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Analysis of heterocyclic aromatic amines in foods by gas chromatography–mass spectrometry as their *tert.*-butyldimethylsilyl derivatives

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Abstract

A derivatization method for the analysis of 12 heterocyclic aromatic amines (HAs) in food, by gas chromatography–electron impact mass spectrometry, was developed. The amines are derivatized in a one-step reaction with *N*-methyl-*N*-(*tert.*-butyldimethylsilyl)trifluoroacetamide. The derivatives are characterized by easy-to-interpret mass spectra due to the prominent ion $[M\text{-}57]^+$ by loss of a *tert.*-butyldimethylsilyl group, allowing quantification in the selected-ion monitoring mode at the picogram level. The effect of temperature, time, and reagents on the formation of the derivatives was monitored in detail. Quality parameters were evaluated in the optimum working conditions. This derivatization method is not applicable to the pyridoimidazoles Glu-P-1 and Glu-P-2 and to the β-carboline harman due to incompletely derivatization. The instability of the imidazolquinoline and imidazoquinoxaline derivatives, requiring their injection on the same working day, is a further drawback. This simple, rapid and accurate derivatization procedure is suitable for routine analysis, as illustrated by the analysis of some common foods.

Keywords: Food analysis; Derivatization, GC; Coffee; Amines, heterocyclic aromatic; Methyl-*tert.*-butyldimethylsilyltrifluoroacetamide

1. Introduction

Several heterocyclic aromatic amines (HAs) are potent mutagenic compounds to which humans are regularly exposed through diet and ambient environments. Some HAs present higher mutagenic activity in the Ames/*Salmonella* assay than typical mutagens/carcinogens, such as aflatoxin B_1 and benzo[*a*]pyrene [1,2]. These compounds are formed in thermally treated protein-rich foods, at the ng/g level, but are also detected in several other environments, including cooking fumes, cigarette smoke, air, rain, and river water [3]. More than twenty HAs have been isolated from several food samples and model systems, and some of their structures determined. Although suspected [4], the presence of HAs on roasted coffee was not yet confirmed [5,6].

The major pathway for the metabolic activation of HAs starts with the hydroxylation of the exocyclic amino group, catalyzed mainly by cytochrome P4501A2, followed by acetylation or sulphation to form direct-acting reactive mutagens that alter DNA and genome. Their carcinogenic potential in humans has not been established, but the polymorphic variations in these metabolizing enzymes could modulate individual susceptibility to the intake of HAs [2,7–10].

Due to the high complexity of most food matrices and the low level of concentration of the HAs, sensitive and selective analytical methodologies are required. For the analysis of these compounds, solid-phase purification techniques are usually used [11–15], followed mainly by chromatographic techniques such as high-performance liquid chromatography (HPLC), gas chromatography (GC), or capillary electrophoresis [3,16,17]. Actually the leading technique for HAs analysis is liquid chromatography–mass spectrometry (LC–MS), but is expensive and may not be available in most laboratories. GC–MS, on the other hand, is becoming more popular in quality control laboratories mainly due to its wide range of applications and relative cost when compared with the above-mentioned LC–MS systems.

However, GC methods are only possible with less polar derivatives of these mutagenic amines, otherwise they adsorb in the GC column and elute as broad, tailing peaks. Several

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derivatization reactions are described, but only for some HAs [16].

The non-polar HAs (Trp-P-1, Trp-P-2, AαC, MeAαC, harman and norharman) (see Table 1 for nomenclature) were determined in meat products by GC–MS without derivatization [18], but contamination of the ion source through the deposition of non-volatile material was observed. Generally, the commonly used derivatization reactions for GC fit into one of three categories: alkylation, acylation, and silylation. Since 1988 Murray's team has been working with the alkylated di(3,5-bistrifluoromethylbenzyl) derivatives (3,5-bisTFMBB) for the quantification of three HAs: MeIQx, 4,8-DiMeIQx and PhIP, by employing negative chemical ionization GC–MS [19–21]. Following these works, Tikkanen et al. [22] also used the 3,5-bisTFMBB derivatives to analyze the same three HAs in several foods and Vainiotalo et al. [23] analyzed cooking fumes by electron impact ionization (EI) and selected ion monitoring (SIM). More recently, Richling et al. [24] extended this methodology to 10HAs, also as their 3,5-bisTFMBB derivatives, and analyzed them by GC–EI-MS. Among the several derivatization reagents tested for HAs by Katatoka et al. [25], the *N*-dimethylaminomethylene derivatives formed by alkylation with *N*,*N*-dimethylformamide dimethyl acetal (DMF-DMA) gave good results for 10 HAs with nitrogen–phosphorus detection (GC-NPD) [25,26]. Traditional acylation with acid anhydrides usually yields derivatives with very poor GC properties [22,25] but Reistad et al. [27] tested the acylation with heptafluorobutyric acid anhydride followed by methylation of the acidic amide proton with diazomethane with good results for three HAs. No further progress in the GC–MS analysis of HAs has been reported to date.

Silylation is probably the most versatile GC derivatization technique. It involves the displacement of an acidic hydrogen on the compound with an silyl group, by nucleophilic attack [28]. Among these, the sterically crowed *tert.*-butyldimethylsilyl (TBDMS) group is presently the most versatile for GC analysis. When compared with other silylating reagents, such as the popular trimethylsilyl group, the TBDMS group is up to 10,000 times more stable to hydrolysis, presents enhanced reactivity towards amines, and origins by-products that provide direct injection of the reaction mixture for GC analysis [28,29]. Besides improving volatility and stability, the introduction of the silyl group can also serve to enhance mass spectrometric properties. The mass spectra are easy-to-interpret, and the characteristic high-mass ions, derived from the molecular ions, can be used in trace analysis in the SIM mode [28].

The objective of this work was to study the derivatization of several HAs to their TBDMS derivatives, in a one-step derivatization procedure, with *N*-methyl-*N*-*tert.*-butyldimethylsilyltrifluoroacetamide (MTBSTFA) and their GC–EI-MS analysis with SIM quantification.

2. Experimental

2.1. Chemicals

The HAs used in this study (Table 1) were purchased from Toronto Research Chemicals (Downsview, Canada). Harman (1-methyl-9*H*-pyrido[3,4-b]indole) was from Aldrich (Milwaukee, WI, USA) and norharman (9*H*-pyrido[4,3-b]indole) was from Sigma (Steinheim, Germany). Stock standard solutions of 100 μg/ml in methanol were prepared and used for further dilution. 2-amino-3,4,7,8-tetramethylimidazo[4,5-f]quinoxaline (TriMeIQx) (Toronto Research Chemicals) was used as internal standard (IS) (1.0 $\mu g\,ml^{-1}$ methanolic solution). MTBSTFA, with 1% *tert.*-butyldimethylchlorosilane (TBDMCS) as a catalyst was obtained from Aldrich.

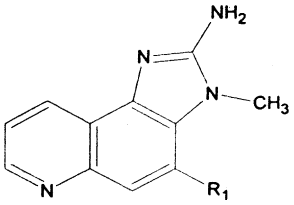
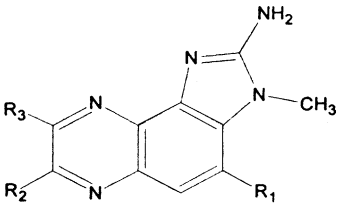
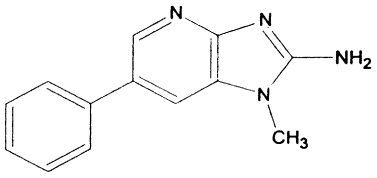
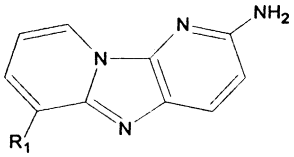
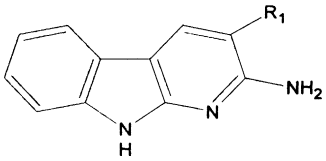
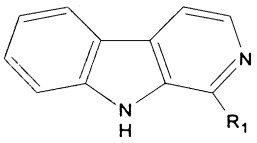
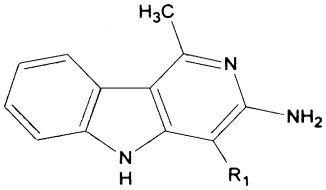
Diatomaceous earth extraction cartridges (Extrelut NT, 20 ml) were provided by Merck (Darmstadt, Germany). Bond Elut propylsulfonyl silica gel (PRS; 500 mg) and octadecylsilane (C_{18}; 100 mg) cartridges were from Varian (Harbor City, USA). The PRS cartridges were preconditioned with dichloromethane (7 ml) and the C_{18} cartridges with methanol and water (5 ml each). Amberlite XAD-2, 20–60 mesh (Supelco, Bellefonte, PA, USA) was conditioned with methanol, followed by acetone and finally water.

2.2. Sample analysis

Food samples, namely fried bacon and meat bouillon concentrates, as well as a sample of commercial roasted ground coffee, were extracted in triplicate according with Gross and Grüter [11], with some modifications implemented by Toribio et al. [32]. Briefly, a 3 g food sample was homogenized in 1 M NaOH (12 ml) and the suspension left for 3 h with occasionally shaking. For coffee, an aqueous extract was prepared (50 g of coffee/500 ml water boiled for 1 min), filtered, and 10.0 ml aliquots were analyzed, after being adjusted to the same 1 M NaOH concentration. The alkaline solutions (food samples and coffee) were mixed with Extrelut NT refill material (15 g) and transferred to an empty Extrelut column coupled to a preconditioned PRS cartridge. To extract the analytes from diatomaceous earth, 75 ml of dichloromethane were passed through the tandem. The PRS cartridge was then dried and successively rinsed with 15 ml of methanol–water (4:6, v/v) and 2 ml water. After being coupled to a preconditioned C_{18} cartridge, the compounds were eluted with 0.5 M ammonia acetate (pH 8.5, 20 ml). The later cartridge was rinsed with water (5 ml) and the HAs desorbed with 2 ml × 0.8 ml methanol–ammonia (9:1, v/v) into PTFE/neoprene-lined silanized screw-capped vials (Supelco). The solvent was gently evaporated under nitrogen and the residue redissolved with 100 μl of the internal standard solution in methanol.

A Supelco Visiprep (Supelco) was used to manipulate the solid-phase extraction cartridges. Evaporations were per-

Table 1
Compounds used in this study

Chemical name	Abbreviation	General structure	R1	R2	R3
Imidazoquinolines					
2-Amino-3-methylimidazo[4,5-f]quinoline	IQ		H		
2-Amino-3,4-dimethylimidazo[4,5-f]quinoline	MeIQ		Me		
Imidazoquinoxalines					
2-Amino-3-methylimidazo[4,5-f]quinoxaline	IQx		H	H	H
2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline	MeIQx		H	H	Me
2-Amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline	4,8-DiMeIQx		Me	H	Me
2-Amino-3,7,8-trimethylimidazo[4,5-f]quinoxaline	7,8-DiMeIQx		H	Me	Me
2-Amino-3,4,7,8-tetramethylimidazo[4,5-f]quinoxaline	TriMeIQx (IS)		Me	Me	Me
Imidazopyridine					
2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine	PhIP				
Pyridoimidazole					
2-Amino-6-methyldipyrido[1,2-a:3′,2′-d]imidazole	Glu-P-1		Me		
2-Amino-dipyrido[1,2-a:3′,2′-d]imidazole	Glu-P-2		H		
Pyridoindole					
α-Carbolines					
2-Amino-3-methyl-9H-pyrido[2,3-b]indole	MeAαC		Me		
2-Amino-9H-pyrido[2,3-b]indole	AαC		H		
β-Carbolines					
1-Methyl-9H-pyrido[3,4-b]indole	Harman		Me		
9H-Pyrido[3,4-b]indole	Norharman		H		
γ-Carbolines					
3-Amino-1,4-dimethyl-5H-pyrido[4,3-b]indole	Trp-P-1		Me		
3-Amino-1-methyl-5H-pyrido[4,3-b]indole	Trp-P-2		H		

formed using an evaporating unit coupled to an "Reacti-therm" heating block (Pierce, Rochford, IL, USA).

2.3. Derivatization procedure for gas chromatography

The solvent from the standard solutions (corresponding to 0.1–50 ng of each HAs) was evaporated under a gentle stream of nitrogen. The residues, from both standards and samples, were dried overnight over phosphorous pentoxide in vacuo. Then, 25 μl of MTBSTFA (with 1% TBDMCS) and 25 μl of anhydrous acetonitrile were added to the dry residue, the mixture sonicated for about 1 min and heated at 90 °C for 90 min on "Reacti-therm" heating block (Pierce). After cooling, 1 μl aliquots were directly injected into the gas chromatograph.

2.4. Gas chromatography–mass spectrometry

GC–MS analyses were performed on a Hewlett-Packard gas chromatograph 6890 equipped with an electronically controlled split/splitless injection port and interfaced to a MSD-5973N mass selective detector (Little Falls, DE, USA). The gas chromatograph was equipped with a DB-5MS fused-silica capillary column (30 m × 0.25 mm, 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA), coupled directly to the mass detector.

The injection was performed at 270 °C in the pulsed splitless mode. Purge-off time was set at 0.7 min. Helium was used as carrier gas, with a constant flow of 1 ml/min. An 18 min two-step temperature gradient was used: 150 °C–10 °C/min–270 °C (1 min)–20 °C/min–290 °C (4 min). The transfer line temperature was 280 °C. Calibration of the GC–MS was performed weekly using the autotune function of the mass selective detector. Electron impact mass spectra were measured at acceleration energy of 70 eV. The detector was switched off in the initial 8 min. Data acquisition was performed in the full-scan mode during the optimisation of the method (mass range initially at 40–480 u, later only 150–400 u). SIM mode based on the ions $[M\text{-}57]^+$ (quantification) and $[M\text{-}15]^+$ (confirmation) was used, monitoring different ions during the chromatographic run, with a dwell time of 30 ms.

The overall system was controlled by Agilent MS Chemstation G2578A software.

2.5. HPLC

The HPLC analyses were performed by means of a Jasco HPLC system (Japan) equipped with an AS-950 autosampler, a PU-1580 quaternary pump, a DG-1580-54-4-line degasser, a MD-950 diode array detector (DAD) and a FP-920 programmable fluorescence detector, connected in series. The amines were separated at ambient temperature using an TSK-gel ODS 80T column (5 μm, 25.0 mm × 4.6 mm i.d.) (TosoHaas, Germany) and a Supelguard LC-8-DB precolumn (Supelco). The mobile phase was a gradient of: 0.01 M triethylamine (pH 3.2) (A); 0.01 M triethylamine (pH 3.6) (B); acetonitrile (C), programmed according with Gross and Grüter [11] with minor modifications.

3. Results and discussion

3.1. Optimisation of the derivatization conditions

The amino group is not particularly reactive to silylating reagents, and its conversion into a silyl derivative through nucleophilic attack is difficult. Therefore, 1% TBDMCS is usually used as catalyst [28]. The standard or sample residues were always dried under a gentle stream of dry nitrogen and kept overnight over phosphorous pentoxide in vacuum [30] in order to prevent the presence of water. Anhydrous acetonitrile was also used as solvent in the derivatization procedure [29]. Since the amines are particularly prone to adsorption on the glassware surface, silanised vials were used.

Several experiments were conducted in order to find the best conditions for the complete and accurate derivatization of the HAs. For this purpose time and temperature combinations as well as the MTBSTFA amount were monitored. After a derivatization protocol of 60 min, ranging from room temperature to 150 °C, it was verified that the reaction yield was proportional to the temperature used. For temperatures higher than 100 °C the results were inconclusive due to the screw-cup liner degradation and/or losses by evaporation. Also, for temperatures as low as 70 °C, about 150 min were necessary to achieve complete derivatization of most HAs. Therefore, we have studied in detail the reaction at 90 °C finding that 90 min were sufficient for most HAs complete derivatization. Nevertheless, and for all the temperatures tested, the derivatization of Glu-P-1 and Glu-P-2 was incomplete (1–10% effective derivatization) and a tailed peak, corresponding to the underivatized compound was observed. Harman was not derivatized under these conditions, though no structural justification was found, when compared to the complete derivatization of norharman at low temperature. Also, AαC and MeAαC complete derivatization was difficult, requiring about 6 h, at 90 °C. Nevertheless, their derivatization was reproducible presenting good quality parameters as will be discussed later on. The amount of MTSTFA (with 1% TBDMCS as catalyst), necessary for the derivatization in the tested range (about 750 ng total compounds), was set to 25 μl.

The derivatives were kept at −18 °C and periodically injected over a 3-day period (data not shown) in order to monitor their stabilities. The carbolines norharman, AαC and MeAαC were relatively stable (93–99% after 24 h), as was PhIP. The imidazoquinolines and imidazoquinoxalines, were greatly reduced (62–90% after 24 h), including the IS. These derivatives should, therefore, be injected on the same working day.

Table 2
Main ions in the EI mass spectra[a] of the heterocyclic amine derivatives

Heterocyclic amine	Mr	*m/z* (Rel. Ab.%)			
		$[M]^+$ confirmation ion	$[M\text{-}15]^+$	$[M\text{-}57]^+$ quantitative ion	$[M\text{-}71]^+$
Norharman	168	282 (31)	267 (2)	225 (100)	211 (7)
AαC	183	297 (31)	282 (2)	240 (100)	226 (7)
MeAαC	197	311 (37)	296 (2)	254 (100)	240 (7)
IQ	198	312 (28)	297 (3)	255 (100)	241 (8)
IQx	199	313 (13)	298 (4)	256 (100)	242 (13)
Trp-P-1	211	325 (8)	310 (2)	268 (100)	254 (7)
Trp-P-2	197	311 (8)	296 (2)	254 (100)	240 (8)
MeIQx	213	327 (14)	312 (4)	270 (100)	256 (14)
7,8-DiMeIQx	227	341 (16)	326 (4)	284 (100)	270 (16)
MeIQ	212	326 (31)	311 (4)	269 (100)	255 (9)
4,8-DiMeIQx	227	341 (16)	326 (4)	284 (100)	270 (16)
PhIP	224	338 (13)	323 (3)	281 (100)	267 (9)
4,7,8-TriMeIQx (IS)	241	355 (16)	340 (4)	298 (100)	284 (21)

[a] Mass range 150–400 u.

Table 3
Ions monitored in the SIM mode

Time (min)	*m/z*
8.0–11.6	225, 240, 254, 282, 297, 311 (for norharman, AαC, and MeAαC)
11.6–13.9	254, 255, 256, 268, 270, 311, 312, 313, 325, 327 (for IQ, IQx, Trp-P-1, Trp-P-2, and MeIQx)
13.9–18.0	269, 281, 284, 298, 326, 338, 341, (355) (for 7,8-DiMeIQx, MeIQ, 4,8-DiMeIQx, PhIP, and TriMeIQx)

3.2. Spectra analysis

The identities of the derivatives were established by EI-MS. The mass spectra were always characterized by an intense $[M\text{-}57]^+$ ions, originated directly from the molecular ion by loss of a *tert.*-butyl group $[C(CH_3)_3]^+$, and used for quantification purposes [28,30]. The $[M\text{-}57]^+$ ion is also indicative of the molecular mass. A relatively intense molecular ion $[M]^+$ is always present and can be used for confirmation purposes. Other ions with low relative intensities, such as $[M\text{-}15]^+$ or $[M\text{-}71]^+$, are also characteristic. See Table 2 for detail. The characteristics ion of the trimethylsilyl group, *m/z* 73 $[(CH_3)_3Si]^+$, and the ion of the *tert.*-butyl group, *m/z* 57 $[C(CH_3)_3]^+$, were always observed, as expected [31]. The mass spectrum of IQ is presented in Fig. 1, as an example.

The internal standard molecular ion (*m/z* 355), used for confirmation purposes in the SIM mode as described in Table 3, can be avoided in the cases where this *m/z* is a high background noise. This is usually originated from desorption of both silicone polymers (decamethylciclopentasiloxane) from the GC septa, or siloxane coatings of the GC capillary columns.

The size of the TBDMS group usually prevents multiple silylation of the nitrogen atom [31] and only a single peak is observed for each compound. The regularity of the mass spectra patterns of the TBDMS derivatives allows direct identification by the appropriate ions $[M]^+$ and

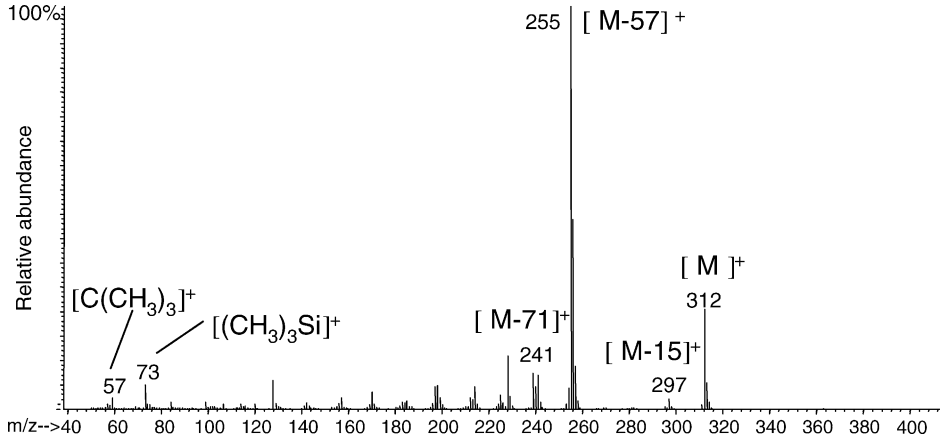


Fig. 1. Mass spectrum of IQ (2-amino-3-methylimidazo[4,5-f]quinoline), as its *tert.*-butyldimethylsilyl derivative.

Table 4
Quality parameters of the HAs derivatives

Heterocyclic amine	t_r (min)	Day-to-day precision (R.S.D.%)		Linearity range (ng)	Correlation coefficient (r^2)	Detection limits	
		t_r	Concentration			Standard solution (pg injected)	Food samples (ng g^{-1})
Norharman	9.9	0.2	5.0	0.1–50.0	0.9996	0.5	0.02
AαC	10.7	0.4	6.9	0.1–10.0	0.9964	1.2	0.05
MeAαC	11.4	0.6	9.2	0.5–10.0	0.9994	2.7	0.07
IQ	12.9	1.6	8.8	0.5–50.0	0.9993	3.3	0.09
IQx	13.3	1.4	12.7	0.5–50.0	0.9985	1.5	0.05
Trp-P-1	13.3	1.2	9.7	1.5–50.0	0.9969	13.4	0.35
Trp-P-2	13.4	1.4	15.0	1.0–50.0	0.9993	11.3	0.29
MeIQx	13.5	1.2	10.2	0.5–50.0	0.9994	1.9	0.05
7,8-DiMeIQx	14.1	0.9	11.8	0.5–50.0	0.9996	2.2	0.06
MeIQ	14.2	0.9	7.1	0.5–50.0	0.9991	3.4	0.09
4,8-DiMeIQx	14.5	1.6	12.6	0.5–50.0	0.9994	3.0	0.08
PhIP	16.1	0.8	20.7	0.5–50.0	0.9989	4.8	0.12

$[M\text{-}57]^+$. Those were the ions monitored in the SIM mode, as represented in Table 3. A detailed chromatogram from the GC–MS analysis of a standard solution with 12HAs analyzed under those conditions is shown in Fig. 2, obtained in the SIM mode The individual mass spectra of the TB-DMS derivatives, obtained in the full scan mode, are also represented.

3.3. Quality parameters

Precision, linearity and limits of detection were determined and are represented in Table 4. The derivatization reproducibility was evaluated over a 3-day period with standard solutions. Calibrations for the derivatized HAs were performed with amounts ranging from 0.1 to 50 ng, and were calculated from the ratio of the $[M\text{-}57]^+$ HA area to the $[M\text{-}57]^+$ area of the internal standard (m/z 198). The relative standard deviations of each HA's peak area ratios for all the calibration points, were 0.2–13.8% ($n = 3$).

In the SIM mode, the detection limits based on a signal-to-noise ratio of 3:1, ranged from 0.5 to 13.4 pg injected. For food samples, the detection limits were established by low-level spiking of reference standards (0.05–0.3 ng/g) and submitting to the entire protocol. For the HAs present in the sample the values were estimated from the calibration curve based on a signal-to-noise ratio of 10. The values varied from 0.02 to 0.4 ng/g, with Trp-P-1 and Trp-P-2 presenting the higher values. These values are comparable or improved when compared to those described in the literature, achieved either by GC–MS or HPLC-MS [17].

3.4. Application

After optimisation of the derivatization and chromatographic conditions, the method was tested with food samples, such as fried bacon and bouillon cubes.

A chromatogram corresponding to a fried bacon extract is given in Fig. 3. Norharman, IQ, Trp-P-1, MeIQ, 4,8-MeIQx and PhIP were positively identified, and its contents (Table 5) were in accordance with those described in the literature [18,20,33–37].

For the coffee extracts, norharman was the only compound whose presence was confirmed but accurate quantification was not possible due to the complexity of the coffee extract. Two complementary cleaning methods were tested. On the first, and according with Gross and Grüter [5], the initial aqueous coffee extract was adjusted to pH 7.5 and mixed with preconditioned XAD-2 (10 g). After being washed with water (100 ml), elution was performed with methanol (30 ml) and methanol–acetone (1:1, v/v; 20 ml). The extract was reduced to about 5 ml on a rotary evaporator; 5 ml of 3 M NaOH were added and the volume adjusted to 15 ml. The process followed the same procedure in experimental, being mixed with the Extrelut material. The second tentative cleaning step was

Table 5
Food samples analyzed by GC–MS

	Chicken bouillon cube (ng/g)	Fried bacon (27.4% fat) (ng/g)
Norharman	43.2 ± 5.1[a]	6.7 ± 2.7
AαC	nd[b]	nd
MeAαC	nd	nd
IQ	nd	1.6 ± 0.1
IQx	nd	nd
Trp-P-1	nd	0.6 ± 0.3
Trp-P-2	nd	nd
MeIQx	nd	nd
7,8-DiMeIQx	nd	nd
MeIQ	0.5 ± 0.2	2.8 ± 0.8
4,8-DiMeIQx	3.7 ± 0.7	1.1 ± 0.4
PhIP	nd	36.4 ± 11.3

[a] Mean ± standard deviation ($n = 3$).
[b] Not detected.

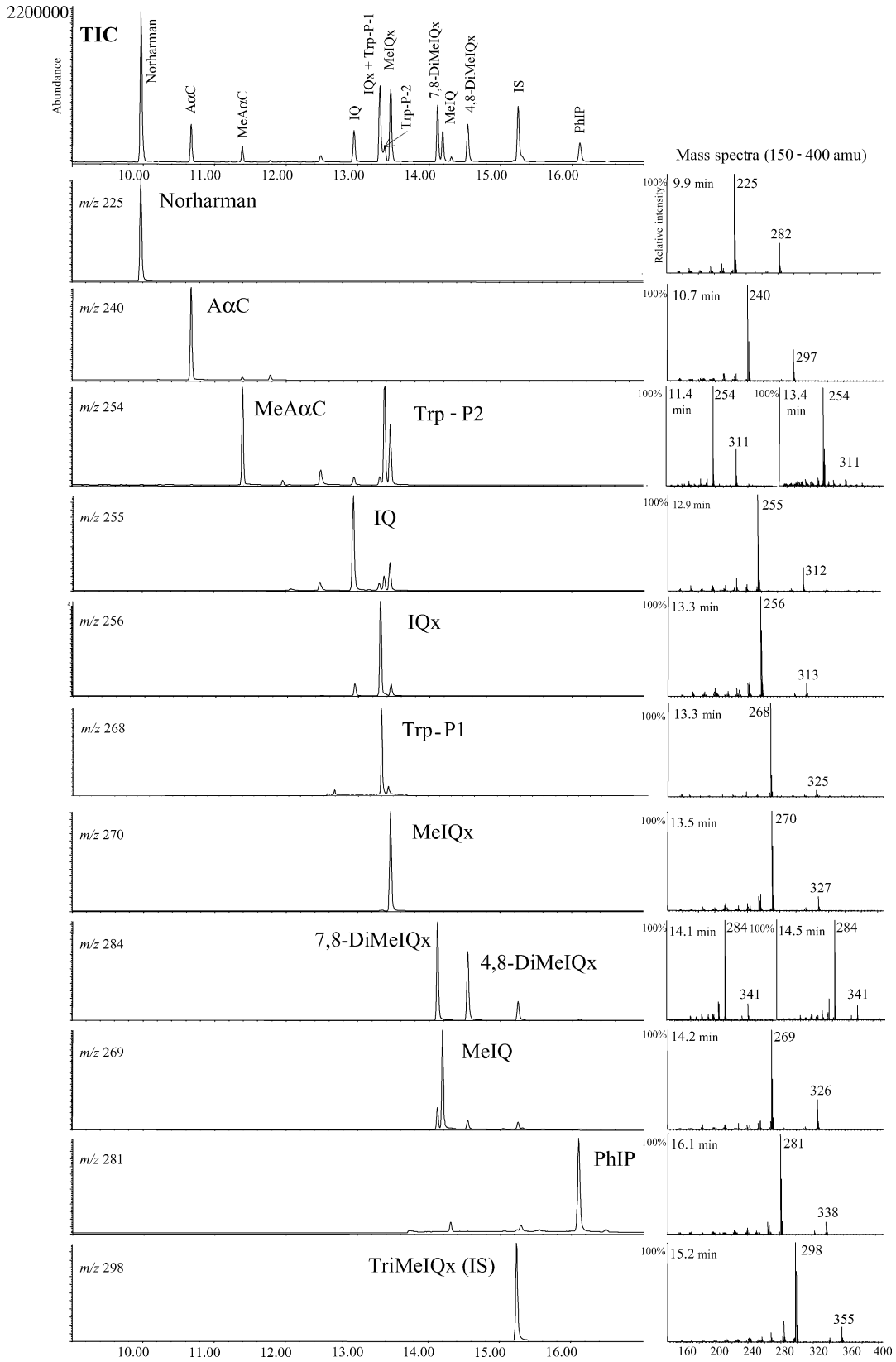


Fig. 2. Total ion compound (TIC) chromatogram of a standard solution with 12 HAs (25 ng each) with the internal standard (above), together with the individual chromatograms in the SIM mode. Each individual mass spectra, obtained in the full-mode, is represented on the right.

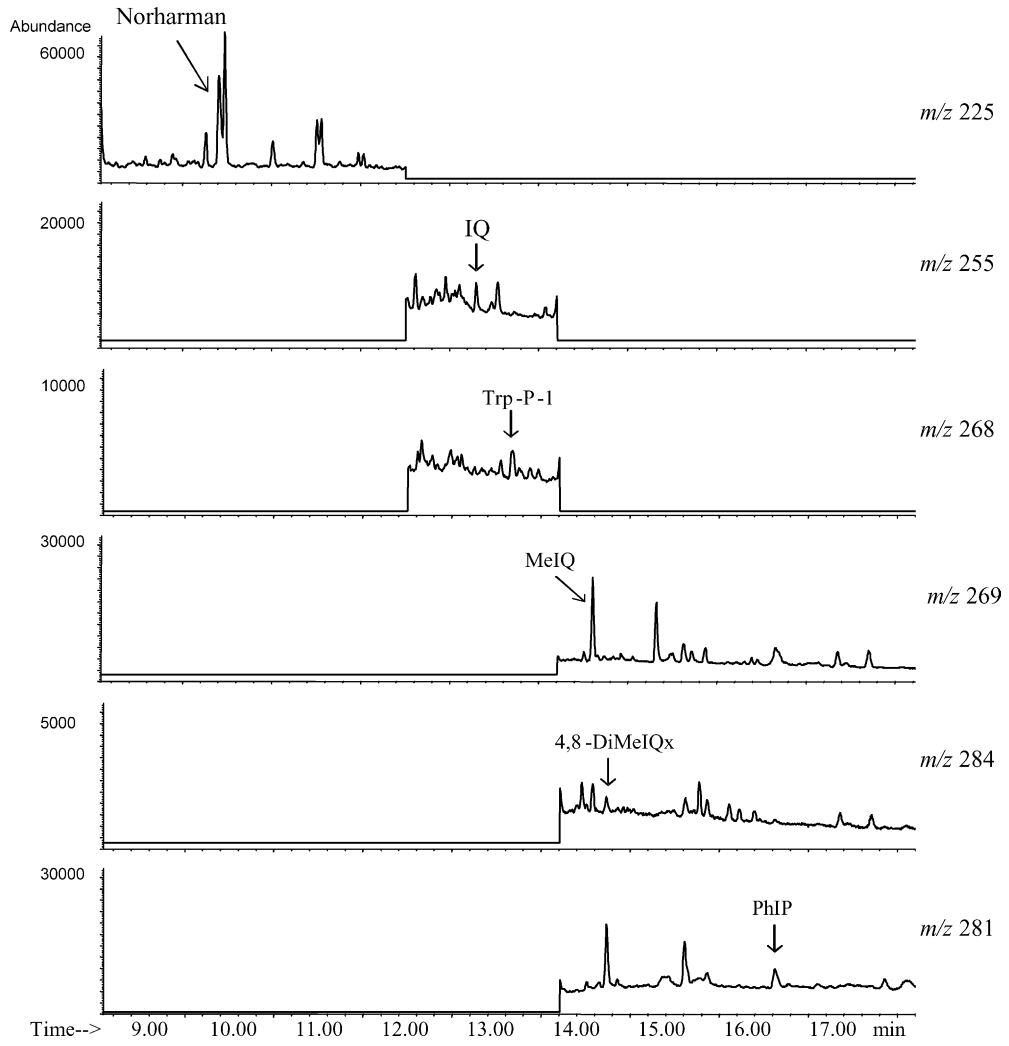


Fig. 3. SIM chromatogram from a fried bacon sample.

tested by applying the method described by Murray et al. [20] on the final extracts, already cleaned as described in Section 2. In this case, the final cleaned residue was taken with 0.1 M HCl (200 μl) and washed with hexane (2 μl × 750 μl). Then, 0.5 M sodium carbonate solution (100 μl) was added to the aqueous phase and the alkaline product extracted with ethyl acetate (2 μl × 750 μl). The organic extracts were combined and taken to dryness under nitrogen.

The derivatization of the dried extracts obtained by these two complementary clean-up procedures gave final extracts still not suitable for GC–MS quantification. Therefore, and only for coffee, the extracts obtained by the PRS/C_{18} method were analyzed by HPLC, following the Gross and Grüter method [11], as described in Section 2.

In Fig. 4 are represented the DAD and fluorescence chromatograms from both coffee extract and standard solution.

The presence of the co-mutagen norharman was confirmed and harman was also detected, but no mutagenic HAs were detected. These two β-carbolines were quantified with the fluorescence detector (excitation: 300 nm, emission: 440 nm) with mean levels of 1.3 ± 0.3 μg/g for norharman and 0.4 ± 0.1 μg/g for harman ($n = 3$). The results are in agreement with the recent results from Herraiz [38], obtained also by HPLC. The levels found in coffee are substantially higher than those found on other cooked foods, but comparable with those described in cigarette smoke [39], making these two habits probably the main exogenous sources of these normal body constituents in mammals.

Harman and norharman are described as co-mutagens. Although in vitro coffee mutagenicity has been mainly verified for individual coffee constituents [6], the presence of high amounts of harman and norharman should not be ignored [40]. Also, coffee drinking and smoking seem to be negatively associated with Parkinson's disease with a 20 and 60% reduction, respectively [41,42]. These β-carbolines have not been taken into account, although being important central monoaminooxidase inhibitors [43]. Their association could give further insight into these epidemiological studies.

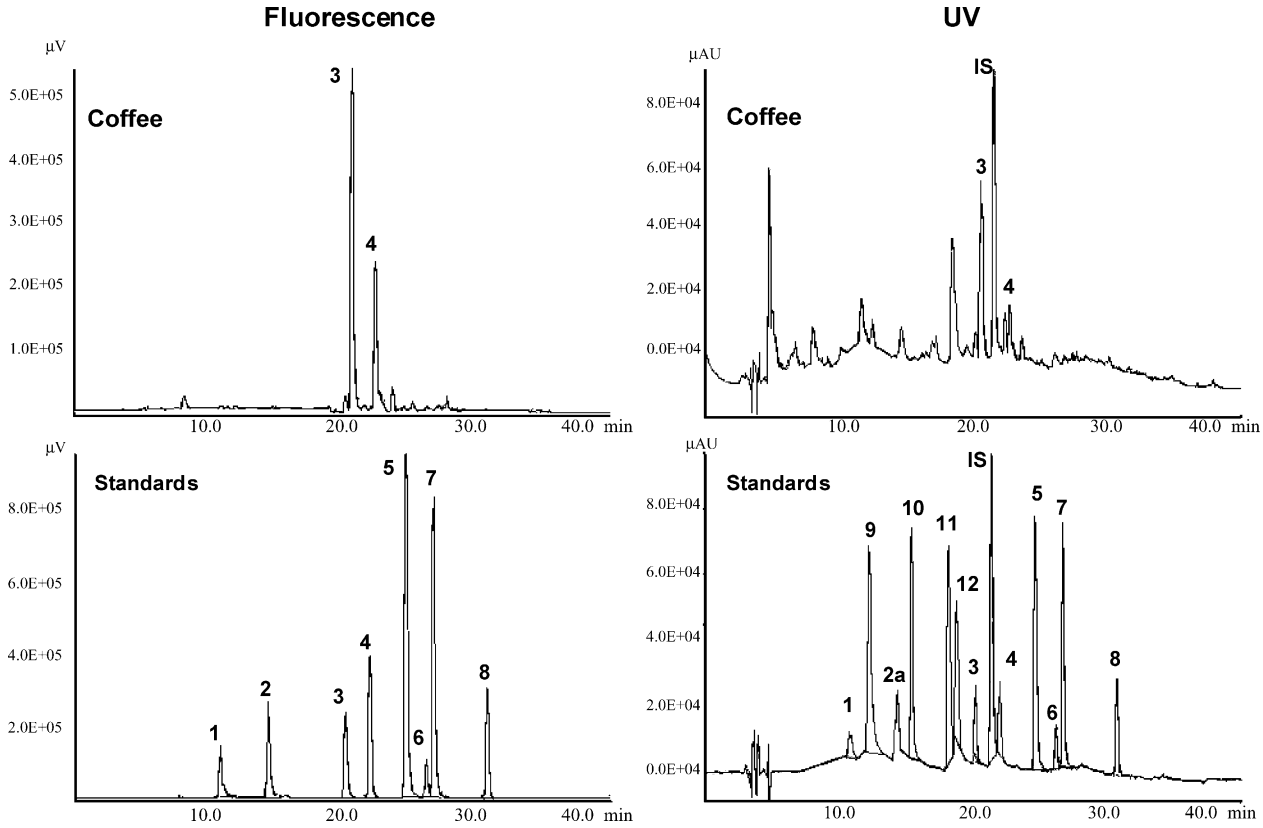


Fig. 4. HPLC chromatograms of a roasted coffee extract and reference standards (50 ng each) on the fluorescence detector (left) and diode array detector (263 nm), obtained under a ternary gradient as specified in Section 2. Peaks identified as follows: (1) Glu-P-2; (2) Glu-P-1; (2a) Glu-P-1 + MeIQ; (3) norharman; (4) harman; (5) Trp-P-2; (6) PhIP; (7) Trp-P-1; (8) AαC; (9) IQ + IQx; (10) MeIQx; (11) 7,8-diMeIQx; (12) 4,8-diMeIQx.

4. Conclusions

These experiments demonstrated that the HAs can be accurately determined in food samples by GC–MS as their *tert.*-butyldimethylsilyl derivatives. The derivatization method is simple, selective and sensitive, allowing the simultaneous quantification of twelve HAs. When applied to some food samples, low limits of detection were found, thus providing a less expensive alternative to the LC–MS techniques to estimate the content of HAs in food samples.

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