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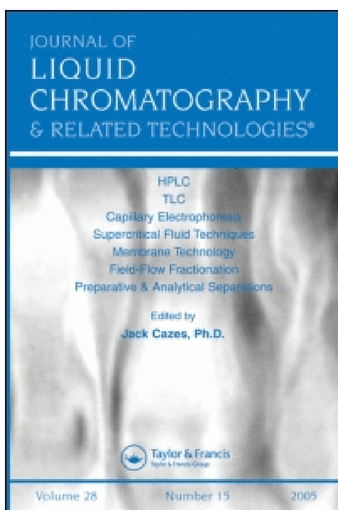

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Extraction, Detection, and Quantification of Heterocyclic Aromatic Amines in Portuguese Meat Dishes by HPLC/Diode Array

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Abstract: The present paper describes the results obtained in the analyses of heterocyclic aromatic amines (HAs) in bovine meat dishes prepared by three different coking methods; usually ingredients such as, salt, garlic, wine, olive oil, onion, and tomato were added. Control meat samples (cooked without ingredients) were also prepared. Analyses were performed by HPLC/diode array and detection of the eluted HAs was performed at 263 nm. The calibration graphs for fourteen amines injected into the column were linear up to approximately 2.0 ng/μL and the detection limits (signal-to-noise ratio 3:1) ranged from 0.06 to 0.4 ng injected.

Nine HAs, namely 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 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-phenylimidazol[4,5]pyridine (PhIP), 3-amino-1,4-dimethyl-5*H*-Pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5*H*-Pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-9*H*-Pyrido [2,3-*b*]indole (AαC) 2-amino-3-methyl-9*H*-Pyrido [2,3-*b*]indole (MeAαC), 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) were encountered in meat samples. A paired Student's t-test showed no significant differences on HAs levels ($p > 0.05$) of control meat samples (cooked without ingredients) and meat samples cooked with ingredients usually in the Portuguese diet and rich in antioxidants. Changes in cooking habits are needed

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to make possible the use of ingredients not only as flavoring but also as protective agents against HAs formation during domestic cooking.

Keywords: Heterocyclic aromatic amines, Meat, Ingredients, HPLC/Diode Array, Solid-phase extraction

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INTRODUCTION

Heterocyclic aromatic amines (HAs) are the major mutagenic compounds isolated from broiled and grilled meats and fish[1,2] and have been shown to induce tumors in multiple organs, including the colon and mammary gland, in rodent bioassays.[3–6] HAs normally have planar structures and consist of three fused aromatic rings with at least one nitrogen atom in the ring structure and with one exocyclic amino group, and up to four methyl groups as substituents. There are, however, some exceptions, for example, PhIP has only two fused rings. Depending on their chemical structure and their mechanism of formation, these xenobiotic genotoxic substances can be grouped into two main families. The first named IQ type or aminoimidazoa-zaarenes (AIAs), includes amines containing a 2-aminoimidazole group generated from the reaction of free amino acids, create(ni)ne and hexoses at ordinary cooking temperatures.[7] The other amines, called non-IQ type or pyrolytic HAs, are formed through the pyrolytic reaction of amino acids and proteins at temperatures between 200 and 300°C. During recent years, there has been a great concern among the scientific community about the risks and health aspects of the intake of those compounds.[8–11] For estimates of the daily intake of HAs and for reliable risk assessments, it is important to determine the levels of HAs in foods cooked under ordinary conditions, which change from country to country, depending on population habits. Therefore, data on HAs from one country cannot be used to assess the human exposure in another country, unless the contents of various precursors and other components in meat and the cooking practices are similar.

It is important to establish databases on the HA content in cooked foods that are representative for the eating habits of the population being studied. Additionally, the accurate assessment of human exposure to HAs requires reliable methods for extraction.

Because of the relatively low amounts of HAs formed in food matrixes (0.1–50 ng/g), the challenge has been to develop rapid analytical methods that isolate and unequivocally identify HAs in these complex matrices at the low ppb level.[12,13] A purification step must be carried out, followed by a separation technique such as liquid chromatography (LC), gas chromatography (GC), or capillary electrophoresis (CE). A number of excellent reviews have been compiled over recent years, to describe the extraction, detection, and quantification of heterocyclic amines and provide a concise appraisal of the relative merits of each approach.[12–16]

Laborious cleanup procedures based on liquid-liquid extraction (LLE),[17] extraction with blue cotton,[18] solid-phase extraction (SPE) with disposable columns[19] or tandem extraction procedures consisting of the coupling of LLE and SPE[20,21] have been developed. Usually, the sample is dispersed using different solvents, namely, methanol, acetone, ethyl acetate, hydro-alcoholic mixtures, or aqueous, like hydrochloric acid, water, or sodium hydroxide.[13] Liquid liquid extraction is the separation method preferred by most of the authors for the first step in the isolation of the analytes from the food matrix. Liquid liquid extraction can be achieved by using inert solid materials such as diatomaceous earth and coupled with solid phase extraction. The use of diatomaceous earth as solid support for liquid liquid extraction is recommended for samples homogenized in sodium hydroxide solution; the aqueous phase distributes itself in the form of a thin film over the chemically inert matrix and macromolecules like proteins and carbohydrates remain adsorbed on the inert material.[13]

The Gross method,[20,21] which uses the coupling of LLE with diatomaceous earth as solid support and two SPE steps with propylsulfonic acid (PRS) and C_{18}, is the most popular tandem method and can yield two extracts clean enough for the determination of polar and less polar heterocyclic aromatic amines. Recently, Toribio et al.[11] proposed a faster method to extract HAs from meat samples on a single extract.

Identification and quantification of HAs has been commonly carried out by means of chromatographic or related techniques using different detection systems.[14] Essential aspects in the analysis of complex matrices are the unequivocal identification of HAs and the accurate quantitative determination of the HAs. This can be achieved with mass spectrometry or diode array.

Liquid chromatography with different detection systems such as ultra-violet,[22,23] electrochemical (ED),[24] fluorescence,[25] and mass spectrometry[26] detection are the most used, because the derivatization step required in GC is avoided. The detection method most commonly used is diode array detection (DAD),[13] which allows on-line identification of the analytes by spectral library matching and has a low cost. Fluorescence detection is sometimes used as a complement to DAD, because unavoidable interferences are frequently produced when using UV detection. To enhance the selectivity of the detection, LC-MS-MS using triple quadrupole,[19,27–29] ion-trap,[26,30] or time-of-flight[31] instruments have been used. In HPLC systems, authors refer that the TSK gel column with a ternary gradient elution system shows the best peak symmetry and separation efficiency. However, good UV spectra were difficult to obtain at pH 3.2 and 3.6. The HPLC at pH 7.0 can be used as an alternative when samples contain multiple interfering peaks such as high temperature meats.[14]

Capillary electrophoresis, either with mass spectrometry (CE-MS),[32] ultra-violet (CE-UV),[33] or electrochemical (CE-ED) detection,[34] has also been proposed, although high detection limits have been obtained.

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Gas chromatography (GC) with nitrogen-phosphorus selective detection (GC-NPD)[12] and gas chromatography-mass spectrometry (GC-MS)[35] have been also used to analyse HAs. However, most of these compounds are polar and non-volatile, and consequently, a derivatisation step is needed.

Other analytical procedures for the determination of HAs are based on the ELISA (enzyme-linked immunosorbent assay) methods. Because of their high sensitivity, high selectivity, and low sample preparation requirements, some monoclonal antibodies have been developed to carry out the analysis of IQ, MeIQ, MeIQx, 4,8-DiMeIQx, and PhIP.[36,37]

Analyte extraction during sample pretreatment is not complete, thus different strategies can be used for the correction of analytical results. Using a single internal standard for this correction is not ideal because HAs include several classes of compounds, which are extracted with varying efficiencies. Even within a class of compounds, significant extraction variation has been reported.[38] When the recovery of HAs was investigated using different commercial brands of solid phase extraction cartridges, the highest variation in the recovery was observed for Trp-P-1 and Trp-P-2, ranging from 3 to almost 60%.[39] External standards can be used or multiple standard addition, which enable determination of the recovery of each analyte individually.[13]

There is extensive literature on the presence of HAs in meats cooked at high temperature; however, in some cases samples were cooked to maximize the production of HAs, and are not representative of the ways meats are usually cooked and eaten by the general population. The presence of HAs in foods depend on many factors, such as cooking method, time, and temperature, the presence of relative amounts of precursors, enhancers, and inhibitors, lipids, antioxidants, and water content;[40] for example, supplementation with antioxidants is considered to be an effective measure to reduce HA exposure. Thus, cooking meat with tomatoes, vegetables, fruits, and spices can be useful to lessen the levels of HAs. However, more studies are needed to find the appropriate concentrations to obtain the desirable effect, because antioxidants are known to exert both anti- and pro-oxidative effects depending on concentrations.

Our objectives were to correlate HAs in three Portuguese meat dishes with known determinants (cooking duration, maximum surface temperature, weight loss due to cooking, and way of cooking), to compare the levels in meat cooked with and without the usual ingredients from Portuguese diet, that are antioxidant rich, such as, garlic, olive oil, onion, tomato, and wine.

EXPERIMENTAL

Chemicals

The compounds studied were 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethyli-

midazo[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]quinoxaline (TriMeIQx), 2-amino-1-methyl-6-phenylimidazol[4,5]pyridine (PhIP), 3-amino-1,4-dimethyl-5*H*-Pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5*H*-Pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-9*H*-Pyrido [2,3-*b*]indole (AαC) 2-amino-3-methyl[2,3-*b*]indole (MeAαC), 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), purchased from purchased from Toronto Research Chemicals, (North York Ontario, Canada). Stock standard solutions of 100 μg/mL in methanol were prepared and used for further dilution.

The methanol, acetonitrile, and dichloromethane were of HPLC grade and were provided by Merck (Darmstadt, Germany). The chemicals used for sample treatment (sodium hydroxide, hydrochloric acid, ammonium acetate) and for mobile phase triethylamine were of analytical grade and were also purchased from Merck. All the solutions were measured using a combined pH glass electrode connected to a pH meter (MicropH 2001, Crison, Barcelona, Spain) and passed through a membranes nylon − 0,2 μm from Teknokrom before injection into the HPLC system.

Extrelut reservoirs and Extrelut HM-N diatomaceous earth refill material were obtained from Merck (Darmstadt, Germany). Bond Elut PRS (500 mg) and endcapped Bond Elut C_{18} (100 and 500 mg) cartridges were from Varian (Harbor City, USA). A Supelco Visiprep and a Visidry SPE vacuum manifold (Supelco, Gland, Switzerland) were used for manipulations with solid phase extraction cartridges and solvent evaporation, respectively.

An Ultra-Turrax® blender (Sotel, Warsvawa, Poland) was used to homogenize cooked meat.

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Meat Samples and Cooking Procedures

The meat dishes were prepared in a way that reflects normal household cooking conditions at two different degrees of doneness. Patties of freshly ground beef (<10% fat content), 10–12 cm diameter, thickness of 1.10–1.30 cm were purchased from a local supermarket in Porto, Portugal. Usual ingredients of Portuguese meat dishes, namely, salt, garlic, and wine were added 10 min before cooking, as usual, in ordinary conditions. Six beef samples were fried on a flat metal, by adding olive oil, the heat source was a gas cooker, and the temperature of the metal surface, monitored with a surface thermometer, was maintained between 160–180°C. Other six beef samples were grilled in a metal skillet at a surface temperature ranging from 180 to 200°C, without adding oil. Two pieces of 500 g of meat with salt, garlic, wine, tomato, onion and olive oil were added and oven cooked at 250–260°C. The duration of cooking time was dependent upon the experiment and is stated in the respective tables. The degree of cooking of the

samples was judged by the colors of the surface and of the center of the products, and was classified as "well done" and "rare". The steaks were cut up using a knife, ground with a food blender, and stored at $-20°C$ until analysis. Similar experiments were performed in meat without any ingredients (control samples); only olive oil was used for fried meat.

A number of conditions in this study were carefully controlled, the same type of bovine meat was used and sample mass and shape was similar. Well done samples were cooked to the level that would be consumed in the home.

Extraction and Purification

Extraction and purification was performed according to the method developed by Gross[20] and modified by Galceran et al.,[41] since this procedure is the reference method in inter-laboratorial exercises.[42]

Sample preparation was as follows: A 5 g sample of beef extract was homogenized in 12 mL 1 M NaOH with sonication (10 min), and the suspension was then shaken for 1 h using a ultra Turray. The alkaline solution was mixed with Extrelut refill material (14 g) and was used to fill an empty Extrelut column. After being preconditioned with 7 mL dichloromethane (DCM), an Isolute PRS column was coupled on-line to the Extrelut column. To extract the analytes from diatomaceous earth, 75 mL of dichloromethane were passed through the tandem. The washing solutions arising from the PRS cartridge, which consisted of 6 mL 0.01 M HCl, 15 mL MeOH-0.1 M HCl (6:4, v/v), and 2 mL of water, were collected for the analysis of the less polar compounds (AαC, MeAαC, Trp-P-1, Trp-P-2). After lowering their organic solvent content by adding 25 mL of water, the acidic washing solutions were neutralized with 500 μL ammonia. The resulting solution was passed through a C_{18} cartridge (500 mg), previously conditioned with 5 mL MeOH and 5 mL water, and less polar HAs were concentrated. Finally, the C_{18} cartridge was rinsed with 5 mL water and the sorbed HAs were eluted using 0.8 mL of methanol-ammonia (9:1, v/v). On the other hand, a 100 mg Bond Elut C18 cartridge was conditioned with 5 mL MeOH and 5 mL water, and was then coupled on-line with the PRS cartridge. After that, the most polar amines (Glu-P-1, Glu-P-2, IQ, MeIQ, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, PhIP) were eluted from the cationic exchanger with 20 mL of 0.5 M ammonium acetate at pH 8.5. Finally, the C_{18} cartridge containing the most polar analytes was rinsed with 5 mL water and the sorbed HAs were eluted using 0.8 mL of methanol-ammonia (9:1, v/v). The extracts containing either the most, or least, polar analytes were gently evaporated under a stream of nitrogen and the analytes were redissolved in 80 μL of methanol. The final extracts from both clean-up procedures were analyzed using the HPLC-Diode Array method described in the next section.

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Quantification and recovery calculation of the amines in meat extracts was carried out by standard addition. Before sample treatment, the meat extract was spiked with 25 or 50 ng by adding a methanolic solution of the analytes (5.6 $\mu g\ g^{-1}$). Thereafter, a sample extraction procedure was applied to spiked and unspiked samples. Recovery was calculated with an external calibration curve, which was constructed using 6 standards with concentrations ranging from 0.03-1 ng/μL for MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, and Trp-P-1, 0.1-1 ng/μL for IQ, IQx, TriMeIQx, Trp-P-2, and AαC, 0.2–2.5 ng/μL for Glup-P1, Glu-P-2, PhIP, MeAαC. Duplicate analyses of all the samples, including the fortified samples, were carried out.

Chromatographic Conditions

The chromatographic analysis was carried out in an analytical HPLC unit (Jasco) equipped with one Jasco PU-1580 HPLC pump, a MD 910 Multiwavelength detector, and a type 7125 Rheodyne Injector with a 20 μL loop. The column was a TSK gel ODS80 (Toyo Soda) (5 μm; 250 mm length; 4.6 mm internal diameter). The Borwin PDA Controller Software (JMBS Developments, Le Fontanil, France) was also used. The mobile phase was as follows: solvent A, 0.01 M triethylamine adjusted with phosphoric acid to pH 3.2, solvent B, same as A but adjusted to pH 3.6, solvent C, acetonitrile. The linear gradient program was: 0–10 min, 5–15% C in A, 10–10.1 min exchange of A with B; 10.1–20 min, 15–25% C in B; 20–30 min, 25–55% C in B; 30–55 min, column rinse and re-equilibration. Separations were carried out at ambient temperature. Peak identification was achieved by comparison of retention time and on-line recorded UV spectra with library entries.

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RESULTS AND DISCUSSION

Performance of the HPLC/Diode Array Method

The linearity has been investigated over the ranges indicated in Table 1 for each amine. Calibration curves were calculated from the representation of the peak area of the analytes versus the ratio of the concentration of each compound. Curves were fitted to a linear function, obtaining regression coefficients higher than 0.9911 in all cases. To evaluate the analytical performance of the HPLC-Diode array method, repeatability or run-to-run precision and medium term or day-to-day precision were determined. To calculate repeatability and medium term precision, six daily replicate analysis of a methanolic solution of all the analytes at 1 ng/μL were carried out on the same day and on different days. Figure 1 shows a typical chromatogram obtained. A study of the variance of one factor for retention and time concentration was then performed. The RSD for retention time varied from 0.12 to 1.84% for

Table 1. Calibration curve parameters determined by external standard method

	Concentration range (ng/μL)	Slope (área units/ng)	r^2
Glu-P-2	0.2–2.5	3.4×10^4	0.9967
IQ	0.1–1	1.7×10^5	0.9926
IQx	0.1–1	1.1×10^5	0.9987
MeIQx	0.03–1	3.2×10^5	0.9987
Glu-P-1	0.2–2.5	5.9×10^4	0.9976
MeIQ	0.1–1	1.1×10^5	0.9911
7,8-DiMeIQx	0.03–1	3.0×10^5	0.9971
4,8-DiMeIQx	0.03–1	3.0×10^5	0.9976
TriMeIQ	0.1–1	1.1×10^5	0.9911
Trp-P-2	0.1–1	1.7×10^5	0.9982
PhIP	0.2–2.5	3.5×10^4	0.9953
Trp-P-1	0.03–1	3.0×10^5	0.9952
AαC	0.1–1	9.7×10^4	0.9985
MeAαC	0.2–2.5	4.2×10^4	0.9925

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run-to-run precision and from 0.33 to 2.75% for day-to-day precision (Table 2). For concentration, run-to-run precision, expressed as RSD, was between 2.54 and 10.03%, and day-to-day precision between 3.12 and 17.61%. Similar values were obtained by other authors.[26,43]

Detection limits (LODs), based on a signal-to-noise ratio of 3:1, were determined in standard solutions and meat extracts (Table 3). In the case of

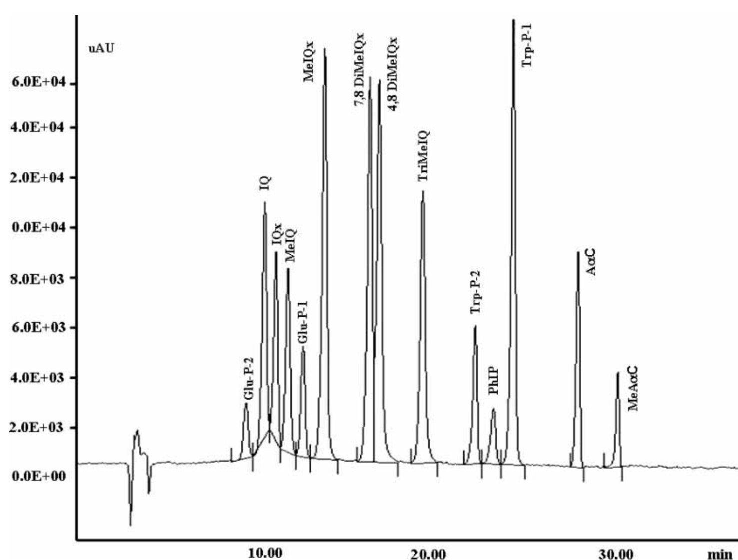

Figure 1. HPLC chromatogram of standard solution (1 ng/μL). For conditions, see text.

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Table 2. Run-to-run precision and day-to-day precision of the method for a standard solution

	Precision RSD %			
	Rt (retention time, min)		Concentration (1 ng/μL)	
	Run-to-run (n = 6, $\alpha = 0.05$)	Day-to-day (n = 10, $\alpha = 0.05$)	Run-to-run (n = 6, $\alpha = 0.05$)	Day-to-day (n= 10, $\alpha = 0.05$)
Glup-P-2	1.84	1.66	2.54	4.78
IQ	0.69	2.54	2.98	3.18
IQx	0.91	1.40	3.02	3.78
MeIQ	0.48	2.75	2.87	3.12
Glup-P-1	1.11	1.99	3.12	4.66
MeIQx	0.84	1.48	5.88	9.22
7,8DiMeIQx	0.34	0.34	9.14	15.44
4,8DiMeIQx	0.74	1.16	9.54	16.45
4,7,8-TriMeIQx	0.39	0.58	9.36	12.65
Trp-P-2	0.59	1.22	10.03	17.69
PhIP	0.39	0.60	9.98	14.15
Trp-P-1	1.57	1.28	6.11	8.21
AaC	0.12	0.76	8.59	16.17
MeAaC	0.18	0.33	4.66	9.70

the meat extracts, LODs were determined by fortifying blank samples at very low levels. Limits of detection in the samples were consistent with those reported by other authors.[44,45] In general, less polar amines have lower detection limits than polar amines. PhIP shows the lowest calibration sensitivity. Quantification limits (LOQs) were established as the amount of analyte that produces a signal-to-noise of 10:1 (Table 3).

Analyses of Meat Samples

Meat samples (spiked and not spiked) were purified using the solid phase extraction procedure described in Materials and Methods, which proved a suitable cleanup of the HAs to permit their analysis by HPLC/diode Array. Two purified extracts, one from most polar amines and another from less polar amines, were obtained. All together, nine HAs, IQ, MeIQx, 4,8-DiMeIQx, PhIP, Trp-P-1, Trp-P-2, AαC, MeAαC, Glu-P-1 were encountered in meat samples; however, in some cases were detected at levels below their limit of quantification. IQx, MeIQ, TriMeIQ, 7,8-DiMeIQx, Glu-P-2 could not be detected in any of the analyzed samples. The identities of amines in the samples were confirmed by on-line recorded UV spectra of the compounds

Table 3. Quality parameters of studied heterocyclic amines in a standard solution, and in a meat extract sample

	Standard	Samples	
	LODs (ng injected)	Meat extract LODs (ng/g)	Meat extract LOQs (ng/g)
Glu-P-2	0.4	1.50	5.00
IQ	0.2	0.50	1.7
IQx	0.2	0.80	2.6
MeIQx	0.06	0.80	2.6
Glu-P-1	0.4	1.00	3.3
MeIQ	0.2	0.26	0.86
7,8-DiMeIQx	0.06	0.17	0.56
4,8-DiMeIQx	0.06	0.17	0.56
TriMeIQ	0.2	0.50	1.7
Trp-P-2	0.2	0.80	2.6
PhIP	0.4	1.50	5
Trp-P-1	0.06	0.30	1
AαC	0.2	0.80	2.6
MeAαC	0.4	0.90	3

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together with reference spectra. Table 4 summarizes the average recoveries and standard deviations obtained in repeated analyses of the spiked meat extract. The extraction recoveries varied with the compound. Average recoveries varied from 27 to 50.6% for the IQx compounds, 60% for PhIP, 31.6% for Glu-P-1, and for the piridoindoles the recoveries varied from 41.2 to 60.3%. The recovery values were comparable to those obtained in previous studies.[41–43] The low extraction efficiencies generally found in the analysis of the cooked meat samples may be due to macrocomponents of

Table 4. Recovery (%) of HAs in spiked meat samples

	Recovery[a] (%)	Standard deviation
IQ	30.6	5.1
Glu-P-1	31.6	4.7
MeIQx	27.2	7.5
4,8-DiMeIQx	50.6	4.0
Trp-P-2	54.1	6.5
PhIP	60.0	5
Trp-P-1	41.2	3.6
AαC	60.3	5
MeAαC	55.0	5

[a]Mean recovery of 3 independent extractions.

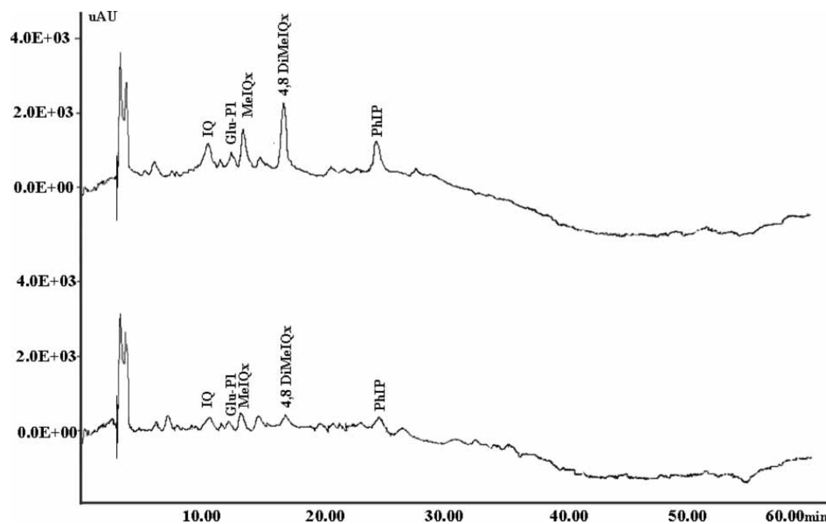

Figure 2. HPLC chromatograms of the analysis of a fried meat sample. Spiked (upper) and unspiked chromatograms of most polar fraction at 262 nm. For conditions see text.

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the matrix, such as lipids. These substances are not only capable of interacting with the analytes but can also modify the selectivity of the different extraction and purification steps.

Figures 2 and 3 show the chromatograms of the spiked and unspiked meat extracts for most polar and less polar amines, respectively. The complexity of the matrix prevents an efficient cleanup and, thus, several interfering substances are present in the final extracts.

Determination of HAs in Portuguese Dishes

HAs were found in "well done" samples of fried, grilled, and oven cooked meat samples with added ingredients, but not in "rare" ones. The results corrected for incomplete recovery, are summarized in Table 5. The amounts of HAs were calculated as ng per g cooked food product and are given as the average of two determinations. The difference between duplicate determinations was generally below 20%. The concentrations of HAs ranged from undetectable levels up to 6.05 ng/g for the IQx compounds, 7.85 ng/g for PhIP, and 2.72 ng/g for the aminocarbolines. As expected, the amount of HAs formed depends on the cooking method and cooking time. Similar profiles of HAs were observed for grilled and fried meat samples with comparable cooking time, which contain higher amounts of IQ type HAs. Results are comparable to others from literature.[10,44–46] Oven cooked meat samples

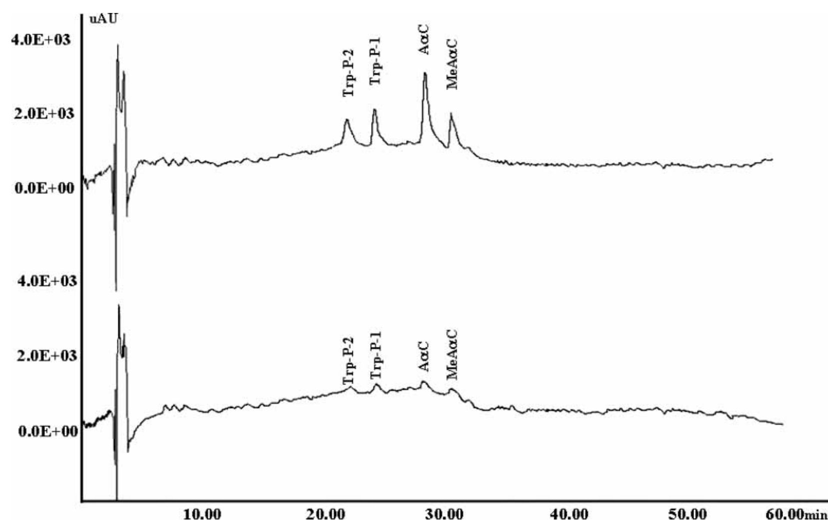

Figure 3. HPLC chromatograms of the analysis of a fried meat sample. Spiked (upper) and unspiked chromatograms of less polar fraction at 262 nm. For conditions see text.

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Table 5. Amounts of HAs in meat dishes cooked in ordinary conditions (ng/g cooked product)

	Grilled meat (180–200°C)		Fried meat (160–180°C)		Oven cooked (250–260°C)	
	Rare[a]	Well done[b]	Rare[a]	Well done[b]	Rare[c]	Well done[d]
IQ	n.d.	6.05	n.d.	4.86	n.d.	n.d.
Glu-P-1	n.d.	Traces	n.d.	Traces	n.d.	n.d.
MeIQx	Traces	4.51	n.d.	2.57	n.d.	n.d.
4,8-DiMeIQx	n.d.	Traces	n.d.	Traces	n.d.	n.d.
TrpP2	n.d.	Traces	n.d.	n.d.	Traces	4.33
PhIP	n.d.	Traces	Traces	7.85	n.d.	n.d.
TrpP1	n.d.	2.11	n.d.	Traces	n.d.	2.01
AC	n.d.	Traces	n.d.	Traces	n.d.	Traces
MeAC	n.d.	Traces	n.d.	Traces	n.q.	2.72

[a]Grilled or fried for 4 minutes (2 minutes each side) the color of the surface was light and of the center of the meat was red; weight loss ranged between 15 and 20%.

[b]Grilled or fried for 8 minutes (4 minutes each side) the color of the surface was dark and of the center of the meat was light brown; weight loss ranged between 35 and 40%.

[c]Oven cooked for 30 minutes the color of the surface was light and of the center of the meat was red; weight loss ranged between 20 and 25%.

[d]Oven cooked for 70 minutes the color of the surface was brown and of the center of the meat was red; weight loss ranged between 38 and 42%.

presented a different profile of HAs, as a result of their different exposures to heat and crust formations together with oven temperatures, no IQ type amines were detected. However, the non-polar HAs, which are formed from amino acids without contribution of creatinine, were higher in over cooked meat.

In several publications it was stated that the presence of antioxidants reduces the content of HAs in meat.[47] Consequently, addition of ingredients rich in antioxidants is considered a promising measure to reduce HA exposure.[40,48,49] The application of spices (rosemary, thyme sage, garlic, brine), olive oil, and wine can reduce the content of the HAs. Additionally, salt confers a better water holding capacity, reducing the transport of precursors towards the surface during cooking, that could lead to the formation of low amounts of HAs. However, a paired Student's t-test showed no significant differences on HAs levels ($p > 0.05$) of control meat samples (cooked without ingredients) and meat samples cooked with ingredients usually in Portuguese diet and rich in antioxidants. Changes in cooking habits are needed to effectively make use of ingredients not only as flavouring, but also as protective agents against HAs formation during domestic cooking.

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