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Research Article

Simultaneous determination of some heterocyclic amines (HCAs) in processed foods by liquid chromatography tandem mass spectrometry (LC-MS/MS)

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Abstract

Heterocyclic amines (HCAs) are a group of more than 25 chemical compounds containing at least one heterocyclic ring, often produced during the processing of foods such as meat and fish at high temperatures. HCAs have been shown to be carcinogenic and mutagenic. The International Agency for Research on Cancer (IARC) has classified IQ as group 2A and 9 substances including MeIQ, MeIQx, PhIP, AαC, MeAαC, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2 as group 2B, the group that can cause cancer in humans. Human health is seriously harmed if food contaminated with HCAs is regularly consumed. Therefore, it is necessary to develop a method to simultaneously analyze HCAs in food to assess the risk of processed foods in Vietnam. In this study, 10 HCAs in the IARC list were studied and identified by liquid chromatography-mass spectrometry (LC-MS/MS) using a C18 chromatographic column, positive ion electrospray ionization source ESI (+), multiple reaction ion monitoring (MRM) mode. The results showed that the method had good specificity, the standard curve was built in the concentration range of 0.05 - 10 µg/kg, the detection limit was from 0.015 - 0.15 µg/kg, the quantification limit was from 0.05 - 0.5 µg/kg; the precision and accuracy met the requirements according to AOAC. The method was used to analyze the HCAs content in 23 processed food samples in Hanoi. The results showed that 22/23 samples detected HCAs, the sample with the highest content was up to 17.9 µg/kg.

Keywords: *Heterocyclic amine, HCAs, LC-MS/MS, processed food.*

1. INTRODUCTION

Heterocyclic amines (HCAs) are a group of chemical compounds that contain at least one heterocyclic ring, in which one or more carbon atoms in the ring are replaced by other atoms [1]. HCAs are compounds produced by the Maillard reaction in meat and fish cooked thoroughly at high temperatures [1-4]. Free amino acids, proteins, creatinine, reducing sugars, and nucleosides are the main precursors involved in the formation of HCAs [5]. The variety and content of HCAs are related to many factors such as the type of food, heating time and temperature, cooking method and equipment, storage time of fresh meat, raw materials and additives, presence of precursors, water activity, and pH [5, 6]. Common foods that can form HCAs have been studied, including meat, fish, and their products [7], coffee [8], food additives, and alcoholic beverages.

Toxicology studies have shown that HCAs are carcinogenic compounds in the colon, breast, pancreas, prostate, stomach, and lungs and have the potential to cause gene mutations [9, 10]. These compounds are highly carcinogenic and mutagenic: nearly ten times more carcinogenic than other toxic compounds such as aflatoxin B1, nitrosamines, and benzo[a]pyrene, more than 100 times more mutagenic than aflatoxin B1, and 2000 times more than benzo[a]pyrene [11]. The International Agency for Research on Cancer (IARC) has classified 9 HCAs

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as 2-Amino-3,4-dimethylimidazo[4,5-f]quinolone (MeIQ), 2-Amino-3,8-dimethylimidazo [4,5-f]quinoxaline (MeIQx), 2-Amino-1-methyl-6-phenyl-imidazo[4,5-b]pyridine (PhIP), 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), 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) are carcinogenic substances in group 2B humans and 2-Amino-3-methylimidazo[4,5-f]quinolone (IQ) are carcinogenic substances in group 2A humans [5, 10]. The Council of Europe (EU) recommends that HCAs intake be lower than 1 µg per day [11].

The identification of HCAs in foods is difficult due to the diversity of substances (more than 25 substances), the formation and presence of HCAs in different foods, and in complex sample backgrounds, such as foods with many compounds that interfere with detection and quantification. Currently, there are many methods in the world to identify HCAs in samples such as bread [6], coffee [8], fish [12], meat [13-15], v/v and use a variety of techniques such as gas chromatography mass spectrometry (GC-MS, GC-HRMS), visible ultraviolet spectroscopy (UV-Vis), enzyme-linked immunoassay (ELISA), high-performance thin-layer chromatography (HPTLC), high-performance liquid chromatography (HPLC), and double mass spectrometry liquid chromatography (LC-MS/MS). However, there is no standard method for identifying HCAs in foods. In today's world, studies mainly use the LC-MS/MS technique because it is possible to analyse many substances simultaneously with high sensitivity, specificity, and short analysis time. In Vietnam, there is no regulation on the maximum limit of HCAs in food nor is there an official method to identify HCAs. Therefore, it is essential to develop a method for determining HCAs in food. This study was conducted to develop an analytical method to simultaneously identify 10 types of HCAs on the list of substances that may cause cancer in humans according to IARC in the background of food samples, using the LC-MS/MS method. The results of the study were used to determine the content of HCAs in some processed foods in the Vietnamese market.

2. MATERIALS AND METHODS

2.1. Objects of study

The analytes selected in the study were 10 HCAs on the IARC list of potentially carcinogenic substances in humans, including IQ, MeIQ, MeIQx, PhIP, AαC, MeAαC, Trp-P-1, Trp-P-2, Glu-P-1, and Glu-P-2.

The sample subjects in the study are a number of processed foods, including instant noodles, grilled sausages, braised eggs, fried pork, grilled fish, braised fish, fried shrimp, and grilled beef, randomly collected at markets and supermarkets in Hanoi. A total of 23 samples of processed foods have been collected as of May 2024. The number and classification of samples collected are presented in **Table 1.**

Table 1. *Quantification and classification of food samples used in the study*

Type of food	Number of samples	How to prepare		
		Frying (C)	Grill (N)	Braised (K)
Instant noodles (M)	01	-	-	-
Sausages (X)	01	-	01	-
Eggs (Tr)	01	-	-	01
Pork (TL)	02	02	02	-
Fish (C)	03	02	-	01
Shrimp (T)	08	08	-	-
Beef (TB)	05	-	05	-

The white samples, according to the sample bases collected, include: vermicelli samples (noodle base), boiled pork (meat base), boiled eggs (egg base), and steamed fish (fish base). These samples were identified using the LC-MS/MS method with no detection of analytes. White samples were used in the specificity evaluation and method validation.

The meat sample used in the sample processing survey was a pork sample that was grilled with charcoal at high temperatures, the surface of which had turned dark brown. Through preliminary analysis by LC-MS/MS, it was found that the sample contained 2 high-content HCAs, AαC and Trp-P-2 (HCAs belong to the pyrolysis group, formed when processing food at temperatures above 250°C).

2.2. Materials and chemical

HCAs and 3 internal standards (AαC-$^{15}N_3$, MeIQx-d_3, PhIP-d_3) were sourced from Toronto Research Chemicals (Canada) with a purity of 95 - 98% (**Table S1**). Other solvents and chemicals supplied by Merck (Germany) include: acetonitrile (ACN), methanol (MeOH), ammonium formate ($HCOONH_4$), formic acid (HCOOH), ammonia 25% (NH_4OH), sodium chloride (NaCl), magnesium sulfate ($MgSO_4$), and deionized water (H_2O).

2.3. Equipment

The analytical equipment used in the study is the LC 20 AXL liquid chromatography system of Shimadzu (Japan), connected to the Sciex Triple Quad 5500 mass spectrometer detector of AB Sciex, the Symmetry C18 chromatography column (150 mm x 3.0 mm x 3.5 µm) of Waters (USA).

Some other auxiliary equipment includes the XS105 (Metter Toledo) analytical balance (with an accuracy of 0.1 mg); Mikro 200R refrigerated centrifuge (Hettich); S 180H ultrasonic device (Elma), and other common laboratory equipment and instruments.

2.4. Method

2.4.1. LC-MS/MS conditions

Liquid chromatography analysis of double mass spectrometry (LC-MS/MS) was used in this study. By referring to the literature [15, 16], the conditions for simultaneous analysis of 10 HCAs compounds were selected as follows: using an electron injection ionization source in positive ion mode (ESI+), multi-reaction selective scan mode (MRM), 1 parent ion and at least 2 child ions (1 child ion is used for quantification and at least 1 child ion is used for qualitative), the dynamic phase in gradient mode (%B: 0 → 2 min: 10%; 2 → 5 min: 10% → 95%; 5 → 7 min: 95%; 7 → 8 min: 95% → 10%; 8 → 10 min: 10%) were set at a flow rate of 0.5 mL/min, injection volume 10 µL, column temperature at room temperature, and dynamic phase composition survey was conducted, chromatographic column size.

2.4.2. Sample preparation

Combined sample processing methods of ultrasonic extraction, QuEChERS, and solid-phase extraction (SPE) were used to enhance the efficacy of HCAs compounds. On the basis of reference to literature [15, 16], the sample handling procedure was selected as follows: Accurate weighing of approximately 2.0 g of homogenized food samples into a 50 mL centrifuge tube. Add 100 µL of AαC-$^{15}N_3$, MeIQx-d_3, PhIP-d_3 10 ng/mL intrastandard solution mixture and 10 mL of deionized water, vortex shake for 10 s, and ultrasound for 30 min at 50 - 60°C in an ultrasonic bath. Add 10 mL of 1% HCOOH solution in ACN, vortex for 1 min. Add a mixture of extracted salts of 3.0 g $MgSO_4$ and 1.0 g NaCl, swirl for 1 min. Centrifuge at 6000 rpm for 5 min using a centrifuge. Take 9 mL of solution layer above the solid-phase extraction on the Oasis MCX column 6 cc/150 mg using a solid-phase extractor according to the procedure: column activation with 3 mL MeOH and 3 mL H_2O, then load the sample through the column at a rate of no more than 5 mL/min and then wash the column with 3 mL H_2O and 3 mL MeOH, and finally elute with 4 mL of 25% MeOH : NH_4OH solution (95 : 5, v/v) into 8 mL glass tube. Blow dry the eluent with N_2 gas and bite with 900 µL of 0.1% HCOOH solution in H_2O : ACN (90 : 10, v/v). Using roast pork samples that have been determined to have Trp-P-2 and AαC content to carry out surveys, including: salt extraction survey (4.0 g $MgSO_4$; 4.0 g NaCl; 3.0 g $MgSO_4$ + 1.0 g NaCl), solid-phase extraction column survey (Oasis MCX 6 cc/150 mg (MCX); Oasis MAX 6 cc/150 mg (MAX); Oasis HLB 3 cc/60 mg (HLB); Discovery DSC-SCX 3 cc/500 mg (SCX); Discovery DSC-C18 3 cc/500 mg (C18)) and reconstituted solvent survey (H_2O:ACN (90 : 10, v/v); ACN; H_2O; MeOH; H_2O:MeOH (90 : 10, v/v), with 0.1% formic acid added, 3 times per analysis.

2.4.3. Method validation

The method was validated with parameters including: specificity (number of IP points, ion intensity ratio, analysis of white samples, standard samples and additional samples), detection limit, quantitative limit (based on signal-to-noise ratio of S/N), standard line (constructed in the concentration range of 0.05 - 10 µg/kg), repeatability and recovery, repeatability and measurement uncertainty. The results were evaluated and compared with the regulations under the AOAC 2016 [17].

2.4.4. Data processing methods

The statistics are processed using Microsoft Excel 2016 software. The concentration of HCAs was calculated using QQQ Quantitative Analysis software on the LC-MS/MS device.

3. RESULTS AND DISCUSSION

3.1. Condition survey of 10 HCAs by LC-MS/MS method

3.1.1. Mass spectrometry condition survey

For the investigation of MS/MS conditions, the tripodod quadrupole mass spectrometry with an electron injection ionization source, positive ion mode, is used to investigate the optimal parent ion and child ion conditions and collision energy. The results are summarized in **Table 2** (the number of IP points calculated according to the EC standard 2021/808 [18]).

Table 2. *MS/MS conditions used in the analysis of 10 HCAs*

Analytes	Precursor ion (m/z)	Product ion (m/z)	CE (eV)	IP Score
IQ	198.8	184.1*	35	6.5
		157.1	45	
		130.2	53	
MeIQ	212.7	198.0*	32	6.5
		197.0	45	
		170.0	55	
MeIQx	214.1	199.2*	35	5
		131.1	47	
PhIP	225.1	210.1*	35	5
		140.0	59	
AαC	184.1	140.2	39	5
		167.1*	29	
MeAαC	197.7	181.0*	23	5
		154.0	38	
Glu-P-1	199.2	92.1*	44	6.5
		65.1	63	
		145.2	39	
Glu-P-2	185.2	78.0*	46	6.5
		131.1	39	
		158.2	33	
Trp-P-1	212.0	167.0*	47	6.5
		195.0	36	
		168.0	47	
Trp-P-2	197.7	181.0	23	5
		154.0*	38	
AαC-$^{15}N_3$**	187.1	169.1	31	-
MeIQx-d_3**	217.1	199.2	33	-
PhIP-d_3**	228.1	210.0	39	-

** Quantitative ions* *** Internal standards*

Based on the survey results, each analyte selects one quantitative ion and at least one qualitative ion, and an IP score of ≥ 5 is achieved for analysis on mass spectrometry (according to AOAC [17]). The ratio of qualitative ionic strength to quantitative ions between the standard sample and the standard extra-white sample is not more than 40% (**Table S2**), which meets the requirements according to EC 2021/808 [18]. Thus, the MS/MS condition has achieved the necessary specificity to be able to use sample processing surveys and simultaneous analysis of 10 HCAs.

3.1.2. Survey of analytical conditions on liquid chromatography equipment

** Dynamic phase survey*

After selecting the molecular ions, daughter ions, and optimal conditions of the mass spectrum, a mixed standard solution of 10 HCAs of 1.0 ng/mL was used to investigate the dynamic phase. Some of the analytical conditions fixed during the survey include: Symmetry C18 column (150 mm x 3.0 mm x 3.5 µm), flow rate 0.5 mL/min, sample pump volume 10 µL. Dynamic phase solvent systems surveyed in gradient mode (section 2.4.1) include: (1) formic acid 0.1% in water (channel A) and methanol (channel B), (2) formic acid 0.1% in water (channel A) and acetonitrile (channel B).

From the results obtained (**Figure S1**), the chromatographic peak of 10 HCAs when using the solvent system (1) is symmetrical, pointed, and does not have peak adhesion, while the solvent system (2) gives a broad chromatographic peak, which is more disproportionate than the solvent system (1). Therefore, a solvent system consisting of 0.1% formic acid in water and methanol was selected for further surveys.

** Chromatographic column size survey*

After selecting the dynamic phase solvent of 0.1% formic acid in water (channel A) and methanol (channel B), the chromatographic column size survey was carried out with three columns: (A) Symmetry C18 (150 mm x 3.0 mm x 3.5 µm), (B) Symmetry C18 (100 mm x 2.1 mm x 3.5 µm) and (C) Eclipse Plus C18 (150 mm x 2.1 mm x 3.5 µm).

The results obtained showed (**Figure S2**) that the signal obtained when analyzed by the chromatographic column (C) was asymmetrical, and there was peak splitting in some substances such as Glu-P-1, Glu-P-2, MeIQ, and MeIQx, while the chromatography columns (A) and (B) gave a symmetrical, pointed, and non-sticky peak. Therefore, the Symmetry C18 chromatography column (150 mm x 3.0 mm x 3.5 µm) was selected for further studies. The chromatography of the 10 HCAs after optimization is shown in **Figure 1**.

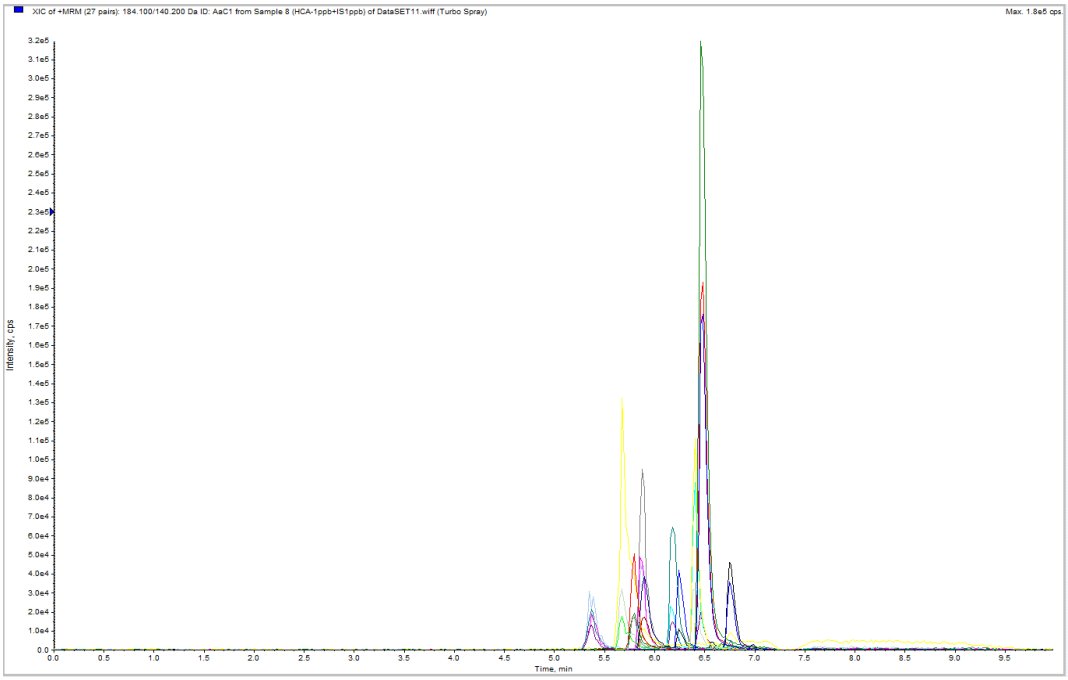

Figure 1. *Optimal results of liquid chromatography conditions of 10 HCAs*

3.2. Investigation of sample preparation

Some of the conditions in the process mentioned in section 2.4.2 are surveyed to improve the efficiency of sample processing. The survey was conducted with real samples identified as containing Trp-P-2 and AαC, comparing peak area signals at different conditions to select the best conditions.

3.2.1. Selection of extraction salts

In order to be able to extract the maximum amount of HCAs from the sample solution, the use of extraction salts helps to ensure that the analyte is maximally transferred to the organic phase. The extracted salts selected for the survey were 4.0 g of $MgSO_4$, 4.0 g of NaCl, and 3.0 g $MgSO_4$ + 1.0 g NaCl; other conditions were kept unchanged. The survey results presented in **Figure 2** show that using a mixture of $MgSO_4$ and NaCl salts gives better results than using salt alone. This can be explained by the fact that $MgSO_4$ has the effect of making the acetonitrile layer separate from the aqueous phase, NaCl is soluble in water, so it increases the ability of the analyte to transition from the aqueous phase to the organic phase. Therefore, a mixture of $MgSO_4$ and NaCl was used for further experiments.

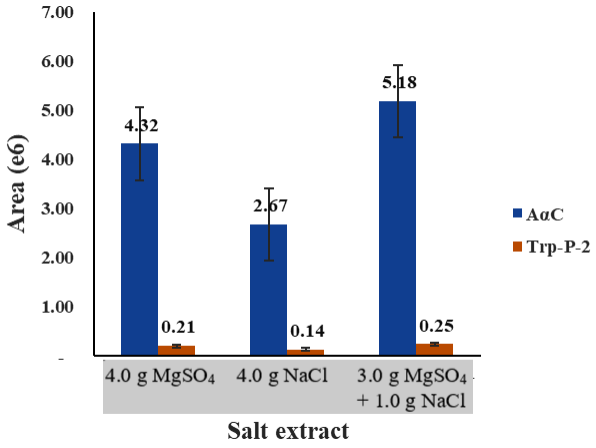

Figure 2. *Results of salt extraction survey*

3.2.2. Solid phase extraction column survey

For complex sample substrates such as food, especially fatty samples such as meat and fish, it is very important to clean the sample before analyzing on the equipment to increase sensitivity and avoid the effect of the sample substrate on the analyte. Different solid-phase extraction columns (according to section 2.4.2) were selected for survey with real samples. The results in **Figure 3** show that when using the Oasis MCX column 6 cc/150 mg, the highest analyte signals. This can be explained by the fact that HCAs contain an NH_2 group, a cation-exchange group, which is suitable for the MCX column with high selectivity and sensitivity when extracting compounds with a cationic exchange group. Therefore, the Oasis MCX 6 cc/150 mg solid-phase extraction column was selected for further surveys.

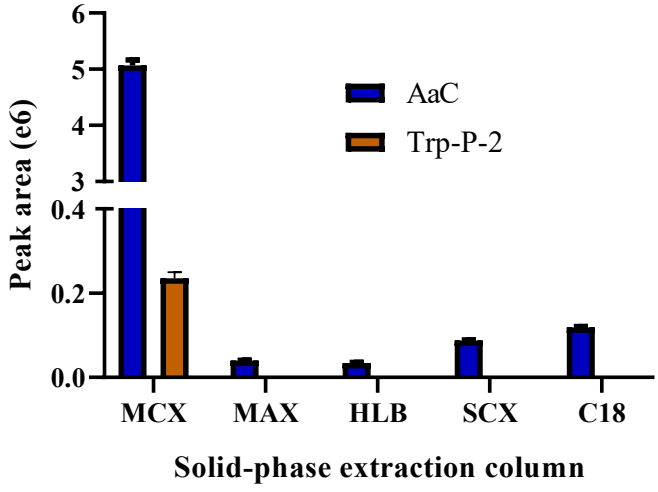

Figure 3. *Survey results of solid-phase extraction columns*

3.2.3. Reconstituted solvent survey

After selecting the extraction salt and the solid-phase extraction column, 4 types of reconstituted solvents were also investigated (according to section 2.4.2). The reconstituted solvent survey is intended to ensure maximum recovery performance and to be compatible with the LC-MS/MS instrument system. The results in **Figure 4** show that, when using a solvent mixture H_2O:ACN (90 : 10, v/v), the highest analyte signals. HCAs compounds are polar because they contain the NH_2 amino group, so they have good solubility in polar solvents such as ACN, MeOH, H_2O,... H_2O:ACN solvent mixtures (90 : 10, v/v) have a close polarity to HCAs compounds, so they have greater recovery efficiency (consistent with the study of Hao *et al*. [15]). Therefore, the solvent mixture H_2O:ACN (90 : 10, v/v) was chosen in this study.

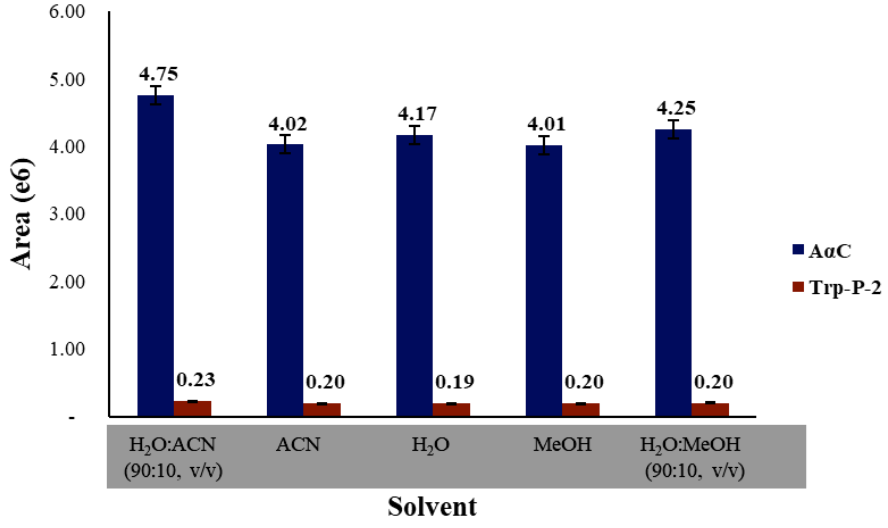

Figure 4. *Reconstituted solvent survey results*

3.3. Method validation

3.3.1. Specificity

The specificity of the method is assessed through the following criteria:

Calculation of IP scores: the results of the IP score calculated according to the EC standard 2021/808 [18], shown in **Table 3**, show that the 10 HCAs all have 1 parent ion and at least 2 child ions, giving an IP score of ≥ 5, meeting the requirements of AOAC [17] when analyzing on the mass spectrum.

Analysis of white samples, standard samples, and additional standard white samples: The results of the specificity assessment showed that the white sample did not show the signal of the analyte, the standard sample (the white sample was supplemented with HCAs at a concentration of 1.0 ng/mL) gave the signal at the same retention time (difference not more than 2%) compared to the standard HCAs solution of 1.0 ng/mL (**Figure S3**). Thus, the specificity method meets the AOAC requirement [17] to analyze 10 HCAs using the LC-MS/MS method.

3.3.2. Limit of detection (LOD) and limit of quantification (LOQ)

To determine LOD and LOQ, additional white samples at progressively lower concentrations are analyzed, and signals may also be produced. The signal-to-background noise ratio (S/N) is then determined based on the device's software with 6 repeated experiments. The detection limit is the concentration at which S/N = 3. The quantitative limit is the limit at which S/N = 10. The LOD results of the 10 HCAs are shown in **Table 3** with values in the range of 0.015 - 0.15 µg/kg and LOQ in the range of 0.05 - 0.5 µg/kg.

3.3.3. Construction of benchmark roads

Based on the selected survey conditions, the benchmark for determining 10 HCAs by the LC-MS/MS method was established at concentration levels of 0.5, 1.0, 2.0, 5.0, and 10 µg/kg for IQ, MeIQ, MeAαC, Trp-P-2, Glu-P-1, Glu-P-2, and 0.05, 0.1, 0.25, 0.5, and 1.0 µg/kg for AαC, MeIQx, PhIP, Trp-P-1. The benchmark

is constructed based on the dependence between the ratio of the peak area of the analyte to the intranor and the corresponding concentration. The results in **Table 3** show that, within the survey concentration range, the signal of the analyte has a linear correlation with the concentration, the linear correlation coefficient $R^2 > 0.995$, and the deviation of less than 15% with all analytes.

Table 3. *Detection limit, quantitative limit, and standard-line equation of 10 HCAs*

Analytes	LOD (µg/kg)	LOQ (µg/kg)	S/N ratio (at LOQ)	Linear curve	R^2	Maximum deviation (%)
IQ	0.15	0.5	10.5	y = 1.33x - 0.0152	0.9982	8.5
MeIQ	0.15	0.5	24.6	y = 2.99x + 0.0591	0.9988	7.0
MeIQx	0.015	0.05	10.3	y = 1.69x + 0.101	0.9998	4.2
PhIP	0.015	0.05	11.8	y = 5.88x + 0.134	0.9982	11.4
AαC	0.015	0.05	15.5	y = 2.09x + 0.00836	0.9998	7.7
MeAαC	0.15	0.5	51.7	y = 0.772x - 0.0233	0.9996	10.0
Glu-P-1	0.15	0.5	25.8	y = 1.06x + 0.129	0.9996	12.8
Glu-P-2	0.15	0.5	10.1	y = 0.915x + 0.00671	0.9994	5.0
Trp-P-1	0.015	0.05	14.1	y = 1.61x - 0.00981	0.9990	14.0
Trp-P-2	0.15	0.5	81.3	y = 1.31x - 0.0412	0.9996	2.8

3.3.4. Accuracy and precision

The accuracy and precision of the method were evaluated, respectively, through the recall (R%) and repeatability (relative standard deviation, RSD_r%) of 4 white samples with additional standards representing 4 sample backgrounds: meat, fish, eggs, and noodles. Samples are added standard at content levels of 0.5, 2.0, and 5.0 µg/kg for IQ, MeIQ, MeAαC, Trp-P-2, Glu-P-1, Glu-P-2, and 0.05, 0.25, and 0.5 µg/kg for AαC, MeIQx, PhIP, Trp-P-1, 6 doses each. The results are shown in **Table 4.**

Table 4. *Recovery (R%) and repeatability (RSD_r%) results of 10 HCAs in sample backgrounds*

Analytes	Meat		Fish		Eggs		Noodles	
	R%	RSD_r%	R%	RSD_r%	R%	RSD_r%	R%	RSD_r%
IQ	75.7-106.4	2.60-6.10	83.3-108.3	5.42-10.9	86.1-108.6	4.52-6.84	77.8-103.4	4.16-7.95
MeIQ	79.1-108.6	4.25-7.76	80.8-107.4	4.72-10.4	77.9-108.2	2.96-11.8	76.6-104.8	4.48-9.48
MeIQx	74.7-102.4	4.45-8.05	70.4-101.2	5.72-14.1	81.4-111.2	3.95-9.73	76.4-102.7	2.56-8.54
PhIP	73.4-106.8	4.21-5.23	83.0-105.9	2.27-6.17	85.6-105.9	4.04-5.80	76.7-107.9	5.29-7.84
AαC	71.8-106.7	2.55-16.8	84.0-107.3	2.88-5.33	86.2-105.6	4.69-6.88	82.8-103.4	4.36-6.88
MeAαC	82.7-109.0	1.95-3.85	85.2-104.3	4.02-6.56	81.0-105.6	5.76-7.93	83.0-107.4	4.29-5.28
Glu-P-1	84.1-108.8	1.93-4.02	82.5-111.2	3.10-8.02	86.3-106.5	4.03-7.90	86.6-108.9	2.85-4.45
Glu-P-2	84.1-108.0	4.32-4.93	83.0-106.1	5.07-9.39	81.8-107.9	4.96-10.5	78.3-108.9	5.13-7.13
Trp-P-1	76.9-103.8	3.33-4.90	86.3-109.9	5.16-8.46	80.9-103.0	3.81-8.67	82.1-109.9	3.58-6.59
Trp-P-2	82.7-108.7	2.81-8.02	89.8-106.4	3.08-6.91	90.6-105.0	2.21-4.69	83.6-109.9	3.44-4.65

The results in **Table 4** show that the recovery values of IQ, MeIQ, MeAαC, Trp-P-2, Glu-P-1, Glu-P-2 are in the range of 75.7 - 111.2%, the relative standard deviation is in the range of 1.93 - 11.8%; the recovery value of AαC, MeIQx, PhIP, Trp-P-1 is in the range of 70.4 - 111.2%, the relative standard deviation is between 2.27 - 16.8%. These results show that the method has a recovery and repeatability that meets the requirements of the AOAC [17] (recovery in the range of 60 - 115%, and relative standard deviation ≤ 21% at concentrations of 0.5 - 10 µg/kg; recovery in the range of 40 - 120% and relative standard deviations of ≤ 30% at concentrations of 0.05 - 1.0 µg/kg).

3.3.5. Intermediate accuracy and measurement uncertainty

The intermediate accuracy of the method was assessed through the repeatability (relative standard deviation RSD_R%) of 4 white samples with additional standards representing the 4 sample backgrounds of meat, fish, eggs, and noodles, and performed similarly to the repeatability analysis, but at a different time. Samples were standardized at 0.5 for IQ, MeIQ, MeAαC, Trp-P-2, Glu-P-1, Glu-P-2, and 0.05 µg/kg for AαC, MeIQx, PhIP, Trp-P-1, with 6 repeats. The measurement uncertainty of the method for sample substrates is calculated through recall and intermediate accuracy. The results of the analysis are shown in **Table 5.**

Table 5. *Intermediate accuracy assessment results (RSD_R%)and measurement uncertainty (U%) of 10 HCAs in sample substrates*

Analytes	Meat		Fish		Eggs		Noodles	
	RSD_R%	U% (k=2)	RSD_R%	U% (k=2)	RSD_R%	U% (k=2)	RSD_R%	U% (k=2)
IQ	4.82	25.0	4.73	17.0	2.56	13.7	3.31	26.2
MeIQ	7.51	25.8	8.52	23.8	6.66	20.9	3.83	23.1
MeIQx	5.13	27.6	3.81	32.3	6.10	19.1	7.26	24.1
PhIP	3.40	28.9	5.95	18.0	7.15	17.6	4.24	23.1
AαC	2.82	22.8	6.59	22.1	6.61	19.7	6.88	22.3
MeAαC	3.81	16.6	6.12	20.2	4.56	21.0	4.98	17.9
Glu-P-1	2.58	16.1	7.46	24.5	3.96	14.1	6.77	19.3
Glu-P-2	8.13	22.1	7.16	21.0	6.02	22.4	6.23	20.1
Trp-P-1	2.64	19.8	5.05	17.6	6.15	17.9	6.02	20.1
Trp-P-2	6.63	19.7	5.76	16.5	5.10	14.1	1.80	16.0

From the results of **Table 5**, it is shown that the intermediate accuracy value of the analytes is in the range of 1.80 - 8.52%, meeting the requirements of the AOAC [17] (RSD_R% ≤ 45% at concentrations of 0.05 - 0.5 µg/kg).

3.4. Analysis of HCAs in food samples

The post-appraisal method was applied to determine the content of HCAs in 23 samples of processed foods collected in Hanoi. The results are presented in **Table 6.**

The results in **Table 6** show that, out of the 23 samples collected, 22/23 (96%) samples detected HCAs, only the instant noodle sample (M1) did not detect HCAs, and none of the samples detected 05 substances: IQ, MeIQ, Trp-P-1, Trp-P-2, and Glu-P-2. 06 samples detected PhIP with a content in the range of < LOQ - 3.85 µg/kg; only the egg sample (TrK1) detected MeIQx at a content of 0.18 µg/kg; 06 samples detected Glu-P-1 but with low content (< LOQ); 12 samples detected MeAαC, of which 04 samples were at < LOQ, the remaining 08 samples had content in the range of 0.62 - 2.23 µg/kg. AαC was detected in 22/23 samples: 02 samples were at < LOQ levels, the remaining 20 were in the range of 0.09 - 14.0 µg/kg. The total HCAs in the quantitative samples ranged from 0.22 to 17.9 µg/kg.

***Table 6**. Total HCAs and HCAs in 23 food samples*

No.	Sample code	HCAs content (µg/kg)					
		MeIQx	**PhIP**	**AαC**	**MeAαC**	**Glu-P-1**	**Total HCAs**
1.	M1	ND	ND	ND	ND	ND	ND
2.	XN1	ND	ND	0.22	ND	ND	0.22
3.	TrK1	0.18	0.08	0.09	ND	< LOQ	0.35
4.	TLN1	ND	ND	6.83	0.95	ND	7.78
5.	TLN2	ND	1.65	14.0	2.23	ND	17.9
6.	TLC1	ND	ND	1.43	< LOQ	ND	1.43
7.	TLC2	ND	ND	1.28	< LOQ	ND	1.28
8.	CK1	ND	< LOQ	< LOQ	ND	< LOQ	< LOQ
9.	CC1	ND	3.85	0.58	ND	ND	4.43
10.	CC2	ND	< LOQ	< LOQ	ND	< LOQ	< LOQ
11.	TC1	ND	ND	4.41	0.62	ND	5.03
12.	TC2	ND	ND	3.16	< LOQ	ND	3.16
13.	TC3	ND	ND	9.35	1.76	ND	11.1
14.	TC4	ND	ND	8.68	1.52	ND	10.2
15.	TC5	ND	0.53	0.87	< LOQ	ND	1.40
16.	TC6	ND	ND	3.81	0.72	ND	4.53
17.	TC7	ND	ND	5.65	0.82	ND	4.47
18.	TC8	ND	ND	4.24	0.70	ND	4.94
19.	TBN1	ND	ND	1.01	ND	ND	1.01
20.	TBN2	ND	ND	0.58	ND	ND	0.58
21.	TBN3	ND	ND	0.90	ND	ND	0.90
22.	TBN4	ND	ND	0.58	ND	ND	0.58
23.	TBN5	ND	ND	0.76	ND	ND	0.76

** ND: Not detected.*

Compared by processing, the largest amount of HCAs is formed by baking, followed by frying in oil, and finally storage. Roast pork samples had a total HCAs content of about 5.6 to 14 times higher than fried pork samples, while stock-based samples typically detected low levels of HCAs. AαC was the HCAs with the highest frequency and accounted for a relatively high proportion in the samples, followed by MeAαC, while MeIQx, Glu-P-1, and PhIP were only detected at low levels. This can be explained by the fact that the samples have been processed by frying and baking at high temperatures, so they tend to form pyrolysis HCAs such as AαC and MeAαC. According to the study of Gross *et al.* [12], the content of pyrolysis HCAs increased significantly when processing salmon by baking at 270°C for 12 min compared to frying in a pan at 200°C for 12 min (AαC content increased by 12.1 times, norharman increased by 6.6 times; harman increased by 8.1 times). In addition, the study by Karpavičiūtė *et al.* [8] also showed that the content of HCAs increased by about 2.5 times when food was processed for a longer period of time. In addition to temperature and time factors, the diversity of amino acid composition in foods has made a difference in the formation of HCAs in different sample backgrounds [5, 6]. It is the diversity of food ingredients and many factors that affect the formation of HCAs (temperature, processing time, spices,...) that make it difficult to control the risk of

processed foods at high temperatures containing HCAs. Currently, in the world and Vietnam, there are no regulations on the maximum allowable residue level for HCAs in processed foods. Therefore, in order to have a basis for promulgating regulations to control the content of HCAs in food, it is necessary to carry out more studies to assess the risk of HCAs compounds to human health.

4. CONCLUSION

The study has succeeded in developing a method to determine the content of 10 HCAs in meat, seafood, eggs and noodles using the LC-MS/MS method to determine the content. Analytical conditions with a dynamic phase in gradient mode include methanol and formic acid 0.1% in water, a Symmetry C18 chromatography column, and sample processing combined with solid-phase extraction with an Oasis MCX column. The methodology has been validated to meet the requirements of the AOAC [17]. The method was applied to analyze HCAs in 23 food samples. The results detected 22 samples containing HCAs, accounting for 92%, and AαC was the most detected HCAs. This result also partly shows that foods processed by baking have a higher risk of containing HCAs than those processed by frying and steaming. However, the research mainly focuses on building analytical methods, so the sample base is still small and not sufficiently representative. Therefore, research will continue to be expanded to analyze the content of HCAs in many processed foods and in many other regions in Vietnam. At the same time, research will also be expanded to investigate the influence of factors such as temperature, time, and spices,... in food processing to limit the formation of heterocyclic amines such as HCAs.

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SUPPLEMENT DATA

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