel time delay: 5 ms.

providing the less-polar extract. The PRS column was then coupled to a C_{18} (100 mg) cartridge, and after that the most polar amines 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 polar extract. The two extracts were evaporated to dryness under a stream of nitrogen and the analytes were redissolved in 100 μl of a solution containing the internal standard TriMeIQx 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 systems.

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

In a preliminary study of the applicability of LC–MS for the analysis of HAs, two API ionisation sources were evaluated using an ion trap instrument. In both cases HAs provide the protonated molecular ion $[M + H]^+$ as base peak. Working under the same chromatographic conditions (Symmetry C8 column, at 300 μl/min), higher sensitivity (5–10 times) was achieved when using electrospray for both standard solutions and meat extract, so this ionisation source was selected for the study.

3.1. Optimisation of MS conditions

3.1.1. Ion trap

To obtain a good separation of the amine compounds, a mobile phase with low content of organic solvent was used during most of the elution time (5–20% in 14.5 min). Under these conditions, ionic evaporation efficiency was poor and low responses were obtained. In order to solve this problem, post-column addition of 0.1% (v/v) of formic acid in acetonitrile was used. Acetonitrile decreases the droplet surface tension in the ESI source and helps ionic evaporation, and the formic acid assures that HAs are fully ionised in the liquid phase. As an example, in Fig. 2A, the chromatograms for MeIQ (2.2 ng injected) with and without post-column addition are given showing an important increase in the signal when it was added. Post-column addition flow rate was varied from 50 to 150 μl/min, and the optimum value was 100 μl/min.

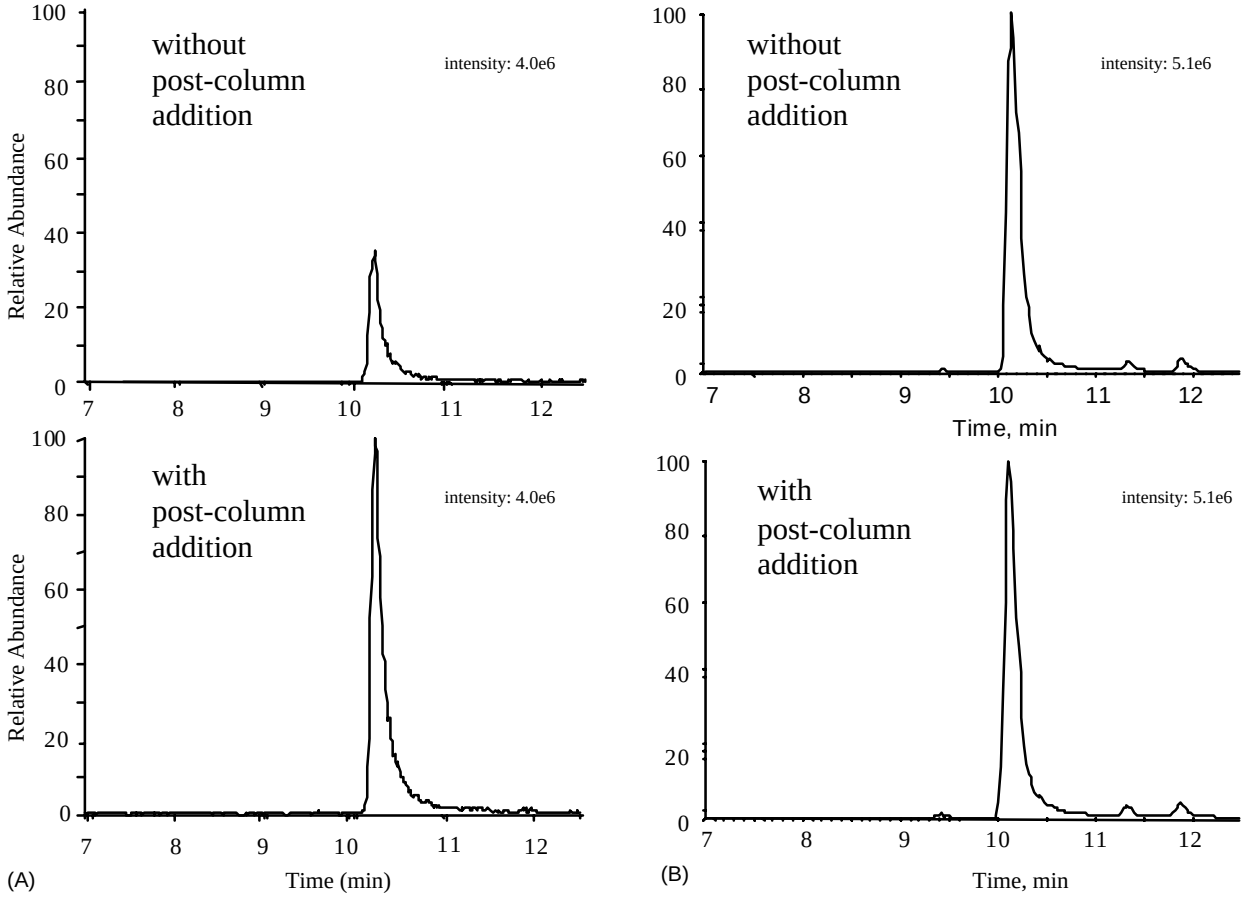

Fig. 2. Effect of post-column addition of 0.1% formic acid in acetonitrile (100 μl/min) in the signal intensity of MeIQ (*m/z* 213). (A) Ion trap LCQ in full scan; (B) triple quadrupole API 3000 in SIM.

Ionisation source working parameters have been optimised by flow injection using two representative HAs which eluted at different mobile phase compositions (IQ and Trp-P-2). Methanolic solutions of these HAs (0.5 μg/g) were injected (5 μl) using a mobile phase of acetonitrile (acetic acid–ammonium acetate 30 mM pH 4.5) (10:90) for IQ, and 50% acetonitrile for Trp-P-2. Spray voltage was studied from 2 to 3.5 kV, and the optimum was 3 kV. At lower values the sample was not effectively ionised and the signal decreased, while at higher values sparkles were produced. To favour ionic evaporation and assist in desolvating ions, sheath gas (or nebuliser gas) and auxiliary gas high flow rates (90 and 50 a.u., respectively) and a capillary temperature of 280 °C were necessary, because the mobile phase flow rate was relatively high (300 μl/min) and the organic modifier content was low.

To optimise the tube lens offset voltage needed to accelerate the ions into the background gas in the capillary-skimmer region, individual methanolic solutions (5 μg/g) were infused at 3 μl/min into the mobile phase at their corresponding elution conditions. The voltage was studied from −20 to 80 V, and the best response was obtained at 9 V. Higher values resulted in a decrease in the signal due to both defocusing and collision-induced dissociation. No significative differences in the optimal source parameters for both amines (IQ and Trp-P-2) were obtained.

For LC–MS and LC–MS–MS analysis, data acquisition was performed in full scan and product ion scan respectively. When using selected ion monitoring or multiple reaction monitoring in the ion trap, no improvement was obtained, so these modes were not employed. Conditions for collision-induced dissociation (CID) of the precursor ion, $[M + H]^+$, have been established and they are given in Table 1. Some ion-molecule reactions were observed into the trap, in agreement with results obtained by Toribio et al. using LC-atmospheric pressure chemical ionisation (APCI) MS–MS [34]. These reactions occurred for carbolines (Trp-P-1, Trp-P-2, AαC, MeAαC, Norharman, Harman, Glu-P-1 and Glu-P-2) between the product ion $[M + H–NH_3]^+$ and a solvent molecule (water and ACN), and adducts of *m/z* higher than parent ion, as shown as an example in Fig. 3A for Trp-P-2, were obtained. The abundance of these product ions is highly dependent on small changes of experimental conditions. So, these ions must be added to the base peak to carry out the quantification of HAs by MS–MS in order to obtain reproducible results.

3.1.2. Quadrupole and triple quadrupole

In contrast with what was observed when using the ionisation source of the ion trap instrument, no post-column addition was needed with the Turbo Ionspray in either single or triple quadrupole instruments. As an example, Fig. 2B shows the chromatogram for MeIQ with and without addition. As can be seen, no significative increase of the signal was observed. This fact can be explained by the higher electrospray ionisation efficiency that provides the Turbo Ionspray compared with the LCQ source.

To optimise the parameters of the source depending on mobile phase, TriMeIQx which eluted at an intermediate mobile phase composition (30% acetonitrile) was used. It was introduced by flow injection in the LC–MS system. As observed in the ion trap instrument, relatively high flow rate values of nebuliser and turbo ionspray gases (11 and 7 a.u., respectively) and also a high temperature of turbo ionspray gas (400 °C) were needed to favour ionic evaporation. Moreover, to prevent contamination of the analyser, a high flow rate value of curtain gas (14 a.u.) was recommended. For the ionspray voltage, the best results were obtained at 2.5 kV, because an increase in the noise and the formation of sparks occurred at higher values.

Optimisation of declustering potential (DP) from 0 to 200 V, was carried out by infusion of individual methanolic solutions of analytes, as for ion trap instrument. For all compounds, the maximum response was obtained at 30 V. At low values, clusters of solvent molecules with the analyte were not fully avoidable, and at high values, in-source fragmentation by collisional-induced dissociation occurred. As a consequence in both cases the signal decreased.

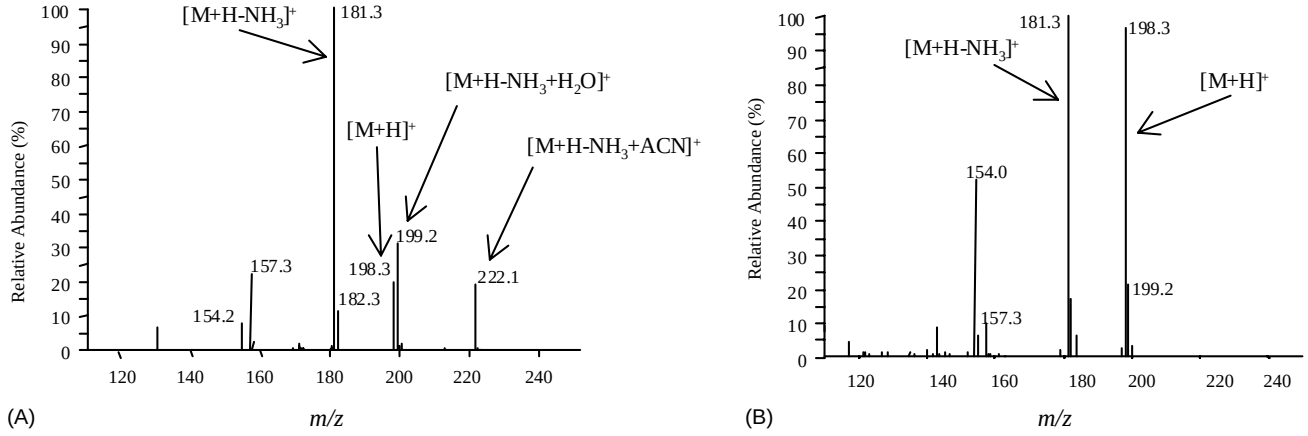

Fig. 3. Comparison of MS–MS spectra of Trp-P-2 in (A) ion trap LCQ, (B) triple quadrupole API 3000.

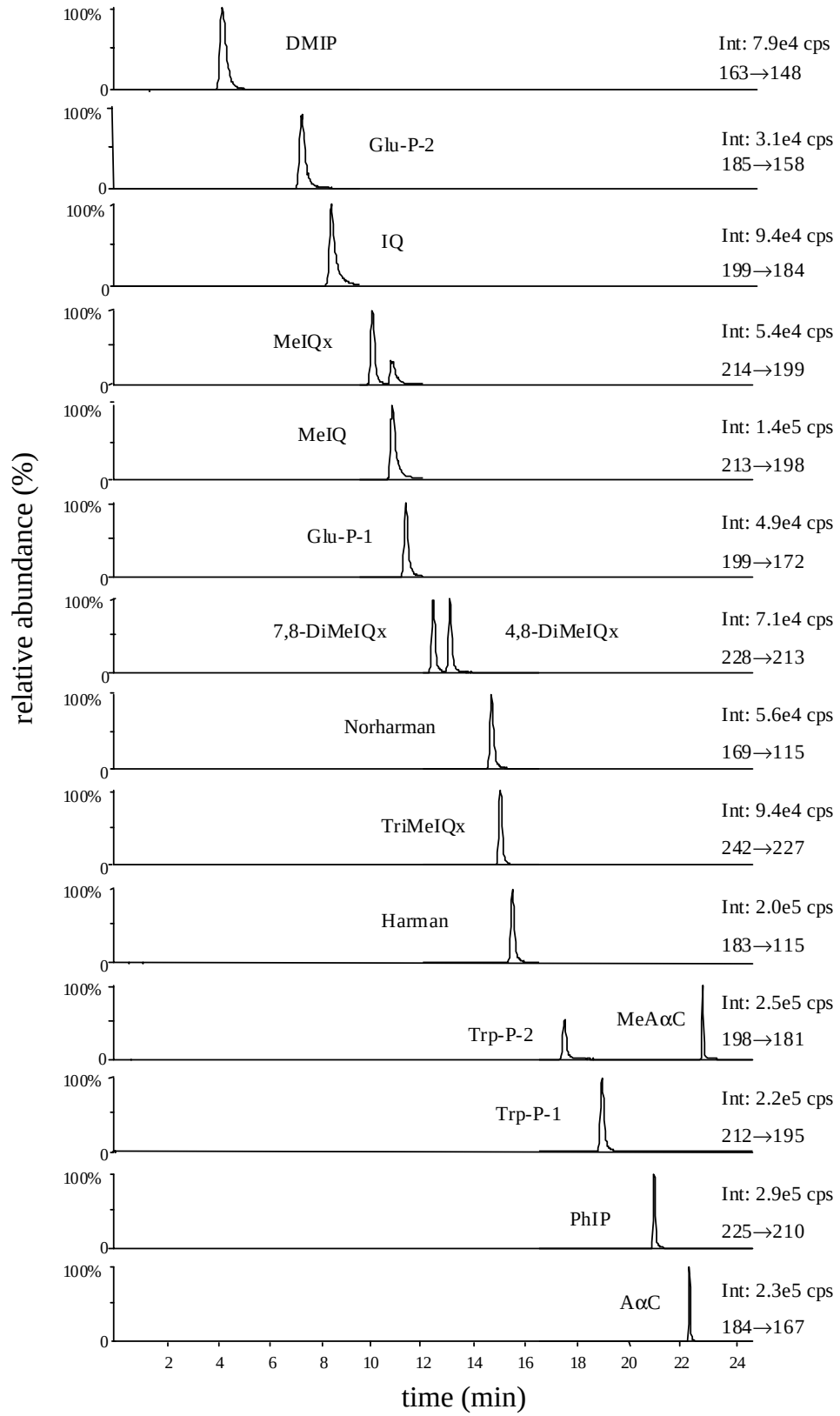

Fig. 4. Chromatogram of a standard solution of 15 HAs (+I.S.) at a concentration of 0.5 μg/g (2.2 ng injected), obtained by triple quadrupole using MRM as acquisition method. Transitions between parent and product ions are indicated in each window.

Data acquisition when working with single quadrupole instrument was performed by SIM, monitoring the *m/z* corresponding to the protonated molecular ion $[M + H]^+$. When working with triple quadrupole, data acquisition was performed in both SIM and MRM modes. SIM conditions were the same as for the single quadrupole, but conditions for MRM were studied. Collision gas pressure for MS–MS experiments was optimised by flow injection of a methanolic solution of IQ, and it was kept at 6 a.u. The collision offset voltage was studied and both the transition precursor to product ion, and the collision offset voltage selected for each amine, are given in Table 2. For carbolines the adducts observed in the ion trap spectra were not present in the MS–MS spectra obtained with the triple quadrupole instrument (Fig. 3B). This fact can be explained by the absence of neutral molecules from the mobile phase inside the collision cell. As an example, Fig. 4 shows a chromatogram corresponding to a standard solution of 0.5 μg/g of 16 HAs (including TriMeIQx as internal standard), obtained by triple quadrupole instrument with MRM acquisition.

3.2. Performance of LC–ESI–MS methods

3.2.1. Quality parameters

To check performance of the methods, quality parameters such as limit of detection, limit of quantitation, repeatability or run-to-run precision, medium term or day-to-day precision, and linearity range were studied. Tables 3 and 4 show the results obtained by LC–MS and LC–MS–MS using the different acquisition modes and the three instruments.

Limits of detection (LODs) and limits of quantitation (LOQs) were established as the amount of analyte that produces a signal-to-noise ratio of 3:1 and 10:1 respectively. LODs are given in Table 3. They were calculated using standard methanol/buffer solutions at low concentration levels, and meat extracts ($n = 3$) free of HAs spiked with very low amounts of analytes in the beginning of the extraction. For standards, the best results were obtained when using quadrupole instruments, with values in the low pg level. Moreover, similar or slightly lower values in MS–MS were achieved. In meat extract, LODs were always higher than those for standards due to matrix complexity that affects both chemical noise and ionisation efficiency. This effect was less important for the Applied Biosystems (API 150 and API 3000) instruments and can be explained by the design of the Turbo Ionspray source that provides a higher electrospray efficiency than the Thermo Finnigan LCQ source. Moreover, the decrease of trapping efficiency of target ions in the presence of a high amount of interferent ones can also affect the LODs in the ion trap instrument. Nevertheless, for both instruments, the high selectivity provided by MS–MS allows a significant reduction of the noise. Consequently, an increase in the signal-to-noise ratio was observed, as can be seen in Table 3, where low LODs are shown for product ion scan using the ion trap and for MRM mode when working with the triple quadrupole instrument. Although triple quadrupole presented better LODs than the ion trap, this last analyser allows to work with product ion scan without reducing the sensitivity, which is important to avoid false peak identification.

Linearity range was studied for all the instruments between limit of quantitation and 2.5 μg/g. For all compounds and instruments, the response was linear up to 1.0 μg/g. As triple quadrupole provided lower limits of quantitation than the other systems, its linearity range was generally two orders of magnitude larger. For instance, for MeIQ linearity range for MS–MS in the ion trap was 0.03–1 μg/g and for MRM with the triple quadrupole was 0.0005–1 μg/g.

Table 3
Limits of detection in standards (pg injected) and lyophilised meat extract (pg injected and μg/kg).

HAs	Standards					Meat extract									
	Ion trap		Quadrupole	Triple Quadrupole		Ion trap				Quadrupole		Triple quadrupole			
	Full scan (pg)	Production scan (pg)	SIM (pg)	SIM (pg)	MRM (pg)	Full scan		Production scan		SIM		SIM		MRM	
						pg	μg/kg	pg	μg/kg	pg	μg/kg	pg	μg/Kg	pg	μg/kg
DMIP	50	19	2	5	0.1	563	8.4	179	3.6	25	0.5	18	0.4	3	0.1
Glu-P-2	27	20	7	8	4	253	5.1	94	1.9	29	0.6	40	0.8	5	0.1
IQ	38	19	6	7	2	180	3.6	30	0.6	25	0.5	37	0.7	2	0.04
MeIQx	26	20	6	7	0.5	400	8.0	147	2.9	85	1.7	56	1.1	4	0.1
MeIQ	17	19	0.4	2	1	68	1.4	37	0.7	61	1.2	21	0.4	2	0.04
Glu-P-1	19	34	6	7	3	213	4.3	69	1.4	14	0.3	32	0.6	5	0.1
7,8-DiMeIQx	23	24	4	2	0.3	136	2.7	54	1.1	7	0.1	25	0.5	3	0.1
4,8-DiMeIQx	29	27	11	6	0.4	141	2.8	59	1.2	9	0.2	26	0.5	3	0.1
Norharman	12	26	8	7	1	214	4.3	14	0.3	18	0.4	10	0.2	2	0.04
Harman	8	15	7	9	3	385	7.7	32	0.6	32	0.6	21	0.4	4	0.1
Trp-P-2	15	17	3	0.2	0.3	225	4.5	28	0.6	43	0.9	9	0.2	1	0.02
Trp-P-1	9	8	1	0.1	0.3	168	3.4	6	0.1	17	0.3	2	0.04	1	0.02
PhIP	15	9	2	0.6	0.1	450	9.0	63	1.3	41	0.8	18	0.4	0.5	0.01
AαC	20	13	2	1	0.3	341	6.8	86	1.7	25	0.5	11	0.2	1	0.02
MeAαC	38	10	4	0.2	0.3	352	7.0	83	1.7	27	0.5	10	0.2	1	0.02

Table 4
Short-term (run-to-run, $n = 6$) and medium term (day-to-day, $n = 6$) precision for all instruments and acquisition methods (R.S.D., %). Low level: 0.05 μg/g; medium level: 0.40 μg/g

HAs	Ion trap								Quadrupole				Triple quadrupole							
	Full scan				Production scan				SIM				SIM				MRM			
	Low level		Medium level		Low level		Medium level		Low level		Medium level		Low level		Medium level		Low level		Medium level	
	Run-to-run	Day-to-day	Run-to-run	Day-to-day	Run-to-run	Day-to-day	Run-to-run	Day-to-day	Run-to-run	Day-to-day	Run-to-run	Day-to-day	Run-to-run	Day-to-day	Run-to-run	Day-to-day	Run-to-run	Day-to-day	Run-to-run	Day-to-day
DMIP	6	8	3	4	6	7	5	5	5	7	4	6	6	7	6	8	0.4	7	1	8
Glu-P-2	6	8	2	10	2	7	2	3	7	9	6	8	6	8	3	5	3	5	2	4
IQ	5	7	1	9	5	12	4	7	8	10	6	8	8	9	3	3	3	5	2	2
MeIQx	3	5	1	5	9	12	3	10	6	2	5	2	7	9	2	4	3	5	1	2
MeIQ	3	5	3	6	8	11	3	8	5	7	4	6	5	6	1	4	2	9	0.4	2
Glu-P-1	4	6	2	6	8	4	7	8	7	9	5	7	8	9	3	5	4	6	4	4
7,8-DiMeIQx	3	5	0.5	3	7	10	4	11	7	9	5	7	6	8	3	9	5	8	1	1
4,8-DiMeIQx	5	7	3	4	7	11	7	4	7	9	5	7	7	9	3	10	4	8	1	1
Norharman	3	5	1	7	8	11	5	5	7	9	5	7	6	8	5	7	3	5	3	3
Harman	4	6	2	2	5	7	3	8	6	8	4	6	6	8	4	5	4	6	2	3
Trp-P-2	5	7	2	3	7	8	4	3	5	7	2	5	5	5	0.4	2	1	7	0.5	1
Trp-P-1	4	6	3	6	9	9	5	6	2	4	2	6	2	8	1	5	1	6	1	1
PhIP	7	9	3	5	8	8	7	4	3	5	5	7	2	4	1	5	3	6	0.4	0.5
AαC	7	9	4	4	7	9	3	5	5	7	3	9	5	8	1	3	3	4	1	2
MeAαC	6	8	2	4	7	8	3	6	5	7	5	9	6	9	0.5	3	4	5	0.5	1

To determine repeatability, six replicate injections of a HAs standard solution at both low (0.05 μg/g) and medium (0.4 μg/g) concentration levels were carried out. Relative standard deviations (R.S.D.) were calculated (Table 4). The best R.S.D. values were obtained when using triple quadrupole with MRM acquisition and they were lower than 5%. For ion trap R.S.D. were slightly higher, <7 and <9% with full scan and product ion scan acquisition modes respectively. Similar values were obtained for the single quadrupole instrument. In terms of relative error, the best results were obtained at a concentration of 0.4 μg/g, although at 0.05 μg/g the calculated bias were lower than 11% and no significative differences among the instruments were observed.

For the determination of medium-term precision, two replicate injections along three consecutive days of an HAs standard solution at low and medium levels of concentration were carried out. R.S.D. were calculated from the six

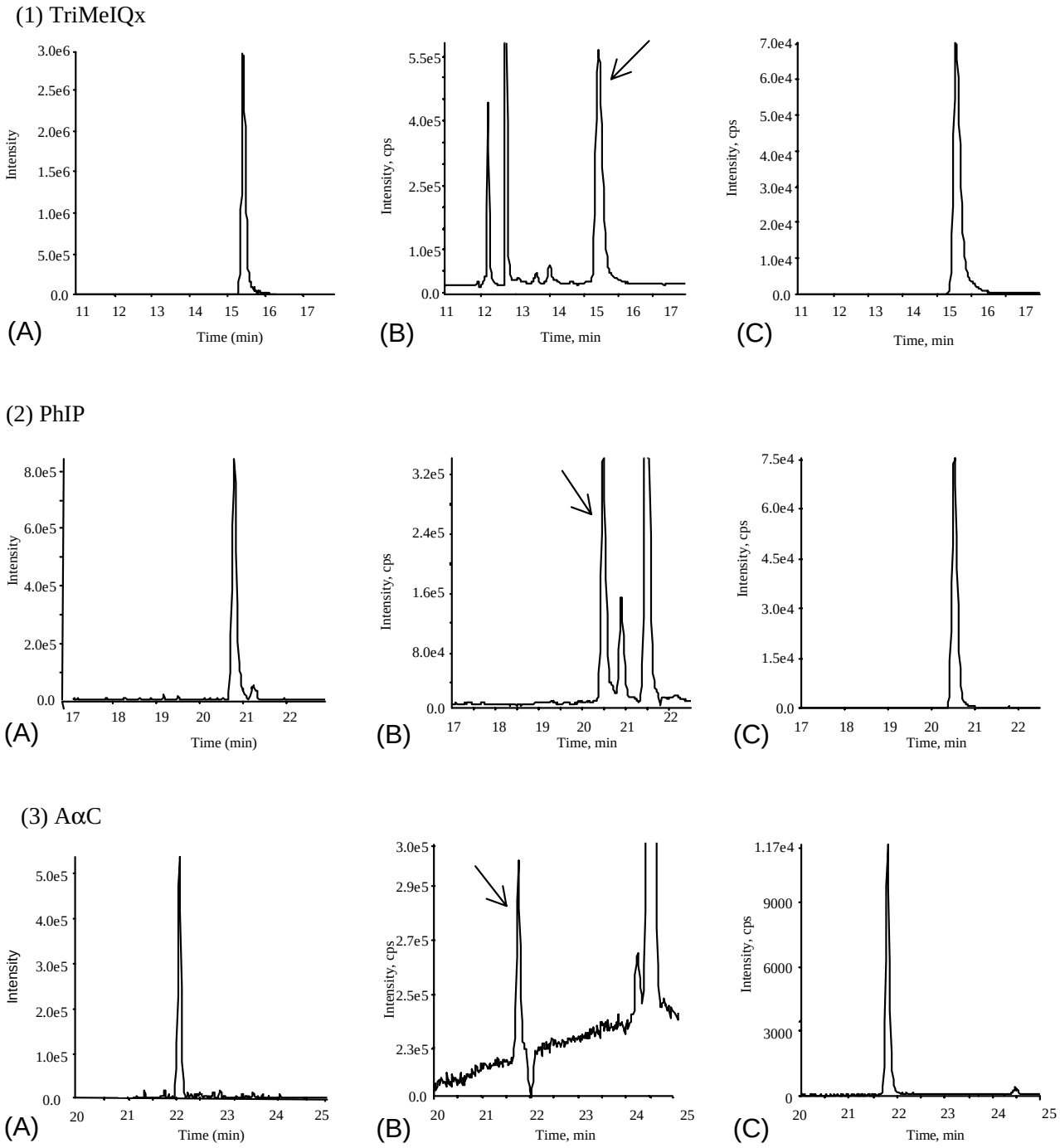

Fig. 5. Chromatograms corresponding to the less polar extract of the lyophilised meat, measuring TriMeIQx, PhIP and AαC by three different instruments and methods of acquisition: (A) ion trap, product ion scan; (B) single quadrupole, SIM; (C) triple quadrupole, MRM.

Table 5
Results of quantification of HAs (μg/g extract) in a meat extract ($n = 6$)

HAs	Ion trap		Quadrupole	Triple quadrupole	
	Full scan	Product ion scan	SIM	SIM	MRM
IQ	54.3 ± 11.2	49.4 ± 5.5	44.9 ± 4.5	43.1 ± 3.6	42.7 ± 3.6
MeIQx	56.3 ± 17.7	46.6 ± 4.7	40.2 ± 5.3	39.7 ± 4.3	41.5 ± 2.9
MeIQ	52.9 ± 12.8	45.0 ± 5.4	41.9 ± 5.9	39.9 ± 4.3	42.9 ± 2.7
PhIP	49.6 ± 12.1	40.3 ± 5.3	37.3 ± 3.6	35.2 ± 5.0	35.2 ± 2.9
AαC	40.2 ± 4.7	37.8 ± 5.1	35.0 ± 5.7	43.6 ± 4.5	42.8 ± 2.0

calculated concentration values, and slightly better results were also obtained using triple quadrupole with MRM acquisition mode (Table 4). R.S.D. for all the instruments and adquisition modes were lower than 12%, showing that all the systems provide satisfactory values of repeatability and reproducibility required for an accurate analysis of heterocyclic amines.

3.3. Quantitative analysis of HAs in a lyophilised meat extract

The LC–MS methods have been applied to the determination of HAs in a meat extract that was used as reference material in a European interlaboratory comparison. The amines to be analysed in this material were IQ, MeIQ, MeIQx, PhIP and AαC. Six individual, fully independent analyses were carried out, on three different days, and from three different bottles. The clean-up procedure is described in Section 2.4. Quantification of HAs was performed by the standard addition method, spiking at four concentration levels around 50, 100, 150 and 200% for each replicate analysis. Besides, TriMeIQx was used as injection internal standard and it was added to the final extract at a concentration of 0.5 μg/g. As an example, Fig. 5 shows the chromatograms of the less-polar extract obtained with ion trap instrument using product ion scan, and single and triple quadrupole instruments, with SIM and MRM acquisition modes respectively. Some differences can be observed in the chromatograms. For instance the high selectivity of MS–MS provided better chromatograms (Fig. 5A and C) and allowed quantification without interferences. Moreover, the signal-to-noise ratio is higher than for the single quadrupole in SIM mode. The results obtained with all the instruments and acquisition modes are in agreement (Table 5), although the more precise ones were obtained with the triple quadrupole instrument. Nevertheless, the results achieved with the ion trap were also good and had the additional advantage of providing spectral information for false positive peak identification.

4. Conclusions

Three LC–ESI–MS instruments using the same chromatographic conditions were evaluated for the determination of HAs. High values of ionisation source parameters such as auxiliary and nebuliser gas flow rates and desolvation temperature were required due to the low amount of organic solvent in the mobile phase. Moreover, post-column addition of formic acid–acetonitrile was needed to increase ionisation efficiency when using the LCQ instrument. In MS–MS experiments in the ion trap, adduct formation due to ion-molecule reactions for carbolines were observed in contrast with their absence in the triple quadrupole. Hence, to obtain reproducible results in the ion trap instrument for these compounds, the main product ion plus adduct ions have to be included in the response used for quantification purposes. For all instruments and compounds, responses were linear from limit of quantitation to 1 μg/g. Nevertheless, triple quadrupole in MRM mode provided a linear range two orders of magnitude higher than ion trap (product ion scan) due to the low LODs achieved. The results obtained for the analysis of HAs in a beef extract showed good precision with all the instruments, but the most precise results were obtained in MRM mode. Nevertheless, the product ion scan mode using an ion trap can be used to prevent false peak identification due to the spectral information provided by this mode.

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