Simultaneous determination of 9 heterocyclic aromatic amines in pork products by liquid chromatography coupled with triple quadrupole tandem mass spectrometry

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Abstract. Heterocyclic aromatic amines (HAAs) are potent mutagens that formed at high temperature in cooked, protein-rich food. Owing to their frequent intake, an accurate method is essential to access human health risk of HAAs exposure through detecting these compounds in various heat-treated meat products. In this study, a liquid chromatography-electrospray tandem mass spectrometry (LC--ESI-MS/MS) method was developed to perform the determination of 9 mutagenic heterocyclic amines (HAAs) in meat samples with multiple reaction monitoring (MRM) mode. Ultrasound assisted extraction and diatomaceous earth was employed to extract HAAs from food samples, and the analytes were purified and enriched using tandem solid phase extraction, with propyl sulfonic acid coupled to a C18 cartridge. Two parameters, extraction time and eluent, were carefully optimized to improve the extraction and purification efficiency. The LC separation was carried out using a Zorbax SB-C18 (3.5 μm particle size, 2.1×150 mm i.d.) column and optimized some parameters, such as pH, concentration and volume. Under the optimal experimental conditions, recoveries ranged from 52.97% to 97.11% with good quality parameters: limit of detection values between 0.02 and 0.24 ng mL\textsuperscript{-1}, linearity (R\textsuperscript{2}>0.998), and run-to-run and day-to-day precisions lower than 9.81% achieved. To evaluate the performance of the method in high throughput analysis of complex meat samples, the LC-MS/MS method was applied to the analysis of HAAs in three food samples, and the results demonstrated that the method can be used for the trace determination of HAAs in pork samples.

1. Introduction
Heterocyclic aromatic amines (HAAs) are considered to be carcinogenic, formed during the thermal processing of meat products which are rich in proteins and creatine/creatinine [1]. Recently lots of studies have indicated that many methods of cooking can bring about the generation of chemicals that are harmful to human health [2]. Epidemiological studies suggest an increased cancer risk of the colon, intestine, breast, prostate and pancreas after high intake of cooked meat, especially red meat [3-5]. Red meat contains beef, goat, pork, and lamb [6]. According to the FAO, meat production increased from
96.31 million tons in 2003 to 111.93 million tons at global level total in 2013 (FAO 2013). Besides, in China, pork is the highest consumption and accounted for nearly 65.1% of overall meat production. Therefore, to develop a method to quantify HAAs in cooked pork products is essential. The aim of this study is to establish an accurate and effective analysis method to detect relatively low-level HAAs in pork samples.

Since detected in cooked meat [7], HAAs have attracted much interest. Now, more than 25 types of HAAs have been found [8]. According to structure, HAAs can be divided into thermic HAAs and pyrolytic HAAs. The thermic HAAs, which include IQ, IQx, MeIQ, MeIQx, 4, 8-DiMeIQx, 7, 8-DiMeIQx, PhiP and DMIP, are formed from precursors such as free amino acids, creatine/ creatinine, and hexoses at common processing temperatures in the Maillard reaction [9]. In contrast, pyrolytic HAAs, which contain Harman, Norharman, AαC, MeAαC, Trp-P-1, Trp-P-2, Glu-P-1 and Glu-P-2, are formed through a pyrolytic reaction at processing temperatures higher than 300°C [10]. This class of HAAs is not directly mutagenic but can enhance the mutagenicity of other HAAs [11]. There are many factors affecting the generation of HAAs, including type of meat, processing methods, content of precursors [12,13].

Since the complexity of the meat matrix and the trace amounts of HAAs, sample pre-treatment is extremely essential. There are several strategies have been employed in reported methods, such as liquid-liquid extraction [14], extraction with blue cotton [15], solid-phase extraction (SPE) [16]. Based on the SPE method, Gross and Grütter [17] developed a tandem SPE method (Extrelut-PRS-C18 coupled cartridges), which is the commonly used method, improving the purification efficiency. As to the identification and quantification of HAAs, chromatographic or electrophoretic techniques are commonly carried out, using different methods of detection [18]. These contain liquid chromatography (LC) in conjunction with ultraviolet (UV) [19, 20], fluorescence (FLD) [21], diode array (DAD) [22], or electrochemical (CE) detection [23], gas chromatography–mass spectrometry (GC–MS) [24], liquid chromatography–mass spectrometry (LC–MS) [25-27] and ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) [28]. In the last few decades, the widespread applied for HAAs analysis in food samples is LC-MS, because of its high selectivity and specificity than UV, DAD and FLD, without requirement of analyte derivatization like GC-MS and its lower equipment demanding than UPLC-MS.

In this work, by optimizing an Extrelut-PRS-C18 coupled cartridges method and LC-MS conditions, a LC-ESI-MS/ MS method for the determination of 9 HAAs in pork sample was established. These HAAs are more common and relatively high concentration in the pork samples [29]. To confirm the suitability of the method, pork samples were analyzed to further demonstrate the applicability of the method, which can offer the assessment of human health risk of HAAs exposure.

2. Materials and methods

2.1. Chemicals

The Heterocyclic aromatic amine standards IQ (2-amino-3-methyl-imidazo[4,5-f]quinoline), MeIQ (2-amino-3,4-dimethyl -imidazo [4,5-f] quinoline), MeIQx (2-amino-3,8-dimethyl-imidazo [4,5-f]quinoline), 4,8-DiMeIQx (2-amino-3,4,8-trimethyl-imidazo [4,5-f] quinoxaline), TriMeIQx (2-amino-3,4,7,8 -tetramethyl-imidazo [4,5-f] quinoxaline), PhiP (2-amino-1-methyl-6- phenylimidazo [4,5-b]pyridine), AαC (2-amino-9H-pyrido[2,3-b]indole), Trp-P-1 (3-amino-1,4-dimethyl -5H-pyrido[4,3-b]indole), Harman (1-methyl-9H-pyrido[3,4-b]indole), Norharman (9H-pyrido[3,4-b]indole) were obtained from Toronto Research Chemicals Inc. (Toronto, Canada). Stock standards solutions of 100 μg mL⁻¹ in methanol were prepared and stored at 4°C for further dilutions. Standard mixtures of all HAAs with 4,7,8-TriMeIQx (500 ng·mL⁻¹) as internal standard (IS) at different concentration levels were prepared using methanol to establish the range of linearity and for the calibration curves. Solutions and samples were filters through 0.22 μm nylon filter (Jin teng, China) before analysis.

Methanol, acetonitrile, ammonium formate and formic acid were HPLC grade, provided by J.T.
Baker (Gross-Gerau, Germany) and Aladdin (Shanghai, China) respectively. Water was purified in a Ro water purification system (Heal Force, China). Other chemicals were obtained from Sinopharm Chemical Reagent Co., Ltd (Beijing, China). Mobile phases were also filtered with a 0.22 μm nylon filter (Jin teng, China). Extrelut NT20 extraction cartridge and refill material (diatomaceous earth) were from Merk (Darmstadt, Germany). Octadecysilane (C18,100 mg and 500 mg) SPE columns and propyl sulfonic acid (PRS,500mg) columns were obtained from Agilent Technologies Inc. (America). Coupling pieces and stopcocks were purchased from Agilent (America).

2.2. Instrumentation and MS conditions
Chromatographic separation of HAAs depended on a liquid chromatography system, equipped with a binary pump system from Agilent technologies model series 1200 (Agilent, USA), using Zorbax SB-C18 (3.5 μm, 2.1×150 mm) (Agilent, USA) column. Solvent A: 7 mM formic acid/ ammonium formate pH 3.2; solvent B: acetonitrile. Optimum separation was achieved at a flow rate of 0.3 mL min⁻¹. The gradient elution program was: 0-1min, 5%B; 1-15 min, 30%B; 15-18 min, 60%B; 18-24 min, 60%B; 24-25 min, return to initial conditions; 25-35 min, equilibration of the column. Volume injected was 10 μL. The LC system was coupled to an Agilent 6410 triple quadrupole mass spectrometer equipped with electrospray ionization (ESI) (Agilent, America). The MS instrument was operated in the positive mode and the data was gained in multiple reaction monitoring (MRM) mode. For quantification, 9 analytes were monitored simultaneously. Source working conditions were as follows: spray voltage, 3.5 kV; nebulizer pressure, 30 ps; drying gas flow rate, 10 L·min⁻¹ heated capillary temperature, 350°C. Data acquisition was performed by Mass Hunter Workstation Software.

2.3. Sample treatment
Raw pork and commercial pork products were acquired from a local market in Beijing. Raw meat samples were cooked in water at 100°C. And all the samples were fully mixed using a meat grinder and stored at -18°C before analysis. HAAs were extracted from food samples and purified by the method developed by Gross and GrÜter [17] with some modification [25]. Meat sample (4 g) was homogenized with 40mL of 1mol/L NaOH by Scattering Machine (FSH-2, Changzhou Guohua, China) for 5 min, and extracted by ultrasonic instrument (KQ-100E, Kunshan Shumei, China) for 20 min. Then the cooked food of which quantity equal to 1 g blended with 13 g diatomaceous earth material. The sample was transferred to a vacant Extrelut column coupled to a Bond Elut PRS cartridge (500 mg) conditioned with dichloromethane containing 5% methylbenzene (4 mL). After being extracted from diatomaceous earth, the HAAs were eluted to the PRS cartridge with the usage of dichloromethane containing 5% methylbenzene (75 mL) through solide phase extraction device (12 Position Vacuum Manifold Set, CNW Technologies GmbH, Germany). The PRS cartridge was vacuum dried and rinsed with HCl (0.1 M, 6 mL), methanol/0.1 M HCl (4:6, v/v, 15 mL) and water (2 mL) to wash out the nonpolar HAAs and others impurities. In order to the further purity, ammonia (0.5 mL) and water (20-30 mL) were added to solution containing nonpolar HAAs to dilute to the content of methanol less than 20%. Then added to a Bond Elut C18 (500 mg) cartridge conditioned with methanol (2 mL) and water (10 mL) and washed with water (2 mL), dried through a vacuuming procedure. And with the usage of methanol/ ammonia (9:1, v/v, 1.4 mL), HAAs were eluted to a vial.

Coupled to a Bond Elut C18 (100 mg) cartridge pretreated with methanol (2 mL) and water (10 mL), the PRS cartridge was desorbed with ammonium acetate (0.5 M, 20 mL) regulated at pH8.0. To end, the C18 (100 mg) cartridge was cleaned with water (2 mL), dried through a vacuuming procedure, and then HAAs were eluted to a vial with methanol/ammonia (9:1, v/v, 0.8 mL). The mixture solvent was evaporated by a flow of nitrogen with Sample Concentrator (BF-2000, Bu Fang Century, China) and the residues were re-dissolved with internal standard 4,7,8-TriMeIQx (500 ng·mL⁻¹, 500 μL) in methanol.

To assess the recovery of method added three levels (10 ng·g⁻¹, 20 ng·g⁻¹, 50 ng·g⁻¹) HAAs to food meat samples, and recoveries could be obtained by analyzing the calibration curves.
3. Results and discussion

3.1. Optimization of the chromatographic separation

For the purpose of gaining the best chromatographic separation, buffer pH, concentration and injected volume were studied. In previous work [30], most of the columns for HAAs analysis were C18 or C8 with small particle size (from 3 to 5 μm) providing high efficiency. Taking into account the results obtained, a Zorbax SB-C18 (3.5 μm, 2.1×150 mm) column was used for the optimization of HAAs separation in this study. In order to protonate the amino groups of the HAAs, the mobile phase pH is required to be lower than the pKa value of the corresponding compound. The pKa values of the HAAs

![Figure 1](image_url)

**Figure 1.** Effect of pH value of mobile phase on HAAs separation. Chromatographic conditions were as follows. Injection of 5 μL of a standard solution of 100ppb. The concentration of mobile phase buffer is 10 mM. 1: IQ, 2: MeIQ, 3: 8-MeIQx, 4: 4,8-DiMeIQx, 5: Norharman, 6: Harman, 7: 4,7,8-TriMeIQx, 8: PhIP, 9: Trp-p-l, 10: AuC.
Figure 2. Effect of concentration of mobile phase buffer on HAAs separation. Chromatographic conditions were as follows. Injection of 5 μL of a standard solution of 100 ppb. pH: 3.2. 1: IQ, 2: MeIQ, 3: 8-MeIQx, 4: 4,8-DiMeIQx, 5: Norharman, 6: Harman, 7: 4,7,8-TriMeIQx, 8: PhIP, 9: Trp-p-1, 10: AαC.

Figure 3. Effect of injected volume of sample on HAAs separation. Chromatographic conditions were as follows. The concentration of mobile phase buffer is 7 mM, pH 3.2. The concentration of standard solution is 100 ppb. 1: IQ, 2: MeIQ, 3: 8-MeIQx, 4: 4,8-DiMeIQx, 5: Norharman, 6: Harman, 7: 4,7,8-TriMeIQx, 8: PhIP, 9: Trp-p-1, 10: AαC.

studied in this study were mostly in the vicinity of 6, such as IQ, MeIQ, 8-MeIQx, 4,8-DiMeIQx, 4,7,8-TriMeIQx, PhIP, Norharman and Harman, whose values of pKa are 6.4, 5.5, 6.5, 6.0, 6.5, 6.3, and 6.9, respectively [31]. In the positive ion detection mode, the mobile phase pH is generally two units lower than the pKa value of the corresponding HAAs [32]. Therefore, the separation effect of 10 kinds of heterocyclic amines was investigated when the pH value was 3.2, 3.7, 4.0 and 4.5. As shown in figure 1, obviously, a complete separation could not be obtained under any condition. Increasing pH varied from 3.2 to 4.5, the retention time of the HAAs was prolonged, although only slightly variated, and the chromatographic peak of 8-MeIQx (peak 3) and Trp-p-1 (peak 9) widened. Bianchi et al [33] also studied the effect of pH on the chromatographic behaviors, as the pH increasing from 2.8 to 4.7,
the retention time and peak width increased, which were consistent with this study. Moreover, there was no change in the elution order, the results were consistent with an earlier research [30]. Comparing with previous study [33] (pH 3.0, 3.5, 4.0, 4.75), the elution order of Trp-p-1, PhIP, MeIQ and 8-MeIQx were identical when buffer pH below 4.75, changing at 4.75. Consequently, pH 3.2 was selected since the highest signal responses and the best resolution between MeIQ (peak 2) and 8-MeIQx (peak 3) were obtained.

In terms of concentration of mobile phase, the separation effect of the formic acid-ammonium/formate buffer solution at the concentration of 1, 3, 7, 10 mM was investigated. Figure 2 shows that with concentration lower than 7 mM, Trp-p-1 (peak 9) and AαC (peak 10) co-eluted. With the increasing of the mobile phase concentration, the retention time was increased. When the concentration was 7 mM and 10 mM, the chromatographic peak separation of 10 kinds of heterocyclic amines was relatively good. Compared with 10mM, the retention time was shorter and baseline separation of all the analytes was better at 7mM, so the concentration of 7mM was the best.

For the injected volume (5, 10, 15, 20 μL), Figure 3 showed that the concentration had little effect on the separation of HAAs, compared to others, the resolution between Trp-p-1(p9) and AαC (peak 10) was worse for 5 μL. As the injected volume varying from 5 to 20 μL, chromatographic peak gained better signal responses. However, as the injection volume increased, the response value did not multiply, 10 μL was established as sufficient.

3.2. Optimization of the MS/MS working conditions

To obtain the best signal responses of all the analytes, we optimized fragmentor of precursor ions and collision energy (CE) of all the product ions in this section. In the positive mode, the precursors ion opted were the protonated molecular ions \([M+H]^+\). Using product scan mode, the product ions were acquired through the process of scanning m/z from 100 to 250 for each HAA at 100ng mL\(^{-1}\). The most abundant product ions were monitored for HAAs quantification, and the second-most abundant product ions were monitored for confirmation of the analytes. Then fragmentor of precursor ions and CE of all the product ions were optimized by MassHunter Optimizer Software, ranged from 70 to 150 eV and between 20 to 50 eV respectively. As shown in table 1, the greatest responses for every precursor and product ion were acquired after optimization.

| Analyte   | Precursor ion (m/z) | Fragmentor (eV) | Quantitation | Confirmation |
|-----------|---------------------|-----------------|--------------|--------------|
| IQ        | 199.1               | 80              | 184.3        | 27           | 157.0        | 40           |
| MeIQ      | 213.0               | 115             | 198.1        | 27           | 197.0        | 39           |
| 8-MeIQx   | 214.2               | 115             | 199.1        | 27           | 131.2        | 40           |
| 4,8-DiMeIQx | 228.1             | 115             | 213.1        | 27           | 186.9        | 23           |
| 4,7,8-TriMeIQx | 242.0          | 115             | 226.8        | 27           | 201.2        | 23           |
| PhIP      | 225.2               | 115             | 210.1        | 31           | 182.8        | 39           |
| Norharman | 169.1               | 140             | 114.8        | 31           | 167.8        | 35           |
| Harman    | 183.0               | 130             | 114.9        | 27           | 168.1        | 39           |
| Trp-P-1   | 212.1               | 135             | 195.0        | 23           | 168.0        | 31           |
| AαC       | 184.0               | 130             | 167.1        | 23           | 139.8        | 35           |

The results agreed with previous studies [34, 35] for the majority ion of all the analytes. For polar
compounds, such as IQ, MeIQ, 8-MeIQx, 4,8-DiMeIQx, 4,7,8-TriMeIQx and PhIP, the product ions \([\text{M+H-CH}_3]^+\) that provided the maximal intensity resulted from the loss of the methyl group, while for nonpolar HAAs, such as Trp-p-1 and AαC, the ammonia-loss ion, \([\text{M+H-NH}_3]^+\), was the predominant fragment. However, for the qualitative ions of 8-MeIQx, 4, 8-DiMeIQx and Norharman, there were some differences between this study and others [18, 25, 36]. The reasons of these results may relate to the difference of buffer pH and concentration [25], mass spectrometer [36], ion source [37] and monitoring mode [18]. Consequently, as shown in figure 4, all compounds were baseline-separated, also, a more accurate quantification could be behaved in MRM mode after optimization.

![Figure 4. The LC-MS/MS chromatograms of HAAs at standard solution of 100ppb in MRM.](image)

### 3.3. Optimization of sample treatment

#### 3.3.1. Ultrasound assisted extraction time

The extracting efficiency were assessed on the recovery that achieved. This procedure influenced by many parameters, such as the concentration of alkaline, the selection of solvents and the time of extraction [37, 38]. The extraction time and solvents were studied. Extraction time is the necessary time to release the HAAs from matrix completely by ways of the treatment of initial alkaline. Several periods (0, 5 and 20 min) of Ultrasound assisted extraction were studied after 5min homogenized with the solution of sodium hydroxide. Figure 5 shows the results. With the prolongation of ultrasound time, the recoveries of IQ, MeIQ, 8-MeIQx, Harman and Trp-P-1 were increased. However, for 4,8-DiMeIQx, Norharman, AαC and PhIP, the recoveries were lowest at 5 min, while the recovery of PhIP was highest at the time of non-ultrasonography. Except
for IQ and MeIQ, there was no significant change on the recoveries of other HAAs between groups (P > 0.05). As to IQ and MeIQ, compared with other groups, their recoveries had significant difference (P <0.05) at 20 min. In short, when extraction time was 20 min, the overall recovery of heterocyclic amines was better, so 20 min was fixed as the optimal ultrasonic extraction time.

Figure 5. Effects of extraction time on the recoveries of HAAs.

3.3.2. Selection of solvents. For the extracted solvent of heterocyclic amines, the more widely used are dichloromethane and ethyl acetate. Related report [39] showed that owing to the presence of benzene ring in the PhIP structure, according to the similar compatibility principle, the addition of 5% methylbenzene to the solvent improved the recovery of PhIP, so this study selected dichloromethane, ethyl acetate, dichloromethane/methylbenzene (v/v = 95/5) and ethyl acetate/methylbenzene (v/v = 95/5) as extraction solvent to estimate the extracting efficiency of heterocyclic amines. As can be seen in figure 6, dichloromethane/methylbenzene (v/v = 95/5) gained the highest recoveries for all HAAs, ranging from 50.92%-87.12%. However, no significant difference in the recoveries of IQ and MeIQ was seen between groups (P > 0.05). Compared with the experimental group without adding 5% methylbenzene, the addition of group could significantly improve the recovery of AαC (P <0.05). Whether 5% methylbenzene was added to dichloromethane or not, for the remaining heterocyclic amines, their recoveries were higher than that of ethyl acetate and ethyl acetate supplemented with 5% methylbenzene, significantly (P <0.05). Compared with the group of dichloromethane, the recovery of Trp-P-1 and AαC was significantly improved by adding 5% methylbenzene in dichloromethane as extraction solvent (P <0.05). Thus, dichloromethane/methylbenzene (v/v = 95/5) was opted as the optimum solvent of extraction.
Figure 6. Effects of extraction solvents on the recoveries of HAAs.

Table 2. Quality parameters of the LC-ESI-MS/MS method.

| Compound    | Regression line | LODs (ng g⁻¹) | LOQs (ng g⁻¹) | RSD% (n=6) |
|-------------|-----------------|---------------|---------------|------------|
|             | Slope           | Intercept     | Correlation coefficient | Spiked level 20 ng/g | Spiked level 10 ng/g |
| IQ          | 7.3038          | -0.0488       | 0.9996        | 0.03       | 0.11       | 7.80 | 7.94 |
| MelIQ       | 7.7372          | -0.0840       | 0.9995        | 0.02       | 0.05       | 4.56 | 8.68 |
| 8-MeiQx     | 4.9773          | 0.0100        | 0.9998        | 0.17       | 0.55       | 1.62 | 9.81 |
| 4,8-DiMeIQx | 5.4622          | 0.0218        | 0.9992        | 0.18       | 0.59       | 2.15 | 8.61 |
| Norharman   | 8.4165          | 0.0704        | 0.9994        | 0.03       | 0.08       | 3.53 | 6.14 |
| Harman      | 5.5082          | -0.0162       | 0.9986        | 0.24       | 0.80       | 1.92 | 8.21 |
| Trp-P-1     | 10.0312         | -0.0084       | 0.9985        | 0.09       | 0.30       | 6.08 | 7.17 |
| AaC         | 6.2248          | -0.0608       | 0.9992        | 0.20       | 0.65       | 6.39 | 7.27 |
| PhIP        | 4.1632          | 0.0126        | 0.9999        | 0.20       | 0.65       | 2.90 | 7.21 |

3.4. Evaluation of the LC-ESI-MS/MS method
To assess the LC-MS/MS method's performance, quality parameters such as linearity, the limits of
detection (LODs), the limits of qualification (LOQs), repeatability (run-to-run precision) and reproducibility (day-to-day precision) were studied. Calibration curves were selected relative concentration ratio of each heterocyclic amine to 4,7,8-TriMeIQx (internal standard) as the abscissa, the relative peak area ratio as the ordinate. The regression equation and the correlation coefficient were obtained. The LODs, based on a signal-to-noise ratio of 3, and LOQs, depending on a signal-to-noise ratio of 10 were calculated by injecting 10 μL of diluted HAAs standard solutions. Table 2 shows the results. PhIP, Trp-P-1 and AαC had good linearity in the concentration range of 10–500 ng/mL under the optimized chromatographic and mass spectrometric conditions, and the other six in the range of 5–500 ng/mL. Correlation coefficients of all curves were over 0.998. The range of LODs was from 0.02 to 0.24 ng·g⁻¹ and LOQs ranged from 0.05 to 0.80 ng·g⁻¹. Run-to-run precision based on concentration was structured via six consecutive injections of a standard solution at spiked levels of 10ng/g and 20 ng/g. To assess reproducibility, repeated the above step with the same solution of both two levels over three consecutive days. And accuracy was established by 6 replicate extractions performed with same solution.

High repeatability, reproducibility and accuracy were gained for each compound with relative standard deviations lower than 12.51% in all cases. Moreover, the recoveries of different spiked levels of pork samples were studied, and the results were shown in Table 3. For all HAAs, the recoveries were higher than 50.33%, and the average recoveries ranged from 52.97% to 97.11%. All of results confirmed that the method is successful in providing reliable values of repeatability and reproducibility essential to an exact HAAs analysis.

Table 3. The recovery (R, %) of different spiked levels of pork samples.

| Compound  | Spiked levels (ng·g⁻¹) | Average recovery (%) |
|-----------|------------------------|----------------------|
|           | 10                    | 20                   | 50                   |
| IQ        | 78.05 ± 3.78           | 82.56 ± 2.27         | 90.78 ± 2.21         | 83.80 |
| MeIQ      | 64.98 ± 5.16           | 59.51 ± 1.84         | 68.49 ± 2.19         | 64.33 |
| 8-MeIQx   | 77.25 ± 2.18           | 77.32 ± 1.91         | 78.58 ± 2.43         | 77.72 |
| 4,8-DiMeIQx | 83.54 ± 6.17       | 85.97 ± 3.80         | 85.73 ± 4.02         | 85.08 |
| Norharman | 77.03 ± 7.31           | 90.44 ± 9.79         | 85.36 ± 10.07        | 84.28 |
| Harman    | 95.77 ± 1.38           | 106.93 ± 11.02       | 88.64 ± 8.31         | 97.11 |
| Trp-P-1   | 52.96 ± 1.44           | 55.93 ± 10.31        | 56.05 ± 9.38         | 54.98 |
| AαC       | 50.33 ± 4.55           | 53.45 ± 5.54         | 54.13 ± 2.47         | 52.97 |
| PhIP      | 68.11 ± 5.41           | 67.08 ± 4.22         | 71.02 ± 4.38         | 68.74 |

Table 4. HAAs found in three food samples.

| HAAs     | Concentration ± SD⁺ (ng·g⁻¹) |
|----------|-----------------------------|
|          | Pork loss | Roast bacon | Roast fillet |
| IQ       | 0.98±0.01 | 4.96±0.26 | nd |
| MeIQ     | 0.54±0.01 | 0.66±0.02 | 0.59±0.02 |
| 8-MeIQx  | 0.05±0.01 | 0.32±0.02 | 0.12±0.03 |
| 4,8-DiMeIQx | 0.02±0.02 | nd        | 0.13±0.05 |
| Norharman| 3.37±0.79 | 7.08±0.37 | 3.66±0.10 |
| Harman   | 11.81±0.51 | 2.79±0.11 | 3.81±0.15 |
| Trp-P-1  | nd        | nd        | nd |
| AαC      | nd        | nd        | 0.02±0.01 |
PhIP  0.09±0.02  0.10±0.01  0.12±0.01

*Standard deviation (n=3); nd: not detected.

For the purpose of evaluating the applicability of the LC-MS method for the analysis of food samples, three pork samples were analyzed (pork loss, roast bacon and roast fillet). Each sample analysis was repeated three times. Table 4 shows the consequences. Low level of HAAs was in most pork products. Among the HAAs detected, Norharman and Harman were found at a high content of all the samples, and the highest concentration of 7.08 and 11.81 ng·g⁻¹ in Roast bacon and food loss respectively.

4. Conclusions
By optimizing the pretreatment conditions, chromatographic conditions and mass spectrometry conditions, the method of simultaneous determination of 9 kinds of heterocyclic amines in pork through solid phase extraction and liquid chromatography tandem triple quadrupole mass spectrometry was established. The samples were extracted with dichloromethane (containing 5% methylbenzene), purified by sodium propane sulfonate (PRS) column and C18 solid phase extraction column, and analyzed through column chromatography Zorbax SB-C18 (3.5 μm, 150 mm) at optimum condition. The results showed that the correlation coefficient of heterocyclic amine compounds was more than 0.998, the detection limit was 0.02-0.24 ng/mL, the limit of quantification was 0.05-0.65 ng/mL. The average recoveries were 52.97% -97.11%, and RSD were lower than 9.81%. Finally, the method was validated by analyzing three food meat products. The results of the quality parameters achieved, confirm that they are sufficient to propose it as a method for the reliable analysis of HAAs on food samples.

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