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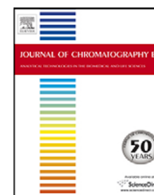

Preparation and characterisation of fried chicken as a laboratory reference material for the analysis of heterocyclic amines

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ABSTRACT

The preparation of a laboratory reference material (LRM) for the determination of naturally occurring heterocyclic amines (HAs) in processed foods is presented in this work. A LRM was prepared from raw chicken breast meat, which was fried under controlled cooking temperature and time. The cooked meat was ground, lyophilised, sieved, homogenised, bottled, and labelled. The HAs DMIP, PhIP, MeIQx, 4,8-DiMeIQx, Norharman and Harman were analysed in the LRM. Homogeneity and stability studies of the bulk LRM were carried out and no statistical differences were observed in the content of the studied HAs in between-bottle and within-bottle comparisons at different storage temperatures (−18, +4, +25 and +40 °C) and times (1, 3, 6 and 9 months) by means of HAs determination and analysis of the results. Consequently, the material can be considered homogeneous and stable and can be used in intercomparison exercises for the determination of HAs as well as for quality control purposes in the routine analysis of HAs in foodstuffs. This is the first LRM for the analysis of HAs where these analytes were naturally formed in the material.

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1. Introduction

HAs constitute a potential health risk due to their potent mutagenic activity and their presence in cooked meat and fish processed at household conditions [1,2]. HAs are formed during the thermal treatment of proteinaceous matrices that contain compounds involved in their formation: creatine, amino acids and carbohydrates [3–5]. The cooking temperature has a great influence on the amount of HAs formed [4,6]. The International Agency for Research on Cancer (IARC) listed eight HAs (MeIQ, MeIQx, PhIP, AαC, MeAαC, Trp-P-1, Trp-P-2 and Glu-P-1) as possible human carcinogens (class 2B) and one (IQ) as a probable human carcinogen (class 2A) [7]. At present, over 25 HAs have been identified and isolated from protein-rich foods and their concentration has been found at ng g^{-1} level [8,9]. Major disadvantages in the analysis of HAs in foods are their low concentration level and tedious sample treatments required as a consequence of the complexity of the heat-treated food matrices. For the analysis of these mutagens in foods, analytical methods have been developed recently with the aim to propose effective, fast, and low sample handling, sample extraction and purification procedures [10–12].

The availability of laboratory reference materials for the analysis of HAs will help in the validation of the developed procedures and inform about the quality of the determination of the analytes, in terms of accuracy and precision, in a matrix very similar to the samples. Validation can be performed using Certified Reference Materials (CRMs) [13–16] but due to the high production and certification costs, these materials cannot be used routinely in laboratory work; therefore, a LRM is preferred [17]. The development of LRM is somewhat labour intensive but they are relatively inexpensive and equally effective in comparison with CRMs [18]. Currently, there are no CRMs for the analysis of HAs commercially available. Various LRM for the analysis of HAs were developed by our group and were used to validate analytical methods applied by different laboratories in intercomparison exercises [19]. These LRM were based on beef flavour spiked with HAs at different levels.

In this work, the preparation of fried chicken breast laboratory reference material is presented, in accordance with recommendations of different experimental protocols and guidelines for the in-house production of reference materials [20,21]. To our knowledge, this is the first report on the preparation of a LRM where HAs have been naturally generated HAs, which can be used to improve the quality of results in the determination of HAs.

2. Experimental

2.1. Chemicals

Solvents and chemicals were of HPLC or analytical grade. Ethyl acetate, acetonitrile and methanol were purchased from Merck

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(Darmstadt, Germany). Water was purified in a Milli-Q Simplicity 185 system (Millipore, Bedford, MA, USA). Ammonia solution (25%), formic acid (98–100%), α-naphthol and diacetyl were obtained from Merck (Darmstadt, Germany). Ammonium formate and zinc acetate dehydrate were purchased from Fluka (Buchs, Switzerland). Sodium hydroxide, sodium sulphate, potassium ferrocyanure trihydrate, ethanol (96%) and sulphuric acid (96%) were supplied by Panreac (Barcelona, Spain). Glucose kit was supplied by Química Clínica Aplicada (Tarragona, Spain); creatine hydrate was purchased from Sigma–Aldrich Chemie (Steinheim, Germany). The studied HAs 2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine (DMIP), 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-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3,4,7,8-tetramethylimidazo[4,5-*f*]quinoxaline (4,7,8-TriMeIQx), were purchased from Toronto Research Chemicals (Toronto, Canada). 1-Methyl-9*H*-pyrido[4,3-*b*]indole (harman) and 9*H*-pyrido[4,3-*b*]indole (norharman) were obtained from Sigma (Missouri, USA). The chemical purity of the reference compounds was higher than 98%.

Extrelut NT20 extraction cartridges were provided by Merck (Darmstad, Germany), and Isolute diatomaceous earth refill material was obtained from IST (Hengoed, Mid-Glamorgan, UK). PRS (500 mg) and C_{18} (100 mg) Bond Elut cartridges and coupling pieces were supplied from Varian (Harbor City, USA).

Individual stock standard solutions of the HAs at 150 $\mu g\,g^{-1}$ were prepared in methanol and used for further dilutions. Standard mixtures of the studied HAs at concentration levels between 0.003 and 1.00 $\mu g\,g^{-1}$ containing 4,7,8-TriMeIQx at 0.5 $\mu g\,g^{-1}$ as internal standard were prepared by weight for calibration purposes. Standards and samples were filtered through a 0.22 μm nylon filter (Scharlab, Sentmenat, Spain) before injection into the LC–MS system.

The LRM was bottled in 40 ml amber bottles (Sigma–Aldrich, Spain) provided with screw cap with PTFE seal.

2.2. Instrumentation

Cooking temperature was measured with type-K insulated-wire probes and monitored with Normadics TC6 software from Cole-Parmer (Vernon Hills, IL, USA).

The cooked food was blended with a Microtron MB 550 (Kinematica AG, Littau, Switzerland), and lyophilised with a freeze dryer Telstar model Liolabor (Telstar, Spain), which had a maximum condenser capacity of 12 kg/ice at −60 °C. Three tempered trays of a total surface area of 0.2 m^2 were used to freeze-dry the ground cooked meat. For sample analysis, the freeze-dried cooked meat was mixed with 1 M NaOH and homogenised with an Ultra-Turrax® T25 basic (IKA, Staufen, Germany). In the SPE method and solvent evaporation, Visiprep™ and Visidry™ vacuum manifolds (Supelco, Gland, Switzerland) were used.

The determination of HAs was performed using a quaternary pump system from Agilent Technologies model Series 1100, (Waldbronn, Germany) which was coupled to a triple quadrupole mass spectrometer PE Sciex API 3000 (PerkinElmer Sciex, Concord, ON, Canada). In the characterisation of the material, a Cecil CE 1020 spectrophotometer, Cecil Instruments (Cambridge, England) was used in the analysis of glucose and creatine. A Selecta Kjeldahl Digestor model Bloc-digest 12P (ElectroScience UK, Sudbury Suffolk, UK) was used for total nitrogen determination. The analysis of free and total amino acids was carried out using a Biochrome 20 amino acid analyser (Cambridge, UK) by ion-exchange chromatography using a column (5 mm, 4.6 mm × 200 mm) with polystyrene–divinylbenzene stationary phase with sulphonate groups (Pharmacia LKB Biotechnology, Biochrome, Cambridge, UK) using ninhidrin as post-column derivatisation [22].

2.3. Preparation of the laboratory reference material

Fresh chicken breasts were obtained from a local supermarket, (Barcelona, Spain) and were made into fillets of thickness 5 mm. A total of 12 kg of raw meat was cooked in different batches. The fillets were pan-fried in a Teflon-coated frying pan (270 mm × 270 mm) over an electric heater (Teka model IZ622, Teka Industrial, Santander, Spain.) under controlled cooking temperature and time using insulated type-K probes and Nomadics TC6 software. Five probes were used to monitor the temperature at 1 mm below the meat surface at both the upper and lower sides and of the fillet; in the centre of fillet; between pan and meat, and in centre of frying pan. Probes were calibrated before the cooking process by immersing them in ultra pure boiling water and assigning 100 °C the measured temperature. The duration of the cooking process was 6 min and the fillets were turned once after 3 min the cooking process began. Temperature was recorded every 5 s. The fried fillets were weighed and the weight loss was measured.

The cooked meat was ground with a Microtron MB 550 blender. To find out adequate density and moisture for the preparation of bulk material, three different lots (A1, A2 and A3) of ground cooked food were prepared for the feasibility study at different water/cooked chicken breast proportions. Each lot, constituted by 30 g of fried chicken, was mixed with water (40, 50 or 60 g) and after blending they were lyophilised. Temperatures ranged between −30 and 30 °C in the lyophilisation cycle. Finally, the bulk material was mixed in a proportion of 50:30 (water/ground cooked meat) before lyophilisation and a total of 6 kg ground fried chicken was prepared.

The ground freeze-dried material was disaggregated by use of a wooden hammer, sieved at 250 μm to control the particle-size distribution. The fibrous material (>250 μm) was separated, ground with the wooden roller and passed through 250 μm sieve again. Finally, the freeze-dried sieved material was thoroughly mixed with a laboratory mill (Fritsch pulverisette, Laval lab, Quebec, Canada), bottled, sealed in amber vials and labelled. Each bottle was filled with nearly 20 g of the freeze-dried meat and stored at −18 °C until analysis. A flow chart summarising the preparation of the laboratory reference material is shown in Fig. 1.

2.4. Characterisation of the laboratory reference material

The characterisation of uncooked and fried chicken breast was performed in terms of the glucose and creatine by an enzymatic method [23,24]; total and free amino acids by cation exchange chromatography and UV detection [22]. The content of ashes, fat, moisture (based on gravimetric methods) and total nitrogen (by the Kjeldahl method) were determined according to the AOAC procedures [25–27]. Determinations were carried out in triplicate. Density was calculated by measuring the volume and weight of the material at 25 °C.

2.5. Homogeneity studies

To select the number of samples to study the homogeneity of the material several guidelines have been recommended [20,21]. In this research work, the expression $3(n)^{1/3}$ was used to select a representative number of bottles to be analysed, where n corresponds to the total number of bottles (in this work, $n = 300$). Then, homogeneity was evaluated by analysing 20 bottles randomly selected from the total batch.

The homogeneity of the materials was tested by comparing the amounts of the HAs determined within a given bottle and among the bottles. Between-bottle homogeneity was determined by analysing one replicate in each of the 20 randomly selected bottles from the total batch. Within-bottle homogeneity was tested in four bottles randomly chosen (four replicates/bottle).

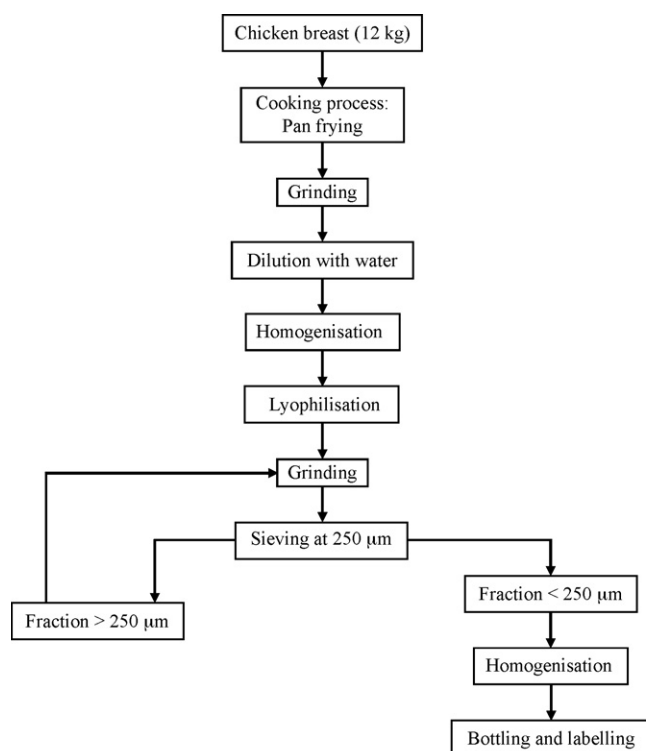

Fig. 1. Flow chart for the preparation of fried chicken as LRM for the determination of HAs.

2.6. Stability studies

Stability studies were performed either for 1, 3, 6 or 9 months of storage at four different storage temperatures (−18, +4, +25 and +40 °C). Two bottles were selected randomly for each storage temperature, using the bottle stored at −18 °C as reference, because, at this storage temperature, stability of the target compounds can safely be assumed. HA concentrations at each period and storage temperature were normalised in relation with the concentration of the samples stored at −18 °C.

2.7. Analysis of HAs

The extraction and clean-up procedure used for the analysis of HAs had been previously developed for cooked foods [28]. In brief, 1 g of ground lyophilised fried chicken breast was homogenised in 12 ml of 1 M NaOH using an Ultra-Turrax T25 basic. The alkaline meat solution was thoroughly mixed with 13 g of Isolute diatomaceous earth refill material and it was packed in an empty extraction cartridge which was coupled on-line with a Bond Elut PRS (500 mg) cartridge, previously preconditioned with 5 ml of 0.1 M HCl followed by 10 ml H_2O and 5 ml MeOH. HAs were extracted with 75 ml of ethyl acetate from the alkalinised meat dispersed on diatomaceous earth and were retained in the preconditioned PRS cartridge. Then, the PRS cartridge was dried and successively rinsed with 15 ml methanol–water (4:6, v/v) and 2 ml water. The PRS cartridge was then coupled with C_{18} (100 mg) cartridge which had previously been preconditioned with 5 ml of MeOH and 5 ml of H_2O. HAs were eluted from the PRS to the C_{18} with 20 ml of 0.5 M ammonium acetate adjusted at pH 8.5 with ammonia. Finally, HAs were eluted from, C_{18} after rinsing with 5 ml H_2O, using 0.8 ml of methanol-ammonia (9:1, v/v). The extract was evaporated to dryness under a gentle stream of nitrogen and the analytes were reconstituted in a suitable volume of a solution of methanol that contained internal standard to control the injection volume (TriMeIQx, 0.5 $\mu g\,g^{-1}$). Then, the purified extracts were filtered through a 0.22 µm nylon membrane and injected into LC–MS system. HAs from LRM were quantified by standard addition method, which comprised two unspiked and three spiked samples. The spiking levels for DMIP and PhIP used were 0, 13, 37, and 125 $ng\,g^{-1}$ LRM and for MeIQx, 4,8-DiMeIQx, harman and norharman were 0, 2, 5 and 15 $ng\,g^{-1}$ LRM.

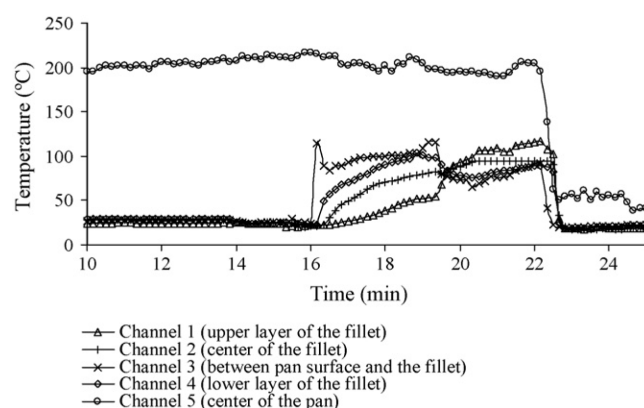

Fig. 2. Variation of temperatures during cooking process.

The chromatographic separation of HAs was achieved using reversed phase column Symmetry® C_8 (5 µm, 150 mm × 2.1 mm) (Waters Corporation, Milford, MA, USA). The gradient elution program and the MS conditions were as previously described [29]. Normalised collision energies (NCE), time schedule and MS/MS parameters are given in Table 1.

3. Results and discussion

3.1. Preparation of the material

Both the distribution and the amounts of HAs formed during cooking depend on the type of meat and the cooking temperature. In order to prepare a material representative of common food, we chose to use chicken, fried under carefully controlled tempera-

Table 1
MRM parameters used with the triple quadrupole instrument[a,b].

HAs	Precursor ion $[M+H]^+$ (*m*/*z*)	Quantification product ion (*m*/*z*) tentative assignation [30]	Confirmation product ion (*m*/*z*) tentative assignation [30]	Collision offset voltage (V)
DMIP	163	148 $[M+H-CH_3]^{+\bullet}$	147 $[M+H-CH_3-H]^+$	37
MeIQx	214	199 $[M+H-CH_3]^{+\bullet}$	173 $[M+H-C_2NH_3]^+$	38
4,8-DiMeIQx	228	213 $[M+H-CH_3]^{+\bullet}$	187 $[M+H-C_2NH_3]^+$	40
4,7,8-TriMeIQx	242	227 $[M+H-CH_3]^{+\bullet}$	201$[M+H-C_2NH_3]^+$	38
Norharman	169	115 $[M+H-2HCN]^+$	–	49
Harman	183	115 $[M+H-C_2NH_3-HCN]^+$	168 $[M+H-CH_3]^{+\bullet}$	49
PhIP	225	210 $[M+H-CH_3]^{+\bullet}$	183 $[M+H-CH_3-HCN]^{+\bullet}$	43

[a] Interchannel time delay: 5 ms.
[b] Dwell time was 150 ms.

ture conditions. Fig. 2 displays the temperature at 4 sites of fillets and pan temperature in one of the cooking processes. The total raw chicken (12 kg) was cooked in several batches. In each batch, the thermal treatment was within the range as shown in Fig. 2. Recording the temperature profiles during cooking is important, when studying Maillard reaction products such as HAs, and this has been performed in earlier works [31–34]. As shown in the temperature profiles (Fig. 2), the material has been cooked under moderate conditions; no charring was visible.

As can be seen in Fig. 2, probe 1, inserted in the fillet at the upper layer (1 mm depth) shows the lowest temperature, up to 52 °C at first half (3 min) of the cooking process. In contrast, probe 4, located in lower layer (1 mm depth) of the fillet, shows the highest temperature up to 104 °C, due to high transfer of heat. After 3 min, the fillet was turned, and probe 4 behaves like probe 1 and then the new upper side experiences less temperature. Probes 2 and 3 show the temperature in the centre of fillet and between pan and fillet respectively. The temperature in probe 2 reached up to 90 °C and in probe 3 up to 115 °C. Probe 5 (centre of the pan), used to measure the cooking temperature, measured an average temperature of 210 °C during the frying process. After cooking, all probes were removed from the cooked meat and the temperature went down to room temperature. Weight loss in the frying process was 47%. High weight loss has been obtained compared with literature data applying prolonged thermal treatments [32] which might be due to the lesser thickness (5 mm) of raw fillets made in this work.

Three different water/ground fried chicken proportions (40/30, 50/30 and 60/30) were assayed to study their influence on density and moisture in the final lyophilised material. In the case of the lot mixed with the higher water content, 60/30, density = 0.42 g ml^{-1} and moisture = 8.4% w/w were observed for the freeze-dried product. The lots mixed with lower content of water, 40/30 and 50/30, yielded higher density (0.49 and 0.52 g ml^{-1} respectively) and lower moisture (4.2 and 4.1%, w/w respectively) in the freeze-dried product. The selected conditions to dilute the ground cooked meat before the lyophilisation were water/ground fried chicken (50/30), conditions that provided higher density, and thus the material is easier to work with, and lower moisture, which is important to prevent microbial degradation. At the selected conditions the weight loss in the freeze drying process was 53%.

To characterise and standardise the final product, for use as a LRM, several parameters were determined in both the raw and the lyophilised fried chicken (Table 2): glucose, creatine, free and total amino acids, total nitrogen, ashes, fat content, and moisture.

3.2. Homogeneity

Lyophilised ground fried chicken breast homogeneity was tested by analysing for HAs in the randomly chosen bottles of the final product. Statistical significance of the differences of between-bottle and within-bottle results was assessed by one-way ANOVA. Table 3 summarises the coefficients of variation (CV%) obtained between-bottle and within-bottle results, calculated and tabulated F-values.

Table 2
Characteristics of raw and cooked material.

Parameters	Raw meat		Cooked and freeze-dried LRM	
	Average	CV (%)	Average	CV (%)
Moisture (%)	80.0	2.0	1.6	3.7
Ashes (%)	0.92	3.1	4.9	2.0
Fat (%)	0.36	6.5	2.7	9.8
Total N (%)	10.0	1.4	50.0	2.6
Glucose (mg g^{-1})	0.27	3.8	0.004	8.2
Creatine (mg g^{-1})	3.10	0.4	0.87	2.3
Free amino acids (mg g^{-1})	25.8	2.4	20.2	7.4
Total amino acids (mg g^{-1})	815.9	5.0	743.7	2.1

Table 3
Concentration of HAs and coefficients of variation (CV%) obtained between-bottle and within-bottle.

Analyte	HAs (ng g^{-1} ± s[a])	CV (%)		F calculated[b]
		Between-bottles[c]	Within-bottles[c]	
DMIP	8.7 ± 0.52	11.8	8.8	1.70
MeIQx	2.4 ± 0.02	5.5	4.7	1.29
4,8-DiMeIQx	1.6 ± 0.01	4.1	3.8	1.68
Norharman	2.8 ± 0.05	4.2	3.8	0.15
Harman	0.9 ± 0.05	7.6	6.4	4.06
PhIP	27.5 ± 0.30	7.7	6.4	0.84

[a] Standard deviation obtained from addition standard calibration (n = 5).
[b] Tabulated F for 95% of confidence: 4.26.
[c] n = 4.

F-calculated, that was in all cases lower than the F-tabulated value (P = 0.05), did not reveal significant differences between within-bottle and between-bottle variances. Considering the results of this study, it was concluded that the material was bottled homogeneously, which is essential for its use as LRM.

Fig. 3 displays the HAs identified in the LRM. The HAs most frequently detected in chicken have been determined. Besides, additional chromatographic peaks that have recently been detected in beef and chicken have been observed in our material. For instance, peak 1 (Fig. 3) could be a novel HA, a DMIP regioisomer, on the basis of their multistage mass spectrometry spectra according to recently published data in our group [35]. A compound (peak 2) with the same MRM transition and similar retention time to MeIQx could be 2-amino-1,7-dimethylimidazo[4,5-g]quinoxaline, an isomer recently identified Turesky et al. [36].

Fig. 4 compares the between-bottle and within-bottle relative standard deviation (RSD). Uncertainties associated to RSD values were calculated using formula RSD/$\sqrt{(2n)}$, where "n" corresponds to the number of replicates [37–39]. It can be observed from Fig. 4 that DMIP presented higher dispersion than the other analytes, which may be consequence of its elution profile at the present chromatographic conditions, leading to broadening peak and major uncertainty in the measurement of the peak area. In addition, Table 3 shows the amount of HAs determined by standard addition in the freeze-dried ground fried chicken by a single analyst by only one analytical method. In order to assess the concentration of HAs in the LRM and to evaluate and improve the performance of the methods currently used by laboratories for the analysis of these compounds, the material is intended to be used in feasibility and interlaboratory exercises.

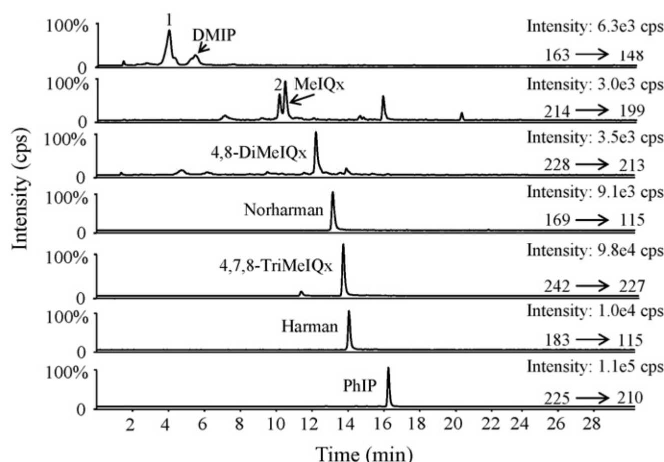

Fig. 3. LC–MS/MS chromatograms of HAs obtained in LRM.

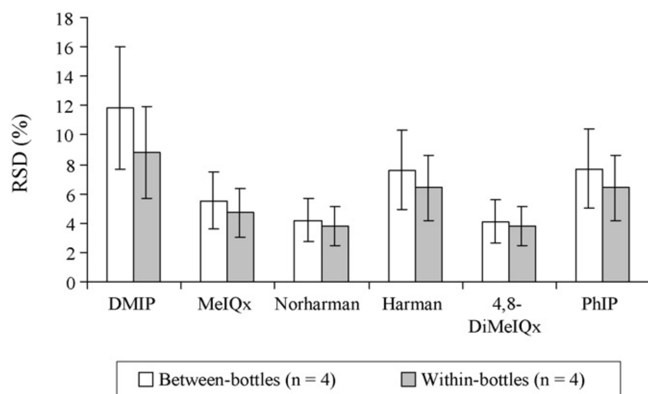

Fig. 4. Relative standard deviations (RSD) obtained for each analyte in between-bottle and within-bottle during homogeneity study.

The measured concentrations of the HAs in the fried chicken used to prepare the LRM were DMIP 8.7 ng g^{-1}; MeIQx 2.4 ng g^{-1}; 4,8-DiMeIQx 1.6 ng g^{-1}; PhIP 27.5 ng g^{-1}, norharman 2.8 ng g^{-1} and Harman 0.9 ng g^{-1}. These amounts are in the same range as those found for fried chicken cooked under similar conditions [40,41]. This demonstrates that the LRM represents fried chicken breast well, in terms of matrix and concentrations of HAs.

3.3. Stability

Stability was studied for a period of 9 months at 4 storage temperatures (−18, +4, +25 and +40 °C). The reference temperature was −18 °C. Four bottles were kept at each temperature for 1, 3, 6 and 9 months. Relative HAs concentration ratios (R_T) were calculated for each storage period by dividing the mean values obtained from duplicate analyses carried out in each of the bottles at each storage temperature, T = +4, +25 or +40 °C (X_T) by the mean of replicates at −18 °C ($X_{-18\,^\circ C}$): $R_T = X_T/X_{-18\,^\circ C}$. The uncertainty of these ratio values, U_T, was calculated with the expression $U_T = (\text{RSD}_T^2 + \text{RSD}_{-18\,^\circ C}^2)^{1/2}\ R_T/100$, RSD_T being the relative standard deviation obtained in the determination of HAs in vials stored at each conditions. A stable compound should in principle yield R_T values of unity at each time–temperature combination, but in practice some variations arise due to analytical procedure. According to the long term reproducibility of the analytical process, involving the SPE clean-up method and the determination by LC–MS/MS, uncertainty below 9% cannot be attributed to instability, thus as the ratios obtained or its uncertainties overlapped the R_T value 0.91–1.90, the HA concentrations were proved to be stable for a period of at least 9 months in the LRM even at temperature of +40 °C. As an example results of the stability of HAs for 9 months at the studied storage temperatures are shown in Fig. 5.

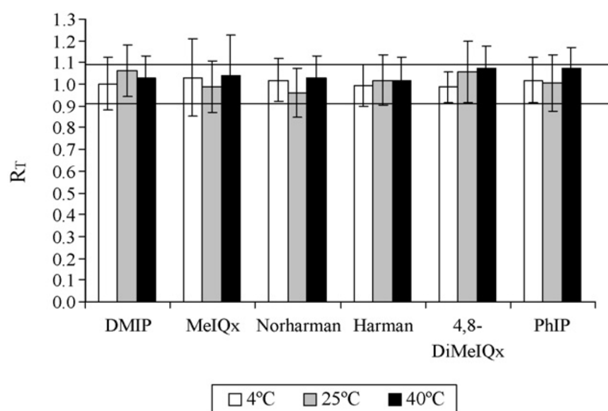

Fig. 5. Results of the stability study (9 months) at different storage temperatures. Error bars correspond to UT values (n = 4).

4. Conclusions

We have prepared a LRM for the analysis of HAs, mutagens found in cooked meat. Raw chicken breast was fried at controlled conditions (average cooking temperature of 210 °C for 3 min/side), ground, diluted (50/30, water/cooked meat), homogenised, lyophilised, sieved, homogenised and bottled, several analytical parameters in both raw and lyophilised fried chicken were determined following the conditions above described. The concentrations of HAs, as well as major components (glucose, free amino acids, creatine, total amino acids, total nitrogen, fat, ashes and moisture) were determined in order to characterise and assure the traceability of the final product as candidate to laboratory reference material. Homogeneity of LRM was studied by single factor ANOVA, finding no significant differences between within-bottle and between-bottle variances, at a confidence level of 95%. Hence, the material was found to be homogeneous at level of concentration of the HAs. All studied HAs were found to be stable in the LRM at common storage temperatures (+4, +25 and +40 °C) for a period of 9 months.

The fried chicken breast LRM fulfils the requirements of homogeneity and stability, and should be suitable for interlaboratory comparisons intended to control the analysis of HAs in foodstuffs.

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