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Preparation of a beef-extract as a laboratory reference material for the determination of heterocyclic amines

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Abstract

The present paper describes the preparation of a suitable laboratory reference material (LRM) to validate analytical methods for the determination of heterocyclic amines (HAs) in foods. Three different lots of reference material were prepared using a beef extract which was contaminated with a well-known quantity of amines at different levels ranging from 10 to 75 ng/g. These materials were then lyophilised under determined conditions and, after grinding and sieving, homogenised and, finally, bottled and labelled. Homogeneity and stability studies were performed and no statistical differences were observed in the analysis of variances for within- and between-bottle results, thus demonstrating the homogeneity of the material. Stability at different storage temperatures (−18, +4, +25 and +40 °C) and times (1, 2, 3 and 6 months) was also tested. Therefore, the material can be considered homogeneous and stable and can be proposed for use in inter-comparison exercises for the determination of HAs.

Keywords: Food analysis; Laboratory reference material; Heterocyclic aromatic amines

1. Introduction

HAs are carcinogenic and/or mutagenic compounds present in protein rich foods, such as meat and fish, when these materials are processed by thermal treatments [1]. Some of them, aminoimidazoazaarenes (AIAs), are generated from the reaction of precursors such as glucose, creatine/creatinine and free amino acids at ordinary cooking temperatures [2]. Other amines, called pyrolitic HAs, are formed at temperatures above 300 °C through a pyrolitic reaction [3]. The main problem found in the analysis of amines in foods is their very low level of concentration (∼ng/g) and the high number of matrix interferences. For the analysis of these compounds, sensitive and selective analytical techniques have been developed [4] which must be validated. This validation can be performed using certified reference materials (CRM). These materials are a powerful and valuable tool that permits a rigorous control of parameters such as accuracy, precision, and traceability of measurements [5–9]. The production and certification of these materials is very expensive [10] and they are currently devoted to final verification of analytical procedures [11–14]. For routine work such as daily quality control, interlaboratory exercises and the evaluation of the reproducibility of methods for the use of laboratory reference materials (LRM) is recommended [9]. These materials are cheaper and equally effective because their composition and/or the analytical parameters to be tested are well known although they are not certified. These materials can be used in interlaboratory exercises or in the establishment of quality control charts. However, the homogeneity and stability of analytes of interest in the materials must be established [12,13,15].

Here, the preparation of a laboratory reference material is described, taking into account the recommendations of different guidelines [16]. First, a feasibility study of lyophilisation conditions such as temperature cycle or water/dried extract ratio was performed. Four lots (B1, B2, B3 and B4), changing temperature cycle, methanol proportion and water/beef extract ratio, were prepared to study lyophilisation conditions. Afterwards, three different lots were prepared as follows: (A) containing IQ, MeIQx, MeIQ, PhIP and AαC at a concentration level of 50 ng/g; (B) containing DMIP, IQ, MeIQx, MeIQ, 4,8-DiMeIQx, Trp-P-2, Trp-P-1, PhIP, AαC and MeAαC at a concentration level of 75 ng/g; and (C) containing DMIP, IQ, MeIQx, MeIQ, 4,8-DiMeIQx, Trp-P-2, Trp-P-1, PhIP, AαC and MeAαC at a concentration

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level of 10 ng/g. The homogeneity and stability of these three lots were then established.

2. Experimental

2.1. Reagents

Solvents and chemicals used were HPLC or analytical grade, and the water was purified through an Elix System coupled to an ultrapure water system Milli-Q plus 185 (Millipore, Bedford, MA, USA). All the solutions were passed through a 0.45 μm nylon filter (Whatman, Clifton, NJ, USA) before injection into the HPLC system.

The compounds studied were 2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine (DMIP), 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-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), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-9H-pyrido[2,3-*b*]indole (AαC), 2-amino-3-methyl-9H-pyrido[2,3-*b*]indole (MeAαC), 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole (Trp-P-2), purchased from Toronto Research Chemicals (Toronto, Canada). Stock standard solutions of 130 $\mu g\,g^{-1}$ in methanol were prepared and used after dilution for the preparation and analysis of the contaminated meat extracts. TriMeIQx and 7,8-DiMeIQx were used as internal standards.

Empty Extrelut-20 extraction cartridges were provided by Merck (Darmstadt, Germany), and Isolute diatomaceous earth refill material was obtained from IST (Hengoed, UK). Bond Elut PRS (500 mg) and endcapped Bond Elut C_{18} (100 and 500 mg) cartridges were from Varian (Harbor, City, USA). Coupling pieces and stopcocks were purchased from Varian.

Zinc acetate dihydrate was purchased by Fluka (Buchs, Switzerland), potassium ferrocyanure trihydrate was provided by Panreac (Madrid, Spain), Glucose Kit and Creatinine Kit was distributed by Quimica Clinica Aplicada (Tarragona, Spain), creatine hydrate was purchased by Sigma–Aldrich Chemie (Steinheim, Germany), and α-naphthol, diacetyl, sodium hydroxide, boric acid and sodium sulphate were provided by Merck.

2.2. Instrumentation

A freeze dryer Liomega 20 (Telstar, Barcelona, Spain) was used to lyophilise the material. It is provided with five tempered shelves of a total surface area of 1.8 m^2. The ice condenser capacity is 20 kg at −60 °C. The shelf temperature can be varied from −30 to 45 °C. An automatic reactor equipped with PTFE scrapers was used to mix the material. A Fritsch pulverisette (Laval Lab, Que., Canada) was used to grind and homogenise the lyophilised material.

For sample preparation a rotating shaker Rotary Mixer 34526 (Breda Scientific, Breda, The Netherlands) was used. A Supelco Visiprep and Visidry SPE vacuum manifold (Supelco, Gland, Switzerland) were used to manipulate the solid-phase extraction cartridges and solvent evaporation, respectively.

The determination of the heterocyclic amines was performed using a Waters 2690 Separation Module (Milford, MA, USA), equipped with a quaternary solvent delivery system and an autosampler. The determination of free and total amino acids was performed using a Pharmacia LKB Biochrom 20 instrument (Uppsala, Sweden) and an autosampler.

MS detection of HAs was carried out with LCQ mass spectrometer (Thermo-Finnigan, San Jose, CA, USA). It was provided with an electrospray ionisation (ESI) source and an ion trap as mass analyser. In all cases the post-column addition of a solution 0.01% of formic acid in acetonitrile was performed using a Pharmacia LKB pump model 2150 (Uppsala, Sweden).

A ATI-Unicam UV-4-100-Spectrophotometer (Thermo Electron Corp., Waltham, MA, USA), Selecta Kjeldahl Digestor model Bloc-digest 12P (ElectroScience UK, Sudbury Suffolk, UK) and Karl Fischer Automat 633 (Methrom, Barcelona, Spain), were used to establish parameters such as glucose, creatine, creatinine, total nitrogen and moisture.

2.3. Analytical procedures

2.3.1. Heterocyclic amines

A previously published purification method [17,18] was used to extract analytes from lyophilised meat extract. A 1 g sample was homogenised in 12 ml 1 M NaOH with sonication, and the suspension was then shaken for 3 h using a rotating shaker. The alkaline solution was mixed with Isolute refill material (13 g) and it was used to fill an empty Extrelut column. After being preconditioned with 7 ml dichloromethane (DCM), Bond Elut PRS column was coupled on-line to the Extrelut column, and extracted with 75 ml of DCM. The PRS cartridge was then dried and washed successively with 6 ml 0.01 M HCl, 15 ml MeOH–0.1 M HCl (6:4, v/v) and 2 ml of water. The washing solutions were collected for the analysis of the less polar compounds (Trp-P-1, Trp-P-2, PhIP, AαC and MeAαC). The acidic washing solutions were neutralised by adding 500 μl ammonia. Then, the preconcentration of these compounds were achieved in a 500 mg Bond Elut C_{18} column which had previously been conditioned with 5 ml of MeOH and 5 ml of water. Finally, the cartridge was washed with 5 ml water and the less polar HAs were eluted with 1.4 ml MeOH–NH_3 (9:1, v/v).

On the other hand, a 100 mg Bond Elut C_{18} cartridge was conditioned identically 500 mg Bond Elut C_{18} cartridge. It was then coupled on-line with the PRS cartridge. The most

polar amines (DMIP, IQ, MeIQx, MeIQ and 4,8-DiMeIQx) were eluted from the cationic exchanger with 20 ml of 0.5 M ammonium acetate at pH 8.5. Finally, 5 ml water and 0.8 ml MeOH–NH_3 (9:1, v/v) was used to rinse and elute, respectively. The extracts were evaporated to dryness under stream of nitrogen and finally redissolved in 100 μl of the internal standard (7,8-DiMeIQx, TriMeIQx) in MeOH–buffer (50:50, v:v).

Amines were separated by reversed-phase LC using a C_8 Symmetry® column (5 μm, 150 mm × 2.1 mm i.d.) (Waters Corporation, Milford, MA, USA) and the separation was achieved with a binary mobile phase at a flow-rate of 300 μl min^{-1}. Solvent A: acetonitrile; solvent B: 30 mM acetic acid/ammonium acetate buffer at pH 4.5. The gradient elution program for less polar HAs was: 0 min 10% A, 0–3 min, 15% A, 3–6 min, 15–30% A; 9–14 min, 30–60% A; 14–22 min, 60% A; 22–25 min, return to initial conditions; 5 min post-run delay. The gradient elution program for polar HAs was: 0–3 min, 5% A, 3–15 min, 5–30% A; 15–16 min, 30% A; 16–18 min, 30–60% A; 18–26 min, 60%, 26–31 min, return to initial conditions; 10 min post-run delay. The sample volume injected was 5 μl. Data acquisition was carried out by Xcalibur™ 1.2 software.

Optimal ionisation source working parameters were: spray voltage, 3 kV; sheath gas, 90 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 full scan, scanning from *m/z* 150–250 in centroid mode, with a maximum injection time of 200 ms, 3 microscans, and automatic gain control activated.

Limits of detection (LOD) and limits of quantitation (LOQ) ranged from 8 to 50 and 24 to 150 pg injected, respectively. Run-to-run precision at low concentration level (0.05 μg/g) and medium concentration level (0.40 μg/g) gave %R.S.D. values of 3–7 and 0.5–4, respectively. Slightly higher values (%R.S.D.), 5–9 and 2–10, were obtained for day-to-day precision [27].

2.3.2. Determination of precursors

In the analysis of glucose, creatinine and creatine, pre-treatment of the sample with pentane, ethanol and Carrez solutions was performed to prevent interferences such as fat and proteins. Creatine, creatinine, and glucose content were determined spectrophotometrically according to the Wong method [19,20] and by using creatinine [21] and glucose [22] enzymatic kits. The determination of free and total amino acids are based on an ion exchange chromatographic separation using a cationic exchange column (5 μm, 200 mm × 4 mm i.d.) and ninhidrine as post-column derivatisation [23] to determine them by UV (570 nm). The sample volume injected was 50 μl.

2.3.3. Other determinations

In order to characterise the raw material used to obtain the final beef extract, several parameters, such as total nitrogen, ashes, and fat were determined using AOAC methods [24–26]. For all lots and raw material, moisture was analysed using the KF method.

2.4. Preparation of lyophilised meat extract

2.4.1. Raw material

A commercial beef extract free from HAs except harman (36 ng/g) and norharman (43 ng/g) was used as raw material for the preparation of the lyophilised meat extract. Several amounts of heterocyclic amines in methanol solution, ranging from 10 to 75 ng/g were added to an aqueous solution (40%) of raw material and thoroughly mixed to homogenise the mixture. Ox meat extract, water, vegetable proteins, and hydrolysed ox meat, yeast extract, colouring (E-150), sugar, salt, and spices are some of the components of this material. Some parameters that characterise the raw material expressed on a dry basis are given in Table 1.

2.4.2. Feasibility study of lyophilisation conditions

Previously to the preparation of lots, a feasibility study of lyophilisation conditions was performed. Specifically four lots (B1, B2, B3 and B4) spiked at 75 ng/g with DMIP, IQ, MeIQx, MeIQ, 4,8-DiMeIQx, Trp-P-2, Trp-P-1, PhIP, AαC and MeAαC were prepared under different conditions of lyophilisation: temperature cycle, methanol proportion and water/meat extract ratio (dry basis) (Table 2).

2.4.3. Preparation and bottling

Conditions corresponding to lot B1 were used for the preparation of three lots: A, B and C (Table 3). Lot A (50 ng/g) contained IQ, MeIQ, MeIQx, PhIP and AαC. Lot B (75 ng/g) and lot C (10 ng/g) contained DMIP, IQ, MeIQ, MeIQx, 4,8-DiMeIQx, Trp-P-2, Trp-P-1, PhIP, AαC and MeAαC. After lyophilisation, grinding, sieving at 250 μm, and finally homogenisation and bottling were performed. Meat extract was placed in amber bottles, which were filled with ca. 21 g of sample. For lots A, B and C 68, 103 and 63 bottles were respectively prepared. The storage temperature was −18 °C (freezer). The last step was the labelling. The information contained in each label was the lot number of each bottle, the type of product contained in the bottle, and the address of the laboratory where the material was pre-

Table 1
Main characteristics of the raw material expressed on dry basis

Parameters	Raw material	CV (%)
Total *N* (%)	11.1	3.0
Fat (%)	0.73	2.0
Moisture (%)	38	0.3
Ashes (%)	26	4.4
Glucose (mg/g sample)	0.98	13.0
Creatine (mg/g sample)	12.2	12.0
Creatinine (mg/g sample)	0.81	7.1
Free amino acids (mg/g sample)	78	0.5
Total amino acids (mg/g sample)	353	0.02

Table 2
Lyophilisation conditions in feasibility study

Lot	*T* cycle (°C)	Meat extract g d.b.[a]	Mixture (water/meat extract (d.b.)) (%)	Conc. HAs spiked (ng/g)	Lyoph yield (%)	MeOH (%)	Density (g/ml)
B1	−40 to 20	690	60/40	∼75	98	1	0.75
B2	−40 to 40	684	60/40	∼75	98	1	0.69
B3[a]	−40 to 20	630	60/40	∼75	98	1.8	0.75
B4	−40 to 20	703	45/55	∼75	98	1	0.92

[a] d.b.: dry basis.

Table 3
Lyophilisation conditions in the preparation of the different lots

Lot	*T* cycle (°C)	Meat extract g d.b.[a]	Mixture (water/meat extract (d.b.)) (%)	Conc. HAs spiked (ng/g)	Lyoph yield (%)	MeOH (%)	Density (g/ml)
A	−40 to 20	2360	60/40	∼50	98	1	0.69
C	−40 to 20	2378	60/40	∼10	98	1	0.74
B	−40 to 20	5406	60/40	∼75	98	1	0.75

[a] d.b. dry basis.

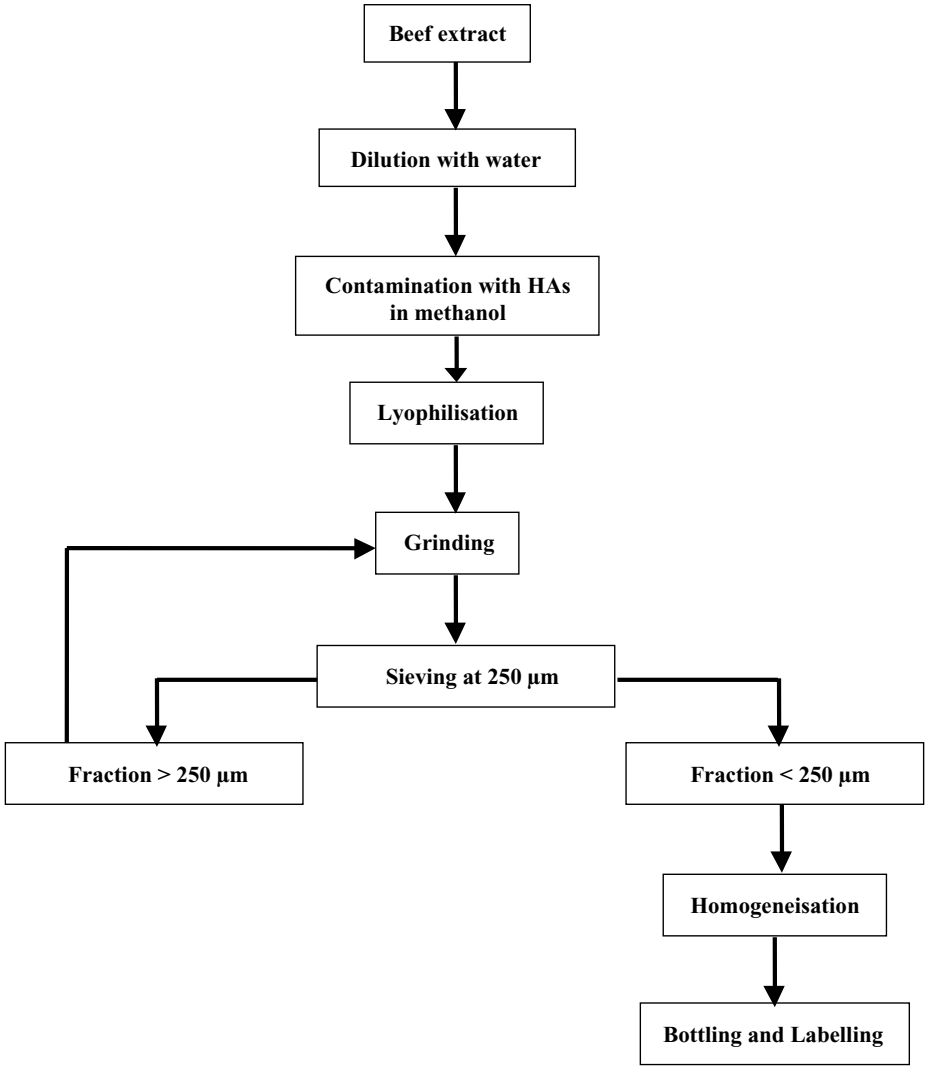

Fig. 1. Flow chart for beef extract preparation.

pared. The flow chart for beef extract preparation is shown in Fig. 1.

2.5. Homogeneity and stability studies

2.5.1. Homogeneity test

There are no definitive rules to select the number of samples for homogeneity [16,23]. In this work, $3(n)^{1/3}$ was the formula used to select a representative number of bottles to be analysed, where n is the total number of bottles of the lot. Respectively, 13, 14 and 12 bottles were selected randomly to study the homogeneity of lots A, B and C.

Firstly, in order to determine the concentration of HAs of each lot, one bottle randomly selected was analysed by duplicate using the standard addition method. Four spiking levels around 50, 100, 150 and 200% were used. TriMeIQx was used as internal standard. The results are given in Table 4. Recoveries ranging from 50 to 80% were obtained.

Homogeneity within bottles was tested to ensure that successive test portions from a bottle would lead to similar results (within-bottle homogeneity). A similar study was performed to verify that there was no difference between test portions taken from various bottles (i.e. between-bottle homogeneity). For within-bottle homogeneity, three bottles randomly chosen and analysed from among the ones selected (five replicates/bottle). Between-bottle homogeneity was determined by analysing one replicate of each of the 13, 14 and 12 selected bottles. The sample intake was 1 g.

2.5.2. Stability test

The stability of the material should be tested at established time intervals in order to ensure that the composition of the sample remains unchanged throughout the shelf-life of the material. The stability studies of HAs in the laboratory reference material were performed at different times (1, 2, 3 and 6 months) and storage temperatures (−18, +4 (except lot A), +25 and +40 °C). For each lot, one bottle was selected randomly for each temperature including −18 °C as reference, and a triplicate analysis of each of these bottles was carried out after each allocated time. Amine concentrations at each time and storage temperature were normalised to the concentration of the samples stored at −18 °C.

3. Results and discussion

3.1. Influence of the lyophilisation conditions

The objective of the preparation of four lots (B1, B2, B3 and B4) was to study the influence of lyophilisation conditions on several parameters of the final meat extract such

Table 4
Study of homogeneity of lots A, B and C

Lot	HAs	Conc. HAs (ng/g)	CV (%)		*F* calc.	*F*th
			Between-bottles	Within-bottles		
A	IQ	51.8	13.3	12.3	0.764	3.020
	MeIQ	49.0	9.8	11.3	0.517	2.943
	MeIQx	49.1	10.4	14.7	0.445	5.050
	PhIP	48.8	14.4	12.4	1.642	3.229
	AαC	51.5	14.4	16.4	1.308	3.293
B	DMIP	76.7	14.6	11.7	1.832	2.660
	IQ	79.6	13.3	14.6	1.437	2.660
	MeIQx	82.1	8.8	10.4	1.525	2.660
	MeIQ	77.0	10.5	10.6	2.139	2.660
	4,8-DiMeIQx	76.7	4.8	5.7	1.121	2.660
	Trp-P-2	76.8	12.8	13.5	1.415	2.660
	Trp-P-1	76.9	10.3	8.0	2.387	2.660
	PhIP	76.7	9.2	11.6	1.296	2.660
	AαC	76.8	15.0	11.9	2.326	2.660
	MeAαC	76.9	14.9	14.2	2.136	2.660
C	DMIP	9.7	9.7	11.1	1.259	2.600
	IQ	9.7	11.5	8.3	0.588	2.600
	MeIQx	9.3	10.1	7.5	0.784	2.600
	MeIQ	9.9	8.3	10.6	1.885	2.600
	4,8-DiMeIQx	9.9	6.9	6.7	1.333	2.600
	Trp-P-2	10.5	10.3	8.7	1.510	2.600
	Trp-P-1	9.8	6.2	5.3	0.848	2.600
	PhIP	9.6	1.0	1.3	1.338	2.600
	AαC	9.6	1.5	3.4	1.864	2.600
	MeAαC	10.3	3.4	4.4	2.190	2.600

as density, moisture, lyophilisation yield, and HAs precursor concentrations (glucose, creatinine and creatine). Table 2 shows the conditions which were modified: %MeOH (B3), $\%H_2O$ (B4) and the temperature cycle (B2). Parameters such as lyophilisation yield and density of the material at the different lots are also given in this table.

Lyophilisation yield was in all cases relatively high (98%). This indicates that this parameter was not affected by the different lyophilisation conditions. The high yield value was achieved by repeating the sieving/grinding steps in the beef extract preparation until exhaustion of the coarse material. Density was affected by the water proportion in the initial raw material solution. The value of this parameter increased to 0.9 g/ml for lot B4 which was obtained from a solution that contained 25% less water than the other lots. Lot B4 was an agglomerate and cohered material that was very difficult to grind.

The concentration of creatinine, creatine, glucose, moisture, and the concentration of HAs in the different lots were also calculated. The results of all these parameters, except moisture, showed no differences between lots; therefore, they were apparently not influenced by the lyophilisation

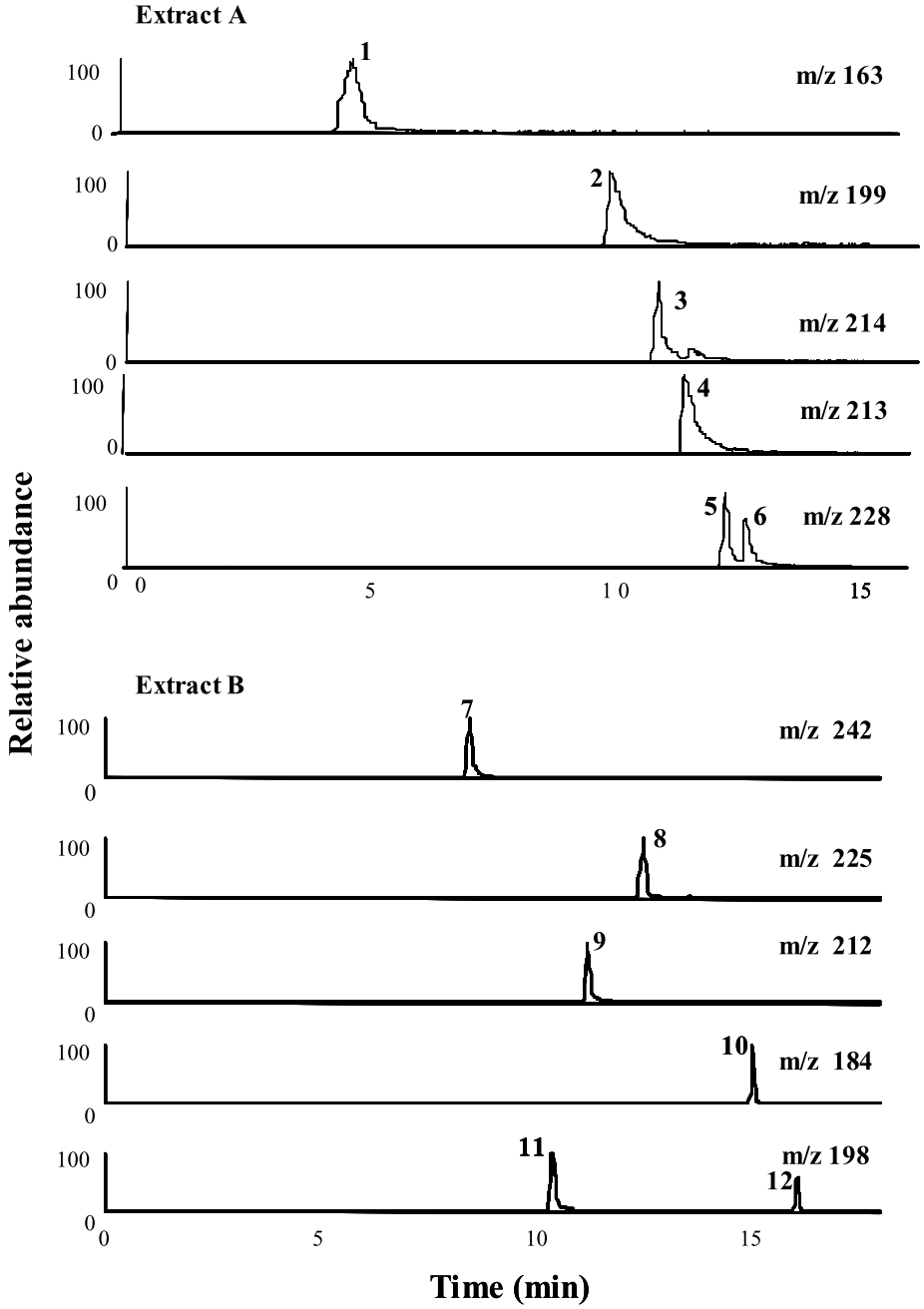

Fig. 2. LC-MS chromatogram corresponding to a sample of lot C. Peaks: Extract A: (1) DMIP; (2) IQ; (3) MeIQx; (4) MeIQ; (5) 7,8-DiMeIQx (IS); (6) 4,8-DiMeIQx. Extract B: (7) TriMeIQx (IS); (8) Trp-P-2; (9) Trp-P-1; (10) PhIP; (11) AαC; (12) MeAαC. Conditions are included in Section 2.

conditions. In contrast, the moisture of lot B2 was lower than the other lots, which is due to the different temperature cycle. While this parameter was −40–40 °C for lot B2, it finished at +20 °C for the rest of lots. Although moisture was lower it showed a poorer reproducibility than for the other lots because this material was more hygroscopic. From these results, lot B1 lyophilisation conditions were selected for the preparation of final LRM materials.

3.2. Homogeneity

In order to check the homogeneity, HAs content in the randomly selected bottles of lots A, B and C was determined by LC-MS. The concentration of HAs in the LRM materials are given in Table 4. As an example in Fig. 2, a LC-MS chromatogram of lot C is shown. A single factor ANOVA was used as statistical method to test the significant difference between variances and coefficients of variation at the within-bottle and between-bottle test results using a confidence level of 95%.

The coefficients of variation (CV, %) corresponding to the within-bottle and between-bottle series were determined. Table 4 shows these data and the calculated and theoretical *F*-values. Analytical method for the determination of HAs was affected for some of the compounds by an uncertainty of around 15%. As a consequence, the within- and between-homogeneity can only be confirmed if the obtained uncertainties are not significantly different with respect to the uncertainty of the analytical method and provided that the variances of between-bottle and within-bottle are not significantly different.

No significant differences could be discerned in the variances between the within-bottle series or the between-bottle series because the calculated *F*-values were always lower than the theoretical *F*-values. Moreover the coefficients of variation were similar to the analytical method. Therefore, it was concluded that the materials were bottled homogeneously.

3.3. Stability

Stability was investigated over a period of 6 months at storage temperatures of −18, +4 (except lot A), +25 and +40 °C. After 1, 2, 3 and 6 months, HAs were determined by triplicate in four bottles from each storage temperature. The stability tests were conducted under the assumption that no instability occurs at −18 °C storage temperature because the possible changes at this temperature should be minimum. The relative HAs concentrations, R_T, were calculated for each storage time by dividing the mean of the replicates at each storage temperature by the mean of the replicates at –18 °C:

$$R_T = \frac{X_T}{X_{-18\,^\circ\mathrm{C}}}$$

The combined uncertainty (U_T) was obtained from the coefficient of variation (CV) of each set of measurements:

$$U_T = \frac{(\mathrm{CV}_T^2 + \mathrm{CV}_{-18\,^\circ\mathrm{C}}^2)^{1/2} R_T}{100}$$

The confidence intervals ($\mathrm{CI}_{\alpha,T}$) at $\alpha = 0.05$ (95% confidence level) were obtained from the combined uncertainty, the number of replicates in both the series for the temperature T (n_T) and the series for −18 °C ($n_{-18\,^\circ\mathrm{C}}$) and the critical values of Student's *t*-distribution (t_α):

$$\mathrm{CI}_{a,T} = \frac{t_\alpha U_T}{(n_T + n_{-18\,^\circ\mathrm{C}})^{1/2}}$$

In theory, all R_T values should equal unity, but in practice differences occur. If the difference remains within the calculated confidence interval, the R_T value does not differ significantly from unity. For all HAs storage temperatures (+4, +25 and +40 °C), R_T values do not significantly differ from unity. It was concluded that HAs concentrations in lots A, B and C are stable for a period of at least 6 months even at +40 °C. As a consequence, the material can be stored at +40 °C and no particular precautions have to be taken with these materials.

4. Conclusions

The lyophilisation of a commercial meat extract has allowed the preparation of a LRM material suitable for the determination of HAs. The lyophilisation conditions were established after studying experimental conditions such as temperature cycle and water/meat extract (d.b.) ratio, showing that moisture and density were affected by these changes. Conditions −40 to +20 °C and 60/40 as water/meat extract (d.b.) ratio were selected for the preparation of LRM materials.

Homogeneity of LRM materials was demonstrated and the stability of the different lots at three temperatures (+4, +25 and +40 °C) for a period of 6 months was assessed.

In conclusion, the prepared meat extracts fulfil the conditions necessary (homogeneity and stability) to be used as laboratory reference materials for intercomparison exercises in order to validate analytical methodology for the determination of HAs in food samples.

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