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Formation and stability of heterocyclic amines in a meat flavour model system
Effect of temperature, time and precursors

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Abstract

A model system based on a commercial meat flavour was used to evaluate the formation of heterocyclic amines, simulating the application of this seasoning in household cooking. The effects of different treatments in both dry and aqueous conditions were studied. The lyophilized meat flavour extract was heated at temperatures ranging between 100 and 200 °C for times ranging from 10 min to 2 h. Similarly, an aqueous suspension of the extract was heated at 175 °C for 1, 2 and 3 h. Precursors of HAs, such as creatinine, glucose, and the amino acids glycine, alanine and phenylalanine were added to the meat extract and their effect was tested by heating the mixture at 200 °C for 30 min, when dry conditions were used, and at 175 °C for 2 h in wet systems. All conditions led to the formation of HAs, PhIP being the amine that was detected at the highest level of concentration in most model systems (i.e. 173 ng g^{-1} at 200 °C, 30 min). Moreover, the addition of creatinine and amino acids to the meat extract flavour produced an important increase in IQ and MeIQx content.

Keywords: Food analysis; Heating temperature; Heating time; Precursors; Heterocyclic aromatic amines

1. Introduction

Heterocyclic amines (HAs) are an important group of food mutagens and potential carcinogens in rodents and primates [1,2]. At present, some 20 HAs have been isolated and identified at part per billion levels in proteinaceous foods after thermal processing, such as household cooking. This fact suggests that the formation of HAs is mainly due to heat-induced nonenzymatic browning, known as the Maillard reaction [3,4]. This reaction involves creatine, free amino acids and monosaccharides, which are present in meat and fish products and depend on precursor concentration, time, and temperature of cooking and water content.

For a better knowledge of heterocyclic amines formation, many authors have developed several theoretical model systems containing different mixtures of amino acids, monosaccharides, such as glucose and fructose, and creatine or creatinine. As a result of these experiments, different HAs have been isolated. Pyrolisis of tryptophan and glutamic acid, leads to the formation of Trp-P-1, Trp-P-2, Glu-P-1 and Glu-P-2 [5]. The comutagens harman and norharman are found in dry-model systems containing tryptophan and glucose and are easily formed, even at temperatures lower than 100 °C [6,7]. Mixtures of glucose, creatinine and amino acids, such as glycine, alanine, lysine, methionine, phenylalanine and threonine heated at different temperatures in diethylenglycol and water, and also in some cases in dry conditions, allow the formation of MeIQ, IQ, IQx, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx and PhIP [8–14]. For instance, Johansson et al. [10] studied different model systems constituted by aqueous solutions of creatinine, glucose and one of the amino acids most frequently found in meat proteins, which were heated at 180 °C for 10 min and showed the presence of the aminoimidazoazaarenes IQx, MeIQx and 7,8-DiMeIQx. In order to investigate and confirm the mechanism of PhIP formation, Murkovic used a model system containing phenylalanine and creatinine in diethyleneglycol, the mixture was heated at temperatures ranging from 120 to 200 °C [13,14]. Other strategies have been directed to study this effect on the formation or inhibition of HAs of other compounds which are naturally present in foods,

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e.g. phenol compounds in virgin olive oil [15], carotenoids in food condiments based on tomato sauces [16] or flavour spices [17]. In contrast with these model systems based on mixtures of chemical reagents, other model systems based on food products, such as meat juice have been developed. These last models show the advantage of having a realistic composition in HAs precursors along with other compounds present in the food matrix and which could make difficult the formation of heterocyclic amines. These model systems can be proposed as a useful tool to improve the quality and safety of industrial and household food processes [18–20].

In this paper, a meat extract flavour has been used as a meat-based model system to study the formation and stability of HAs in food processing. The meat extract flavour was chosen because it is a foodstuff used as a condiment in several bouillons and sauces and therefore it can represent an additional contribution to amines intake. In order to simulate different cooking practices, the influence on HAs formation of several parameters, such as temperature, time, and water content was studied. Moreover, the addition of several HAs precursors, such as glucose, creatinine, and the amino acids glycine, alanine and phenylalanine was also studied in order to check their influence on HAs generation. In all cases, the model systems were studied using both dry conditions and aqueous media.

2. Experimental

2.1. Chemicals

HPLC grade dichloromethane and gradient grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany); water was purified in an Elix-Milli Q system (Millipore, Bedford, MA, USA). Acetic acid, ammonia solution and ammonium acetate were analytical grade (Merck, Darmstadt, Germany). D-Glucose monohydrate, creatinine, glycine, L-alanine and L-phenylalanine were purchased from Fluka (Buchs, Switzerland). Helium of high purity and N_2 (N1) were supplied by Air Liquide (Madrid, Spain).

The heterocyclic amines 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-6-methyldipyrido[1,2-*a*:3′, 2′-*d*]imidazole (Glu-P-1), 2-aminodipyrido[1,2-*a*:3′, 2′-*d*]imidazole (Glu-P-2), 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoline (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-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC), 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), provided by Toronto Research Inc. (Toronto, Canada), and 1-amino-9*H*-pyrido[3,4-*b*]indole (harman) and 9*H*-pyrido[3,4-*b*]indole (norharman) were from Sigma (Steinheim, Germany). The chemical purity of the reference compounds was higher than 99%. Stock standard solutions of 20 μg g^{-1} in methanol were prepared and used for further dilutions. Standard mixtures of all amines with TriMeIQx as internal standard at different concentration levels were prepared by weight. Samples were passed through a 0.22 μm filter before injection into the LC–MS system.

Diatomaceous earth extraction columns (Extrelut-20) as well as refill material were purchased from Merck; propylsulphonic acid (PRS, 500 mg) and octadecylsilane (C_{18}, 100 mg), Bond-Elut cartridges, stopcocks and coupling pieces were obtained from Varian Associates (Harbor City, CA, USA). PRS-columns were preconditioned with dichloromethane (7 ml) and C_{18} columns with methanol (5 ml) and water (5 ml).

2.2. Instruments

A quaternary pump system from Waters (Milford, MA, USA) model Alliance 2690 was coupled to a LCQ (Finnigan MAT, San Jose, CA, USA) MS instrument equipped with an electrospray (ESI) as ionization source and an ion trap as analyzer.

For the sample homogenization, a rotating shaker Rotatory Mixer 64526 (Breda Scientific, Breda, The Netherlands) was used. A Supelco Visiprep and a Visidry SPE vacuum manifold (Supelco, Gland, Switzerland) were used, respectively, for the SPE extraction and solvent evaporation. For samples filtration a centrifuge Capsule HF-120 (Tomy Seiko Co., Ltd., Tokyo, Japan) was used.

2.3. Chromatographic conditions and MS parameters

The chromatographic separation of heterocyclic amines was carried out using a C_8 Symmetry column (15.0 mm × 2.1 mm i.d., 5 μm) (Waters Corporation, Milford, MA, USA). The separation was performed by means of a binary mobile phase at a flow rate of 0.3 ml min^{-1}. Solvent A: 30 mM acetic acid-ammonium acetate adjusted with ammonia solution to pH 4.5; solvent B: acetonitrile. The gradient program was: 5% B in A, 0–0.5 min; 5–20% B in A, 0.5–15 min; 20–60% B in A, 15–18 min; 60% B in A, 18–27 min; return to the initial conditions, 27–30 min; 8 min post-run delay. The amount injected was, in all cases, 5 μl.

Data acquisition was carried out by Xcalibur™1.2 software. Ionization source working parameters, optimised previously [21] 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 chromatogram was segmented into three windows and acquisition was performed using product ion scan mode. In Table 1, the time schedule and the MS/MS parameters, such as the

Table 1
MS/MS parameters used with ion trap LCQ instrument

Segment	Time (min)	Analyte	Precursor ions (*m*/*z*)	NCE (%)	Product ions used for quantification (*m*/*z*)	Product ion scan range (*m*/*z*)
1	0–9	DMIP	163	41	148	(140–170]
		Glu-P-2	185	43	158	(150–190)
		IQ	199	41	184	(150–205)
2	9–14	MeIQx	214	41	199 173	(165–220)
		MeIQ	213	40	198	(165–220)
		Glu-P-1	199	44	184 172	(165–210)
		7,8-DiMeIQx	228	42	213 187	(180–235)
		4,8-DiMeIQx	228	41	213 187	(180–235)
3	14–17	Norharman	169	45	167 142 115	(110–175)
		TriMeIQx	242	41	227 201	(195–250)
		Harman	183	44	181 168 115	(110–190)
4	17–25	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)
		AαC	184	39	208 185 167	(165–215)
		MeAαC	198	37	222 199 183 181	(175–225)

precursor ions ($[M+H]^+$), product ions used for quantification, product ion scan range, the normalized collision energy (NCE%) are given. Moreover, an isolation width (IW, *m*/*z*) of 1.5, an activation time (AT) of 30 ms, and an activation Q (AQ) of 0.45 were applied.

2.4. Model systems

A meat extract flavour was prepared by lyophilization of a commercial meat concentrate, as described previously [22]. The freeze-dried meat extract was characterized for free amino acids, creatine, creatinine and glucose. The contents of these compounds in the lyophilized extract were: 78 mg g^{-1} total free amino acids (glycine 3.2 mg g^{-1}, alanine 8.3 mg g^{-1} and phenylalanine 4.6 mg g^{-1}), 12.2 mg g^{-1} of creatine, and 0.81 mg g^{-1} of creatinine and 0.98 mg g^{-1} of glucose.

To reproduce the effect of baking, the meat extract flavour was heated in open dishes in an oven at 100 °C for 1 and 2 h, at 150 °C for 30 min and 1 h and at 200 °C for 10 and 30 min; this kind of experiments constitute the so-called dry systems or systems processed in dry conditions. Other experiments were carried out by heating a water suspension of the meat extract 1:2 (extract:water) in order to reproduce processes that occur in bouillon. Four ml of the suspension was heated in closed tubes in an oven at the temperature of 175 °C for 1, 2 and 3 h. This kind of experiment constitutes the wet systems or systems treated in aqueous media.

To study the effect of various precursors in the formation and stability of HAs in both dry and wet systems, different amounts of glucose, creatinine and amino acids were added to meat extract flavour. The concentration of glucose was increased 10 and 50 times the native composition. For creatinine, the final concentration was five and 25 times the initial concentration and, finally, a mixture of alanine, glycine and phenylalanine in a proportion 50-fold the concentration found in the meat extract was added. The thermal treatment applied to model systems was 200 °C/30 min for dry systems and 175 °C/2 h for the wet systems.

2.5. Clean-up procedure

The extraction of heterocyclic amines was performed as described by Gross and Grüter [23] with some modifications [24]. Briefly, 1–2 g of meat extract were homogenised in 12 ml of 1 M sodium hydroxide and mixed with diatomaceous earth (13 g). The mixture of alkaline solution and refill material was placed in a Extrelut extraction column. A Bond-Elut PRS (500 mg) cartridge was preconditioned with 5 ml 0.1 M HCl, 10 ml water and 5 ml methanol. The column was vacuum-dried, and afterwards conditioned with 7 ml dichloromethane. The amines were eluted from the Extrelut column directly to the PRS cartridge using 75 ml dichloromethane. The cationic exchanger column was dried and washed with 15 ml of methanol:water (4:6, v/v) and 2 ml of water, coupled to a conditioned C_{18} and eluted with 20 ml of 0.5 M ammonium acetate pH 8.5. After washing with 5 ml of water, the retained analytes were eluted with 0.8 ml of methanol/ammonia (9:1, v/v). The solvent was evaporated with a stream of nitrogen and the analytes were dissolved in 300 μl of methanol:mobile phase (1:1) containing the internal standard (TriMeIQx).

Extraction recoveries were determined by spiking one sample of meat extract at three different levels (20, 40 and 60 ng g^{-1}) with a methanol solution containing the 15 HAs. The recoveries were calculated from the slope of the linear regression obtained between the added analyte concentration and the measured analyte concentration. Quantitative results were corrected for incomplete analyte recoveries, which ranged from 48 to 99%. Limits of detection (MDL) and limits of quantitation (MQL) of the method, defined as the amount of analyte that produces a signal-to-noise ratio of 3 and 10, respectively, were estimated by analysing meat flavour samples spiked at very low concentration levels.

3. Results and discussion

Formation of HAs occurred when the meat flavour was processed at all the different conditions tested. In general, the number and amount of these contaminants was higher with increasing temperature and time. Only the comutagens harman and norharman were present in the not processed meat flavour at concentrations of 86 and 79 ng g^{-1}, respectively.

Up to eight heterocyclic amines were detected in meat flavour processed using dry systems, which were: DMIP, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, norharman, harman, PhIP and MeAαC (Table 2). In some cases, especially at mild conditions, very low amounts of HAs, below quantitation limits of the method were formed. When the meat flavour was heated at 100 and 150 °C for 30 min, DMIP was formed in low amounts near to limit of detection, but an increase of processing time, i.e. 150 °C for 1 h, or the use of higher temperatures, i.e. 200 °C for both 10 and 30 min, produced an important formation of DMIP up to 14 ng g^{-1}. Concerning DMIP, in chemical model systems any information about its occurrence has been found in the literature, nevertheless, some authors described the presence of this amine in meat-based model systems [20,25].

Other amines were detected in the studied samples, for instance, MeIQx was determined in all the samples processed at dry conditions above its limit of quantification. MeIQx concentration ranged from 3.5 to 10.7 ng g^{-1} (Table 2), values that are in agreement with the results obtained in other studies that used either theoretical or realistic model systems [9,10,19,20,25]. 4,8-DiMeIQx and 7,8-DiMeIQx were also present in all dry treated samples, although their quantitation was only possible in the samples corresponding to the strongest conditions, i.e. 200 °C and 30 min. A similar behaviour was observed for MeAαC, but in this case at 200 °C/10 min, this amine was not formed. In agreement with these results, occurrence of carbolines in meat based model systems has also been reported in other studies [8,19]. PhIP was also found in all studied conditions, but the highest amount (173 ng g^{-1}) was formed at 200 °C 30 min, in agreement with literature data that reported these conditions as those that most favour PhIP formation [18]. Harman and norharman were detected in the unprocessed meat extract flavour, as mentioned above, and the heating at temperatures above 100 °C, produced an increase of their concentrations up to values ranging between 0.1 and 2.6 μg g^{-1}.

Table 2
Formation of HAs in the meat flavour model system in dry conditions (ng HA per g extract)

Analyte	100 °C/1 h	100°/2 h	150 °C per 30 min	150 °C per 1 h	200 °C per 10 min	200 °C per 30 min
DMIP	~1.3[a]	~1.3[a]	~1.3[a]	<4.2[b]	~1.3[a]	14
MeIQx	3.5	3.6	4.2	3.8	3.7	10.7
4,8-DiMeIQx	~1.2[a]	<3.9[b]	<3.9[b]	~1.2[a]	<3.9[b]	8.9
7,8-DiMeIQx	~0.96[a]	~0.96[a]	~0.96[a]	<3.2[b]	~0.96[a]	4.0
Norharman	120	154	301	445	209	2200
Harman	130	140	305	455	175	2640
PhIP	~0.84[a]	<2.8[b]	<2.8[b]	7.8	<2.8[b]	173
MeAαC	<3.6[b]	<3.6[b]	~1.1[a]	~1.1[a]	ND	<3.6[b]

ND: not detected.
[a] MDL.
[b] MQL.

Table 3
Formation of HAs in the meat flavour model system in wet conditions (ng HA per g extract)

Analyte	175 °C per 1 h	175° per 2 h	175 °C per 3 h
DMIP	<4.2[a]	8.5	5.8
4,8-DiMeIQx	~1.2[b]	<3.9[a]	<3.9[a]
7,8-DiMeIQx	~0.96[b]	4.8	5.6
Norharman	1990	2680	2300
Harman	1700	2400	2000
PhIP	9.3	19.5	16.7

[a] MQL.
[b] MDL.

In wet systems, only six heterocyclic amines were detected: DMIP, 4,8-DiMeIQx, 7,8-DiMeIQx, harman, norharman and PhIP (Table 3). The highest amounts were observed at 175 °C and 2 h, and were higher than those found at 175 °C and 3 h, with the exception of the amine 7,8-DiMeIQx. Moreover, some other amines, such as MeIQx and MeAαC, in contrast with that observed in dry systems, were not detected. This fact suggested that an excess of time, especially in aqueous medium, may cause the degradation of the formed HAs which is in agreement with the results obtained by other authors from kinetic studies [11,26]. It is important to note that the studied conditions for wet systems produced an important increase in the amount of harman and norharman, which rose to 2.7 $\mu g\,g^{-1}$.

As the most favorable conditions that allowed us to produce both a high quantity and a high variety of HAs were 200 °C and 30 min for dry systems and 175 °C and 2 h for wet systems, these conditions were chosen to study the influence of several precursors, such as creatinine, glucose and the amino acids glycine, alanine and phenylalanine in the formation of HAs. The concentrations of IQ ranged from 10 to 43 $ng\,g^{-1}$, showing that in both dry and wet systems the formation of IQ was enhanced when creatinine and amino acids were present in contrast to what happened in samples without the addition of precursors, where this amine was not detected (Tables 4 and 5). This observation is in agreement with the mechanism described by Jägerstad [3], who proposes that the imidazole part of IQ arises from creatinine and glycine through Strecker degradation. Regarding other amines, DMIP was detected at levels near the limit of detection and the formation of MeIQx was evident in all tested systems. Moreover, as expected, MeIQx content increased, and values 10 times higher than those obtained without precursors were found. The precursors and their studied amounts seemed to produce no significant effects in the occurrence of 7,8-DiMeIQx and 4,8-DiMeIQx, since responses obtained for these two analytes were low in all cases, not exceeding their limit of quantitation. For harman and norharman, their formation in wet conditions was also very high. Finally, in all conditions the most important effect of the addition of precursors was the formation of PhIP at levels higher (256 $ng\,g^{-1}$) than the other amines. In Tables 4 and 5 the concentration of HAs determined in the samples treated at the different conditions are given and, as an example, Fig. 1 shows the chromatogram obtained after

Table 4
Formation of HAs in the meat flavour model system in dry conditions (ng HA per g extract) with addition of precursors

Analyte	Creatinine × 5	Creatinine × 25	Glucose × 10	Glucose × 50	Amino acids × 50
DMIP	~1.3[a]	ND	~1.3[a]	~1.3[a]	~1.3[a]
IQ	28	ND	ND	ND	42
MeIQx	33	19	24	31	28
7,8-DiMeIQx	~0.96[a]	~0.96[a]	~0.96[a]	~0.96[a]	~0.96[a]
4,8-DiMeIQx	11	~1.2[a]	~1.2[a]	~1.2[a]	~1.2[a]
Norharman	1500	970	1140	1230	830
Harman	1700	1130	1300	1450	1000
PhIP	94	199	36	78	256

ND: not detected.
[a] MDL.

Table 5
Formation of HAs in the meat flavour model system in wet conditions (ng HA per g extract) with addition of precursors

Analyte	Creatinine × 5	Creatinine × 25	Glucose × 10	Glucose × 50	Aminoacids × 50
DMIP	~1.3[a]	Interferred	~1.3[a]	~1.3[a]	~1.3[a]
IQ	32	10	ND	ND	43
MeIQx	42	40	12	14	278
7,8-DiMeIQx	~0.96[a]	~0.96[a]	5	~0.96[a]	~0.96[a]
4,8-DiMeIQx	7	~1.2[a]	~1.2[a]	~1.2[a]	~1.2[a]
Norharman	2250	1580	1790	2100	1400
Harman	1700	1200	1360	1200	1500
PhIP	9	7.5	Interferred	Interferred	83

ND: not detected.
[a] MDL.

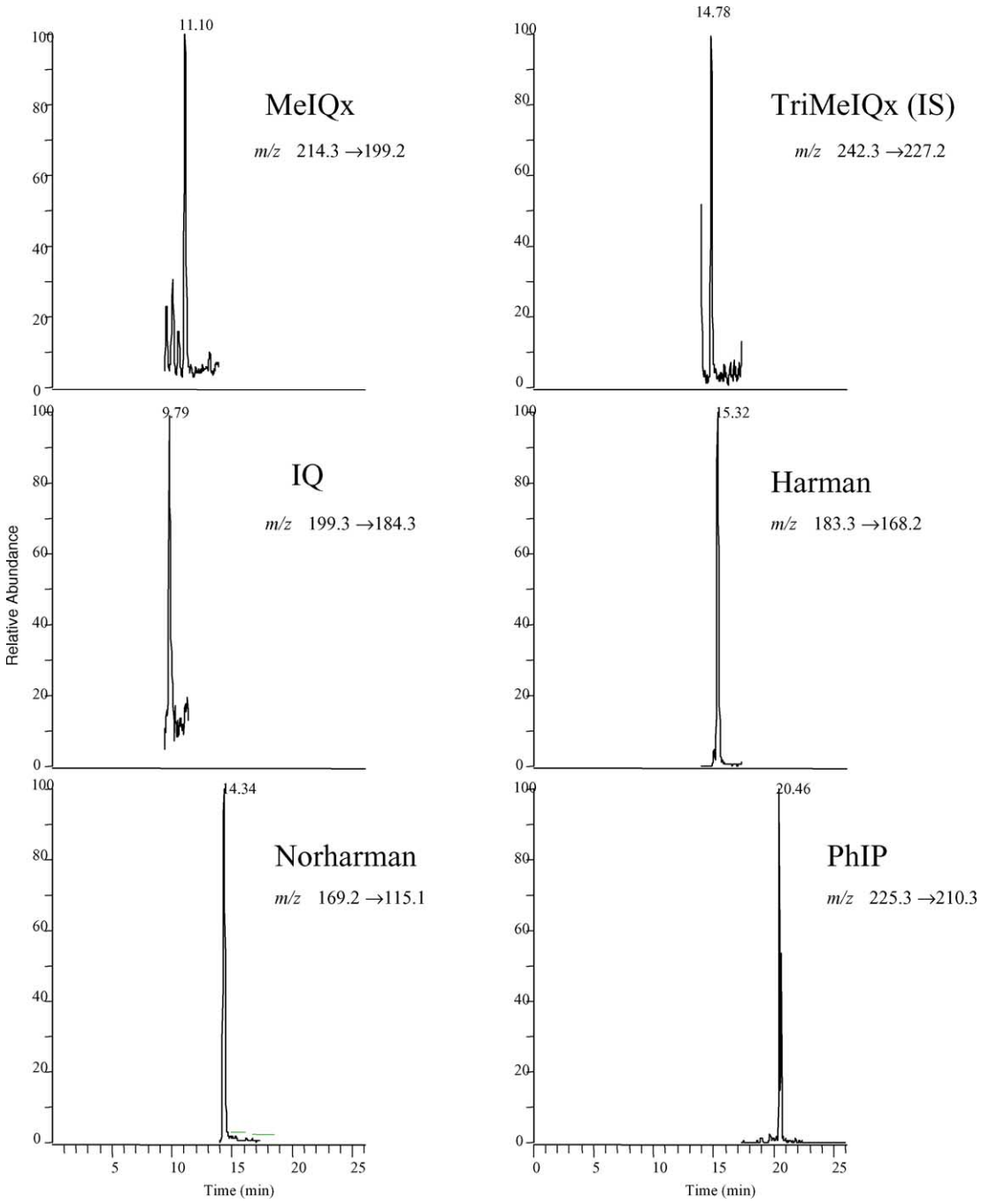

Fig. 1. Chromatogram of heated meal flavour extract (200 °C for 30 min) in presence of glycine, alanine and phenylalanine.

heating the meat flavour extract at 200 °C for 30 min in the presence of glycine, alanine and phenylalanine as potential precursors.

In dry model systems, the addition of creatinine at a concentration five times higher than the native one led to the generation of IQ, MeIQx and PhIP in relatively high amounts. These results agree with the mechanisms of formation described by Jägerstad [3] and Zöchling and Murkovic [14], who indicate that the creatinine is the principal precursor of these amines together with the amino acids glycine, alanine and phenylalanine. Nevertheless, when the increase was 25 times the native amount of creatinine only increased the PhIP content up to 199 ng g^{-1}, whereas in contrast, the concentration of most of IQ-type amines was not as high as expected, showing that a high molar ratio between creatinine and glucose is not a favorable condition to yield IQ-type amines [12]. In wet systems, a similar behaviour regarding the influence of creatinine on IQ formation was observed. Moreover, when glucose was added to both dry and wet model systems, IQ was not detected and the formation of the other mutagens

decreased in accordance with other studies that show the inhibitory effect of an excess of monosaccharides, such as glucose and fructose over amine formation [27,28]. Finally, we note that a high amount of the amino acids glycine, alanine and phenylalanine (50 times the native one), affected the formation of MeIQx and PhIP in aqueous medium and in dry conditions respectively. In both cases the concentrations of these amines raised up to 278 and 256 ng g^{-1} for MeIQx and PhIP, respectively.

4. Conclusions

The commercial meat flavour used in this work has proved to be a suitable model system to study the formation of HAs as an alternative to other models based on chemical mixtures of precursors. This realistic model system was treated at similar conditions to those employed in household cooking practices, showing that high temperatures and long cooking/preparation times led to major HAs formation even when no additional precursors were added to the meat flavour extract. However, the generation of HAs was especially enhanced when creatinine and the amino acids glycine, alanine and phenylalanine were added. The relationship between the generation of HAs and the temperature, time, and the amount of precursors, agrees well with the results obtained using other model systems based on chemical mixtures of precursors. Both dry and wet conditions generated HAs at trace levels, although in wet systems the detection of some amines, such as quinoxaline derivatives was prevented by degradation processes with increasing heating time. These facts confirm that cooking practices that provide foods that receive large processing in terms of time and/or temperature must not be used because of the risk of exposure to these contaminants. Moreover, the use of meat flavour as a model system allows us to state that these kinds of foodstuffs used as condiments can also produce HAs even when heated at relatively mild conditions, such as 100 °C/30 min.

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