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Evaluation of reversed-phase columns for the analysis of heterocyclic aromatic amines by liquid chromatography—electrospray mass spectrometry

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Abstract

Liquid chromatography coupled to mass spectrometry (LC–MS), especially by the use of electrospray ionisation source (ESI), is currently used for the analysis of heterocyclic aromatic amines (HAs) in complex samples. The present paper describes the study of the performance of different narrow-bore reversed-phase columns to achieve the best chromatographic separation for the determination of 16 HAs by LC–ESI-MS in food samples. Different parameters such as peak symmetry, resolution and number of theoretical plates have been evaluated for each column, using different chromatographic conditions. The column that provided the best results was TSK Gel® Semi-Micro ODS-80TS of Tosohaas. Quality parameters have been established, obtaining good short-term precision in all cases (relative standard deviation (R.S.D.) lower than 7.7%) and low limits of detection (<13 pg injected in MS and <16 pg injected in MS/MS). The content of HAs in two beef extracts have been determined.

Keywords: Food analysis; Heterocyclic aromatic amines

1. Introduction

The discovery that cooked fish and beef showed highly mutagenic activity, as detected 25 years ago by the Ames/*Salmonella* test system [1,2], prompted the researchers to begin an extensive search for mutagens present in foods. Since then, more than 20 compounds, known as heterocyclic aromatic amines (HAs) have been isolated and identified from cooked foods [3–5]. Some HAs have also been detected in environmental and biological samples (such as cigarette smoke and human urine), and also in soy sauce, wine, vinegar, beer and in river water [6–11]. Some of these mutagenic compounds have shown to be carcinogens in rodents and nonhuman primates [12–15].

A need for a routine analysis method able to quantify low amounts of HAs in complex matrices such as cooked foods have generated interest from many laboratories. In the last years, liquid chromatography–mass spectrometry (LC–MS) has shown to be one of the most powerful analytical techniques because of the capacity of separation of nonvolatile, thermolabile and polar compounds provided by LC and also because of the selectivity and sensitivity of MS [16–23].

For HAs analysis by LC–MS, electrospray ionisation source (ESI) has been the most frequently used ionisation technique in the last few years, due to its high sensitivity. Narrow-bore liquid chromatography columns are the most compatible ones with ESI, due to the low mobile phase flow rate used. Besides, these columns provide higher efficiency and shorter analysis time than conventional LC columns. The aim of this study was to compare the performance of different narrow-bore columns to achieve the best chromatographic separation of 16 HAs, and to establish an LC–ESI-MS analysis method for their determination in food samples. Six columns were studied: Symmetry® C8 (Waters), Zorbax® SB-C8 (Agilent Technologies), TSK Gel® Semi-Micro ODS-80TS (Tosohaas), Discovery® H5 C18 (Supelco), Synergi™ Max-RP (Phenomenex) and Purospher® Star RP-C18 (Merck). Buffer composition, pH, organic modifier and gradient elution program have been studied for each column in order to obtain the best HAs separation. Different parameters such as peak asymmetry, resolution, number of theoretical plates, analysis time, maximum injection volume and peak height have been evaluated for each column. Taking into account these parameters,

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the column that provided the best results has been used to carry out the validation of a LC–ESI-MS method in full scan and product ion scan acquisition mode. Finally, this methodology has been applied to the analysis of HAs in two lyophilised meat extracts.

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-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), which were obtained from Toronto Research Chemicals Inc. (Toronto, Canada), and 1-methyl-9*H*-pyrido[4,3-*b*]indole (Harman) and 9*H*-pyrido[4,3-*b*]indole (Norharman), which were from Sigma (Missouri, USA). Stock standards solutions of 80 $\mu g\,g^{-1}$ in methanol were prepared and used for further dilutions.

Gradient grade acetonitrile (ACN) and methanol, and HPLC grade dichloromethane, were purchased from Merck (Darmstadt, Germany), and water was purified in an Elix-Milli Q system (Millipore Co., Bedford, MA, USA). Ammonia solution, hydrochloric acid, formic acid and sodium hydroxide were analytical grade and were provided by Merck (Darmstadt, Germany). Ammonium acetate and ammonium formate analytical grade were purchased 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 endcapped C18 (100 mg) Bond Elut cartridges, and coupling pieces and stopcocks were from Varian (Harbor City, USA).

Helium of high purity was used as damper gas for the ion trap, and N_2 (N1) was used as nebuliser and auxiliary gas for

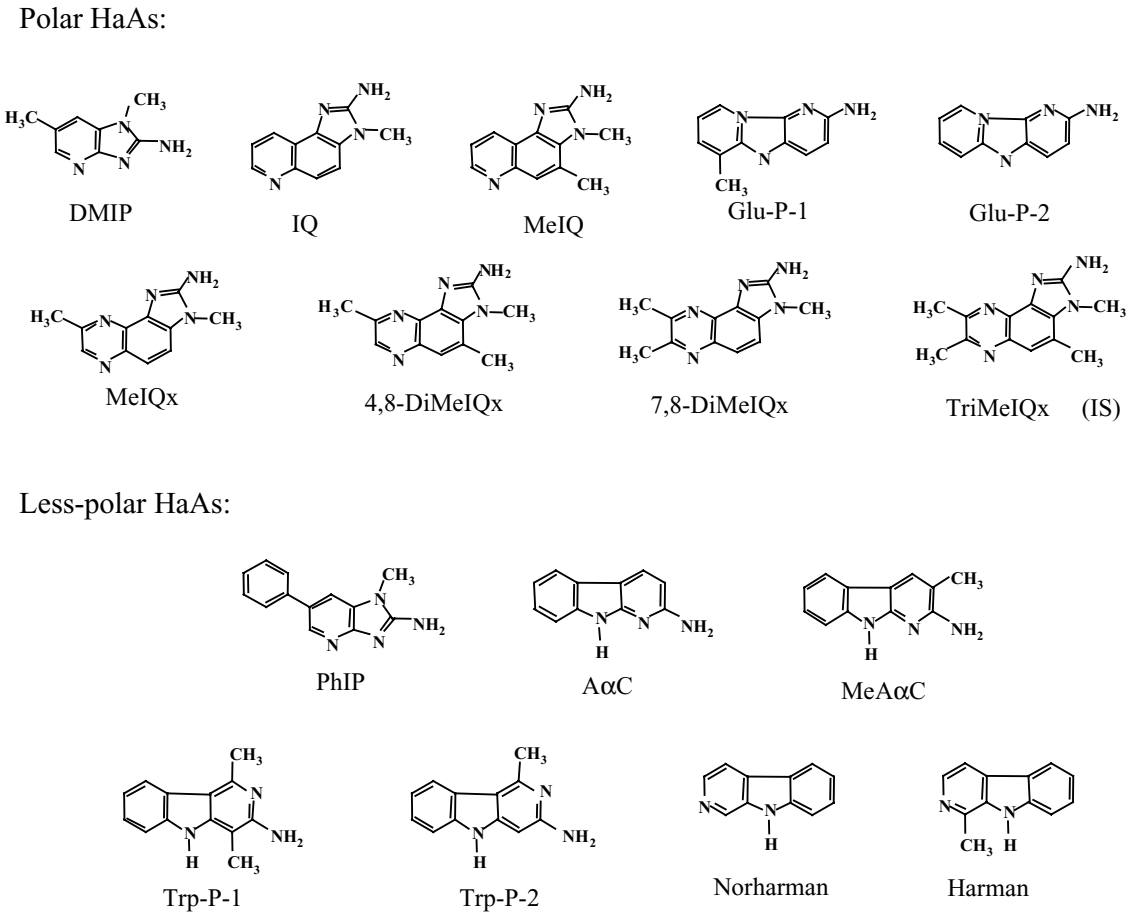

Fig. 1. Structure and abbreviated names of the 16 HAs used in this study.

Table 1
Characteristics of columns selected for this study

Column	Abbreviated name	Stationary phase	Particle size (μm)	TMS endcapping	Pore size (Å)	Length (cm)
Symmetry® C8	Symmetry	C8	5	Yes	80	15
Zorbax® SB-C8	Zorbax	C8	3.5	No	80	15
TSK Gel® Semi-Micro ODS-80TS	TSK Gel	C18	5	Yes	80	15
Synergi™ Max-RP	Synergi	C12	4	Yes	80	15
Discovery® H5 C18	Discovery	C18	3	Yes	80	15
Purospher® Star RP-C18	Purospher	C18	5	Yes	80	12

the ESI source. Both helium and nitrogen were purchased from Air Liquide (Madrid, Spain). Mobile phase, standard solutions and samples were passed through a 0.45 μm filter provided by Whatman Inc. (Clifton, NJ, USA) before LC–ESI-MS analysis.

2.2. Liquid chromatography columns

Six narrow-bore (2.1 mm i.d.) reversed-phase liquid chromatography columns have been evaluated: Symmetry® C8 (Waters, USA), Zorbax® SB-C8 (Agilent Technologies, USA), TSK Gel® Semi-Micro ODS-80TS (Tosohaas, Japan), Synergi™ Max-RP (Phenomenex, USA), Discovery® H5 C18 (Supelco, USA) and Purospher® Star RP-C18 (Merck, Germany). These columns embrace a range of different stationary phases, particle size and endcapping treatment, and these characteristics are given in Table 1.

2.3. Chromatographic conditions

LC was performed in a Waters 2690 separations module (Milford, MA, USA), quaternary pump equipped with an autosampler. The chromatographic separation of amines was carried out using a gradient elution program of a binary mobile phase at a flow-rate of 300 μl min^{-1} composed by acetonitrile (solvent A) and 30 mM buffer (solvent B). Formic acid and ammonium formate were used to prepare buffer solutions of pH 3.2, 3.7 and 4.0, and acetic acid and ammonium acetate were used when preparing buffer solutions of pH 4.5. The optimal gradient elution programs obtained for each column are detailed as follows. Symmetry (solvent B: pH 3.7): 5% A, 0–1 min; 5–30% A, 1–15 min; 30–60% A, 15–18 min; 60% A, 18–24 min; return to the initial conditions, 24–27 min. Zorbax (solvent B: pH 3.7): 5% A, 0–1 min; 5–20% A, 1–15 min; 20–60% A, 15–18 min; 60% A, 18–24 min; return to the initial conditions, 24–27 min. TSK Gel (solvent B: pH 4.0): 5% A, 0–1 min; 5–30% A, 1–15 min; 30–60% A, 15–18 min; 60% A, 18–24 min; return to the initial conditions, 24–27 min. Synergi (solvent B: pH 3.7): 5% A, 0–1 min; 5–20% A, 1–15 min; 20–60% A, 15–18 min; 60% A, 18–24 min; return to the initial conditions, 24–27 min. Purospher (solvent B: pH 4.5): 5% A, 0–1 min; 5–30% A, 1–15 min; 30–60% A, 15–18 min; 60% A, 18–24 min; return to the initial conditions, 24–27 min. The amount injected was 5 μl.

2.4. Mass spectrometric conditions

Identification and determination of analytes were carried out using a LCQ mass spectrometer (Thermo-Finnigan, San Jose, CA, USA) equipped with an electrospray ionisation source and an ion trap mass analyser (IT). Data acquisition was performed using Xcalibur™ 1.2 software.

LC-MS coupling parameters were: spray voltage, 3 kV; nebuliser gas, 90 a.u.; drying gas, 60 a.u.; heated capillary temperature, 280 °C. A solution of 0.01% of formic acid (v/v) in acetonitrile was used as post-column solvent at a flow rate of 100 μl min^{-1}.

For the evaluation of the different columns and also for the quantification of HAs in a meat extract of high concentration level (sample A), data acquisition was performed in full scan mode, scanning from *m/z* 150 to 250 in centroid mode (three microscans), with a maximum injection time of 200 ms and automatic gain control activated. However, for the quantification of HAs in another lyophilised meat extract (sample B) of low concentration level, product ion scan acquisition was performed using the MS/MS conditions given in Table 2. Normalised collision energies (NCE, %) for the fragmentation of the precursor ion $[M + H]^+$ of each compound ranged from 37 to 45%. The spectra of product ions were obtained scanning *m/z* from 110 to 250. For quantification purposes the chromatogram of the most intense product ions were used. Some ion-molecule reactions were observed into the trap, in agreement with results obtained by Toribio et al. using LC–APCI–MS/MS [24]. These reactions occurred only 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 (H_2O and ACN), and adducts of *m/z* higher than parent ion were obtained. These ions had a very high signal, so they were used for the quantification of carbolines by MS/MS in order to obtain reproducible results.

2.5. Sample treatment

Two lyophilised meat extracts candidate to laboratory reference material were analysed. They were prepared from a commercial beef meat extract (Bovril®) that was spiked with HAs [25] at a different levels (sample A: ∼70 ng g^{-1} extract; sample B: ∼10 ng g^{-1} extract). To extract the analytes, a previously described purification method [26] was

Table 2
MS/MS parameters used with ion trap LCQ instrument

Segment	Time (min)	Analyte	Precursor ions $[M + H]^+$ (*m/z*)	NCE (%)[a]	Product ions used for quantification (*m/z*)	Product ion scan range (*m/z*)
1	0–9.3	DMIP	163	41	148	[140–170]
		Glu-P-2	185	43	158	[150–190]
		IQ	199	41	184	[150–205]
2	9.3–11	MeIQx	214	41	199 + 173	[165–220]
		MeIQ	213	40	198	[165–220]
		Glu-P-1	199	44	184 + 172	[165–210]
3	11–14.5	7,8-DiMeIQx	228	42	213 + 187	[180–235]
		4,8-DiMeIQx	228	42	213 + 187	[180–235]
		Norharman	169	45	167 + 142 + 115	[110–175]
		TriMeIQx	242	41	227 + 201	[195–250]
		Harman	183	44	181 + 168 + 115	[110–190]
4	14.5–18	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	18–25	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.
[a] NCE: normalised collision energy.

used. Briefly, 1 g beef extract sample was homogenised in 12 ml NaOH (1 M) and mixed with 13 g diatomaceous earth. The amines were eluted from the extraction column, containing the diatomaceous earth mixture, directly to a propylsulphonic acid (PRS) cartridge using 75 ml dichloromethane. This cartridge was dried and rinsed with 15 ml MeOH–H_2O (4:6) and 2 ml of water. The amines retained were eluted through a C18 (100 mg) cartridge using 20 ml of 0.5 M ammonium acetate solution at pH 8.5. It was rinsed with 5 ml H_2O, and the adsorbed HAs were then eluted using 0.8 ml of methanol–ammonia solution (9:1 (v/v)). The extract was evaporated to dryness under a stream of nitrogen and the analytes were redissolved in 500 μl of a solution containing the internal standard in methanol–ammonium formate 30 mM at pH 4.0 (1:1 (v/v)). Finally, the extract was 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

3.1. Optimisation of chromatographic conditions

The aim of this study was to obtain the best HAs separation, providing high resolution values, low analysis time, high efficiency, and the highest signal in order to decrease the detection limits. For that reason, six reversed-phase columns have been studied. All of them were neither endcapped or specially deactivated for bases. Narrow-bore columns (2.1 mm) compatible with LC–ESI-MS with different reversed stationary phases (C8, C12 and C18) were used. Moreover, particle size was relatively small (from 3 to 5 μm) to achieve high efficiency values. The selected columns are indicated in Table 1.

In order to establish the best chromatographic separation for each narrow-bore reversed-phase liquid chromatography column, buffer composition, pH and gradient elution program were studied. ESI source requires analytes to be ionised in the liquid phase, so for HAs analysis the pH of the mobile phase should be lower than pK_a of the HAs (pH < 5) to protonate the amino group. Volatile buffers as formic acid/ammonium formate (pH 3.2, 3.7 and 4.0) and acetic acid/ammonium acetate (pH 4.5) were selected to cover the pH range between 3 and 5. Buffer concentration was set at 30 mM as a compromise between buffer capacity and ionic strength that must be low to reduce surface tension. Moreover, in all cases acetonitrile was used as organic modifier. Each column was tested using the four buffers proposed for the different pH values, and the gradient elution program was optimised for each case to obtain the best separation of a standard mixture of 16 HAs (0.4 μg g^{-1}). All gradient elution programs started at 5% of acetonitrile, and after an isocratic stage of 1 or 2 min, the percentage of acetonitrile (% ACN) was increased until 20 or 30% in 14 min to achieve a good separation of the most polar analytes. After that, an increase of the % ACN until 60% in 3 or 4 min was performed and then an isocratic stage at 60% ACN was carried out to achieve the separation of the less-polar HAs. Due to the low organic modifier concentration at the initial conditions of the gradient elution program, standards and samples were prepared in 1:1 methanol/buffer to avoid peak broadening.

In a preliminary evaluation the Discovery column was rejected because AαC and MeAαC suffered a strong

Table 3
Peak height values normalised respect the maximum value obtained for each amine using five columns and different pH conditions

HAs	Symmetry				Zorbax				TSK Gel				Synergi				Purospher			
	pH 3.2	pH 3.7	pH 4.0	pH 4.5	pH 3.2	pH 3.7	pH 4.0	pH 4.5	pH 3.2	pH 3.7	pH 4.0	pH 4.5	pH 3.2	pH 3.7	pH 4.0	pH 4.5	pH 3.2	pH 3.7	pH 4.0	pH 4.5
DMIP	27	59	64	58	47	50	61	38	55	92	73	69	100	68	15	28	24	12	3	33
Glu-P-2	46	58	60	32	36	43	62	37	58	100	88	68	84	40	34	23	19	8	4	18
IQ	17	31	45	17	43	66	95	29	55	100	87	58	87	57	82	11	17	16	7	28
MeIQx	19	59	50	24	55	66	59	29	74	100	84	52	97	62	28	15	38	24	11	37
MeIQ	18	45	69	24	37	45	73	35	53	96	100	66	80	61	84	33	19	20	8	33
Glu-P-1	24	55	64	39	50	37	66	40	78	100	87	67	87	57	63	30	31	18	8	32
7,8-DiMeIQx	20	62	56	25	57	65	60	28	83	100	86	49	87	62	34	17	36	22	11	37
4,8-DiMeIQx	23	79	57	29	58	66	61	25	85	89	77	57	100	63	26	15	40	24	11	31
Norharman	29	69	71	36	57	71	80	53	88	100	79	70	66	64	73	30	37	22	10	34
TriMeIQx	27	80	62	32	55	73	67	31	73	96	83	53	100	69	38	16	71	51	19	59
Harman	39	76	90	48	58	70	93	66	82	100	90	84	86	70	95	46	35	24	13	44
Trp-P-2	24	55	66	31	52	67	89	50	64	85	68	55	60	62	100	39	29	23	11	33
Trp-P-1	21	52	57	27	50	100	97	43	52	71	60	50	51	60	69	54	23	18	10	28
PhIP	16	51	47	65	44	88	100	58	52	78	50	50	56	59	29	44	34	26	13	41
AαC	17	36	66	38	46	100	85	49	50	54	31	46	45	80	87	36	30	20	16	56
MeAαC	30	49	40	28	47	100	80	45	58	76	41	33	36	74	30	10	28	29	25	52

Table 4
Peak asymmetry factors, calculated at 10% peak height, obtained for each amine using five columns and different pH conditions

HAs	Symmetry				Zorbax				TSK Gel				Synergi				Purospher			
	pH 3.2	pH 3.7	pH 4.0	pH 4.5	pH 3.2	pH 3.7	pH 4.0	pH 4.5	pH 3.2	pH 3.7	pH 4.0	pH 4.5	pH 3.2	pH 3.7	pH 4.0	pH 4.5	pH 3.2	pH 3.7	pH 4.0	pH 4.5
DMIP	1.2	1.3	1.1	1.6	1.8	1.3	1.7	1.9	1.2	1.3	1.3	1.5	1.0	1.3	1.3	1.8	1.1	1.0	1.2	1.1
Glu-P-2	1.2	1.5	1.1	1.5	2.2	1.8	2.4	2.5	1.4	1.3	1.6	1.6	1.7	2.0	2.3	3.0	2.0	2.1	1.9	3.6
IQ	2.7	2.0	1.5	2.8	0.9	1.6	2.7	2.4	2.4	2.2	1.8	1.8	2.5	2.0	2.0	2.0	3.1	1.9	1.6	1.9
MeIQx	1.4	1.5	1.0	2.0	1.5	1.6	2.0	2.0	1.7	1.5	1.3	2.0	1.6	1.0	1.3	2.3	1.0	1.3	1.2	0.9
MeIQ	2.5	1.8	2.0	3.1	3.6	3.5	3.3	3.2	2.4	2.0	2.0	2.5	2.8	3.0	2.3	4.0	2.9	2.5	1.3	1.9
Glu-P-1	1.4	1.2	1.2	1.5	2.0	2.7	2.5	2.5	1.5	1.9	1.3	1.5	1.1	1.5	2.0	2.0	1.7	1.2	2.0	1.7
7,8-DiMeIQx	1.3	1.4	1.3	1.6	1.1	1.7	3.0	2.5	3.0	1.5	1.5	1.5	1.7	2.0	1.7	2.7	1.1	1.2	1.1	1.2
4,8-DiMeIQx	1.0	1.4	1.5	1.3	2.0	2.1	2.5	2.4	2.0	1.5	1.5	1.8	1.5	2.0	1.8	3.0	0.8	1.3	2.0	1.6
Norharman	1.4	1.1	1.0	1.3	1.5	1.3	1.5	1.8	1.5	1.5	1.3	1.5	1.6	1.5	1.5	2.0	1.8	3.0	1.8	2.7
TriMeIQx	1.1	1.3	1.4	2.1	1.2	2.0	2.1	2.0	1.2	1.1	1.1	1.6	1.5	1.5	1.2	1.9	1.0	1.2	1.6	1.5
Harman	1.1	1.0	1.2	1.4	1.3	1.4	1.4	1.5	1.1	1.2	1.3	1.3	1.2	1.4	1.4	1.8	2.3	1.8	1.6	1.9
Trp-P-2	1.1	1.1	1.5	1.6	1.3	1.2	1.5	1.8	1.8	1.4	1.3	1.6	1.6	1.6	1.0	1.7	1.8	1.1	1.5	1.9
Trp-P-1	1.0	1.4	1.7	1.8	1.0	1.6	1.0	1.8	1.5	1.4	1.5	1.6	1.3	1.5	1.4	2.5	1.5	1.3	1.2	1.8
PhIP	1.0	1.0	1.6	1.5	1.1	2.0	1.6	1.5	1.8	1.2	1.0	1.5	1.1	1.5	1.3	2.0	0.9	0.9	0.9	1.5
AαC	1.5	1.3	1.0	1.5	1.0	1.3	1.0	1.5	1.7	1.5	1.5	2.0	2.0	1.6	1.5	1.5	2.8	1.4	1.3	1.2
MeAαC	1.0	2.0	1.0	1.0	1.5	1.2	1.0	1.0	2.0	1.3	1.5	1.0	1.8	2.0	1.0	2.0	2.4	1.8	1.5	1.3

Table 5
Number of theoretical plates ($N = 5.54\ (t_R/w_{0.5})^2$) normalised respect the maximum value obtained for each amine

HAs	Symmetry				Zorbax				TSK Gel				Synergi				Purospher			
	pH 3.2	pH 3.7	pH 4.0	pH 4.5	pH 3.2	pH 3.7	pH 4.0	pH 4.5	pH 3.2	pH 3.7	pH 4.0	pH 4.5	pH 3.2	pH 3.7	pH 4.0	pH 4.5	pH 3.2	pH 3.7	pH 4.0	pH 4.5
DMIP	6	8	6	5	13	17	13	24	15	51	34	30	100	14	14	11	13	8	10	18
Glu-P-2	5	6	6	8	15	29	32	67	30	100	69	94	80	8	9	17	19	8	15	18
IQ	1	3	7	3	40	75	60	46	20	86	100	81	77	25	37	30	14	37	58	77
MeIQx	16	27	24	9	62	59	39	44	71	88	75	67	100	44	54	23	57	55	44	46
MeIQ	1	11	17	9	21	46	40	29	23	100	68	68	76	30	45	37	18	43	40	67
Glu-P-1	4	14	15	23	30	26	32	56	100	100	89	81	91	41	46	36	55	38	47	43
7,8-DiMeIQx	27	31	44	13	70	45	64	49	94	99	74	67	100	52	47	34	53	51	47	42
4,8-DiMeIQx	28	40	46	16	77	65	55	44	95	81	75	69	100	41	48	29	46	42	42	38
Norharman	51	62	64	33	70	69	71	53	88	88	79	60	100	57	72	38	24	29	31	24
TriMeIQx	38	52	40	18	61	64	59	44	67	86	70	42	100	72	54	37	44	45	37	36
Harman	46	69	52	31	52	75	71	57	85	79	90	86	100	77	65	49	37	39	38	37
Trp-P-2	100	32	27	21	39	47	64	32	58	41	42	32	47	50	39	31	23	27	21	22
Trp-P-1	11	16	14	9	75	92	100	14	21	19	22	13	25	96	17	69	10	10	11	8
PhIP	11	12	13	60	12	44	69	41	20	17	18	22	21	25	21	100	9	8	14	8
AαC	22	8	52	66	44	57	100	48	8	12	19	33	7	62	48	68	4	3	7	25
MeAαC	36	14	53	42	46	40	100	50	12	21	35	35	13	43	34	47	3	14	19	26

Table 6
Resolution values ($R_s = 2(t_{R2} - t_{R1})/(w_1 + w_2)$) between consecutive peaks on five columns and different pH conditions

No.	HAs	Symmetry								Zorbax								TSK Gel							
		pH 3.25		pH 3.7		pH 4.0		pH 4.5		pH 3.25		pH 3.7		pH 4.0		pH 4.5		pH 3.25		pH 3.7		pH 4.0		pH 4.5	
1	DMIP	Peaks	R_s	Peaks	R_s	Peaks	R_s	Peaks	R_s	Peaks	R_s	Peaks	R_s	Peaks	R_s	Peaks	R_s	Peaks	R_s	Peaks	R_s	Peaks	R_s	Peaks	R_s
2	Glu-P-2	1–2	0.5[a]	1–2	1.0[a]	1–2	2.0	1–2	5.9	1–2	3.9	1–2	5.8	1–2	6.8	1–2	10.7	1–2	2.7	1–2	3.7	1–2	4.4	1–2	7.4
3	IQ	2–3	2.1	2–3	4.5	2–3	6.4	2–3	2.3	2–3	8.4	2–3	10.3	2–3	8.4	2–4	3.8	2–3	3.6	2–3	7.8	2–3	8.3	2–3	5.4
4	MeIQx	3–5	2.8	3–6	3.8	3–6	3.2	3–4	3.3	3–6	0.9[a]	3–6	2.3	3–6	2.2	4–3	0.5[a]	3–5	3.9	3–6	6.3	3–6	5.6	3–4	6.4
5	MeIQ	5–6	2.7	6–5	1.3	6–5	2.4	4–5	2.2	6–5	3.8	6–4	4.2	6–4	1.7	3–6	2.2	5–6	3.1	6–5	0.6[a]	6–5	1.8	4–5	1.3
6	Glu-P-1	6–4	8.0	5–4	3.7	5–4	1.1[a]	5–6	0.6[a]	5–4	3.3	4–5	2.4	4–5	4.6	6–7	1.8	6–4	7.1	5–4	4.8	5–4	1.6	5–6	1.2[a]
7	7,8-DiMeIQx	4–7	12.6	4–7	9.0	4–7	10.3	6–7	5.3	4–7	9.9	5–7	4.8	5–7	1.7	7–5	1.5	4–7	11.1	4–7	11.0	4–7	9.9	6–7	6.4
8	4,8-DiMeIQx	7–8	1.9	7–8	1.5	7–8	2.0	7–8	2.1	7–9	0.8[a]	7–9	1.4	7–9	2.4	5–8	1.5	7–8	2.1	7–8	1.9	7–8	1.9	7–8	2.3
9	Norharman	8–9	1.6	8–9	4.0	8–9	5.1	8–9	4.9	9–8	3.6	9–8	2.8	9–8	2.0	8–9	2.2	8–9	3.0	8–9	3.6	8–9	3.7	8–9	4.9
10	TriMeIQx	9–11	8.3	9–10	4.1	9–10	3.8	9–10	2.5	8–11	3.8	8–11	5.7	8–11	5.6	9–10	1.6	9–10	5.6	9–10	4.8	9–10	4.4	9–10	1.9
11	Harman	11–10	0.5[a]	10–11	2.1	10–11	2.0	10–11	1.0[a]	11–10	2.0	11–10	1.7	11–10	1.2[a]	10–11	1.3	10–11	1.3	10–11	1.8	10–11	1.7	10–11	2.0
12	Trp-P-2	10–12	15.9	11–12	10.3	11–12	9.9	11–12	9.8	10–12	9.5	10–12	10.2	10–12	10.7	11–12	6.2	11–12	10.5	11–12	8.8	11–12	9.3	11–12	7.7
13	Trp-P-1	12–14	1.1[a]	12–14	2.8	12–14	5.0	12–13	6.5	12–14	1.6	12–14	4.9	12–13	6.1	12–13	6.5	12–14	0.5[a]	12–14	1.6	12–14	3.5	12–13	5.6
14	PhIP	14–13	6.0	14–13	2.3	14–13	0.5[a]	13–14	7.6	14–13	5.1	14–13	1.2[a]	13–14	3.6	13–15	22.7	14–13	6.9	14–13	4.8	14–13	3.2	13–14	3.3
15	AαC	13–15	14.1	13–15	22.0	13–15	26.9	14–15	13.8	13–15	1.5	13–15	7.4	14–15	6.5	15–14	1.0[a]	13–15	4.1	13–15	12.7	13–15	19.5	14–15	22.6
16	MeAαC	15–16	6.9	15–16	6.0	15–16	7.0	15–16	5.2	15–16	8.6	15–16	6.5	15–16	8.2	14–16	7.2	15–16	11.2	15–16	8.4	15–16	8.2	15–16	7.9
		Synergi								Purospher															
		pH 3.25		pH 3.7		pH 4.0		pH 4.5		pH 3.25		pH 3.7		pH 4.0		pH 4.5									
1	DMIP	Peaks	R_s	Peaks	R_s	Peaks	R_s	Peaks	R_s	Peaks	R_s	Peaks	R_s	Peaks	R_s	Peaks	R_s								
2	Glu-P-2	1–2	1.0[a]	1–2	0.9[a]	1–2	2.2	1–2	8.4	1–2	2.3	1–2	2.7	1–2	3.5	1–2	5.2								
3	IQ	2–3	3.8	2–3	7.0	2–3	7.0	2–3	6.3	2–3	2.8	2–3	4.7	2–3	5.2	2–3	3.0								
4	MeIQx	3–5	5.8	3–6	6.2	3–6	5.0	3–4	9.4	3–5	2.9	3–6	3.9	3–5	3.9	3–4	4.9								
5	MeIQ	5–6	4.4	6–5	1.4	6–5	2.6	4–5	2.5	5–6	2.1	6–5	1.5	5–6	2.1	4–5	2.2								
6	Glu-P-1	6–4	8.2	5–4	5.6	5–4	2.0	5–6	0.7[a]	6–4	6.1	5–4	2.9	6–4	0.7[a]	5–6	1.1[a]								
7	7,8-DiMeIQx	4–7	11.9	4–7	12.7	4–7	11.2	6–7	11.8	4–7	9.1	4–7	9.4	4–7	8.2	6–7	3.7								
8	4,8-DiMeIQx	7–8	1.6	7–8	1.6	7–8	1.5	7–8	3.1	7–8	1.4	7–8	1.4	7–8	1.4	7–8	1.7								
9	Norharman	8–9	5.2	8–9	4.3	8–9	5.5	8–9	6.5	8–9	2.2	8–9	2.6	8–9	2.9	8–9	3.9								
10	TriMeIQx	9–10	4.3	9–10	5.7	9–10	3.4	9–10	1.7	9–10	3.0	9–10	3.2	9–10	2.7	9–10	0.8[a]								
11	Harman	10–11	2.6	10–11	1.7	10–11	2.4	10–11	1.6	10–11	1.1[a]	10–11	1.2[a]	10–11	1.2[a]	10–11	1.5								
12	Trp-P-2	11–12	10.3	11–12	12.9	11–12	10.0	11–12	8.5	11–12	6.9	11–12	7.3	11–12	6.5	11–12	5.9								
13	Trp-P-1	12–14	1.4	12–14	2.8	12–14	5.0	12–13	5.8	12–14	0.0[a]	12–14	1.1[a]	12–14	2.4	12–13	4.5								
14	PhIP	14–13	5.1	14–13	3.8	14–13	1.7	13–14	4.1	14–13	5.0	14–13	3.7	14–13	3.1	13–14	2.8								
15	AαC	13–15	6.9	13–15	11.2	13–15	19.9	14–15	11.5	13–15	3.8	13–15	9.1	13–15	14.4	14–15	17.9								
16	MeAαC	15–16	10.4	15–16	5.9	15–16	6.5	15–16	5.2	15–16	7.3	15–16	6.8	15–16	6.6	15–16	7.2								

[a] Values lower than 1.2.

adsorption over all the working conditions tested, giving very wide peaks that prevent their detection. For the other columns, peak height (normalised values, Table 3), peak asymmetry factor (Table 4), and number of theoretical plates (normalised values, Table 5) have been calculated at the different pH values. Differences between columns have been observed. These three parameters are related: a low peak height may be indicative of a tailing or a wide peak and tailing peaks provide high asymmetry factor values. Moreover, wide peaks provide a low number of theoretical plates. So, as can be deduced from Tables 3–5, the TSK Gel column with buffers of pH 3.7 and 4.0 gave the best results for most of the polar amines, while for the less-polar the asymmetry factor values were below 2.0 and the peak height and number of theoretical plates were lower than those obtained with the Zorbax column. This last column showed the best performance when using a buffer of 3.7 and 4.0 for the less-polar amines, but for most of the polar analytes asymmetry factor values were higher than 2.0. As a consequence, the peak height and number of theoretical plates decrease. This fact can be explained by the absence of endcapped treatment in this column that favoured the adsorption of basic analytes. The Synergi column, using a buffer of pH 3.2, gave results similar to the TSK Gel at pH 3.7 and 4.0. But when the buffer pH increased, the peak height and number of theoretical plates decreased. Moreover, the interaction with silanol groups produced tailing peaks, giving high asymmetry factor values in spite of the endcapped treatment of this column. For the Symmetry column using buffer at pH 3.7 and the Purospher column using buffer at pH 4.5, generally the peak height and number of theoretical plates values were lower than for the other columns, while the asymmetry factor values were always below 2.0 for most of the amines. These facts are the consequence of the wider peaks obtained at these separation conditions.

In relation to the resolution (R_s) between closed peaks (Table 6), there are some aspects to comment on. First, some changes in the elution order of MeIQ, MeIQx, Glu-P-1 and PhIP were observed when pH increased. This behaviour could be related to the pK_a values of HAs. For instance, pK_a of PhIP is ∼5.5 [27,28], which is lower than the values of Trp-P-1 and Trp-P-2 (pK_a ∼8.5). So PhIP is partially deprotonated at pH 4.5 and consequently its retention is higher, affecting the elution order. Moreover, the best separations were obtained on the TSK Gel and Synergi columns using a buffer at pH 4.0, because the lowest R_s value was always >1.5. For the Symmetry column the best resolved peaks were obtained using a buffer at pH 3.7 although R_s value for DMIP/Glu-P-2 was 1.0. The highest R_s values (≥1.2) for the Zorbax column were obtained using a buffer at pH 3.7 and 4.0. The Purospher column gave acceptable R_s values (≥1.1) at pH 3.7.

Taking into account peak height and peak symmetry the Zorbax® SB-C8 column was discarded. For the other columns, the best separation conditions for each one were: for TSK Gel, gradient program with buffer at pH 4.0; Synergi with buffer at pH 3.7; Purospher with buffer at pH 4.0; and Symmetry with buffer at pH 3.7, because at these conditions they gave the best column performance. Fig. 2 shows the LC–MS(IT) chromatograms of a standard HAs solution (0.4 μg g^{-1}) obtained at the optimal conditions for these columns.

Other parameters such as equilibration time, maximum injection volume and limits of detection were studied in order to select the best column for HAs analysis. A short equilibration time produces a decrease in the total analysis time. To study this parameter, different equilibration times (between 3 and 10 min) were tested for each column and three consecutive injections of a standard solution (0.15 μg g^{-1}) were performed. A short equilibration time produced a decrease in the retention and the resolution values, especially for the most polar amines that eluted at the first part of the chromatogram. For values higher than 8 min no increase of the retention time (t_R) was observed for all columns. The retention time of each analyte at an equilibration time of 8 min was taken as reference and used to calculate the normalised t_R differences for each compound and column. Fig. 3 shows the normalised differences with respect to the reference retention time for equilibration times between 3 and 7 min, for the most polar amines. For all the compounds except DMIP, it can be observed that the equilibration time for the TSK Gel and the Purospher columns can be reduced without a significant variation in retention times. Using 5 min as equilibration time, the retention times with the TSK Gel column showed only a variation between 0.5 and 2.2% except for DMIP, where the variation was 4.4%. For the Purospher column, the retention time variation was slightly higher.

Maximum injection volume for all columns was studied because LODs values are inversely proportional to the injection volume. Four consecutive injections of a standard solution (0.15 μg g^{-1}) were carried out, varying the volume from 5 to 25 μl. Wider and fronting peaks were observed as the volume increased, and this effect was more important for analytes that eluted in the first part of the gradient. Peak width at 10% height was measured in order to check the variations in the peak shape as the volume injected increased. Peak width at 5 μl injected was taken as the reference value, and differences of peak width between this value and those obtained at 10–25 μl injected are shown in Fig. 4. It can be observed that these differences are more important for all amines (except DMIP) when using the Symmetry and the Synergi columns, and they can reach 1 min when injecting 25 μl. The TSK Gel column showed the lowest peak broadening mainly when 10 μl were injected, because the differences of peak width were lower than 2 s. For compounds which eluted at the last part of the gradient (less-polar amines), the effect of increasing injection volume was only significant when 25 μl were used, so for these cases 15 μl of sample could be injected without having fronting peaks.

Limit of detection (LODs) were established for each column as the quantity of analyte that produces a signal-to-noise ratio of 3:1 (Table 7). Standard solutions at very low con-

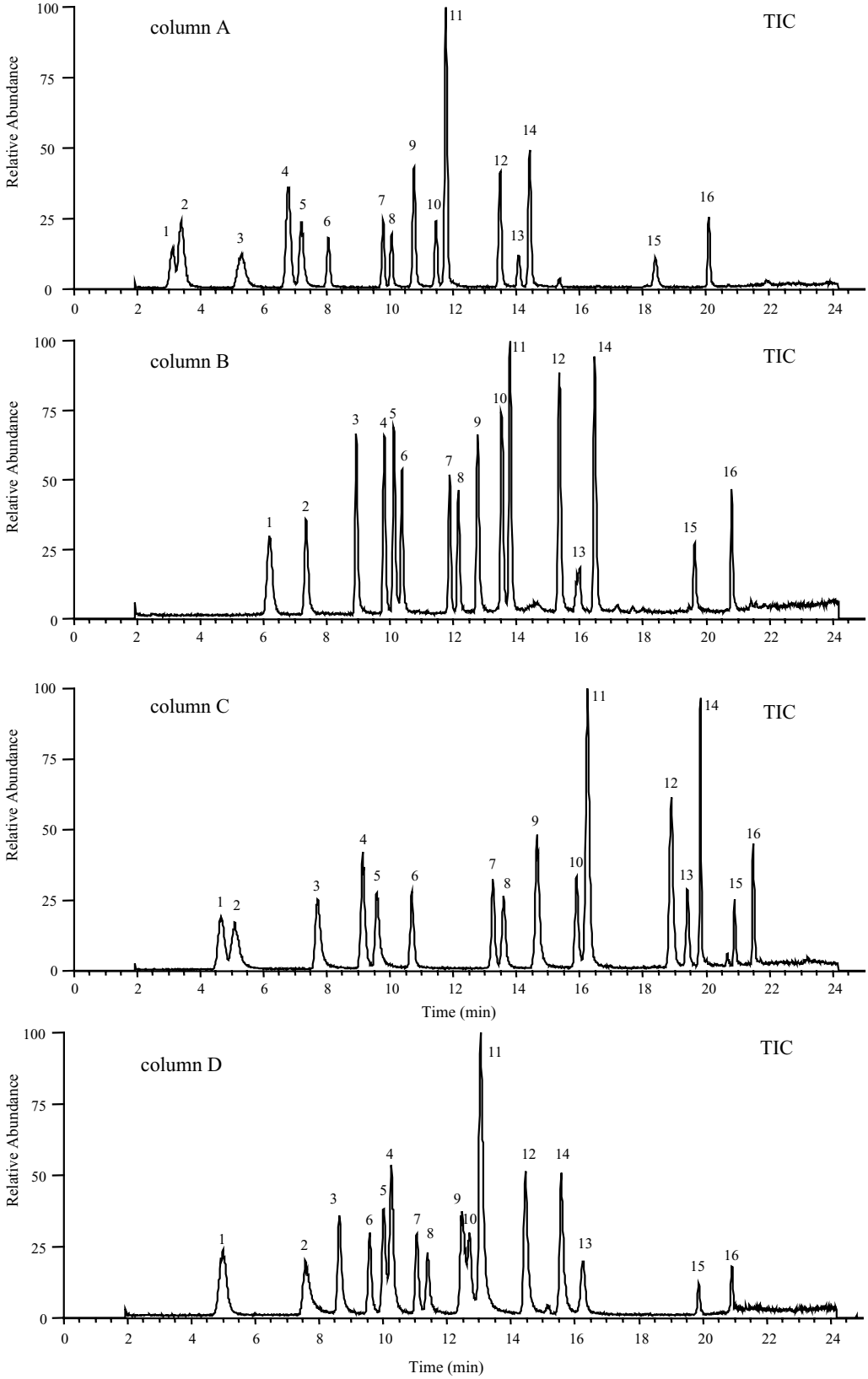

Fig. 2. LC–MS(IT) chromatograms (total ion chromatogram, TIC) of 16 HAs with four columns (optimised separation conditions c.f. Section 2), column A: Symmetry® C8, column B: TSK Gel® Semi-Micro ODS-80TS, column C: Synergi™ Max-RP and column D: Purospher® Star RP-C18. Standard solution of HAs: 0.4 μg g^{-1}, injection volume: 5 μl. Analytes—1: DMIP; 2: Glu-P-2; 3: IQ; 4: Glu-P-1; 5: MeIQ; 6: MeIQx; 7: 7,8-DiMeIQx; 8: 4,8-DiMeIQx; 9: Norharman; 10: TriMeIQx; 11: Harman; 12: Trp-P-2; 13: PhIP; 14: Trp-P-1; 15: AαC and 16: MeAαC.

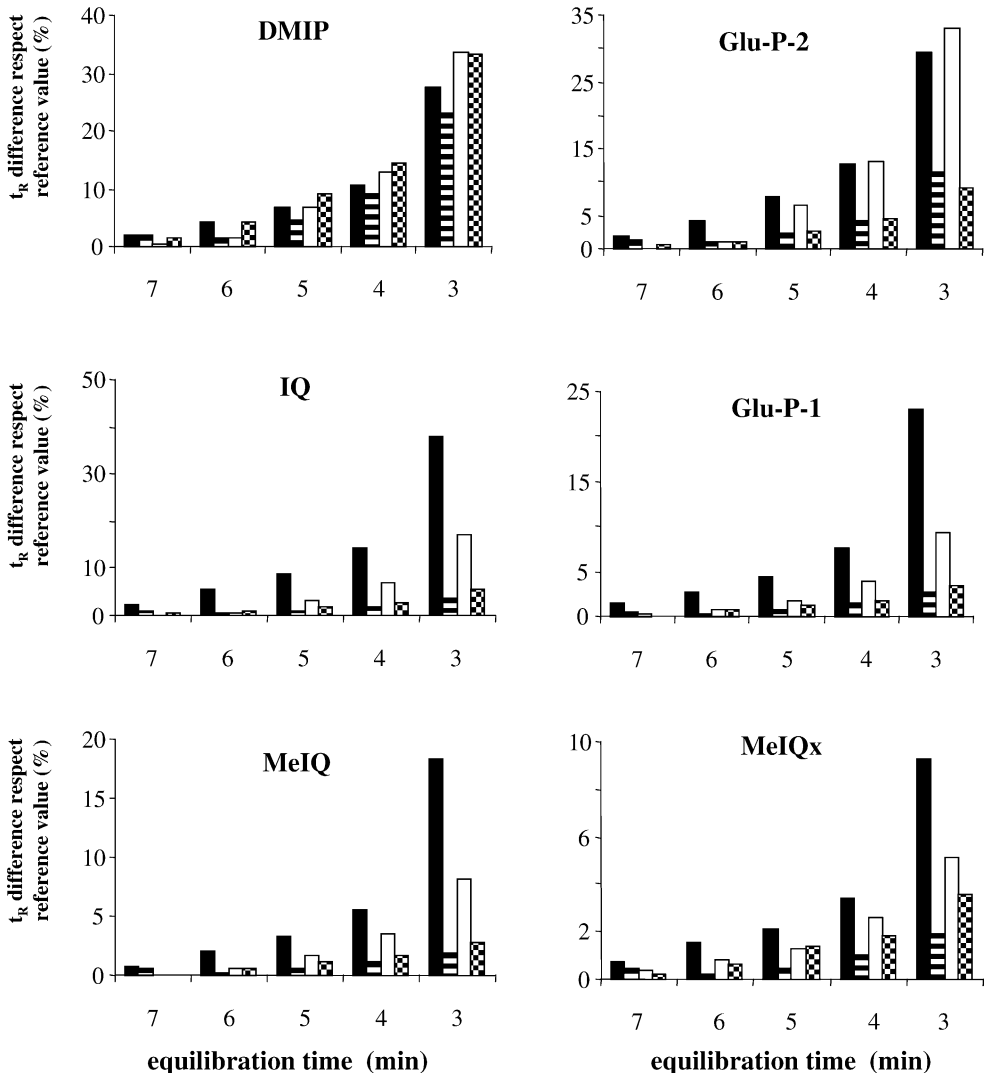

Fig. 3. Bar charts showing the normalised differences of retention times respect to the reference value (t_R at an equilibration time of 8 min), when equilibration time was varied from 7 to 3 min. (■) Symmetry, (▤) TSK Gel, (□) Synergi, (▦) Purospher.

Table 7
Limits of detection (pg injected) of HAs using the best four columns at the optimal conditions (see Section 2)

HAs	Symmetry	TSK Gel	Synergi	Purospher
DMIP	13	13	13	17
Glu-P-2	6	3	13	7
IQ	11	2	11	22
MeIQx	6	6	5	7
MeIQ	5	5	8	5
Glu-P-1	5	2	4	17
7,8-DiMeIQx	1	3	2	9
4,8-DiMeIQx	2	5	8	11
Norharman	3	3	5	6
Harman	2	3	4	9
Trp-P-2	4	4	6	8
Trp-P-1	2	3	2	3
PhIP	4	5	4	5
AαC	12	8	6	17
MeAαC	12	8	8	22

centration levels were injected. Purospher column gave the highest values for most of the compounds. Among the other three columns there were no significant differences, although the TSK Gel gave slightly lower LODs, mainly due to the narrow and symmetrical peaks obtained.

From these studies we can conclude that the TSK Gel® Semi-Micro ODS-80TS column at the working conditions indicated in Section 2 have been selected. This column provided the best separation for HAs determination by LC–ESI-MS in combination with the best values of peak height, peak symmetry, and number of theoretical plates. Moreover, a low equilibration time was needed.

3.2. Performance of the LC–ESI-MS method

To check the method's performance using the TSK Gel column, quality parameters such as limits of detection, linearity range and short-term precision were studied. Limits of detection are given in Table 7 and ranged from 2 to 13 pg

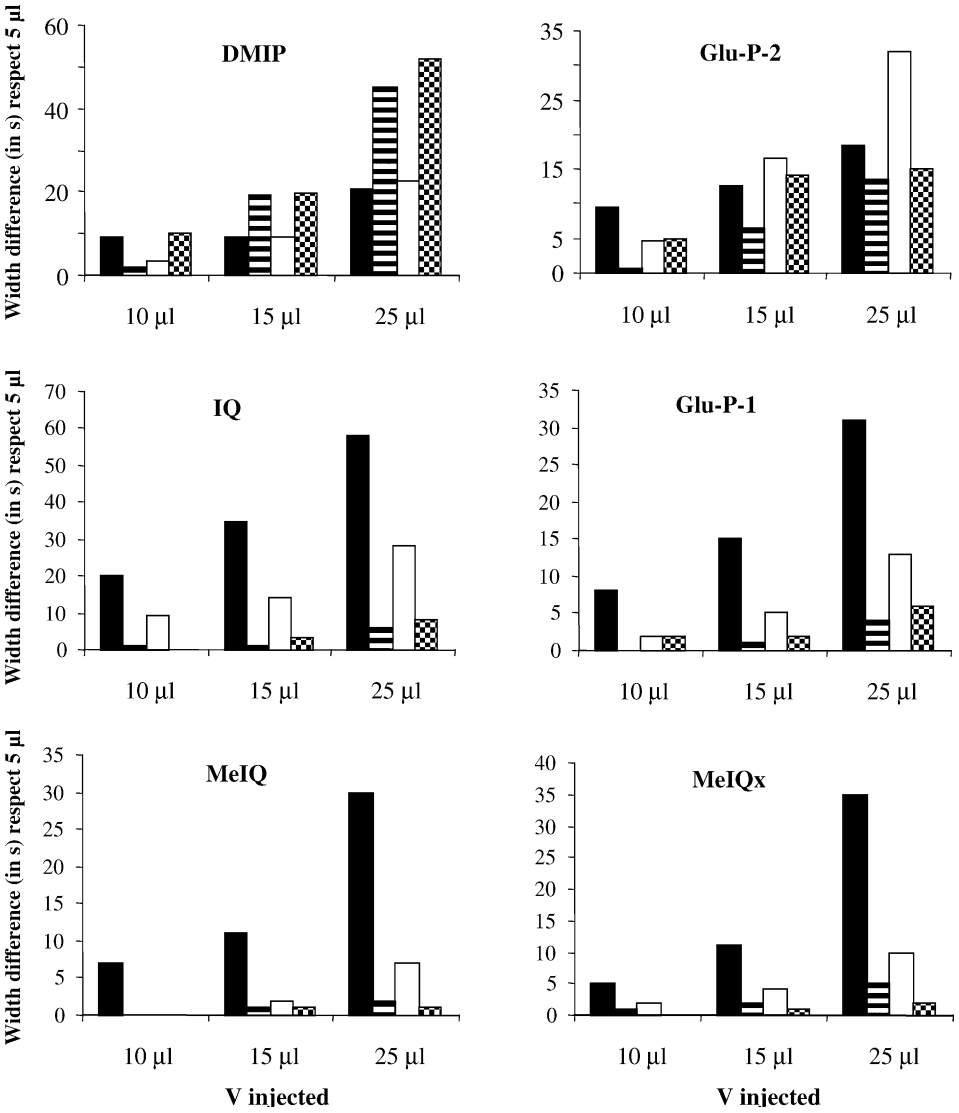

Fig. 4. Bar charts showing the peak width difference (in seconds) when the injected volume was increased from 5 to 10–25 µl. (■) Symmetry, (▤) TSK Gel, (□) Synergi, (▩) Purospher.

injected. The highest LOD corresponds to DMIP because it is the widest peak in the chromatogram.

The linearity range was determined, and was found to be 0.02–1.0 $\mu g\,g^{-1}$ for all compounds. Calibration curves for the amines using TriMeIQx as internal standard (0.2 $\mu g\,g^{-1}$) were established at six concentration levels in the linearity range. The curves were fitted to a linear function, which gave regression coefficients better than 0.99 for all the analytes. To determine repeatability or short-term precision, six replicate injections of a HAs standard solution at a concentration of 0.15 $\mu g\,g^{-1}$ were carried out. Relative standard deviations (R.S.D.) of the concentration values of the six replicates were calculated for all analytes, and they were ranged from 1.8 to 6.5%.

In order to evaluate the applicability of the LC–ESI-MS method to the analysis of real samples, two lyophilised

Table 8
Results of quantification of sample A (~70 $ng\,g^{-1}$) and B (~10 $ng\,g^{-1}$) using full scan and product ion scan modes, respectively

HAs	Sample A ($ng\,g^{-1}$ meat extract)	Sample B ($ng\,g^{-1}$ meat extract)
DMIP	66.9 ± 4.2	12.4 ± 1.7
IQ	70.3 ± 5.6	10.5 ± 1.3
MeIQx	76.2 ± 5.8	11.0 ± 1.8
MeIQ	77.5 ± 5.0	9.9 ± 1.7
4,8-DiMeIQx	77.4 ± 4.6	11.5 ± 1.8
Trp-P-2	63.2 ± 5.0	9.3 ± 3.0
Trp-P-1	69.8 ± 4.8	8.5 ± 1.6
PhIP	70.1 ± 5.6	9.0 ± 2.5
AαC	75.4 ± 4.2	7.3 ± 2.6
MeAαC	70.9 ± 5.5	9.2 ± 1.7

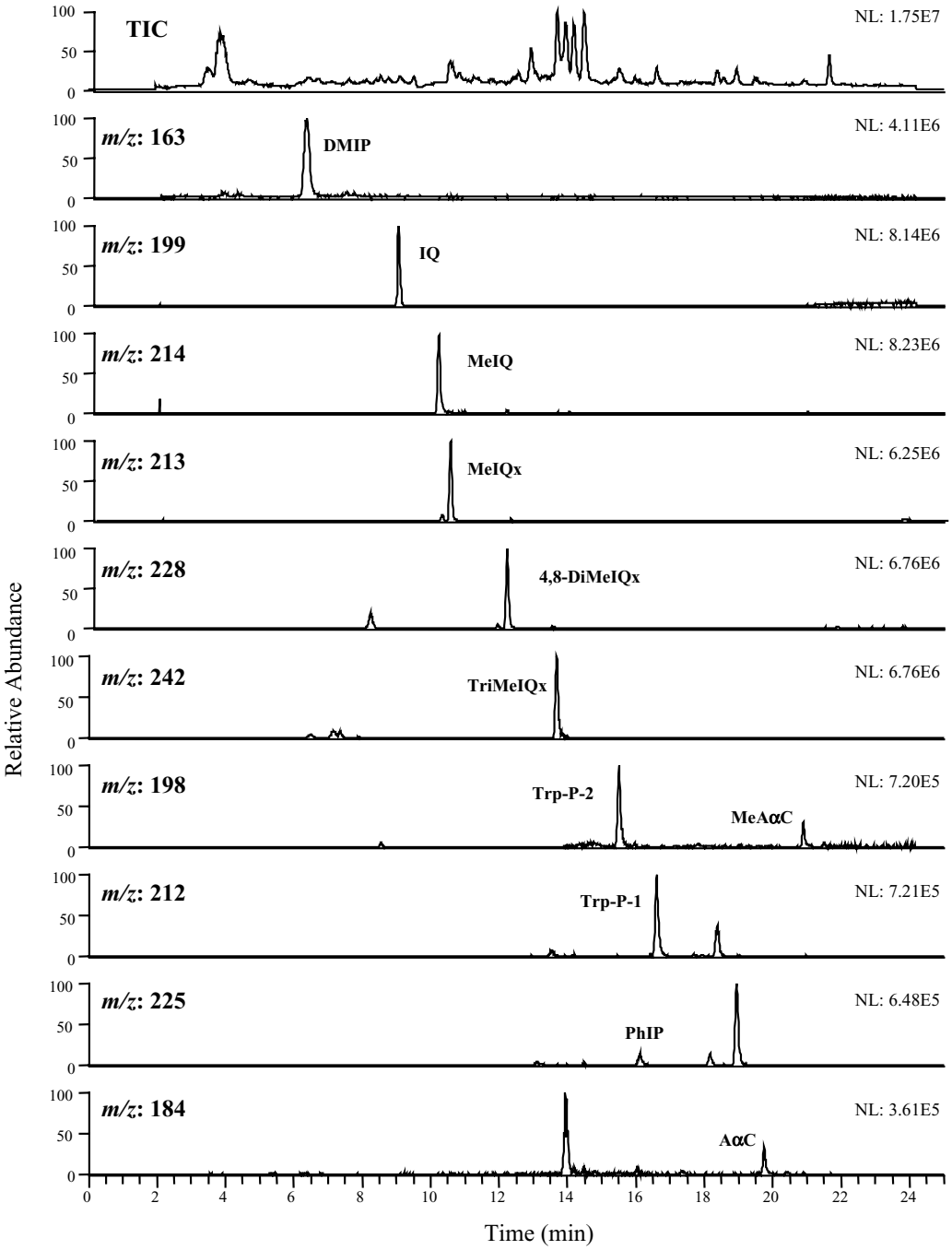

Fig. 5. LC–MS(IT) chromatogram corresponding to sample A in full scan mode.

meat extracts that contain different concentration levels of HAs (sample A and B) were analysed. For each extract, six individual, fully independent analyses were carried out, on three different days. The clean-up procedure used is described in Section 2.5. Quantification of HAs was performed by standard addition method, at four spiking levels around 50, 100, 150 and 200%. TriMeIQx was used as internal standard in order to correct signal variations on ESI-MS.

Results of quantification of HAs in sample A in full scan mode are given in Table 8, where it can be observed that good precision (<8% R.S.D.) was obtained for all the studied compounds. As an example, a LC–MS chromatogram of an extract is shown in Fig. 5. In order to improve sensitivity, for sample B that contains a lower concentration of HAs ($\sim$10 ng g^{-1}), the acquisition was carried out in product ion scan mode instead of full scan mode. LODs and short-term precision using standard solutions were very

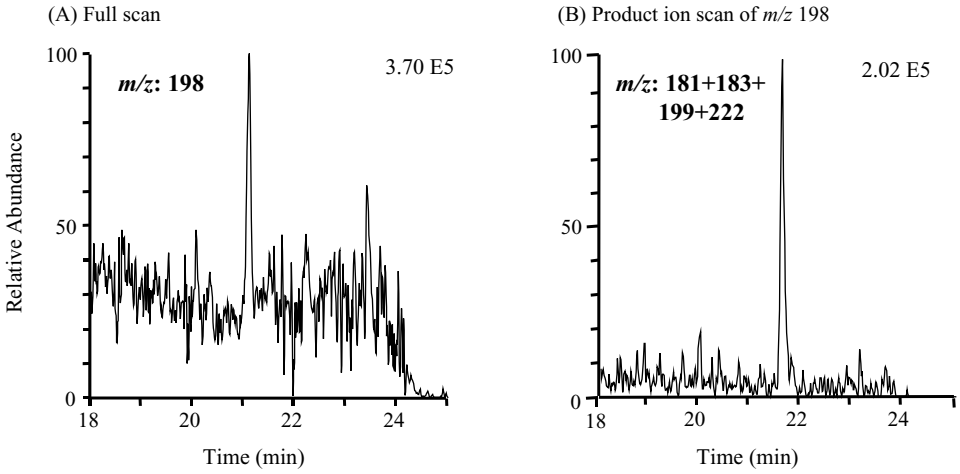

Fig. 6. Ion chromatograms of MeAαC in sample B: (A) full scan LC-MS(IT), (B) product ion scan LC–MS/MS(IT).

similar to those achieved in full scan mode (<16 pg injected and <7.5% R.S.D., respectively). However, in this complex sample LODs decreased over six times in MS/MS compared to MS mode. As an example, Fig. 6 shows the LC-MS chromatograms of MeAαC acquired in both acquisition modes. As can be seen, the improvement of the signal is significant when using the product ion scan mode, due to its higher selectivity in complex samples. Results of quantification of HAs in sample B using product ion scan mode are given in Table 8. A decrease in the precision values (<18% R.S.D.) in sample B due to the lower concentration of HAs was observed.

4. Conclusions

In this work, a comparison of the performance of six narrow-bore reversed-phase liquid chromatography columns has been carried out in order to propose a chromatographic column for the separation of 16 HAs using LC–ESI-MS. The strong adsorption of AαC and MeAαC on the Discovery column, giving very wide peaks that prevent their detection, forced us to reject it for further studies. Moreover, the Zorbax column was discarded because of the low values of peak symmetry and peak height, probably due to the absence of endcapped treatment in its stationary phase. Among the rest of columns, the TSK Gel® Semi-Micro ODS-80TS was selected because it provided the shortest equilibration time and permitted the highest injection volume and the lowest limits of detection. Quality parameters in full scan and in product ion scan modes were established using this column, obtaining low LODs (<16 pg injected) and short-term precision values <6.5% for full scan and <7.5% for product ion scan mode. These methodologies were used for the determination of HAs in two lyophilised meat extracts of different concentration, showing the applicability of this column for the analysis of these compounds in complex food samples.

Acknowledgements

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