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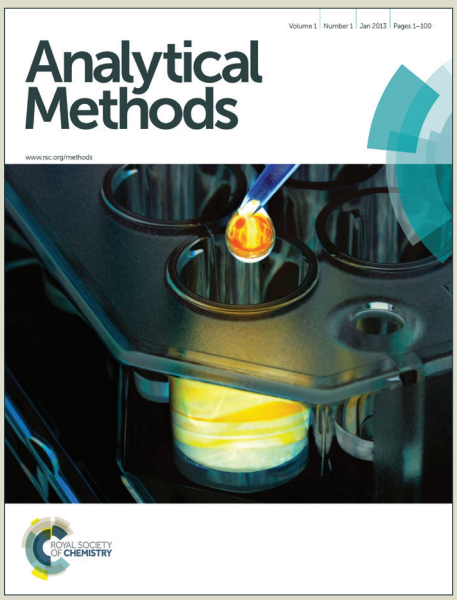

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A reliable and sensitive LCMS-IT-TOF method coupled with accelerated solvent extraction for the identification and quantitation of six typical heterocyclic aromatic amines in cooked meat products

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A reliable and sensitive liquid chromatography-ion trap-time of flight tandem mass spectrometric assay (LCMS-IT-TOF) coupled with accelerated solvent extraction (ASE) was developed to identify and quantify six typical heterocyclic aromatic amines (HCAs), including 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-1-methyl-6-pheny- limidazo [4,5-b] pyridine (PhIP), 2-amino-9*H*-pyrido [2,3-b] indole (AαC) and 2-amino-3-methyl-9*H*-pyrido [2,3-b] indole (MeAαC) in cooked meat products. The effects of various experimental factors on separation and detection were investigated, and the fragmentation patterns of six HCAs were discussed. The method has shown high reproducibility with intra-day and inter-day precision (RSD, %) less than 6.16% across three quality control levels for the six analytes. The assay was linear over the concentration range of 10 to 1000 μg L^{-1} for IQ, MeIQ and 5 to 500 μg L^{-1} for MeIQx, PhIP, AαC and MeAαC ($r^2 \geq 0.996$). The experimental results showed that the proposed method can be used successfully to identify and determine six typical HCAs at ultra-trace levels (μg kg^{-1}) in cooked meat products.

Introduction

Heterocyclic aromatic amines (HCAs) are substances with high mutagenic and carcinogenic potential.[1] They are formed during heating protein-rich food items.[2,3] To date, more than 25 different HCAs have been identified and isolated from various cooked food products such as fish, meat and poultry.[4-6] They are usually divided into two main classes: aminoimidazoazaarenes and aminocarbolines. The first group is formed at the ordinary household cooking temperatures of 100 – 225 ℃ and are sometimes termed thermic mutagens. The aminoimidazoazaarenes commonly reported in cooked foods are: 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), 2-amino-3,4-

dimethylimidazo[4,5-f]quinolone (MeIQ), 2-amino-3,4-dimethylimidazo[4,5-b]quinoxaline (MeIQx), 4,8-DiMeIQx and 7,8-DiMeIQx and 2-Amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP). The aminocarbolines, called pyrolitic HCAs, are formed at temperatures above 300 ℃ through a pyrolitic reaction, and includes the amines: 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), Glu-P-1 and Glu-P-2. [7] In 1993, the International Agency for Research on Cancer (IARC) considers eight of the HCAs tested (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) and recommends a reduced exposure to these compounds. [8] Some human diseases, especially cancers, are associated with the daily intake of various meat products containing these compounds. To assess the exposure to these HCAs and risk to human health, it is important to unambiguously identify and precisely quantify these compounds in different classes of processed meat product since humans are chronically exposed to these compounds in low doses.[9] It is a matter of urgency to develop an effective analytical method that can reliably identify and quantify HCAs in cooked meat products.

Usually, the analytical procedures can involve a variety of purification and pre-concentration steps, followed by various separation and detection techniques. And great efforts are needed to improve existing procedures of clean-up and pre-concentration, which usually occupy most time of the whole analytical steps. Until now, the extraction and purification methods for HCAs were developed by Gross[10] and modified by Messner[11] have been widely used as reference methods. However, such procedures expend considerable organic solvents and involve laborious

purification steps. In order to overcome these limitations, an alternative extraction technique, accelerated solvent extraction (ASE) was developed. Compared to traditional extraction methods, ASE has similar or sometimes even higher extraction efficiencies but consumes less solvent and labour time.[12] Nowadays, ASE has been widely used for the extraction of different food samples such as fish tissue, animal tissue, pork and chicken meat.[13-15] Furthermore, it has been applied to the extraction of 10 HCAs in meat extracts.[16] Hence, this technique is suitable for the routine analysis of HCAs in cook meat samples. The benefits of this method are simplicity, speed of analysis, and a degree of automation that allows the analysis of large numbers of samples with minimal labor.

After the purification and pre-concentration steps, the determination of HCAs is performed commonly using HPLC coupled with UV-VIS, fluorescence, or photodiode array (PDA) detectors.[17-19] Nevertheless, co-extracted compounds from the food sample matrix frequently appear and can interfere or lead to false peak identification in the analysis of HCAs. To solve this problem, the coupling of more selective techniques like mass spectrometry that allows the unambiguous identification of the compounds, can be used. Gas chromatography-mass spectrometry (GC-MS) can be used to identify and quantify some heterocyclic amines,[20] but it requires derivatization before the GC analysis. Liquid chromatography-mass spectrometry (LC-MS) has proven to be a very convenient and efficient technique for identification of HCAs in food products in recent years.[21,22] Identification and characterization of HCAs may be enhanced by high mass resolution and multiple fragmentations using liquid chromatography-ion trap-time of flight tandem mass spectrometry (LCMS-IT-TOF). High mass accurate measurements of HCAs appear to be limited in the literature.

Due to their mutagenic and carcinogenic characteristics, and their high rate of formation in cooked meat products, IQ, MeIQ, PhIP, MeIQx, AαC and MeAαC have been selected as the target analytes (Fig.1). The aim of this study is to establish an accurate and reproducible LCMS-IT-TOF method combined with accelerated solvent extraction that can successfully detect these species in various cooked meat products. The application of LCMS-IT-TOF can yield empirical chemical formula based on the accurate masses of molecular ions and detailed fragmentation information, remove ambiguities out of the interpretation, confirm the identities of the fragment ions and facilitates structural elucidation. It also provides an excellent approach for rapid screening of HCAs in food products.

Figure 1: Chemical structures of studied HCAs.

Materials and Methods

Chemicals and reagents

Analytical standards of IQ, MeIQ, MeIQx, PhIP, AαC and MeAαC (99.5 %) were purchased from Toronto Research Chemicals Inc. (Toronto, Canada). Dionex ASE ™ Prep DE Diatomaceous Earth, HPLC-grade acetonitrile and formic acid were purchased from Fisher Scientific Co. (New Jersey, USA). The HPLC-grade acetone, ethyl acetate, dichloromethane, and analytical reagent grade acetic acid, sodium hydroxide (NaOH), anhydrous sodium sulfate were all obtained from ANPEL Scientific Instrument Co., Ltd. (Shanghai, China).

Stock standard solutions (100 mg L^{-1} in methanol) for IQ, MeIQ, MeIQx, PhIP, AαC and MeAαC were prepared and stored at -20 ℃. The mixed working solutions were freshly prepared by a series of dilutions with 30 mM formic acid-acetonitrile (90:10, v/v). The ultrapure water used

in the work was produced by a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA).

Instrumentation settings

Chromatographic separation was carried out on a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) equipped with an Alltima C18 column (3.2 mm×150 mm, 5μm, Grace, USA). Mobile phase A and B were 30 mM formic acid and acetonitrile. The gradient program for the separation was 0-1 min, 90 % A; 1-3 min, 90-80 % A, 3-5 min, 80-60 % A, 5-7 min, 60-40 % A and 7-9 min, 40-90 % A. Finally, phase A was held at 90 % until end of the run at 10 min. The flow rate during analysis was 0.6 mL min^{-1} and the injection volume was 5 μL. The column oven temperature was maintained at 30 ℃.

The identification of analytes was performed by using an ion trap-time of flight tandem mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI) source, operating in positive ionization mode. Shimadzu's LCMS Solution software was used for data analysis. The formula predictor function of LCMS solution was utilized in identification and confirmation of unknown signals.

A Dionex ASE 150 accelerated solvent extractor (CA, USA) equipped with 66 ml stainless extraction cells was applied for sample extraction. A Turbo Vap II Concentration Workstation (Caliper Life Sciences, Inc., USA) was used for solvent concentration.

Sample preparation using ASE

Chicken breast, duck breast, pork fillet and bream loin were taken as samples in this work. The raw meat products were obtained from a local "TESCO" supermarket store in Changzhou. Chicken breast, duck breast and pork fillet were sliced into 150 g, 2 cm thick portions, then placed

in the oven preheated to 230 ℃ and roasted at 230 ℃ for 20 min. Bream loins were sliced into 5 cm long, 4 cm wide portions, then were placed in fat preheated to 200 ℃ and were fried without cover for 20 min. Temperature during frying ranged between 150–160 ℃. The cooked meat products were homogenized in a high-speed food blender, then they were stored below −20 ℃ in a freezer until the time of analysis.

The extraction of all samples was carried out using the Dionex ASE 150 accelerated solvent extractor. A 66 mL stainless steel extraction cell and a 200 mL glass collection bottle were used. Five grams of the blank/spiked sample material was dissolved in 12ml of 0.5M NaOH (70/30 methanolic/aqueous solution) and mixed for 1 h until completely homogenised. Then, the sample solution was mixed with 12 g of diatomaceous earth and loaded into a stainless extraction cell which preloaded 10 g neutral alumina and extracted with dichloromethane/acetonitrile (1:1, v/v). Twenty-five grams of anhydrous sodium sulphate was added into the collection bottle to adsorb moisture during the process. The ASE conditions were as follows: set the static time for 5 min, static cycles for 2 times, flush ratio at 50 %, purge time for 160 s, extraction temperature at 80 ℃ and extraction pressure at 10.3MPa. The extracted analytes were evaporated to dryness under a stream of nitrogen at 40℃and the residues were dissolved in 1 ml 30 mM formic acid-acetonitrile (90:10, v/v), then filtered through a filter (0.45 μm) and finally identified and quantified using LCMS-IT-TOF system. Further cleanup using solid phase extraction or liquid extraction did not exhibit any significant improvements for the subsequent chromatographic analysis.

Quality parameters

To check performance and reliability of the proposed methods, quality parameters such as regression equation, linear range, limit of detection, limit of quantitation, accuracy, intra-day and

inter-day precision were studied.

The linearity of the proposed method was assessed by direct injection of seven working solutions, prepared in the concentration range from 5 to 1000 μg L^{-1}. Each solution was analyzed in triplicate. The calibration curves were constructed by a least squares linear regression analysis. This method was used to determine the slope, intercept, and correlation coefficient (r^2) of the linear regression equation. Limit of detection (LOD) and limit of quantitation (LOQ) were established as the amount of analyte that produced signal-to-noise ratio of 3:1 and 10:1, respectively. They were calculated using standard solutions at low concentration levels. LOD is the lowest concentration of the analyte that the analytical process can reliably differentiate from background levels, while LOQ is the lowest concentration of analyte that can be quantified.

The intra-day and inter-day precision of the analyses was estimated in terms of repeatability. These parameters were expressed as relative standard deviation (RSD, %). The accuracy (RE, %) was expressed by [(mean observed concentration)/(spiked concentration)] × 100.

Results and discussion

Optimizing the ASE parameters

ASE is a technique that involves extraction using liquid solvents at elevated temperature and pressure, which enhance the extraction performance as compared to those techniques carried out at near room temperature and atmospheric pressure. The merits of enabling the use of solvents at temperatures above their atmospheric boiling point are the enhanced solubility and mass transfer properties. However, some studies have shown that pressure has usually played a minor role in the resulting efficiency and it is only required to maintain the extractant in the liquid phase.[23] The parameters such as the static time, flush volume, purge time and static cycles were optimized

firstly after a series of preliminary studies and maintained constantly at 5 min, 50%, 160 s and 2 times, respectively, throughout this study. The investigation focused mainly on the three most influential parameters, including in extraction solvent, temperature and addition of aluminum oxide.

The extraction solvent must be able to solubilize the target analytes, minimizing the co-extraction of other matrix components. The polarity of the solvents should be close to that of the target compound. Taking into account the solvent or solvent mixtures currently used in ASE methods for food samples,[24] two pure organic solvent, ethyl acetate and acetonitrile, and two solvent mixtures dichloromethane/acetonitrile (1:1, v/v) and dichloromethane/acetone (1:1, v/v) were tested as extraction solvents. As can be seen in Fig.2, dichloromethane/acetonitrile (1:1, v/v) produced relatively high response values for all HCAs and the other three solvent and solvent mixtures produced relatively low response values for one or several HCAs. Take acetonitrile as a example, it achieved good recovery in polar amines such as IQ, MeIQ, while achieved bad recovery in the less polar HCAs, including AαC and MeAαC in this study. Thus, dichloromethane /acetonitrile (1:1, v/v) was selected as the optimal extraction solvent.

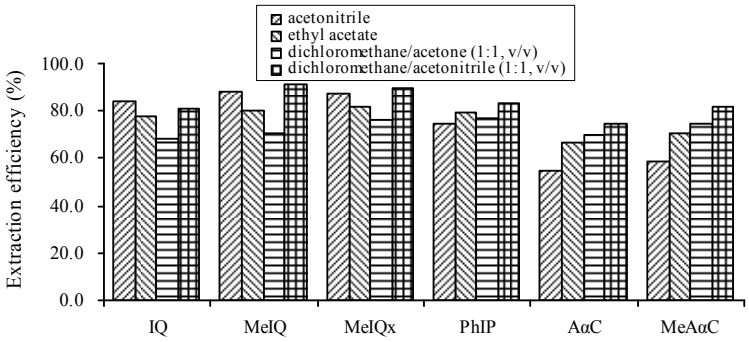

Figure 2: Effect of different solvent on extraction efficiency. Spiked at 20 μg kg^{-1} for 6 HCAs; pressure, 10.3 MPa; static time, 5 min; static cycles, 2; purge time, 160 s; temperature, 80 ℃

Temperature is one of the most important parameters for ASE. High temperatures will help the disruption of analyte - sample matrix interactions caused by van der Waals forces, hydrogen bonding and dipole attraction, which results in an improved extraction efficiency. On the other hand, high temperatures might decrease selectivity of extraction and affect thermo-labile compounds that are subject to disintegration and hydrolytic degradation.[12] The effect of temperature on the extraction efficiency of three HCAs was investigated by varying the temperature in the range 60–120 ℃ with increments of 20 ℃. Extraction efficiency showed an optimum at 80 ℃. Over 80 ℃, recoveries decreased, probably due to degradation of target compounds. Another problem was that in high temperature, the extract was not clear which may be due to the matrix dispersion of the tissues. Recoveries were also low at below 80 ℃, mostly because of the low efficient desorption and dissolution of the HCAs. In all cases, the best recoveries were obtained at 80 ℃, so this temperature was selected for subsequent extraction.

It is reported that aluminum oxide could be used as a sorbent to eliminate grease, pigments and other impurities from food samples.[25] Tests were carried out by adding 0, 5, 10, 15 g neutral aluminum oxide in the extraction cell, respectively. It turned out that poor recovery was got and the extract was not clear before adding aluminum oxide. When adding aluminum oxide up to 10 g, the extract was clear and extraction efficiency of HCAs reached the highest. However, when the amount of aluminum oxide increased to 15 g, extraction efficiency decreased instead because of the increased adsorption from the target compounds. So, 10 g was selected as the adding amount of aluminum oxide.

Optimizing HPLC and MS parameters

For the MS/MS detection, the result showed that electrospray operation in positive ionization

mode (ESI) was better and had excellent signal sensitivity. In order to achieve the quantification of HCAs, the HPLC and mass spectrometric parameters, such as acid concentration, column temperature, flow rate, detector voltage, heat block temperature, curved desolvation line (CDL) temperature, drying gas pressure, scan range and ion accumulative time, were optimized to attain the maximum sensitivity for the detection of the analyte. The optimum conditions obtained were 30 mM formic acid, 30 ℃ column temperature, 0.6 ml min^{-1} flow rate, 1.75 kV detector voltage, 230 ℃ heat block temperature, 250 ℃ CDL temperature, 100 kPa drying gas pressure, 150~250 scan range and 70 ms ion accumulative time.

Identification of 6 HCAs by LCMS- IT-TOF

Compounds with minor molecular weight were highlighted by extracted ion chromatograms (EICs). Figure 3 shows the EICs of 6 HCAs, indicating the high precision and selectivity of the developed method. One of the main attributes of TOF (time of flight) instrument is its accurate mass measurement, which gives the elemental composition of parent and fragment ions and can be used for the identification of unknown compounds and the differentiation of isotopic compounds. And IT (ion trap) may produce multistage tandem mass spectral data, which is very useful for interpretation of molecular structure. Table 1 summarized accurate masses and assigned elemental compositions of fragment ions from MS^n spectra of 6 HCAs. The errors between the measured and calculated values range from -0.7 to 1.0 mDa (-3.3 to 5.8 ppm) in MS^n (n=1-3). The result indicated that for all MS stage, the errors were less than 6 ppm. Thus, all the calculated elemental compositions in Table 1 can be considered to be reliable, which is the basis of the identification of elemental composition and fragmentation pathways.

Figure 3: Extracted ion chromatograms (EICs) obtained from the analysis of IQ (*m/z* 199.09), MeIQx (*m/z* 213.11), MeIQx (*m/z* 214.10), PhIP (*m/z* 225.11), AαC(*m/z* 184.08) and MeAαC(*m/z* 198.10) standard at 100 μg L^{-1}

Table 1 Exact masses of 6 HCAs with their assigned elemental composition as determined from tandem mass spectra, MS^n (*n*=1-3)

According to these multistage tandem mass spectral data, the fragmentation patterns were proposed. The M^1 spectra of 6 HCAs were observed as their protonated molecule $[M+H]^+$ at *m/z* 199.0974, 213.1128, 214.1083, 225.1135, 184.0879, 198.1024, respectively. In the MS^2 spectra, only AαC corresponded to the loss of a molecule of HCN from the parent ion and the other 5 HCAs corresponded to the loss of one methyl unit ($-CH_3$) from the parent ion. In addition, MS^2 of MeIQx exhibited the other major product ion at *m/z* 173, which corresponded to the elimination of one aminoimidazole moiety (C_2NH_3) from the parent ion at *m/z* 214.1083 (calculated for $C_{11}H_{11}N_5$ 214.1087) and MS^2 of MeAαC exhibited the other major product ion at *m/z* 181, which corresponded to the loss of a molecule of NH_3 from the parent ion at *m/z* 198.1024 (calculated for $C_{11}H_9N_3$ 198.1026). In the MS^3 spectra, a common fragmentation pattern of losing a molecule of HCN had been seen from MS^2 spectra of the other 5 HCAs except for MeIQ. The MS^3 of MeIQ exhibited the major product ion at *m/z* 170, which formed by the loss of one $-C-NH_2$ unit from the MS^2 at *m/z* 198.0905(calculated for $C_{11}H_9N_4$ 198.0900). Besides, the MS^3 of PhIP exhibited the other major product ion at *m/z* 168, which corresponded to the loss of a molecule of HN=C=NH from the MS^2 at *m/z* 210.0907 (calculated for $C_{12}H_9N_4$ 210.0900). The ESI-MS^n spectra of 6 HCAs in positive ion mode are shown in Fig.4.

Figure 4: ESI-MS^n spectra of IQ(A), MeIQ(B), MeIQx(C), PhIP(D), AαC(E) and MeAαC(F) in positive ion mode

Linearity and detection limit

Under the optimum conditions, the assay was linear over the concentration range of 10 to 1000 μg

L^{-1} for IQ and MeIQ, 5 to 500 μg L^{-1} for MeIQx, PhIP, AαC and MeAαC. The quality parameters of 6 HCAs were provided in Table 2. As shown in Table 2, the LOD and LOQ of the 6 HCAs were within the range of 1.5–3 μg L^{-1} and 5–10 μg L^{-1}, respectively, and correlation coefficients of linearity (r^2) were higher than 0.996 for all compounds which means good correlation between peak areas and concentrations.

Table 2 Quality parameters of 6 HCAs

Precision and accuracy

The intra-day precision, inter-day precision, and accuracy of the method were evaluated by spiking analyte at three quality control levels (5, 10 and 40 μg kg^{-1}). The intra-day precision and inter-day precision were less than 5.32% and 6.16% (RSD, %), respectively. The accuracy ranged from 69.5% to 93.6%. The detailed values of intra-day, inter-day precision, and accuracy are shown in Table 3. All the values are within the acceptable range. Therefore, the LCMS-IT-TOF method proved to be precise and accurate.

Table 3 Precision and accuracy of the method for the determination of 6 HCAs using LCMS-IT-TOF

Analytical Application

Under the optimum conditions, the proposed method was applied to the determination of IQ, MeIQ, MeIQx, PhIP, AαC and MeAαC in four cooked meat products, including roast chicken, roast duck, roast pork and fried bream. By a standard addition method and comparing the retention times, accurate molecular ion (*m*/*z*), molecular formula and MS^n data of target compounds with those of the mixed standard solution, the above HCAs were identified and quantified. The results showed that PhIP was detected in all four tested meat products, their contents were between 3.9

and 5.4 μg kg^{-1}. MeIQ, AαC and MeAαC were not detected or quantified in four tested meat products. IQ was quantified in the other three samples except for roast pork products and MeIQx was found in roast chicken and fried bream products. The detailed data for the tested meat products are summarized in Table 4, and the contents are consistent with the reported values in previous publications,[26,27] which demonstrated the validity and reliability of the method.

Table. 4 HCAs contents in four cooked meat samples (*n*=3)

Conclusions

HCAs are normally found in low amounts in a complex matrix, which necessitates high efficient extraction methods and sensitive detection systems. A reliable HCAs extraction method of ASE was developed. The method offers advantages in eliminating the extensive clean-up process of extracts prior to analysis, saving solvent amount, reducing the sample manipulation and total extraction time. LCMS-IT-TOF as a powerful tool has been applied to the identification and analysis of six typical HCAs including IQ, MeIQ, MeIQx, PhIP, AαC and MeAαC in cooked meat products. Quality parameters such as regression equation, linear range, limit of detection, limit of quantitation, accuracy, intra-day and inter-day precision were checked for accurate determination. In summary, the results of a series of accuracy, verification, and application tests confirm that the proposed method is reliable，sensitive, and can be used to identify and quantify mutagenic HCAs in complex matrices such as cooked meat products.

Acknowledgments

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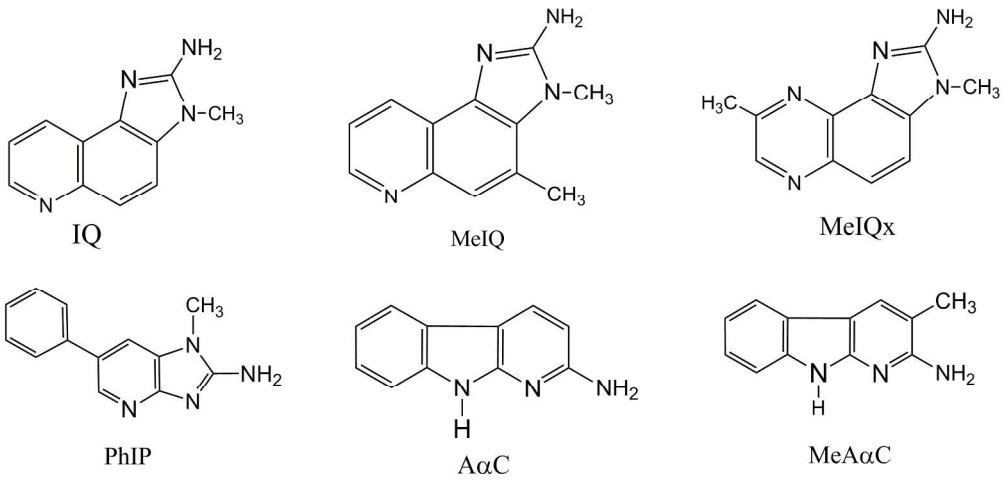

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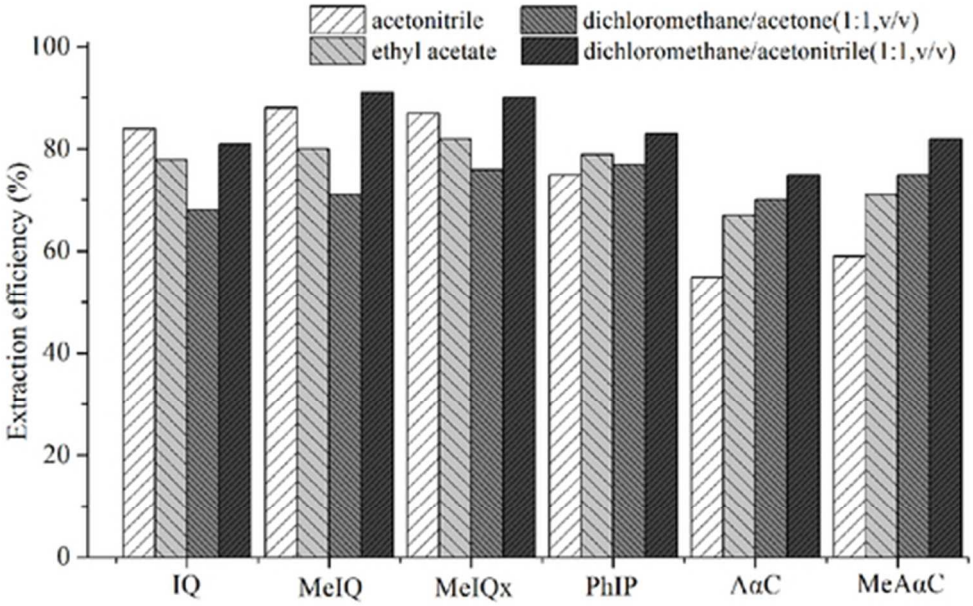

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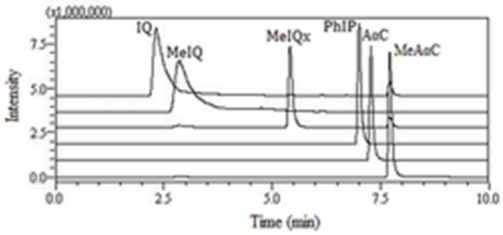

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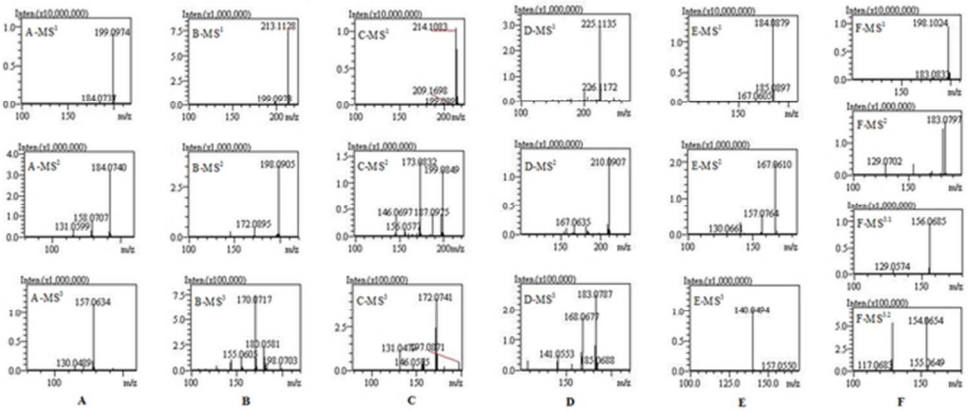

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Tables

Table 1 Exact masses of 6 HCAs with their assigned elemental composition as determined from tandem mass spectra, MS^n (n=1-3)

HCAs	Assignment	Ion	MS^n	Elemental composition	Product ion (m/z)	Measured (m/z)	Calculated (m/z)	Error (mDa)	Error (ppm)
IQ	$[M+H]^+$	1a	MS^1	$C_{11}H_{10}N_4$	199	199.0974	199.0978	-0.4	-2.0
	1a-15	2b	MS^2	$C_{10}H_7N_4$	184	184.0740	184.0743	-0.3	-1.6
	2b-27	3c	MS^3	$C_9H_6N_3$	157	157.0634	157.0634	0	0
MeIQ	$[M+H]^+$	1a	MS^1	$C_{12}H_{12}N_4$	213	213.1128	213.1135	-0.7	-3.3
	1a-15	2b	MS^2	$C_{11}H_9N_4$	198	198.0905	198.0900	0.5	2.5
	2b-28	3c	MS^3	$C_{10}H_7N_3$	170	170.0717	170.0713	0.4	2.4
MeIQx	$[M+H]^+$	1a	MS^1	$C_{11}H_{11}N_5$	214	214.1083	214.1087	-0.4	-1.9
	1a-15	2b	MS^2	$C_{10}H_8N_5$	199	199.0849	199.0852	-0.3	-1.5
	1a-41	2c	MS^2	$C_9H_8N_4$	173	173.0832	173.0822	1.0	5.8
	2b-27	3d	MS^3	$C_9H_7N_4$	172	172.0741	172.0743	-0.2	-1.2
PhIP	$[M+H]^+$	1a	MS^1	$C_{13}H_{12}N_4$	225	225.1135	225.1135	0	0
	1a-15	2b	MS^2	$C_{12}H_9N_4$	210	210.0907	210.0900	0.7	3.3
	2b-27	3c	MS^3	$C_{11}H_8N_3$	183	183.0787	183.0791	-0.4	-2.2
	2b-42	3d	MS^3	$C_{11}H_7N_2$	168	168.0677	168.0682	-0.5	-3.0
AαC	$[M+H]^+$	1a	MS^1	$C_{11}H_9N_3$	184	184.0879	184.0869	1.0	5.4
	1a-17	2b	MS^2	$C_{11}H_6N_2$	167	167.0610	167.0604	0.6	3.6
	2b-27	3c	MS^3	$C_{10}H_5N$	140	140.0494	140.0495	-0.1	-0.7
MeAαC	$[M+H]^+$	1a	MS^1	$C_{12}H_{11}N_3$	198	198.1024	198.1026	-0.2	-1.0
	1a-15	2b	MS^2	$C_{11}H_8N_3$	183	183.0797	183.0791	0.6	3.3
	1a-17	2c	MS^2	$C_{12}H_8N_2$	181	181.0768	181.0760	0.8	4.4
	2b-27	3d	MS^3	$C_{10}H_7N_2$	156	156.0685	156.0682	0.3	1.9
	2c-27	3e	MS^3	$C_{11}H_7N$	154	154.0654	154.0651	0.3	2.0

Table 2 Quality parameters of 6 HCAs

HCAs	Linear regression equation	Linear range (μg L^{-1})	r^2	LOD (μg L^{-1})	LOQ (μg L^{-1})
IQ	y = 297145.9 x + 2729193.6	10–1000	0.9981	3	10
MeIQ	y = 238595.2 x + 2305547.0	10–1000	0.9985	3	10
MeIQx	y = 145211.8 x+ 1041553.9	5–500	0.9987	1.5	5
PhIP	y = 172103.4 x + 2410340.2	5– 500	0.9963	1.5	5
AαC	y = 170499.8 x + 924899.3	5– 500	0.9994	1.5	5

MeAαC	$y = 177793.3\,x + 1922727.0$	5– 500	0.9964	1.5	5

Table 3 Precision and accuracy of the method for the determination of 6 HCAs using LCMS-IT-TOF

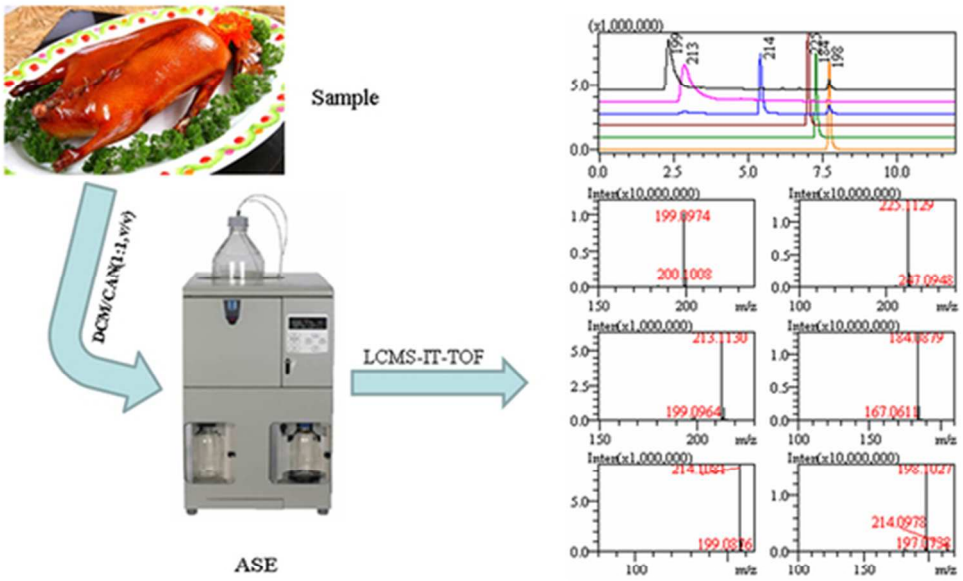
Compounds	Spiking levels (μg kg^{-1})	Intra-day [a]		Inter-day [a]	
		(RSD, %)	(RE, %)	(RSD, %)	(RE, %)
IQ	5	3.86	77.1	4.36	74.6
	10	5.28	79.3	6.16	77.8
	40	3.65	82.6	4.12	80.5
MeIQ	5	3.92	85.2	4.52.	81.6
	10	3.35	89.4	4.18	87.2
	40	2.86	92.5	3.53	91.3
MeIQx	5	4.35	85.8	5.58	83.8
	10	3.46	90.2	4.25	88.5
	40	2.65	93.6	3.16	91.2
PhIP	5	3.82	81.2	4.86	79.5
	10	4.24	83.6	4.75	82.1
	40	3.06	85.5	3.92	84.8
AαC	5	5.25	71.8	5.96	69.5
	10	5.08	75.5	5.45	73.8
	40	5.32	74.6	5.88	72.1
MeAαC	5	4.35	78.4	4.96	76.6
	10	4.87	81.6	5.25	80.5
	40	5.22	83.5	5.75	83.0

[a] $n = 6$.

Table 4 HCAs contents in four cooked meat samples (n=3)

Samples	Content (μg kg^{-1})					
	IQ	MeIQ	MeIQx	PhIP	AαC	MeAαC
Roast chicken	1.1	nd	1.2	5.4	nd	nq
Roast duck	2.8	nd	nd	4.2	nd	nq
Roast pork	nq	nd	nd	3.9	nd	nd
Fried bream	3.5	nd	1.4	4.6	nd	nd

nd not detected *nq* not quantified



Graphical Abstract

47x31mm (300 x 300 DPI)