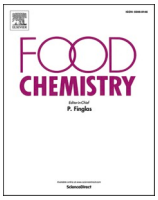
Food Chemistry 447 (2024) 138930

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Simultaneous determination of advanced glycation end products and heterocyclic amines in roast/grilled meat by UPLC-MS/MS

Mingyu Li [a,b], Chunjiang Zhang [a], Zhenyu Wang [a], Na Liu [c], Ruiyun Wu [a], Jiajing Han [a], Wenhan Wei [a], Christophe Blecker [b], Dequan Zhang [a,*]

[a] Integrated Laboratory of Processing Technology for Chinese Meat Dishes, Institute of Food Science and Technology, Chinese Academy of Agricultural Sciences, Beijing 100193, China
[b] Gembloux Agro-Bio Tech, Unit of Food Science and Formulation, University of Liège, Avenue de la Faculté d'Agronomie 2, Gembloux B-5030, Belgium
[c] Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing 100193, China

ARTICLE INFO

Keywords:
Advanced glycation end products
Heterocyclic amines
UPLC-MS/MS
Roast/grilled meat

ABSTRACT

Advanced glycation end products (AGEs) and heterocyclic amines (HAs) are main harmful Maillard reaction products of meat products. Simultaneous quantification of both with high sensitivity, selectivity and accuracy remains a major challenge due to inconsistencies in their pre-treatment and instrumental methods and the different polarity of AGEs and HAs. We developed a method for the simultaneous determination of AGEs and HAs in roast/grilled meat by ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) using dynamic multiple reaction monitoring (D-MRM). The instrument parameters and pre-treatment method were optimized to achieve reasonably good separation and high response for the 11 target analytes within 8 min. From 10 to 200 ng/mL, the limits of detection (LODs) and limits of quantitation (LOQs) ranged from 0.3 to 5.5 μg/L and 0.9 to 6.3 μg/L, respectively, and the correlation coefficient (R^2) was >0.99. It was acceptable to recoveries, standard deviations (RSDs), and matrix effects. Six types of roast/grilled meat samples were then tested using the developed method.

1. Introduction

Favorable aroma is one of the vital factors for the palatability and attraction of roast/grilled meat by consumers. The Maillard reaction was described in 1912 by Louis-Camille Maillard, in which carbonyl compounds react with amino compounds to impart unique flavors and colors to foods (Maillard, 1912). However, the Maillard reaction involves a complex series of chemical reactions that can also produce some harmful Maillard reaction products (Wei et al., 2024). AGEs, which can be produced endogenously or ingested from food, are a class of harmful Maillard reaction products formed by complex interactions between the active carbonyl groups of reducing sugars and the amino groups of nucleic acids, fats, and proteins, with meat products accounting for up to 80% of dietary AGEs (Chen, Lin, Bu, & Zhang, 2018; Uribarri et al., 2010). HAs are polycyclic aromatic molecules that are usually formed as a group of harmful Maillard reaction products after thermal processing with amino acids, sugars and creatinine as precursors in protein-rich foods (especially meat and fish) (Nadeem et al., 2021; Wang et al., 2023). Thermal processing of meat products appears to produce and accumulate AGEs and HAs simultaneously. There is growing evidence that the accumulation of AGEs or HAs may be a causative factor in several types of chronic diseases, and HAs are also potent teratogens and mutagens (Carvalho et al., 2015; Di Pino et al., 2017; Twarda-Clapa, Olczak, Białkowska, & Koziołkiewicz, 2022; Zhang, Zou, Mao, & Chen, 2022). In addition, Quan et al. (2021) found that the co-accumulation of acrylamide, N^{ε}-(carboxymethyl)lysine (CML) and harman had different effects on the liver, kidney, gastrocnemius and metabolic pathways compared to the compounds that accumulated alone, based on serum biochemical analysis, histopathological examination and metabolomic analysis. Single monitoring alone is no longer sufficient to meet public demand for food safety, while simultaneous selective, rapid and reproducible determination of the these harmful Maillard reaction products is important for analytical applications and diagnostic studies.

Since the polarity of AGEs and HAs is different, their levels are μg/g or ng/g in different foods (Lu et al., 2022; Wang et al., 2023; Xu et al., 2021), and the food matrix is generally complex, the selection of a suitable pre-treatment method is crucial and challenging for the robust and simultaneous detection of AGEs and HAs in roast/grilled meat.

* Corresponding author.
E-mail address: dequan_zhang0118@126.com (D. Zhang).

https://doi.org/10.1016/j.foodchem.2024.138930
Received 13 December 2023; Received in revised form 26 February 2024; Accepted 1 March 2024
Available online 4 March 2024

Liquid-liquid extraction (LLE), pressurized liquid extraction (PLE), supercritical fluid extraction (SFE), dispersive liquid-liquid microextraction (DLLME), solid-phase extraction (SPE), solid-phase microextraction (SPME) and microwave-assisted extraction (MAE) have been used to extract AGEs or HAs (Barzegar, Kamankesh, & Mohammadi, 2019; Wang et al., 2023). There are currently two methods of extracting AGEs, enzymatic hydrolysis and acid hydrolysis. Acid hydrolysis is the most commonly used, while enzymatic hydrolysis is mainly aimed at high temperatures and acidic conditions that easily destroy AGEs such as pyrraline (PYR) (Zhu, Huang, Cheng, Khan, & Huang, 2020). The AGEs are then enriched and purified by SPE (Li et al., 2022; Yuan et al., 2021). To date, the most recognized classic method for the extraction of HAs was developed by Gross and Grüter in the 1990s: base-hydrolysed samples are extracted with an organic solvent such as dichloromethane and then passed through an SPE column for extraction and purification (Gross & Grüter, 1992). A new acid hydrolysis method has also been proposed for more efficient extraction of protein-bound HAs (Chen, He, Qin, Chen, & Zeng, 2017). In addition, the QuEChERS method is increasingly being used for the extraction and purification of HAs because it is quick, easy, cheap, effective, rugged, and safe (Chang, Zhang, Wang, & Chen, 2019; Hsiao, Chen, & Kao, 2017; Lai, Lee, Cao, Zhang, & Chen, 2023). LC-MS/MS has become the most widely used detection method for the independent determination of AGEs and HAs in recent years due to its high detection sensitivity, few restrictive conditions and no derivatization step (Jian, Yeh, Wang, Chen, & Chen, 2019; Wei, Liu, & Sun, 2018). However, due to different pre-treatment methods and instrument parameters, they can only be performed separately, doubling the time and cost of testing. As far as we know, there are few studies measuring AGE and HA simultaneously (Wei et al., 2024), and the effectiveness of co-extraction and co-purification of AGEs and HAs in roast/grilled meat matrices has not been thoroughly investigated. The development of a simultaneous separation and quantification method is therefore essential to reduce the cost and increase the efficiency of the assay.

Therefore, the main objective of this study was to establish an accurate, robust and efficient analytical method for the simultaneous determination of AGEs and HAs in roast/grilled meat. Several instrumental parameters were optimized to achieve high responding and separating efficiencies. We compared the performance of the acid hydrolysis SPE method and the QuEChERS method. In the end the chosen method was validated and applied to evaluate the AGEs and HAs content of different roast/grilled meat categories. This method provides time- and cost-saving technical support for the detection of AGEs and HAs, more accurately reflects the comprehensive assessment of food quality and food safety, and facilitates studies of the interaction of multiple harmful Maillard reaction products.

2. Materials and methods

2.1. Chemicals and materials

Methanol and formic acid (HPLC grade) were purchased from Merck & Co Inc. (Darmstadt, Germany), ammonia (LC-MS grade) was obtained from Sigma-Aldrich Trading Co., Ltd. (Saint Louis, Missouri, USA), and sodium borohydride ($NaBH_4$) and borax ($Na_2B_4O_7{\cdot}10H_2O$) were bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), as analytical grade reagents. CML (98%), CEL (98%), d_4-CML (96%), d_4-CEL (96%), PYR (99%), IQ (98%), Harman (98%), Norharman (98%), MeIQ (98%), MeIQx (98%), 7,8-DiMeIQx (98%), IQx (98%), PhIP (98%), 4,8-DiMeIQx (98%), and 4,7,8-TriMeIQx (98%) were purchased from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). Solid phase extraction cartridges (Oasis MCX, 3 cc, 60 mg) were purchased from Waters Co. (Milford, Massachusetts, USA). Ultrapure water was obtained from a BDP-Millipore water purification unit (Nanjing Quan-Kun bio-technology Co.,Ltd., Nanjing, China). Roast chicken breast, thigh, and skin was purchased from Pingdu, Shandong, China. Fresh sirloin for roast steaks was bought from a beef cattle farm in Henan province. Fresh silverside for grilled lamb patties, mutton shashliks were bought from Little Sheep Food Co., Ltd. (Inner Mongolia, China).

2.2. Preparation of standard solution

5 mg of d_4-CML was added to a 5 mL volumetric flask, and 10 mg of CML, CEL, PYR, IQ, MeIQ, IQx, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, PhIP, Harman, Norharman, d_4-CEL and 4,7,8-TriMeIQx were transferred to 14 volumetric flasks (10 mL), then a stock solution (1 mg/mL) was prepared with ultrapure water. Dilute the stock solution with ultrapure water to prepare a mixed standard solution.

2.3. Preparation of sample

The detection of AGEs and HAs was based on the methods of my previous study (Li et al., 2022) with some modifications. In detail, 50 mg of the crushed sample was taken, deproteinised with 5 mL of hexane, centrifuged at 4800 r/min for 10 min and repeated three times. The residue was dried, and reduced with 1.5 mL sodium borate buffer (0.2 mol/L, pH 9.2) and 1 mL sodium borohydride (1 mol/L) at 4 °C for about 8 h, followed by hydrolysis with HCl (12 mol/L, 2.5 mL) at 110 °C for 24 h. The hydrolysate was filtered into a 25 mL volumetric flask and made constant volume with ultrapure water. 1 mL of the above hydrolysate was pipetted and 100 μL of a mixed internal standard (1 μg/mL) of d_4-CML, d_4-CEL and 4,7,8-TriMeIQx was spiked, loaded onto the Oasis MCX column and eluted with 5% methanol-ammonium. The eluate was dried under a nitrogen stream and then dissolved in 1 mL methanol for UPLC-MS/MS analysis.

2.4. UPLC-MS/MS analysis

A triple quadrupole UPLC-MS/MS system (Agilent 1290–6470, Agilent Technologies, Santa Clara, California, USA) equipped with an ACQUITY UPLC HSS T3 column (2.1 × 100 mm, 1.8 μm, Waters Co., Milford, Massachusetts, USA) was utilized to concurrently quantify AGEs and HAs. An injection volume of 2 μL was employed, while the flow rate used was 0.2 mL/min. The mobile phase employed consisted of methanol (B) and an aqueous solution containing 0.1% (*v*/v) formic acid (A). The gradient elution started with 95% A (0 min) and decreased from 0 to 2.0 min to 85% A, 3.0 min to 80% A, then 20% A every minute, directly to 10% A at 6.0 min, to 0% A at 7.0 min and finally returned to 95% A at 7.1 min, equilibrating to 8 min. The detection (mass spectrometric) was performed in the positive mode of ESI, employing dynamic multiple reaction monitoring (D-MRM). The optimal ionization source working parameters were as follows: nitrogen was used as the nebulizer gas; the drying gas temperature was set at 280 °C; the drying gas flow rate was adjusted to 12 L/min; the nebulizer pressure was set at 25 Psi; and a capillary voltage of 3500 V was applied.

2.5. Method verification

According to the SANTE/2020/12830 guidelines, validation of the proposed method was performed to assess its compliance with method validation parameters. The coefficient of determination, R^2, was used to express the linearity of the calibration curve. Calibrators were prepared at 10, 20, 50, 100 and 200 ng/mL (five concentration levels) of AGEs and HAs. The calibration curve can be plotted, the external standard methods, the concentration (abscissa) is plotted against the response (ordinate), the internal standard methods, the ratio of the analyte area to the internal standard area (y-axis) is plotted against the concentration of the calibrator (x-axis). Matrix effects (ME) may also affect the slope of the calibration curve. The ME is particularly dependent on the degree of sample clean-up and chromatographic separation of the analyte and was therefore corrected with stable internal standards (d_4-CML, d_4-CEL and 4,7,8-TriMelQx) and calculated as the ratio of the response values for the

Table 1
MS/MS parameters and retention times of 11 analytes and 3 internal standards.

Compounds	Precursor ion	Product ion[a]	Fragmentor (V)	Collision Energy (eV)	Retention time
4,7,8-TriMeIQx[b]	242.1	201.2 (C)	100	32	6.630 ± 0.5
4,7,8-TriMeIQx[b]	242.1	145.1 (Q)	100	40	6.630 ± 0.5
4,8-DiMeIQx	228.1	213.1 (Q)	128	32	6.358 ± 0.5
4,8-DiMeIQx	228.1	187.1 (C)	128	28	6.358 ± 0.5
7,8-DiMeIQx	228.1	187.2 (C)	100	32	6.366 ± 0.5
7,8-DiMeIQx	228.1	131.1 (Q)	100	40	6.366 ± 0.5
PhIP	225.1	210.1 (Q)	90	32	6.687 ± 0.5
PhIP	225.1	140.1 (C)	90	40	6.687 ± 0.5
d_4-CEL[b]	223.0	134 (C)	70	12	1.239 ± 0.5
d_4-CEL[b]	223.0	88.0 (Q)	70	28	1.239 ± 0.5
CEL	219.1	130.3 (C)	80	12	1.239 ± 0.5
CEL	219.1	84.1 (Q)	80	20	1.239 ± 0.5
8-MeIQx	214.1	173.2 (C)	100	24	6.032 ± 0.5
8-MeIQx	214.1	131.1 (Q)	100	40	6.032 ± 0.5
MeIQ	213.1	198.1 (Q)	85	32	5.362 ± 0.5
MeIQ	213.1	145.2 (C)	85	28	5.362 ± 0.5
d_4-CML[b]	209.1	134.0 (C)	70	10	1.206 ± 0.5
d_4-CML[b]	209.1	87.8 (Q)	70	20	1.206 ± 0.5
CML	205.2	130.1 (C)	55	12	1.206 ± 0.5
CML	205.2	84.0 (Q)	55	20	1.206 ± 0.5
IQx	200.1	185.1 (Q)	103	32	5.552 ± 0.5
IQx	200.1	132.1 (C)	103	40	5.552 ± 0.5
IQ	199.1	184.1 (Q)	60	32	4.994 ± 0.5
IQ	199.1	131.1 (C)	60	24	4.994 ± 0.5
Harman	183.1	115.2 (Q)	55	40	6.496 ± 0.5
Harman	183.1	89.1 (C)	55	40	6.496 ± 0.5
Norharman	169.1	115.1 (Q)	55	40	6.287 ± 0.5
Norharman	169.1	89.1 (C)	55	40	6.287 ± 0.5

[a] Q, quantification ion; C, confirmation ion.
[b] Internal standard.

same amount of analyte added to the sample matrix to the response values for analytes in pure solvents, followed by × 100%. By calculating the signal-to-noise ratio to evaluate the LODs (S/N = 3) and LOQs (S/*N* = 10). The precision and recovery of the method were validated by measuring samples spiked with low, medium and high concentration standards (20, 100 and 200 μg/kg for AGEs and HAs), with precision measured six times and recovery measured three times. The formula for calculating recovery percentage is as follows: Recovery (%) = (amount of analytes added - amount of analytes tested) / (amount of analytes in sample matrix - amount of analytes tested) × 100.

2.6. Statistical analysis

All experiments were conducted independently at least three times. The UPLC-MS/MS system was controlled and the data was processed using MassHunter Workstation (version B.10.0, Agilent, Lake Forest, CA, USA). Significant differences ($p < 0.05$) were analyzed using SPSS 17.0 statistical analysis software. GraphPad Prism (version 8.0) was used for all data plots except principal component analysis plots (SIMCA-P+ version 13.0).

3. Results and discussion

3.1. Optimization of MS/MS conditions

We employed positive electrospray mode for detection because both the target compounds and their internal standards contain primary and secondary amine groups that tend to attract protons to form [M + H] molecular ion peaks. Firstly, by directly injecting standard substances into the mass spectrometer, we monitored and optimized the MS/MS parameters to obtain the strongest response for the $[M+H]^+$ ions of the target analytes. Subsequently, the fragmentor and collision energy have been optimized to enhance the response of the molecular ions. The parent ion was then scanned by secondary mass spectrometry and the most responsive fragment ion was selected for quantification and the second most responsive fragment ion for characterization. In addition, due to the presence of isomers within the 11 target compounds and 3 internal standards analyzed, there might be overlap in the parent or daughter ions of the quantified ions, which could cause interference. Therefore, the D-MRM mode was chosen for more accurate quantification. The optimized MS/MS parameters are listed in detail in Table 1.

3.2. Optimization of UPLC conditions

To ensure accurate quantification and good baseline separation of compounds with diverse properties, it is crucial to optimize the UPLC conditions. ZORBAX Eclipse Plus C18 (2.1 mm × 50 mm, 1.8 μm), ACQUITY UPLC BEH Amide (2.1 mm × 100 mm, 1.7 μm), and ACQUITY UPLC HSS T3 (2.1 mm × 100 mm, 1.8 μm), three different chromatographic columns available in the laboratory, were compared in this study. As shown in Supplementary Fig. 1, the peak shapes of CEL, CML, Harman, Norharman, PhIP were poor on the BEH Amide column, and the peak shapes of IQ, MeIQ, IQx, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, Harman, Norharman were poor on the Eclipse Plus C18 column, whereas the peak shapes on the HSS T3 column were sharp and symmetrical. This may be directly related to the fact that the ACQUITY HSS T3 is the first choice when developing separations for polar and non-polar compounds, and the ursal siliniveca-based bonded phase used for its sorbents is compatible with 100% aqueous mobile phase. It also features ultra-low MS bleed and provides balanced retention of polar and hydrophobic molecules without the use of ion-pairing reagents. Moreover, the ZORBAX Eclipse Plus C18 and ACQUITY UPLC BEH Amide columns failed to separate the target compounds (Supplementary Fig. 1). Although the ZORBAX Eclipse Plus C18 column packed with a silica matrix can provide excellent peak shapes for the separation of highly challenging basic compounds, pH 6–8 conditions are generally preferred. ACQUITY UPLC BEH Amide column is a neutral amide column primarily used for glycosylation analysis, and it is capable of efficiently separating polar compounds under normal phase or hydrophilic conditions. The HSS T3 column was therefore chosen for the simultaneous separation of AGEs and HAs.

If two adjacent peaks are not well resolved by isocratic elution,

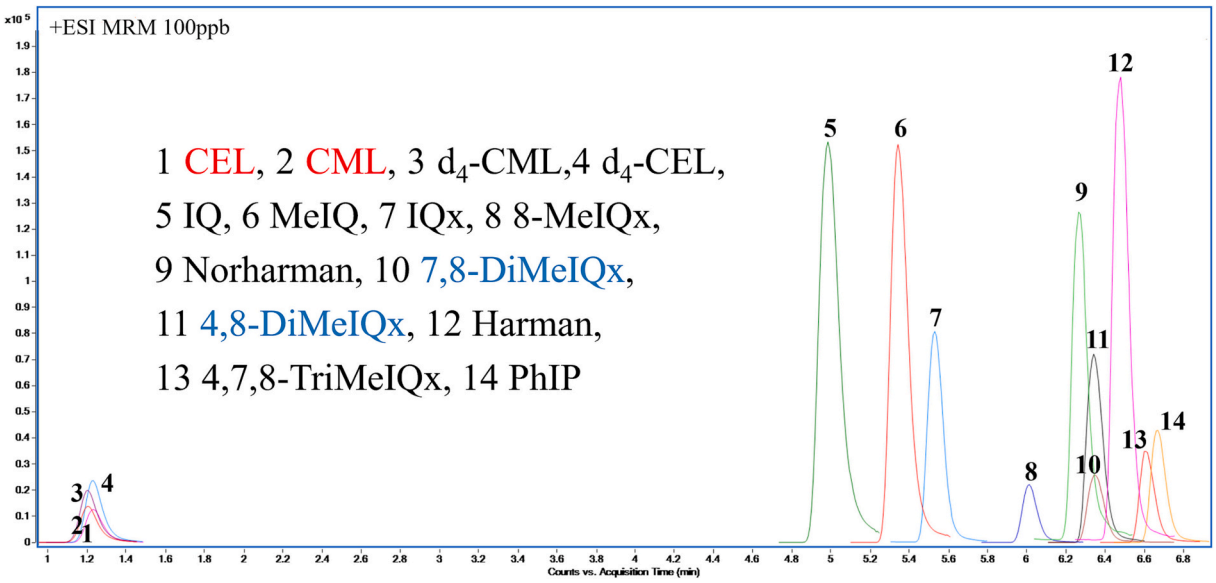


Fig. 1. UPLC-MS/MS chromatograms of analytes and internal standards.

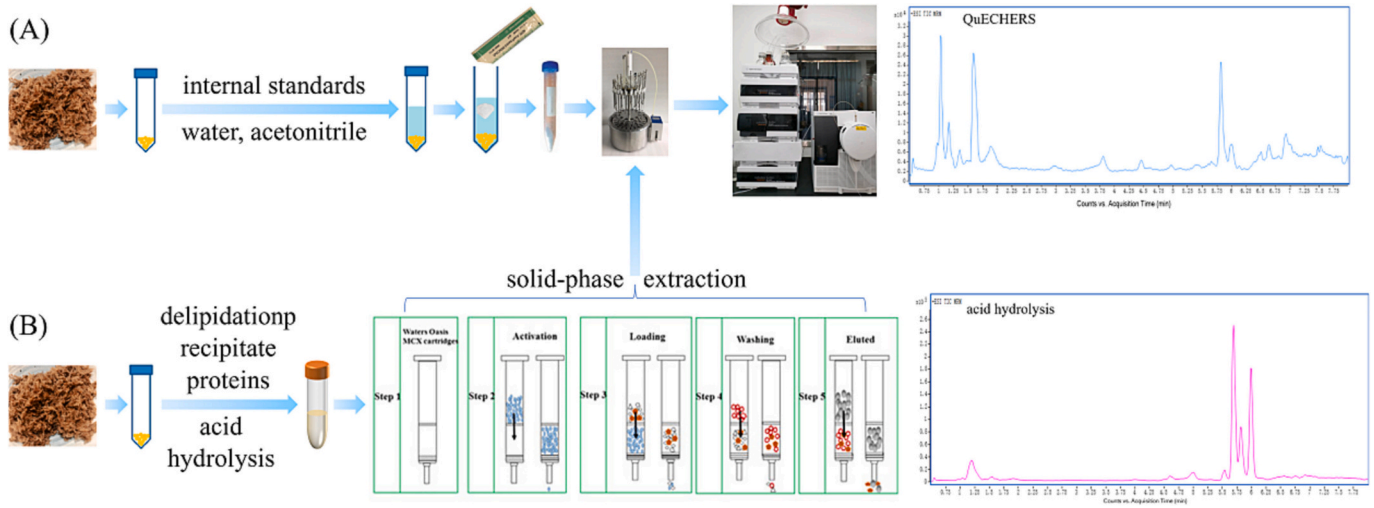


Fig. 2. Schematic diagram and UPLC-MS/MS chromatogram of the different method.

gradient elution may help, also resulting in faster analysis. The mobile phase elution procedure was adapted according to our previous work (Ding, Zhang, Liu, Wang, & Hui, 2022). Finally, an 8 min gradient elution procedure (Supplementary Table 1) was used to elute each target analyte into a single and sharp peak without any interfering peaks. Fig. 1 provides the chromatogram of a 100 ng/mL analytical standard solution under the optimized conditions.

3.3. Optimization of sample preparation

The aim of this research was to establish a simultaneous pre-treatment approach for extracting both AGEs and HAs from roast/grilled meat samples. The usual pre-treatment methods for AGEs (acid hydrolysis + SPE - B) (Li et al., 2022) and the usual pre-treatment methods for HAs (QuEChERS - A) (Ding et al., 2022) were considered first. However, it has been reported by Cheng et al. (2021) that acid hydrolysis (110 °C ± 5 °C, 24 h) could convert Amadori products, such as fructoselysine, into new AGEs, leading to an overestimation. To address this issue, $NaBH_4$ was added to reduce Amadori products (Li et al., 2021). In contrast, high-temperature hydrolysis does not generate additional HAs due to their structural and physicochemical properties. Wei et al. (2024) also found that while there was new formation of AGEs, there was little new formation of HAs when monitoring hydrolyzed samples of raw pork. Furthermore, hydrolysis with the addition of $NaBH_4$ not only did not interfere with HAs conversion but also corrected the overestimation of AGEs. Method (A) exhibited poor extraction efficiency and low response. It was also prone to interference in quantification due to the proximity of the signal-to-noise ratio. Conversely, Method (B), demonstrated improved response and good extraction efficiency (Fig. 2). Therefore, the pre-treatment method selected for this study involved acid hydrolysis and SPE.

After acid hydrolysis, the optimal activation and washing solutions have been optimized for more efficient purification and enrichment of AGEs and HAs using the Oasis MCX column. The SPE column was activated and washed with ultrapure water and 0.1 M HCl, respectively, and it was found that the recoveries using 0.1 M HCl as the activation and washing solution were significantly higher than those using ultrapure water (Supplementary Table 2). Unfortunately, the recovery of the PYR is very low (0.2%). The optimum elution volume was then investigated and the chromatogram (Fig. 3) showed that the optimum elution volume

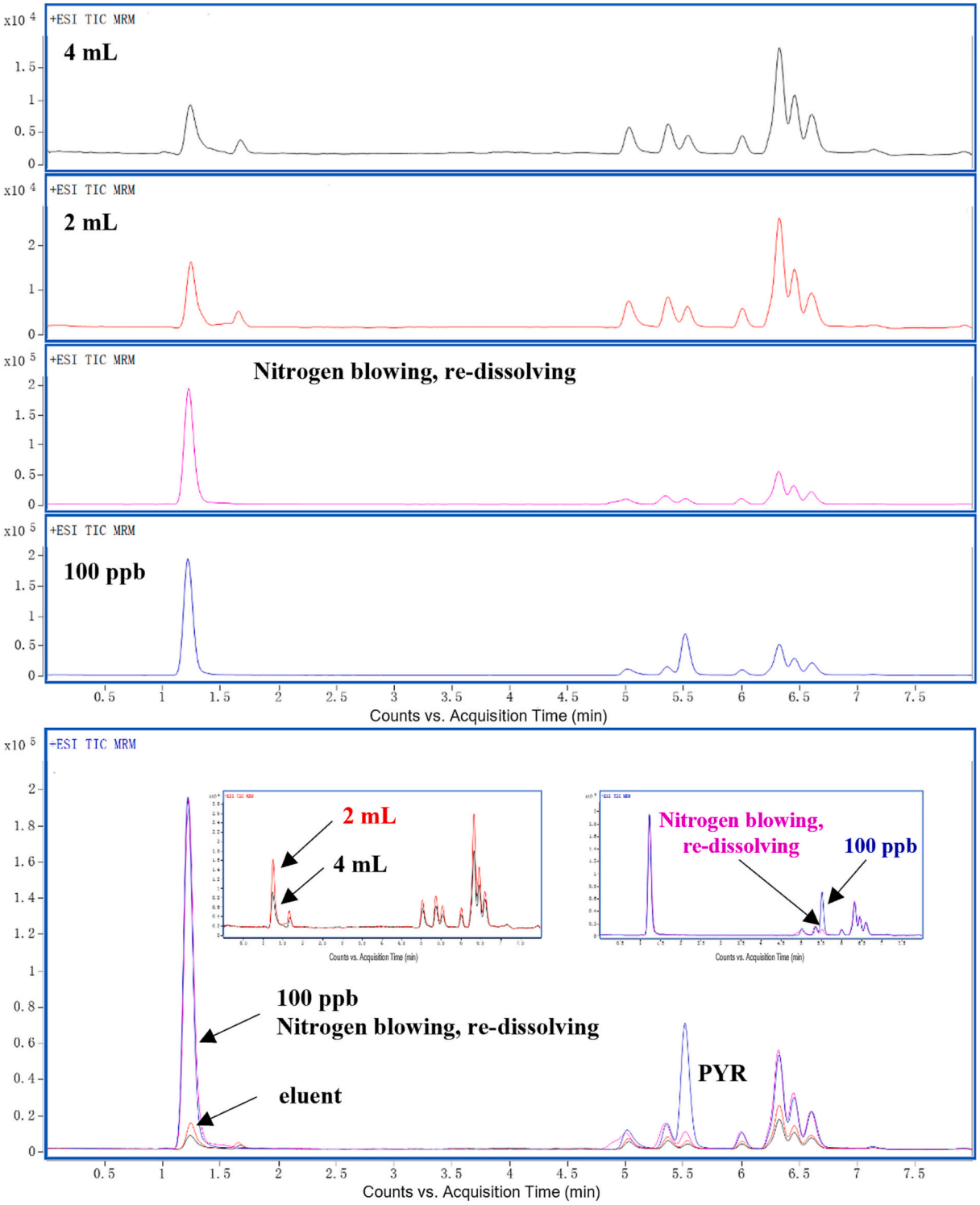


Fig. 3. UPLC-MS/MS chromatogram of solid phase extraction optimization.

was 2 mL, which required nitrogen blowing to concentrate and then redissolve, but the recovery of PYR was still not improved. Therefore, this pre-treatment method was only used for the simultaneous extraction of CML, CEL, IQ, MeIQ, IQx, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, PhIP, Harman, and Norharman.

3.4. Method validation

3.4.1. Linearity and sensitivity

4,8-DiMeIQx, 7,8-DiMeIQx, 8-MeIQx, CEL, CML, MeIQ, and PhIP were quantified by the internal standard method, IQ, IQx, Harman and Norharman were quantified by the external standard method, then standard curves of AGEs and HAs were constructed to determine the dynamic linear range. The calibration curves showed good linearity over a wide range (10–200 ng/mL) between concentration and peak areas

Table 2
Recoveries and RSDs of AGEs and HAs in six categories of roast/grilled meat ($n = 6$).

Samples		Roast steak		grilled lamb patty		Mutton shashlik		Roast chicken breast		Roast chicken thigh		Roast chicken skin	
Compounds	Spiked	RSD/ %	Recoveries/ %	RSD/ %	Recoveries/ %	RSD/ %	Recoveries/ %	RSD/ %	Recoveries/ %	RSD/ %	Recoveries/ %	RSD/ %	Recoveries/ %
CML	L	0.27	81.07	1.52	71.59	0.71	75.24	0.18	75.58	0.52	79.15	0.32	79.79
	M	0.94	92.66	0.39	88.30	0.21	84.51	0.33	88.58	0.10	82.22	0.16	73.89
	H	0.29	102.92	0.47	77.32	0.64	90.93	0.51	87.84	0.48	88.66	0.45	92.72
CEL	L	0.78	85.05	0.39	116.11	0.56	72.82	2.13	71.13	2.21	79.86	0.35	112.52
	M	0.44	95.59	1.51	110.00	0.64	108.75	0.76	109.86	0.72	109.84	0.20	112.94
	H	1.17	80.34	0.53	89.52	1.30	90.07	0.91	77.72	0.42	93.43	0.51	79.15
IQ	L	0.97	76.30	2.08	93.56	1.32	103.68	1.75	80.67	1.20	94.48	0.57	105.37
	M	1.31	77.32	0.17	79.73	0.30	79.84	0.23	87.71	0.86	75.78	0.53	85.51
	H	0.49	108.81	0.29	94.32	0.29	97.76	2.34	94.03	0.82	87.54	0.10	99.94
MeIQ	L	1.26	114.61	3.37	94.37	2.22	100.81	3.93	82.06	0.77	106.48	4.16	86.57
	M	0.59	78.24	0.36	78.29	0.66	77.39	0.99	80.44	0.72	77.27	0.41	88.99
	H	0.18	107.43	0.31	113.69	1.11	99.40	0.76	100.30	0.14	86.03	0.41	95.73
IQx	L	1.37	70.10	1.40	104.01	2.07	71.09	2.84	84.81	5.50	90.81	3.32	104.99
	M	0.17	76.80	0.99	91.02	1.22	70.57	1.45	86.10	0.27	95.83	1.07	86.84
	H	0.15	99.05	0.37	98.53	0.29	94.70	1.17	90.83	0.28	96.98	0.28	96.98
8-MeIQx	L	1.26	91.83	1.19	117.69	2.46	95.40	4.94	96.53	3.99	97.62	1.64	93.72
	M	0.37	114.00	0.68	106.19	0.67	103.21	0.42	115.05	0.62	80.67	0.79	76.42
	H	0.24	109.34	0.48	108.00	0.46	101.18	0.54	87.89	0.25	106.13	0.53	94.07
Norharman	L	0.38	83.35	4.60	104.60	2.67	88.55	1.75	83.60	2.33	88.76	0.38	89.61
	M	0.20	79.80	0.51	92.66	1.05	76.03	0.82	83.70	0.74	88.93	0.86	87.52
	H	0.69	118.12	0.64	100.09	0.32	112.92	0.66	91.29	1.30	100.55	0.39	120.91
4,8-DiMeIQx	L	3.50	91.78	1.09	119.53	1.81	112.00	0.29	93.81	2.01	84.32	3.13	98.97
	M	0.06	107.13	0.49	110.37	0.16	101.08	0.82	109.20	0.52	88.77	0.60	74.23
	H	0.39	99.07	0.98	100.81	0.40	89.42	1.51	104.88	0.23	110.08	0.05	99.34
7,8-DiMeIQx	L	0.20	91.67	1.43	119.90	0.73	105.05	1.58	90.06	3.94	76.97	3.67	105.04
	M	0.50	110.85	0.26	110.58	0.93	107.22	0.59	109.74	0.49	93.85	0.38	73.54
	H	0.73	119.33	1.19	116.33	1.28	121.06	0.38	113.66	1.34	115.10	0.12	120.05
Harman	L	1.19	93.20	1.85	111.49	2.42	121.31	0.34	117.05	7.58	82.00	0.67	97.06
	M	0.39	82.88	0.45	99.28	0.73	70.79	0.84	91.30	1.34	110.23	1.19	90.42
	H	0.43	110.49	0.42	97.02	0.69	114.95	0.55	84.92	0.63	88.69	0.83	105.95
PhIP	L	0.03	86.97	0.98	105.58	1.33	112.91	0.86	104.59	2.79	104.39	2.82	93.41
	M	0.74	93.86	1.27	91.84	0.91	98.34	0.28	100.51	0.83	98.27	0.73	75.02
	H	1.21	95.80	0.30	101.94	0.88	103.27	0.32	109.52	1.16	103.77	1.17	106.65

with correlation coefficients (R^2) > 0.99. The LODs and LOQs range from 0.01 to 0.27 ng/mL and 0.04–0.91 ng/mL, respectively, suggesting that this approach is more sensitive.

3.4.2. Precision and recovery

To demonstrate the stability of the validated method and its suitability in routine analysis, six roast/grilled meat samples, namely roast steak (superheated steam roasting), grilled lamb patty (electrically grilled), mutton shashlik (electrically grilled), roast chicken breast (fruit tree wood roasting), roast chicken thigh (fruit tree wood roasting), and roast chicken skin (fruit tree wood roasting), were monitored using the optimized pre-treatment and instrumental parameters of this study. As shown in Table 2, the recoveries of AGEs and HAs ranged from 71.59% to 102.92% (CML), 71.13% to 116.11% (CEL), 75.78% to 108.81% (IQ), 77.27% to 114.61% (MeIQ), 70.1% to 104.99% (IQx), 76.42% to 117.69% (8-MeIQx), 74.23% to 119.53% (4,8-DiMeIQx), 73.54% to 121.06% (7,8-DiMeIQx), 76.03% to 120.91% (Norharman), 70.79% to 121.31% (Harman), and 75.02% to 112.91% (PhIP), respectively, meeting the standards described in the AOAC guideline (Hsiao et al., 2017). The precision (RSDs) ranged from 0.10% to 1.52% (CML), 0.20% to 2.21% (CEL), 0.10% to 2.34% (IQ), 0.14% to 4.16% (MeIQ), 0.15% to 5.50% (IQx), 0.24% to 4.94% (8-MeIQx), 0.05% to 3.50% (4,8-DiMeIQx), 0.12% to 3.94% (7,8-DiMeIQx), 0.20% to 4.60% (Norharman), 0.34% to 7.58% (Harman), and 0.03% to 2.82% (PhIP), indicating that the method can meet the daily monitoring requirements for the simultaneous analysis of AGEs and HAs. The ME ranged from 0.6 to 1.4, comprising 19.7% of the medium ME and 80.3% of the weak ME (Huang et al., 2019). However, given the complex food matrix and the mostly weak ME, the results are generally acceptable.

3.4.3. Comparison to previous studies

As shown in Table 3, the LODs and LOQs obtained in the current study using acid hydrolysis with SPE are compared with those published in previous studies using similar or different preparation methods. For AGEs, the LODs and LOQs of this developed method are higher than those obtained by other methods (Lee, Ha, Kim, & Hur, 2024; Li et al., 2021; Xue et al., 2022; Yu et al., 2022) than the Wei et al. (2024) method. Although the Wei et al. (2024) method gave lower LODs and LOQs, a two-step pre-processing was required to identify multiple hazards simultaneously, and the separation of CML and CEL was lower than that of the present method, in addition to differences in food matrices. Moreover, the elution time required for the determination of AGEs by dansyl derivatization with UPLC-MS (30 min) was significantly longer than that required by the present method (8 min) (Lee et al., 2024). For HAs, the LODs and LOQs of the current study method are comparable to those of Wei et al. (2024) (ultrasonic extraction with acetone, HCl hydrolysis, SPE) and Xu et al. (2021) (NaOH and acetonitrile extraction, SPE). However, the method of Wei et al. (2024) involves two steps and the HAs determined are not identical to those determined here, The HPLC-Q-Orbitrap-HRMS used by Xu et al. (2021) was more expensive and only detected HAs. Higher results have been obtained with other previous (Chevolleau, Bouville, & Debrauwer, 2020; Li et al., 2023; Xue, Deng, et al., 2022) studies such as LLE-SPE and QuEChERs. In summary, the sensitivity for AGEs and HAs determined simultaneously in this study are similar to or better than previous published articles. Besides, Poojary et al. (2020) used microwave-assisted acid hydrolysis with LC-quadrupole-Orbitrap-MS for the analysis of AGEs in pork and calculated recoveries of 71% - 110%. Yu et al. (2022) applied acid hydrolysis in combination with HPLC-MS/MS to analyse CML and CEL in commercial meat products, and the recoveries ranged from 85.9% to 105.1%. Chen et al. (2017) applied acid hydrolysis in combination with

Table 3
Comparisons of the previous reported method for AGEs/HAs analysis.

Samples	Sample preparation method Column	LODs of AGEs (ng/mL)	LOQs of AGEs (ng/mL)	LODs of HAs (ng/mL)	LOQs of HAs (ng/mL)	Analytical methods Targets compounds Elution Time	Reference
roast/grilled meat	HCl hydrolysis, SPE (MCX) ACQUITY UPLC HSS T3	0.27	0.91	0.01–0.17	0.04–0.56	UPLC-MS/MS, D-MRM AGEs, HAs 8 min	current study
biscuits, potato chips, roasted almonds, candied apricot slices, raw ground pork, plant-based meat alternatives	Ultrasonic extraction with acetone, HCl hydrolyze, SPE (MCX) – two-step ACQUITY UPLC BEH C18	0.04–0.16	0.14–0.53	0.01–0.11	0.02–0.38	UPLC-MS/MS, MRM AA, 5-HMF, HAs, AGEs 7 min	Wei et al. (2024)
coffee, beer, sausage	Dansyl derivatization ACQUITY UPLC BEH C18	3.2–8.7	9.8–26.3	–	–	UPLC-MS, DAD AGEs 30 min	Lee et al. (2024)
heat processed meat products	Ultrasonic extraction with acetic acid-acetonitrile, purification with $MgSO_4$-PSA-C_{18} EcipsePlus C18 RRHD	–	–	0.05–10	3×10^3–4.5×10^5	UPLC-MS/MS, MRM HAs, AA, 5-HMF 17 min	Li et al. (2023)
Beef patties	Ultrasonic extraction with ethyl acetate/ HCl, SPE (MCX) ACQUITY UPLC BEH C18	–	–	0.02–1.64	0.07–5.52	UPLC-MS/MS, MRM HAs 17 min	Xue, Deng, et al. (2022)
Beef patties	HCl hydrolysis, SPE (MCX) ACQUITY UPLC C18	5.77–6.23	8.65–9.35	–	–	UPLC-MS/MS, MRM AGEs 7 min	Xue, Deng, et al. (2022)
commercial meat products	HCl hydrolysis, SPE (MCX) Waters X-Bridge C18	9.2–10.5	25–28			HPLC-MS/MS, MRM AGEs 10 min	Yu et al. (2022)
Milk	HCl hydrolysis, SPE (MCX) Waters T3	39–44	130–150	–	–	LC-MS/MS, MRM AGEs 9 min	Li et al. (2021)
roasted and pan-fried meat	NaOH and acetonitrile extraction, Strata-X-C SPE Poroshell 120-EC C18	–	–	0.02–0.15	0.05–0.5	HPLC-Q-Orbitrap-HRMS, full MS scan HAs 12 min	Xu et al. (2021)
cooked beef	QuEChERS Scientific Hypersil Gold C8	–	–	0.25–0.5	1–5	UHPLC-MS/MS, MRM HAs 4.5 min	Chevolleau et al. (2020)

LODs: the limits of detection; LOQs: the limits of quantitation; LODs/LOQs ranges include only targets identified in this study.

UPLC-MS/MS to detect HAs in roast beef patties, and achieved recoveries in the range of 51.28% - 104.15%. Li et al. (2023) used a modified QuEChERS method with UPLC-MS/MS for the determination of HAs in heat processed meat products with recoveries ranging from 82.10% to 106.47%. The recoveries for AGEs and HAs determined simultaneously in this study are, in most cases, better than those reported in previously published articles.

3.5. Analysis of real samples

The total levels of AGEs and HAs were highest in roast chicken skins (182.6138 μg/g), which may be due to the large heating area and high heating temperature of chicken skins, and the sugar hanging process and high temperature are more conducive to the occurrence of Maillard reaction (Yan et al., 2023); followed by roast chicken thighs (94.2714 μg/g), mutton shashliks (86.3784 μg/g), grill lamb patties (74.2066 μg/g), roast chicken breasts (69.2539 μg/g), whereas the lowest levels were found in roast steaks (57.4120 μg/g), which may be attributed to the fact that superheated steam roasting has the lowest temperature and the food is cooked by heating the water to steam, which prevents the formation of HAs in the roast/grilled meat (Fang et al., 2022; Suleman, Hui, Wang, Liu, & Zhang, 2019). It can be concluded that the processing technology, in particular the heating surfaces and heating time, has a significant effect on the formation of AGEs and HAs (Chen & Smith, 2015; Dong, Xian, Li, Bai, & Zeng, 2020). CML, CEL, MeIQ, 8-MeIQx, IQx, 4,8-DiMeIQx, 7,8-DiMeIQx, harman, Norharman, and PhIP were found, and AGEs levels (52.45–177.48 μg/g) were higher than HAs levels (4.96–5.13 μg/g) in six roast/grilled meat samples, which is in general agreement with the experimental data from previous studies (Wei et al., 2024; Xue et al., 2022; Zhang, Wang, Chu, Sun, & Lin, 2023). However, the levels of HAs measured in this study were generally higher than those measured by acid extraction or QuEChERS combined with UPLC-MS/MS (Lai, Lee, Cao, Zhang, & Chen, 2023; Shi et al., 2023), and there are three possible explanations for this phenomenon: 1) the amino acid residues in proteins react with other HAs precursors or intermediates to form protein-bound HAs; 2) the amino groups in the free HAs react with the carboxyl groups in proteins to form stable amide bonds; 3) the selective adsorption of free HAs on the surface of protein polymers, not only the free HAs (Chen, He, Qin, Chen, & Zeng, 2017; Szterk, 2013).

4. Conclusion

In this study, we have set up a UPLC-MS/MS method was developed for the simultaneous extraction and purification of 11 kinds of AGEs and HAs in roast/grilled meat by acid hydrolysis and SPE. The validation data demonstrated that the developed method had good linearity,

sensitivity, precision, stability and recoveries for the simultaneous trace analysis of AGEs and HAs in roast/grilled meat. Finally, this approach was successfully applied to the simultaneous quantification of AGEs and HAs in different types of roast/grilled meat. The results showed that the profile of AGEs and HAs differed in most types of roast/grilled meat, being highest in chicken skins and lowest in roast steaks, and that total AGEs and HAs were detected at μg/g levels. This method greatly reduces the time and cost of detecting AGEs and HAs, respectively, and provides a new approach to monitoring AGEs and HAs in food. The method significantly reduces the time and cost of detecting AGEs and HAs and provides a new approach to monitoring AGEs and HAs in food, which in turn helps to comprehensively assess food quality and health risks, and facilitates the development of research into the interactions between multiple hazards.

CRediT authorship contribution statement

Mingyu Li: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Chunjiang Zhang:** Writing – review & editing, Resources. **Zhenyu Wang:** Writing – review & editing, Resources. **Na Liu:** Methodology, Formal analysis. **Ruiyun Wu:** Writing – review & editing. **Jiajing Han:** Investigation. **Wenhan Wei:** Investigation. **Christophe Blecker:** Writing – review & editing, Supervision. **Dequan Zhang:** Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgements

The authors acknowledge the financial support from Shandong Province Key R&D Program (Action Plan for Revitalizing Science and Technology Innovation and Revitalization of Rural Areas) [Grant No. 2022TZXD0021], and Taishan Industrial Experts Programme of China.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.138930.

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