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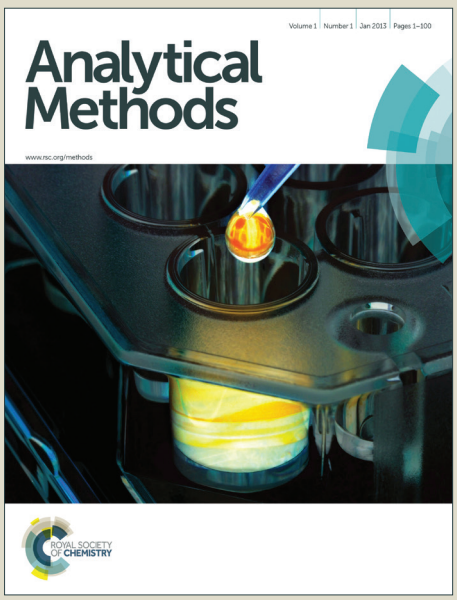

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A novel one-step extraction method for simultaneous determining eleven polar heterocyclic aromatic amines in meat products by UHPLC–MS/MS

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Abstract

A new preparation procedure allowing the analysis of 11 polar heterocyclic aromatic amines (HAAs) in meat samples is proposed and applied to commercial meat products. It is based on ultra high-performance liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS). The procedure involves a one-step extraction using hydrochloric acid coupled with a solid-phase extraction cleanup using Oasis MCX cartridges. Significantly higher recoveries was obtained (ranging from 58.2% to 107.6%) for every target polar HAAs from roasted pork sample prepared in a laboratory, compared with a commonly used reference method based on multiple extractions. The UHPLC-MS/MS parameters were optimized to simultaneously analyze all the 11 HAAs within 17 min, with limits of quantification (LOQs) $< 4.3\ \mu g\ L^{-1}$ by using standard solutions. LOQs for the spiked meat extract (0.026–0.659 ng g^{-1}) and adequate day-to-day precision ranging from 6.17% to 12.03% (N = 15) were obtained. Five commercial meat products (smoked fish, roasted chicken wing, roasted pork, grilled beef, and sausage) were spiked with three concentrations (low, medium and high) of the HAAs standards. Satisfactory recoveries (about 50–110%) were obtained, verifying the applicability and advantage of the proposed method.

1. Introduction

Heterocyclic aromatic amines (HAAs) are highly carcinogenic chemicals which could be formed during high-temperature processing of protein-rich foods, such as meat.[1-3] Since they are discovered by Japanese researchers three decades ago,[4] more than 25 HAAs have been identified.[5] HAAs have been proved to induce tumors at multiple sites of rodents and non-human primates in

several long-term feeding studies.[6] Several epidemiological studies have also revealed that frequent consumption of cooked food containing HAAs may lead to an increased risk of various types of human cancer.[7] Nowadays, population is continuously exposed to the risks of HAAs through diet, as demonstrated by their detection in a wide variety of commercial or homemade meat products.[8, 9] Therefore, more and more attention has been paid to research on formation mechanism and inhibition of HAAs in meat,[10-13] and this situation make accurate quantitative measurement of these mutagens essential.

Many analytical techniques have been described to detect trace HAAs in complex food. High-performance liquid chromatography (HPLC) coupled with ultraviolet or fluorescence detector is considered to be a conventional technique used to separate and detect known HAAs in cooked meat.[14, 15] However, due to the interfering effects caused by co-extracted compounds from sample matrix, more selective techniques such as mass spectrometry (MS) should be applied. Over the last few years, several laboratories have accomplished the determination of HAAs in food by using different MS analyzers.[16-18] Comparing these analyzers, triple quadrupole detector (TQD) in multiple reaction monitoring (MRM) mode has higher selectivity, which has been proved to produce more precise results since lower detection limits, extended linearity ranges and improved repeatability has been reported.[18] Therefore, for simultaneously determining the trace HAAs in complex cooked meat, this type of spectrometers is preferred.

For achieving authentic results with TQD, proper pre-treatment including extraction and clean-up steps have to be employed. However, complex sample matrix from meat, where HAAs

always present on the levels of ppb or ppt, makes the establishment of a proper pre-treatment procedure difficult. The most popular method used for extracting HAAs in foods was first introduced by Gross and Grüter.[19] In this procedure, samples are usually first homogenized by sodium hydroxide (NaOH). Then the HAAs are extracted from alkalified samples with dichloromethane and finally purified by tandem solid-phase extraction (SPE) procedures with three cartridges (Extrelut, propylsulfonic acids and RP-18). As a conventional method, the Gross method has been published in a large number of works in recent years.[13, 14, 20] However, since such an approach takes considerable time, it is not convenient enough in its application, especially when large amounts of samples need to be analyzed. Besides, recovery rates obtained from this conventional method often range from 25% to around 50% for polar HAAs and from 50% to 60% for less-polar HAAs in published studies,[21-23] which still could be improved. Over the past few years, progresses have been made to reduce the extraction and clean-up time as well as to improve the recovery. A pressurized liquid extraction method has been used for meat extracts producing almost a four-fold decrease in extraction time, while limited improvement for the HAAs recoveries was acquired which were in the range of 45–79%.[24] Another method involving acetone extraction followed by a SCX solid phase extraction has been employed to analyze HAAs in meat-based infant food.[25] In this study, a recovery range of 78–98% was obtained for seven HAAs, including IQ, MeIQ, MeIQx, PhIP, AαC, Harman and Norharman. However, some polar HAAs such as DMIP, TMIP and 4,8-DiMeIQx, which have always been proved to exist in cooked meat [26, 27] and are important for the mechanism research of HAAs generation in meat product, have not been

involved.

HAAs are compounds that present hydrophilic under acidic conditions,[28] especially for the HAAs with high polarity. Therefore, an extraction method using acid solution as extraction solvents are supposed to extract multiple polar HAAs in a single step. In addition, the interference of macro-components matrix (such as lipids) was reported as a reason for the lost of the HAAs during the extraction.[22] In an attempt to avoid the recovery lost of the HAAs, an extraction solvent which may not co-extract such interference are inferred to enhance the recovery rates. The objective of this study was to develop an one-step acid extraction pretreatment method plus ultra high performance liquid chromatography (UHPLC)-TQD-MS detection to simultaneously determine 11 polar HAAs in meat samples. To evaluate method performance, a comparison study between this one step method and the conventional Gross method was implemented. The study also validated the one-step method in terms of its linearity, limit of detection, matrix effects and precision. Furthermore, the applicability of this method has been assessed by analyzing HAAs in several meat commodities.

2. Materials and Methods

2.1 Standards, chemicals, and reagents

The following 11 HAAs were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and their standard stock solutions (2.5 mg mL^{-1}) were prepared in methanol and stored in the dark at 4 ℃. Fresh working standard solutions were prepared daily by mixing and diluting each stock standard solution in methanol to obtain different concentrations with similar peak areas:

DMIP (566 μg L^{-1}), 1,5,6-TMIP (127 μg L^{-1}), IQ (68.5 μg L^{-1}), IQ[4,5-b] (77.3 μg L^{-1}), IQx (527.0 μg L^{-1}), MeIQ (552.0 μg L^{-1}), MeIQx (517.0 μg L^{-1}), PhIP (295.0 μg L^{-1}), 7,8-DiMeIQx (1,092.0 μg L^{-1}), 4,8-DiMeIQx (131.5 μg L^{-1}) and 4,7,8-TriMeIQx (519.0 μg L^{-1}). Caffeine (CAF) was used as the internal standard (IS) and was provided by J&K Chemical Ltd. (Shanghai, China). The purity of all of the standards was greater than 99% according to the manufacturers' specifications.

HPLC-grade acetonitrile from Thermo Fisher Scientific (Waltham, MA, USA) was used as the mobile phase. Distilled water was purified using a Milli-Q filtration system from Millipore Corp. (Bedford, MA, USA). All of the reagents were filtered through a 0.22 μm nylon or cellulose filter before being injected into the UHPLC–MS/MS system. The SPE used Oasis MCX cartridges (60 mg, 3 cm^3), which were obtained from Waters Corp. (Milford, MA, USA). The PRS cartridges (500 mg, 6 cm^3) and C_{18} SPE columns were ordered from Supelco Corp. (St. Louis, MO, USA). The Xtrack BCX and STYPR SCREEN DBX columns were offered by Sepax Technologies (Newark, NJ, USA). All of the other chemicals used in the study were of analytical grade and purchased from Sinopharm Chemical Reagent Beijing Co. Ltd. (Beijing, China).

2.2 Instrumentation and UHPLC-MS/MS analytical conditions

The chromatographic separation of the 11 HAAs was performed at 35 ℃ on a Waters Acquity UPLC system equipped with a quaternary pump system, coupled with a Waters Acquity UPLC BEH C_{18} column (100 × 2.1 mm i.d., 1.7 μm particle size) or a Waters Acquity UPLC BEH phenyl column (100 × 2.1 mm i.d., 1.7 μm particle size) (Milford, MA, USA). The gradient elution was achieved with a binary mobile phase of acetonitrile (A) and 5 mM ammonium acetate solution (pH

6.8) (B) at a flow rate of 0.3 mL min^{-1}. The gradient elution program was 0–0.1 min, 10% A; 0.1–10 min, 10–15% A; 10–11 min, 15–100% A; 11–12 min, 100 % A; 12–13 min return to initial composition; 4 min post-run delay. The single injection volume was set at 1 μL.

Analysis of the HAAs was carried out on a Waters Acquity triple quadrupole mass spectrometer (TQD) (Milford, MA, USA) using the positive electrospray ionization (ESI^+) mode. Multiple reaction monitoring (MRM) conditions (collision energy, cone voltage and compound transitions) were automatically optimized with 1 μg mL^{-1} HAAs using the Intellistart function system. The optimized MRM parameters and formation methods of the diagnostic ions for each HAA are summarized in Table 1.

Typical instrument tuning parameters used were as follows: capillary voltage, 3.5 kV; source temperature, 120 ℃; desolvation temperature, 350 ℃; cone gas (Nitrogen, 99.9% purity) flow rate, 60 L h^{-1}; desolvation gas (Nitrogen, 99.9% purity) flow rate, 650 L h^{-1}; collision gas (argon, 99.9% purity) flow rate, 0.13 mL min^{-1}.

2.3 Roasted pork preparation

The roasted pork sample for method testing was prepared in laboratory according to following procedure: Pork meat purchased from local market was cut into small pieces and made to meat pie with fixed size. These pies were then roasted in an oven for 20 min under 250 ℃ (10 min for each side). Then, the roasted pies were crushed to powder.

2.4 Extraction and clean-up procedures

4g of roasted pork powder was homogenized (four times for 15 s) in 25 mL of 0.1 M hydrochloric acid (HCl) solution, extracted for 15 min using ultrasound. After centrifugation of the mixture (11,363 g for 10 min at 4 ℃), the supernatant was collected. The extraction procedures were repeated twice, and the extracting solutions were then combined. A trichloroacetic acid (TCA) solution was added to the acidic solution with an end concentration of 5% to remove the protein. The pH value of the protein-free sample extract was adjusted to around 3 using a 4 M NaOH solution before being applied to the SPE column. An Oasis MCX cartridge activated continuously with 6 mL of methanol, 6 mL of distilled water, and 6 mL of 0.1 M HCl solution was used for separation. The cartridges were rinsed sequentially with 6 mL of 0.1 M HCl solution and then 6mL of methanol. The retained analytes were finally eluted by a 6 mL mixture of methanol and ammonium hydroxide (25%) at a ratio of 95:5. The collected fraction was then evaporated to dryness using a stream of nitrogen at 45 ℃ and dissolved in 0.6 mL of acetonitrile containing the IS before being injected into the UHPLC-MS/MS system. A similar process was applied using Xtrack BCX and STYPR SCREEN DBX columns.

For comparison, a modified Gross method [19] was employed as a conventional method and applied to the same samples, in which Extrelut and dichloromethane were replaced with diatomaceous earth and ethyl acetate, respectively.

2.5 Method validation

A linear regression was performed using the concentrations and peak area ratios of the standards and IS obtained from blank sample spiked with nine various concentrations of the

standards in respective range (DMIP 2.2–566.0 μg L^{-1}, TMIP 0.5–127.0 μg L^{-1}, IQ 0.27–68.5 μg L^{-1}, IQ [4,5b] 0.3–77.3 μg L^{-1}, IQx 2.1–527.0 μg L^{-1}, MeIQ 2.2–552.0 μg L^{-1}, MeIQx 2.0–517.0 μg L^{-1}, PhIP 1.2–295.0 μg L^{-1}, 7,8-DiMeIQx 4.3–1092.0 μg L^{-1}, 4,8-DiMeIQx 0.51–131.5 μg L^{-1} and 4,7,8-TriMeIQx 2.0–519.0 μg L^{-1}) using 125.0 μg L^{-1} of caffeine as IS. The linear ranges were decided according to the regression correlation coefficient for each concentration ranges.

The limit of detection (LODs) and quantification (LOQs) were determined by diluting the standard solutions or the blank sample spiked with HAAs standards at the same concentration levels to a specified signal to noise (S/N) ratio (3 and 10 for the LODs and LOQs, respectively).

The matrix effects were evaluated using the slope comparison method.[29] The blank samples, which were spiked after extraction with diminishing concentrations of the standards, were used to establish standard addition calibration curves. The slopes of these curves (A1, A2...An) were then compared with the slopes (B1, B2...Bn) obtained from the pure standards at the same concentration levels, and the slope ratios (Rn) were equal to An/Bn. The matrix effects could then be determined with Rn as the ionization suppression (< 1) or enhancement (> 1)effect or without matrix effects (= 1).

The recoveries of the sample preparation were determined by analyzing a blank sample spiked with certain levels of standards. All of the recoveries were determined via triplicate analyses to calculate the standard deviation (SD).

The precision was tested using sample extracts obtained from samples spiked with 30 μL of working standard solutions. The run-to-run precision was determined by injecting five sample

extracts (n = 5) preparing on the same day, while the day-to-day precision was evaluated by injecting five daily sample extracts preparing on three different days (N = 15).

2.6 Statistical analysis

The system-provided MassLynx 4.1 SCN 805 software was used to carry out the data acquisition. A least-significant difference test with a one-way analysis of variance was performed using Statistics 9.0 to make the comparison ($P < 0.05$). External calibration curves, obtained by the linear regression of a plot of standard/IS peak-area HAAs ratios against HAA concentrations, were used to calculate the compound concentrations in the samples. In a spiked test, the original HAA content of roasted pork was subtracted to calculate the recoveries.

3. Results and discussion

3.1 Comparison with conventional extraction method

The one-step extraction method investigated in the current study demonstrates superior recoveries over the conventional method. As presented in Fig. 1, the recoveries range from 25.0% to 79.2% by the conventional method, while 58.2–107.6% by the one-step method. The increase is significant for all of the 11 HAAs based on the variance analysis of one factor ($P < 0.05$).

Relative low recovery based on the conventional method in our paper are in accordance with previous studies that employed similar pretreatment methods. For example, the average recoveries were reported as approximate 20% for DMIP and TMIP, below 50% for IQ type HAAs, and around 25% for PhIP by some authors.[30, 31] As for the one-step method, it follows the principle that most

HAAs are hydrophilic and soluble in acidic mixtures under acidic conditions (pH < 3),[28] and allows the analytes to be extracted by 0.1 M HCl (pH ~ 1) using a single step. This method avoids the incomplete absorption of HAAs from alkali solvents, low elution efficiency from diatomaceous earth support and the interference of macro-components matrix (such as lipids), which were reported as the reason why the HAAs was lost during the extraction in the conventional method,[22, 30] and thus make significantly improvement on the method recovery. Furthermore, the new method with a single step HAA extraction can significantly simplify the operating steps of the conventional method, and thus shorten its sample preparation time by nearly 50%.

In the mean time, the one-step method is believed to be superior not only to the conventional method, but also to some recent studies that aimed at improving the current used pretreatment methods for HAAs analysis. An analytical method more precise and accurate than the Gross method was published by Szterk et al.[32] Compared to our one-step method, similar recoveries for most of the HAAs were obtained except for PhIP (46.4% as reported). In addition, due to the using of the acid solvent, a wider range of polar HAAs could be analyzed by using the one-step method in comparison with the method using other extraction solvents. For instance, seven HAAs, including IQ, MeIQ, MeIQx, PhIP, AαC, Harman and Norharman, were analyzed using a published acetone extraction method.[25] Though this method was also convenient and obtained high recovery (78–98%), some polar HAAs including the HAAs that often found in cooked meat at high levels, such as DMIP, TMIP and 4,8-DiMeIQx, have not been involved.

3.2 SPE purification

The use of different extraction solvents and procedures was expected to produce different impurities that would interfere with subsequent detection by UHPLC-MS/MS. At the same time, the concentration of the HAAs after extraction are not high enough to be detected. Therefore, a suitable purification process should be conducted and optimized to purify and concentrate these targets. In this paper, two polymer-based mixed sorbents (sytrene-divinyl benzene polymer for STYPR SCREEN DBX, divinylbenzene-co-*N*-vinylpyrrolidone polymer for Oasis MCX), a silica-based alkyl chain with ion exchange terminal group sorbents (Xtrack BCX), and a combination of PRS and C_{18} cartridges were evaluated as sorbent materials. A spiked roasted meat extract (before SPE) was used to test the interference-purified efficiency and HAA recoveries of these four sorbents, and the results are shown in Table 2. As shown in the table, the DBX cartridge provides high recovery for highly polar HAAs, but relatively lower for less-polar HAAs, such as PhIP (around 50%). The BCX cartridge shows a high degree of absorption for all HAAs (> 79.7%) except DMIP and IQx, which cannot be quantified because they are unable to be isolated from interference. The PRS-C_{18} combined cartridges method makes the procedure tedious and time-consuming. The Oasis MCX cartridge provides high recoveries for all of the 11 HAAs with the cleanest extracts, and is ultimately selected for the cleanup purpose.

3.3 Chromatographic conditions

The analytes involved in this study included analogs with similar structure and polarities. Thus, two columns with different separation principals (BEH C_{18} and BEH phenyl) which were frequently used to isolate HAAs have been evaluated using UHPLC separation and MS detection to

determine the signal intensity and separation efficiency of HAAs.[15, 32] Fig. 2 displays the MRM chromatograms for each of the 11 HAAs using these two columns. For BEH phenyl column, tailing and wide peaks were obtained no matter what gradient was applied using the combination of acetonitrile and 5 mM ammonium acetate (pH 6.8) as the mobile phase. Furthermore, this BEH phenyl column failed to isolate two isomeric HAAs, named 7,8-DiMeIQx and 4,8-DiMeIQx, which have the same molecular weights and fragment ions (Fig. 2A). In contrast, Fig. 2B shows that symmetrical peaks with reasonable widths could be obtained using BEH C_{18} column. Moreover, the BEH C_{18} column can provide acceptable isolation for all of the 11 HAAs, and thus was ultimately chosen for simultaneous detection of these 11 polar HAAs.

3.4 Method validation

The method validation results are summarized in Table 3. Good correlation coefficients were obtained for each of the 11 polar HAAs ($r^2 > 0.9957$) in a wide linear range. The LOQs ranges for spiked meat extracts are from 0.026 to 0.659 ng g^{-1}, which were similar to those reported in other studies.[14, 33] In terms of the matrix effects, Rn values between 0.89 and 1.18 were obtained for the 11 HAAs. The recoveries obtained by the one-step method were in the range of 58.2–107.6%, as mentioned in Section 3.2. The day-to-day precision, expressed as RSD (%), are lower than 12.03% for peak area.

3.5 Adaptability of the one-step method to commercial samples

To evaluate the applicability of the new pretreatment procedure developed in this study, the

method was applied to five commercial meat products (smoked fish, roasted chicken wing, barbecued pork, grilled beef and sausage) purchased from a local supermarket. levels of all 11 HAAs (Table 4) in the five commercial products determined using current one-step method were in line with that of other reported data,[24, 27, 34] indicating that the one-step method could be successfully applied to the determination of HAAs in commodity. Meanwhile, relative recoveries of 11 HAAs in the five commodities were calculated and listed in Table 4. The recoveries range from 85.3% to 116.0%, demonstrating the accuracy of the proposed one-step method for different commercial products.

Standards at three concentrations (low, medium, high) were spiked into the five commercial products to perform recovery studies, and the results are shown in Table 5. The recoveries of the 11 HAAs in the five commercial products at different spiked levels mainly exceed 50% (41.7–106.3% for the smoked fish, 46.4–105.4% for the roasted chicken wing, 51.4–98.5% for the barbecued pork, 46.8–101.2% for the grilled beef and 54.2–96.1% for the sausage). These recoveries were better than those obtained by other authors, who obtained the recoveries of 60.8% for PhIP, 82.1% for MeIQx, 86.9% for 4,8-DiMeIQx and 94.6% for IQ from chicken and duck breast,[35] and 31–68% for eight HAAs (IQ, MeIQ, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, TriMeIQx, PhIP) from barbecued sardines and Atlantic salmon.[36] The superior recoveries obtain from the one-step method further suggest that this method could be successfully used for accurate quantification of HAA in meat commodities.

4. Conclusions

A pretreatment method for analyzing 11 polar HAAs in meat products based on direct extraction using HCl and single step purification by Oasis MCX cartridge has been proposed and applied to five commercial meat products. This one-step method offers more convenient pretreatment procedures which could significantly shorten the sample preparation time with superior recoveries over the conventional method. The proposed method has been proved to be a simple and accurate method for determining HAAs in meat products, and may also be applied to other food systems.

Abbreviations

DMIP: 2-amino-1,6-dimethylimidazo [4,5-b] pyridine,

1,5,6-TMIP: 2-amino-1,5,6-trimethylimidazo [4,5-b] pyridine,

IQ[4,5b]: 2-amino-1-methyl-imidazo [4,5-b] quinoline,

IQ: 2-amino-3-methyl-3H-imidazo [4,5-f] quinoline,

IQx: 2-amino-3-methyl-imidazo [4,5-f] quinoxaline,

MeIQ: 2-amino-3,4-dimethyl-imidazo [4,5-f] quinoline,

MeIQx: 2-amino-3,8-dimethyl-imidazo [4,5-f] quinoxaline,

PhIP: 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine,

7,8-DiMeIQx: 2-amino-3,7,8-trimethyl-imidazo [4,5-f] quinoxaline,

4,8-DiMeIQx: 2-amino-3,4,8-trime-thyl-imidazo [4,5-f] quinoxaline,

4,7,8-TriMeIQx: 2-amino-3,4,7,8-tetramethyl–imidazo [4,5-f] quinoxaline,

Norharman: 9H-pyrido [3,4-b] indole,

Harman: 1-methyl-9H-pyrido [4,3-b] indole,

AαC: 2-amino-9H-pyrido [2,3-b] indole,

CAF: caffeine, 1,3,7-trimethyl-1H- purine-2,6 (3H,7H) -dione.

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Table 1 Parameters and diagnostic fragment ions used for the quantification and confirmation of eleven HAAs on the triple quadrupole instrument

HAAs	Precursor ion $[M+H]^+$ (m/z)	Collision voltage (V)	Cone voltage (V)	Dwell time (ms)	Diagnostic product ions (m/z)	Proposed assignment
DMIP	163	34	46	100	**148**	**$[M+H-CH_3]^+$**
					147	$[M+H-CH_3-H]^+$
1,5,6-TMIP	177	30	54	100	**162**	**$[M+H-CH_3]^+$**
					161	$[M+H-CH_3-H]^+$
IQ[4,5b]	199	30	52	100	**184**	**$[M+H-CH_3]^+$**
					157	$[M+H-CH_3-HCN]^+$
IQ	199	30	58	100	**184**	**$[M+H-CH_3]^+$**
					157	$[M+H-CH_3-HCN]^+$
IQx	200	38	50	100	**185**	**$[M+H-CH_3]^+$**
					158	$[M+H-CH_3-HCN]^+$
MeIQ	213	42	52	100	**198**	**$[M+H-CH_3]^+$**
					197	$[M+H-CH_3-H]^+$
MeIQx	214	40	50	100	**199**	**$[M+H-CH_3]^+$**
					173	$[M+H-CH_3-CN]^+$
PhIp	225	42	54	100	**210**	**$[M+H-CH_3]^+$**
					183	$[M+H-CH_3-HCN]^+$
7,8-DiMeIQx	228	38	50	100	**187**	**$[M+H-CH_3-CN]^+$**
					160	**$[M+H-CH_3-HCN-CN]^+$**
4,8-DiMeIQx	228	34	52	100	**213**	**$[M+H-CH_3]^+$**
					187	$[M+H-CH_3-CN]^+$
4,7,8-TriMeIQX	242	40	54	100	**227**	**$[M+H-CH_3]^+$**
					201	$[M+H-CH_3-CN]^+$
CAF	195	26	38	100	**138**	**$[M+H-2CH_3-HCN]^+$**
					110	$[M+H-2CH_3-2HCN-H]^+$

Table 2 Absolute recoveries of the eleven polar HAAs with four SPE sorbents

HAAs	Recovery (%) ± SD			
	SCREEN DBX	Xtrack BCX	PRS-C18	Oasis MCX
DMIP	104.6 ±14.3	------ [a]	85.6 ±9.2	98.5 ±5.3
1,5,6-TMIP	79.6 ±5.9	94.4 ±9.2	91.8 ±16.5	115.1 ±5.1
IQ[4,5b]	107.9 ±10.7	109.3 ±9.3	90.8 ±14.2	94.0 ±1.1
IQ	61.7 ±10.7	97.4 ±12.6	81.2 ±13.0	101.1 ±3.6
IQx	99.6 ±8.8	------ [a]	86.1 ±4.5	104.2 ±11.0
MeIQ	92.2 ±2.4	96.9 ±6.4	85.4 ±8.0	97.6 ±6.4
MeIQx	98.2 ±13.1	87.7 ±11.8	83.2 ±8.0	90.7 ±6.1
PhIp	52.0 ±5.4	90.9 ±5.6	91.7 ±5.0	114.4 ±3.9
7,8-DiMeIQx	94.9 ±7.9	87.5 ±6.6	87.8 ±3.2	102.1 ±3.8
4,8-DiMeIQx	93.2 ±5.9	79.7 ±8.3	82.4 ±8.3	91.6 ±3.7
4,7,8-TriMeIQX	62.5 ±6.5	87.6 ±1.1	80.7 ±9.0	94.8 ±2.4

[a] not quantified in, because of interaction of interference

Table 3 Analytical characteristics for the eleven polar HAAs standard solutions and spiked pork sample

HAAs	Linear range ($ng\ mL^{-1}$)	Coefficients (r^2)	LOD [a] ($ng\ g^{-1}$)	LOQ [a] ($ng\ g^{-1}$)	Run-to-run precision (RSD [b] %)	Day-to-day precision (RSD [b] %)	Matrix effects [c] (Rn±SD)
DMIP	8.8–283.0	0.9991	0.095 (0.332)	0.179 (1.105)	5.43	6.17	0.94 ±0.03
1,5,6-TMIP	0.5–127.0	0.9977	0.026 (0.149)	0.100 (0.496)	8.20	10.96	0.97 ±0.17
IQ[4,5b]	1.2–77.3	0.9996	0.023 (0.091)	0.049 (0.302)	5.27	10.65	1.18 ±0.13
IQ	0.5–68.5	0.9957	0.013 (0.040)	0.026 (0.134)	6.71	6.71	0.89 ±0.17
IQx	2.1–263.5	0.9967	0.118 (0.618)	0.333 (2.059)	4.96	12.03	0.93 ±0.11
MeIQ	4.3–552.0	0.9980	0.056 (0.324)	0.169 (1.078)	7.81	7.72	0.99 ±0.06
MeIQx	2.0–517.0	0.9996	0.109 (0.606)	0.361 (2.020)	6.80	6.56	0.96 ±0.15
PhIp	1.2–295.0	0.9987	0.122 (0.692)	0.359 (2.305)	4.00	8.21	1.06 ±0.17
7,8-DiMeIQx	4.3–1092.0	0.9982	0.275(1.281)	0.659 (4.266)	4.42	10.10	0.89 ±0.01
4,8-DiMeIQx	0.5–131.5	0.9963	0.029 (0.154)	0.080 (0.514)	8.65	6.64	0.95 ±0.21
4,7,8-TriMeIQx	8.11–519.0	0.9972	0.059 (0.304)	0.161 (1.014)	6.38	9.73	0.92 ±0.22

[a] LOD and LOQ evaluated by standard solutions (ugL^{-1}) are given in brackets.

[b] RSD: Relative standard deviation

[c] Matrix effects was expressed as the slope ratios (Rn) of standard spiked calibration curve to pure standard calibration curves at same analyte concentration. A value of > 1.00 shows ionization enhancement, while < 1.00 indicates ionization supression (n = 3).

Table 4 The levels and relative recoveries of the eleven HAAs (ng g^{-1}) detected in the five commodity meat samples

HAAs	Added (ng g^{-1})	Smoked fish		Roasted wing		Barbecued pork		Grilled beef		Taiwan sausage	
		Levels (ng g^{-1}) ±SD [a]	Recovery (%) ±SD [a]	Levels (ng g^{-1}) ±SD [a]	Recovery (%) ±SD [a]	Levels (ng g^{-1}) ±SD [a]	Recovery (%) ±SD [a]	Levels (ng g^{-1}) ±SD [a]	Recovery (%) ±SD [a]	Levels (ng g^{-1}) ±SD [a]	Recovery (%)±SD [a]
DMIP	8.84	0.71 ±0.26	102.6 ±1.5	n.d. [b]	113.5 ±3.2	n.d. [b]	112.8 ±3.2	2.61 ±0.42	106.6 ±8.9	n.d. [b]	90.8 ±14.9
TMIP	1.98	0.18 ±0.06	106.8 ±4.5	n.d. [b]	103.9 ±12.7	n.d. [b]	98.8 ±2.2	1.74 ±0.17	91.2 ±13.6	n.d. [b]	102.2 ±9.1
IQ[4,5b]	1.21	0.59 ±0.07	94.7 ±8.2	0.12 ±0.05	101.3 ±9.6	0.13 ±0.01	91.5 ±19.7	0.40 ±0.06	85.9 ±13.7	n.d. [b]	90.1 ±24.6
IQ	1.07	0.10 ±0.01	100.1 ±9.4	n.d. [b]	103.7 ±10.2	n.d. [b]	105.1 ±4.2	0.14 ±0.02	88.6 ±13.5	n.d. [b]	92.5 ±20.8
IQx	8.23	n.d. [b]	87.3 ±1.5	n.d. [b]	94.4 ±9.7	n.d. [b]	98.3 ±18.8	3.40 ±0.48	108.0 ±2.8	n.d. [b]	104.4 ±12.8
MeIQ	8.63	n.q. [c]	106.0 ±4.6	n.d. [b]	110.7 ±2.6	n.d. [b]	86.4 ±11.8	n.q. [c]	104.6 ±17.0	n.d. [b]	94.1 ±1.4
MeIQx	8.08	1.22 ±0.30	85.3 ±0.6	n.d. [b]	116.0 ±9.2	n.d. [b]	111.9 ±3.5	4.46 ±0.49	93.8 ±8.4	n.d. [b]	107.6 ±5.1
PhIp	4.61	0.51 ±0.04	108.7 ±2.0	n.d. [b]	103.2 ±4.8	n.d. [b]	97.6 ±8.6	3.67 ±0.45	97.7 ±13.5	n.d. [b]	101.9 ±1.3
7,8-DiMeIQx	17.06	n.d. [b]	105.9 ±6.9	n.d. [b]	98.4 ±10.5	n.d. [b]	108.6 ±0.6	n.d. [b]	100.7 ±3.6	n.d. [b]	91.5 ±13.5
4,8-DiMeIQx	2.05	0.12 ±0.03	112.1 ±8.3	n.d. [b]	90.9 ±2.1	n.d. [b]	101.9 ±9.7	0.43 ±0.07	102.4 ±3.5	n.d. [b]	102.3 ±2.7
4,7,8-TriMeIQx	8.11	n.d. [b]	106.3 ±14.0	n.d. [b]	104.5 ±1.6	n.d. [b]	102.3 ±21.3	n.d. [b]	97.2 ±8.4	n.d. [b]	106.5 ±8.1

[a] SD: Standard deviation

[b] not determined

[c] not quantified

403 **Table 5** Absolute recoveries of the eleven polar HAAs in the five different meat samples

HAAs	Added [a]	Recovery (%) ±SD				
		Smoked fish	Roasted chicken wing	Barbecued pork	Grilled beef	Taiwan sausage
DMIP	Low (16.98)	51.1 ±3.2	88.6 ±4.5	96.8 ±7.9	69.1 ±4.9	54.2 ±6.9
	Medium (42.45)	88.4 ±2.9	105.4 ±7.8	96.7 ±6.5	101.2 ±3.8	85.6 ±4.2
	High (67.92)	83.0 ±1.0	96.1 ±6.7	98.5 ±13.8	95.5 ±5.8	89.0 ±3.3
TMIP	Low (3.81)	59.9 ±3.1	60.0 ±14.2	59.7 ±4.4	55.8 ±3.9	79.9 ±9.8
	Medium (9.53)	73.0 ±8.1	66.9 ±3.3	67.2 ±5.9	68.8 ±1.3	90.0 ±4.3
	High (15.24)	68.6 ±3.1	66.1 ±2.5	66.4 ±3.6	69.3 ±0.9	89.1 ±9.1
IQ[4,5b]	Low (2.32)	45.8 ±7.8	84.8 ±7.2	82.2 ±13.8	74.2 ±9.6	82.2 ±9.6
	Medium (5.79)	58.2 ±1.4	79.8 ±3.6	73.0 ±6.3	54.4 ±3.0	72.1 ±3.0
	High (9.27)	57.0 ±0.9	73.5 ±5.1	69.4 ±2.8	62.6 ±8.1	71.7 ±7.4
IQ	Low (2.06)	41.7 ±12.2	97.4 ±2.8	92.5 ±7.7	74.1 ±9.3	75.3 ±12.2
	Medium (5.14)	77.5 ±2.5	79.0 ±5.4	72.7 ±3.4	58.2 ±8.5	53.7 ±6.9
	High (8.22)	53.3 ±1.8	79.5 ±5.9	68.2 ±5.0	57.1 ±2.3	59.0 ±4.2
IQx	Low (15.81)	95.8 ±2.0	86.1 ±10.5	94.9 ±8.5	91.7 ±5.2	90.3 ±3.3
	Medium (39.53)	88.2 ±3.8	101.0 ±11.2	75.1 ±13.8	77.4 ±6.4	61.6 ±3.6
	High (63.24)	85.5 ±6.1	92.9 ±2.5	83.6 ±18.1	80.9 ±12.6	68.3 ±3.6
MeIQ	Low (16.56)	71.5 ±2.5	72.0 ±1.6	51.4 ±3.0	53.0 ±8.4	63.9 ±4.4
	Medium (41.40)	97.4 ±5.8	77.6 ±5.7	65.0 ±1.8	54.0 ±3.1	62.9 ±3.3
	High (66.24)	88.5 ±4.1	69.6 ±4.1	69.4 ±4.6	53.2 ±4.8	68.6 ±2.6
MeIQx	Low (15.51)	60.4 ±8.1	98.6 ±5.1	91.4 ±7.1	90.0 ±9.1	96.1 ±13.6
	Medium (38.78)	106.3 ±10.9	93.1 ±11.9	98.4 ±12.5	89.7 ±11.1	89.2 ±5.8
	High (62.04)	105.0 ±4.8	96.3 ±5.3	91.4 ±7.5	99.6 ±12.1	91.5 ±10.1
PhIp	Low (8.85)	42.4 ±2.5	58.3 ±1.4	52.2 ±7.2	57.7 ±4.1	66.7 ±3.7
	Medium (22.13)	54.1 ±1.8	67.8 ±4.6	59.4 ±5.4	69.8 ±3.5	62.9 ±4.2
	High (35.40)	47.5 + 1.5	61.1 ±2.5	55.4 ±6.7	67.6 ±4.3	65.5 ±3.8
7,8-DiMeIQx	Low (32.76)	45.1 ±3.3	46.4 ±6.5	53.5 ±5.3	46.8 ±6.0	59.7 ±8.3
	Medium (81.90)	87.2 ±4.5	66.3 ±4.1	75.8 ±6.4	61.1 ±4.6	88.8 ±5.0
	High (131.04)	76.1 ±4.3	56.4 ±3.3	63.8 ±3.8	59.3 ±6.8	92.1 ±4.7
4,8-DiMeIQx	Low (3.95)	80.0 ±8.6	73.7 ±6.0	66.6 ±5.6	54.2 ±6.7	91.1 ±10.4
	Medium (9.86)	95.3 ±1.5	90.0 ±6.9	74.3 ±5.6	64.2 ±9.1	80.5 ±5.8
	High (15.78)	90.6 ±8.0	97.8 ±3.6	74.8 ±5.4	60.1 ±7.3	83.1 ±4.3
4,7,8-TriMeIQx	Low (15.57)	58.3 ±7.1	69.7 ±2.1	62.4 ±5.4	57.0 ±11.2	57.3 ±6.6
	Medium (38.93)	71.0 ±4.9	89.6 ±2.8	72.5 ±2.5	53.6 ±11.8	72.2 ±5.6
	High (62.28)	67.2 ±5.8	81.6 ±3.7	70.3 ±4.5	50.4 ±14.9	65.3 ±7.1

404 [a] Added levels (ng g^{-1}) are given in parentheses

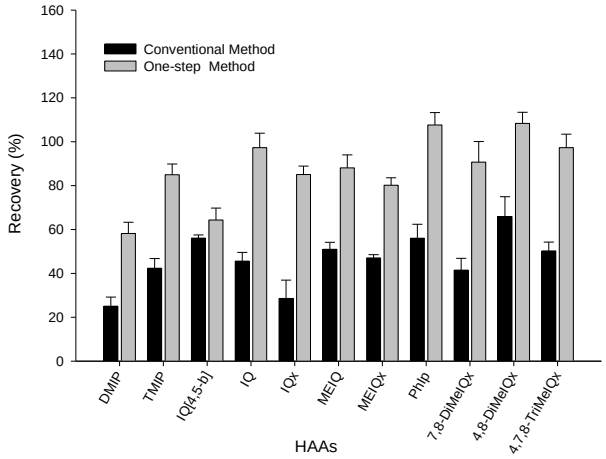

Fig. 1 Absolute recoveries of 11 HAAs using the reference method and the new extraction methods and Oasis MCX cartridge purification after adding 0.1 mL working standards (6.85–109.2 ng) (means with significant different by variance analysis of one factor, $P < 0.05$, n = 3)

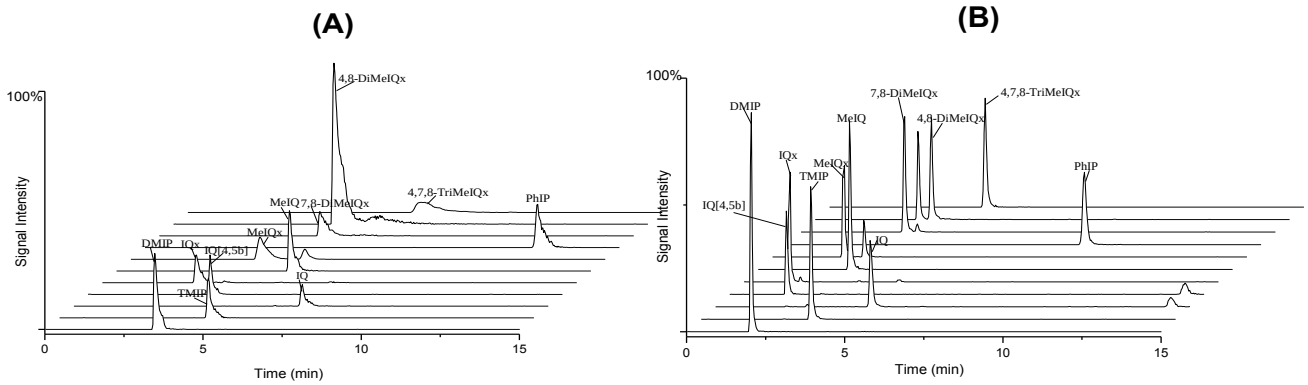

Fig. 2 Isolation characteristics of 11 HAAs obtained using (A) Acquity BEH phenyl column and (B) Acquity BEH C_{18} column at the concentration of working standards using UHPLC-MS/MS