

Determination of N^ε-(1-Carboxymethyl)-L-Lysine in brewing soy sauce by ultrahigh performance liquid chromatography coupled with tandem mass spectrometry

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Abstract

A method for the determination of N^ε-(1-Carboxymethyl)-L-Lysine (CML) in brewing soy sauce was developed using ultrahigh performance liquid chromatography-tandem mass spectrometry. A mixed cation exchange solid phase extraction cartridge was used for purification, while gradient elution of a hydrophilic interaction chromatography column was performed with a 0.1% formic acid aqueous solution-acetonitrile. The adding standard recovery rate of this method ranged from 86.94 to 92.61%, and the relative standard deviation (RSD) was between 3.69 and 6.18%. The limit of detection (LOD) was 0.038 ng/mL, and the limit of quantification was 0.127 ng/mL. The RSD values of the repeatability test and the parallel test were 3.44 and 6.56%, respectively. Using this method, the CML contents determined in eight brewing soy sauce samples were $3.16 \pm 0.18 - 15.10 \pm 1.19$ µg/mL. This method is simple, accurate, sensitive, and precise, and it is an effective method for monitoring the CML content in brewing soy sauce.

Keywords: brewing soy sauce; hydrophilic interaction chromatography; mixed cation-exchange; N^ε-(1-Carboxymethyl)-L-Lysine; ultrahigh performance liquid chromatography-tandem mass spectrometry

Introduction

The Maillard reaction of protein and carbohydrate in food can produce a group of stable products, which are called advanced glycation end-products (AGEs) (Ahmed, 2005; Goldberg *et al.*, 2004; Lee *et al.*, 2022; Singh *et al.*, 2001). More than 20 kinds of AGEs have been discovered to date. N^ε-(1-Carboxymethyl)-L-Lysine (CML) was the first one to be isolated and identified in food, and it is also one of the most significant AGEs (Ames, 2010; Büser *et al.*, 1987; Erbersdobler and Somoza, 2010; Fu *et al.*, 1996). CML exhibited higher acid stability than other AGEs and can be used as a vital indicator to evaluate protein chemical modification, carbonyl stress, and lipid oxidation during Maillard reaction within a food system (Thorpe and Baynes, 2002). Moreover, CML is

closely related to the occurrence of many diseases in the human body and can accelerate diabetes, nephrosis, cardiopathy, atherosclerosis, as well as the rapid aging of human organs (Alison *et al.*, 2006; Baynes, 2001; Begieneman *et al.*, 2015; Zhang *et al.*, 2016). When CML accumulates in the wall of blood vessels, it can lead to a thickening of the blood vessels, a decrease in elasticity, and dysfunction. CML accumulates in cerebrovascular and nerve tissue, which reduces the regeneration ability of nerve cells, and inevitably leads to Alzheimer's disease (Sasaki *et al.*, 2002). Furthermore, accumulation of CML in the lens and retina can cause cataracts (Kumar *et al.*, 2007), while CML aggregation in bone joints can induce arthritis (Mccarthy *et al.*, 2004). Free radicals produced by CML can also damage human DNA (Yen and Liao, 2002) and can accumulate and increase in the human body with

age. CML cross-links with collagen protein resulting in the visible signs of aging displayed in humans (Kasper and Funk, 2001).

Therefore, these findings have caused considerable concern regarding the content of CML in food. Quantitative detection of CML is of great significance in guiding people to reduce their CML intake and mitigating the risk of related diseases. Current research increasingly focuses on the rapid and efficient detection of CML content in food. The traditional methods for CML detection are enzyme-linked immunosorbent assay (ELISA), gas chromatography-mass spectrometry (GC-MS), reversed-phase high-performance liquid chromatography (RP-HPLC), liquid chromatography-mass spectrometry (LC-MS), and high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) (Chao *et al.*, 2009; Charissou *et al.*, 2007; Hegele *et al.*, 2008; Niquet-Leridon and Tessier, 2011; Tauer *et al.*, 1999). However, these methods have several limitations. The ELISA method is easily interrupted by impurities making it challenging to select specific antibodies, and rendering the quantitative analysis results unreliable. Both the GC-MS and RP-HPLC methods require complicated pre-column derivation, which is tedious and not extremely sensitive. Considering the LC-MS and the HPLC-MS/MS techniques, their column efficiency is reduced due to the presence of the “out-of-column effect.” In addition, since CML exhibits poor retention in the C₁₈ chromatography column, it is necessary to increase the retention by adding trifluoroacetic acid, nine-fluorine pentanoic acid, and other ion-pairing reagents in the mobile phase (Han, 2020). However, these reagents will remain in the mass spectrometer and lead to a decrease in detection sensitivity (Annesley, 2003). No recommended standard method currently exists either domestically or globally for the detection of CML in food. The ultrahigh performance liquid chromatography (UPLC) system consists of a small particle filler (particle size < 2 μm) chromatography column and an extra high voltage system (pressure >100 MPa), making it an efficient, sensitive, and specific separation method.

Brewing soy sauce is a kind of liquid condiment with special color, aroma, and taste, which is mainly made from beans rich in protein, grains rich in starch, water, salt, etc., under the action of microorganism and enzyme fermentation. It is a commonly used seasoning for our three meals and its consumption is huge globally every year. As the system is rich in protein and carbohydrates, the long-term fermentation process causes the formation of large quantities of CML, causing potential harm to human health. At present, there are few reports on CML detection methods in brewing soy sauce. In order to monitor the content of CML in soy sauce, N^ε-carboxymethyllysine-D₄ (CML-D₄) was used

as the isotope internal standard and the ultrahigh performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) detection system was used to establish a method for the determination of CML in soy sauce by optimizing the elution conditions of solid phase extraction (SPE) column, screening the chromatographic column, optimizing the conditions of liquid chromatography and mass spectrometry.

Materials and Methods

Reagents and materials

CML standard (98.0% purity) and CML-D₄ isotope internal standard (98.0% chemical purity, 97.9% isotope purity) were supplied by TRC (Toronto, Canada). Methanol, acetonitrile, formic acid, and ammonium acetate were all of mass spectra purity and supplied by Thermo Fisher Scientific (Waltham, MA, USA). The analytical reagents of formic acid (content ≥ 88.0%) and methanol (content ≥ 99.5%) were obtained from the Kemiou Chemical Reagent CO, Ltd (Tianjin, China). Ammonia (content ≥ 25%) was an analytical reagent from the Iron Tower Reagent Factory (Kaifeng, China). Ultra-purified water was prepared using a Milli-Q Advantage A10 system (Millipore, Bedford, MA, USA). The brewing soy sauce samples were all purchased from the local supermarket. The working standard solutions for linear calibration were prepared by diluting the stock solution to a concentration sequence of 1.0, 2.0, 5.0, 10.0, 20.0, and 50 ng/mL spiked with the CML-D₄ isotope internal standard (10.0 ng/mL).

Pretreatment of the brewing soy sauce samples

A 100 μL volume of brewing soy sauce sample and a 625 μL volume of 400 ng/mL CML-D₄ were mixed and diluted with 5.0% of the aqueous formic acid solution to 5.0 mL. A mixed cation-exchange (MCX) SPE cartridge (3.0 mL, 60.0 mg, 30.0 μm, Waters, USA) was activated with 3.0 mL of methanol and equilibrated with 3.0 mL of water in the VacElut SPS 24 vacuum SPE unit (Agilent Technologies, Santa Clara, CA, USA). A 400 μL volume of the sample solution passed through the MCX cartridge at a rate of 1–2 drops/second. After the sample solution was discharged entirely, the MCX cartridge was washed with 3.0 mL of 5.0% aqueous formic acid solution and dried with a pumping vacuum. The MCX cartridge was then washed with 3.0 mL of methanol, dried with a pumping vacuum, and all effluent solutions were discarded. Finally, the MCX cartridge was eluted with 5.0 mL of 15.0% ammonia methanol solution and dried with a pumping vacuum. The effluent solution was collected in a test tube and blow-dried with 40°C

nitrogen by the N-EVAP-24 nitrogen-blowing instrument (Organomation, New York, NY, USA). The residue was dissolved with 2.0 mL of water and mixed using vortices. A 1.0 mL volume of the final solution with the CML-D₄ concentration of 10 ng/mL was filtered into a 2.0 mL sample vial with a 0.22 μm polyethersulfone needle filter, and CML content was determined using the UPLC-MS/MS method.

UPLC-MS/MS detection method

The liquid phase chromatography instrument was an ACQUITY ultrahigh performance liquid chromatograph (Waters, Milford, MA, USA), and the chromatography column was a UPLC BEH Amide hydrophilic interaction chromatography (HILIC) (2.1 mm × 100 mm, 1.7 μm, Waters, Milford, MA, USA). The mobile phases were phase A that consisted of an aqueous solution containing 0.1% formic acid and phase B that contained acetonitrile. Column temperature, injection volume, and running time were 35°C, 5 μL, and 7 min, respectively.

The mass spectrometry instrument was an XEVO TQ triple four-bar tandem mass spectrograph (Waters, Milford, MA, USA). The ion source was used in electrospray positive ion mode (ESI⁺), while multi-response monitoring (MRM) denoted the monitoring mode. The capillary voltage, conical hole voltage, and source temperature were 3.5 kV, 20 V, and 150°C, respectively, while the desolventizing gas temperature, desolventizing gas flow velocity, and collision energy were 400°C, 700 L/h, and 15 V, respectively. The MRM mode included the quantitative analysis of daughter ion CML at m/z 205.22 – m/z 84.00, the qualitative analysis of daughter ion CML at m/z 205.22 – m/z 130.00, and the qualitative analysis of daughter ion at CML-D₄ m/z 209.00 – m/z 87.70.

Method optimization

The main objective of sample pretreatment in this experiment was to reduce the interference of impurities and improve the recovery rate of CML. This process allowed the analysis results to resemble the real values more closely. The realization of the objective mainly depended on the optimization of the SPE conditions. The MCX cartridge is an MCX reverse phase adsorptive extraction cartridge. It is formed by the polymerization of hydrophilic N-vinyl pyrrolidone and lipophilic divinylbenzene in specific proportions and SO³⁻. During this experiment, both the sample solvent and the first washing solvent consisted of an aqueous formic acid solution, while the eluting solvent was an ammonia methanol solution. The adsorption and elution effects of the formic acid concentration levels in the sample solvent, as well as

the ammonia concentration in the eluting solvent, were investigated by comparing the recovery rate of CML.

The chromatography column acts as a carrier for the separation and purification of substances with different adsorption capacities to the stationary phase. The separation and detection capacity of different chromatography columns varies significantly due to their structure, filler, and size. The CML separation and detection efficiency of the ACQUITY UPLC BEH C₁₈ and the ACQUITY UPLC BEH Amide HILIC column were individually investigated during the experiment. The mobile phase significantly influences the peak shape, detection sensitivity, separation degree, and retention time. The CML elution effects of different mobile phases (water-methanol and water-acetonitrile) were investigated. The variation in the CML chromatography peak was also investigated by adding buffer salt and changing the acidity of the mobile phase during the experiment.

Mass spectrometric analysis involves ionizing each component of the sample in the ion source and generating charged fragment ions with different mass-to-charge ratios (m/z). The corresponding peak of a fragment ion in the mass chromatogram is called a fragment ion peak, and the abscissa represents the m/z value of the ion. The ordinate represents the intensity of the ion flow and is usually expressed by the relative intensity. The m/z value and relative intensity of these peaks depend on molecular structure, the type of instrument used, and test conditions. By monitoring the ion flow intensity of two fragment ions that are characteristic of CML, the ion exhibiting a stronger ion flow intensity was used as the quantitative ion, while the other was used as the qualitative ion during the experiment.

Method validation

Both the external standard method and the internal standard method were applied during the experiment. The external standard method was used to optimize the conditions of SPE and liquid chromatography. Furthermore, the internal standard method (CML-D₄ was the isotope internal standard) was used to optimize the conditions of mass spectrometry and verify the accuracy and precision of the detection method, and determine the CML content in the brewing soy sauce samples.

Signal-to-noise ratio (S/N) refers to the ratio of signal intensity and baseline noise. The mean value of S/N (X) was obtained by automatically analyzing three parallel samples of 1.0 ng/mL CML chromatogram. The LOD is defined as the concentration of S/N = 3, and the limit of quantification (LOQ) is specified as the concentration of S/N = 10. LOD and LOQ are usually calculated by a

chromatogram of the lowest concentration in the standard curve. When $S/N = X$, then $D = 3 C/X$. In this formula, C stands for the analyte concentration, X stands for S/N , and D stands for the LOD. When $S/N = X$, then $Q = 10 C/X$. In this formula, C stands for the analyte concentration, X stands for S/N , and D stands for the LOQ.

All quantitative detection methods need to be verified for accuracy. Therefore, a model sample system was established during the experiment for adding the standard test. The CML concentrations when adding the standard test were 10.0, 20.0, and 30.0 ng/mL, respectively. Six parallel tests were performed for each addition of the standard concentration. Since the sample without the standard concentration contained CML, analysis was conducted to determine these content levels. The calculation formula of the standard recovery rate is $P = \frac{(C_2 - C_1)}{C_0} \times 100\%$.

In this formula, P is the standard recovery rate, C_2 is the detected value after adding the CML standard, C_1 is the background value, and C_0 is the added standard quantity.

Precision indicates the size of the random deviation and is usually expressed in terms of the relative standard deviation (RSD). It is the ratio of standard deviation (S) and the arithmetic mean of the detected results.

$S = \sqrt{\frac{\sum_{i=1}^n (x_i - \bar{x})^2}{n-1}}$, $RSD = \frac{S}{\bar{x}} \times 100\%$. In this formula, S is the standard deviation, \sum denotes sum, \bar{x} stands for the arithmetic mean, and $\sqrt{\quad}$ stands for square root. Both the repeatability test and the parallel test were applied to evaluate the accuracy of the detection method during the experiment.

Statistical analysis method

Three parallel tests were conducted for each sample, and the test data were expressed in the form of “mean \pm standard deviation.” The significance of the test data was tested by Dunnett’s T3 method of ANOVA in SPSS statistics 17.0. $P < 0.05$ indicates that there is a significant difference and $P \geq 0.05$ indicates that there is no significant difference.

Results and Discussion

Optimization of the SPE conditions

The MCX cartridge was activated with 3.0 mL of methanol and equilibrated with 3.0 mL of water. A 50.0 ng/mL 1.0 mL volume of CML standard solution was prepared with the aqueous formic acid solution and passed through the MCX cartridge at a rate of 1–2 drops/second. After the solution was completely discharged, the MCX cartridge was washed with 3.0 mL of the aqueous formic acid solution and dried with a vacuum pump. The MCX cartridge was washed with 3.0 mL of methanol and dried with a vacuum pump. In addition, all the effluent was discarded. Finally, the MCX cartridge was eluted with 5.0 mL of the ammonia methanol solution and dried using a vacuum pump. The final elution effluent was collected in a test tube.

A 50.0 ng/mL 1.0 mL volume of the CML standard solution was the sample for analysis. The sample was solved by 1.0, 3.0, 5.0, 7.0, and 9.0% aqueous formic acid solution, respectively, and the result is shown in Figure 1A.



Figure 1. The recovery rate of CML through the MCX cartridge, the sample solvent of 1.0, 3.0, 5.0, 7.0, and 9.0% aqueous formic acid solution (A) and eluting by different concentrations of ammonia methanol solution of 5.0, 10.0, 15.0, 20.0, and 25.0% (B). In the figure, different letters indicate significant differences in data ($P < 0.05$), and the same letter indicates no significant differences in data ($P \geq 0.05$). CML, N^ε-(1-Carboxymethyl)-L-Lysine; MCX, mixed cation-exchange.

Therefore, it is evident that the CML recovery rate of minimum value reached $81.67 \pm 0.87\%$. The recovery rate was $94.28 \pm 1.01\%$ when the formic acid concentration was 5.0%. The statistical analysis showed that there was no significant difference in CML recovery among the three kinds of formic acid concentration (5.0, 7.0, and 9.0%) ($P \geq 0.05$), which indicated that the change in CML recovery was not significant after the formic acid concentration exceeded 5.0%. Consequently, the CML without a positive charge could easily be washed out during the early stage, resulting in a low concentration of eluent during the later phases of the experiment. Moreover, when the concentration of the formic acid exceeded 5.0%, the recovery rate displayed no significant change ($P \geq 0.05$). Therefore, an aqueous formic acid solution with a concentration of 5.0% was applied for the sample solvent during the experiment.

The MCX cartridge was eluted with 5.0 mL of 5.0, 10.0, 15.0, 20.0, and 25.0% ammonia methanol solution, and the results are shown in Figure 1B. Therefore, it is evident that the CML recovery rate of minimum value reached $94.28 \pm 1.01\%$. The recovery rate was $99.92 \pm 1.13\%$ when the ammonia concentration was 15.0%. The statistical analysis showed that there was no significant difference in CML recovery among the three kinds of the ammonia concentration (15.0, 20.0, and 25.0%) ($P \geq 0.05$), which indicated that the change in CML recovery was not significant after the ammonia concentration exceeded 15.0%.

When the ammonia concentration dropped below 15.0%, the negative charge in the eluent was unable to neutralize the positive charge completely, and the CML was not entirely dissociated, leading to a low recovery rate in the elution effluent. When the ammonia concentration exceeded 15.0%, no significant change was evident in the recovery rate ($P \geq 0.05$). Therefore, an ammonia methanol solution with a concentration of 15.0% was applied as the elution solvent in the experiment.

Optimization of the UPLC-MS/MS conditions

A 50.0 ng/mL 1.0 mL volume of the CML standard solution was used for analysis. The CML separation and detection capacity of the C_{18} and the HILIC column are shown in Figure 2A and 2B. The retention time of the CML was only 0.84 min in the C_{18} column, while it reached 4.68 min in the HILIC column. Since the CML displayed poor retention in the C_{18} chromatography column, it was necessary to increase the retention by adding trifluoroacetic acid, nine-fluorine pentanoic acid, and other ion-pairing reagents in the mobile phase. However, these reagents remained in the mass spectrum and led to a decrease in detection sensitivity. Furthermore, these reagents inhibited both the positive and negative ions in

the electrospray. To achieve effective retention without an ion pair reagent, the HILIC chromatography column was applied during the experiment.

A 50.0 ng/mL 1.0 mL volume of CML standard solution was used for analysis. The CML elution effect of water-methanol and water-acetonitrile was compared. The results indicated that when the mobile phase was methanol, the retention time was only 1.50 min, and the tailing peak phenomenon was serious as illustrated by Figure 2C. However, the retention time of acetonitrile extended to 4.78 min displaying a considerable improvement in the peak shape as shown in Figure 2D.

The acidity of the aqueous phase was adjusted with formic acid at levels of 0.05, 0.10, 0.15, 0.2, 0.25, and 0.30%, respectively. The results indicated that the peak shape had improved significantly after adjusting the acidity of the aqueous phase. Therefore, the peak shape was optimal at a 0.10% formic acid concentration as is evident from Figure 2B.

Ammonium acetate was added to 0.1% aqueous formic acid solution and the concentrations were 1.0, 2.0, 3.0, 4.0, and 5.0 mmol/L. After the addition of buffer salt containing ammonium acetate, the CML peak exhibited no significant improvement, and the retention time was not prolonged. However, an apparent reduction in the strength of the total ion chromatogram (TIC) was evident in Figure 2E. Finally, the mobile phases were determined to be 0.1% aqueous formic acid solution in phase A and acetonitrile in phase B. Under these conditions, the peak shape of the CML was optimal with the most cogent response as indicated by Figure 2B.

The primary and secondary mass chromatogram of the 50.0 ng/mL, 1.0 mL of CML standard, and 10.0 ng/mL of CML- D_4 isotopic internal standard solution are shown in Figure 3A and 3B. When the conical hole voltage was 20 V, it is clear from Figure 3A that two ions were present. These were the respective parent ions of CML ($m/z = 205.22$) and CML- D_4 ($m/z = 209.00$). The daughter ions in Figure 3B were $m/z = 84.00$, $m/z = 87.70$, and $m/z = 130.00$. When the optimal collision energy was 15 V, the ion flow intensity of $m/z = 84.00$ was relatively stronger. The $m/z = 84.00$ ion was applied as the quantitative ion of CML, while the $m/z = 130.00$ ion was used as the qualitative ion of CML during the experiment. The $m/z = 87.70$ ion was the daughter ion of CML- D_4 as shown in Figure 3B.

Method validation

The external standard curve was drawn regarding the peak area as the vertical axes (Y) and the content as the horizontal axes (X), which is illustrated in Figure 4A.

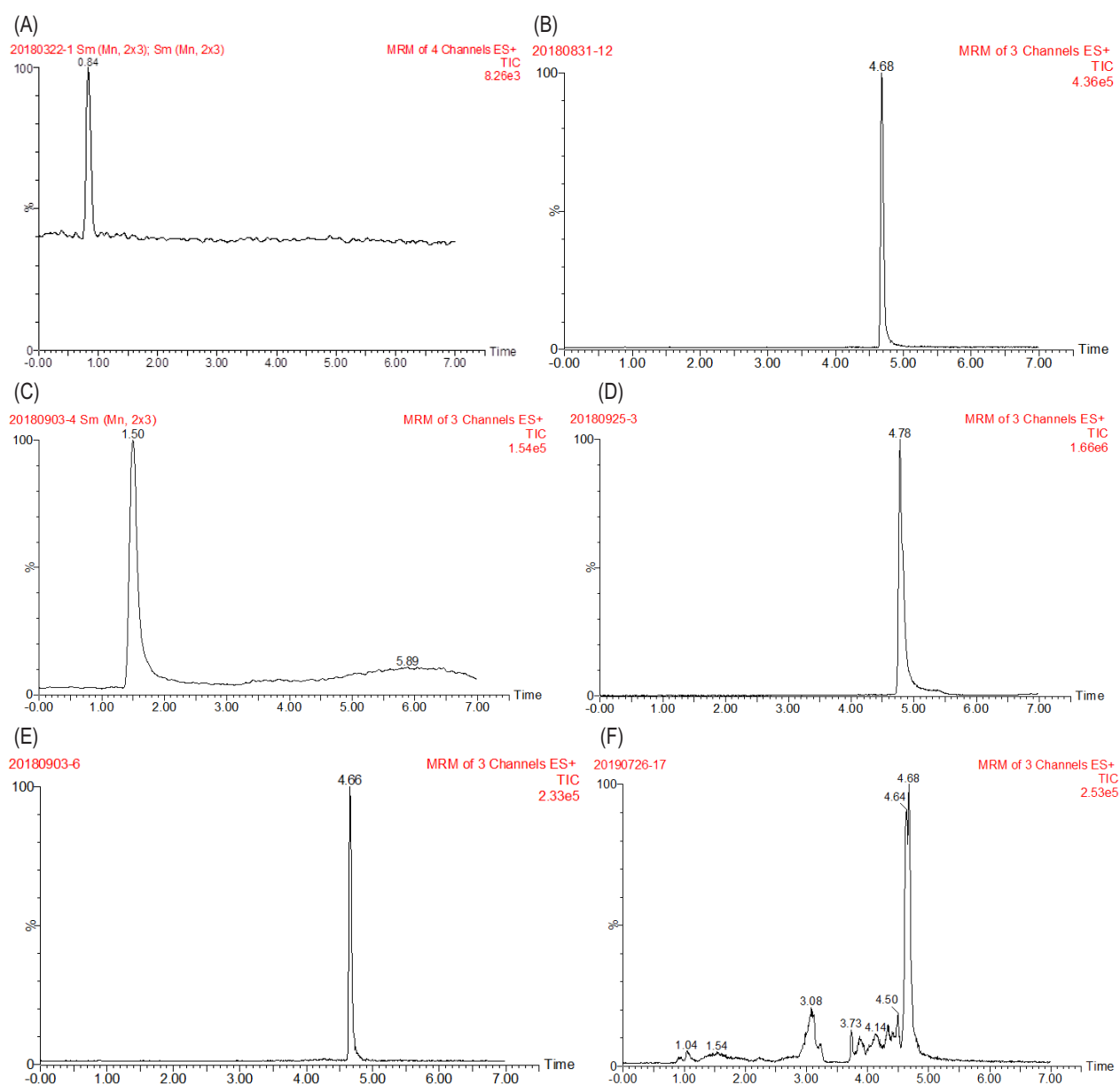


Figure 2. Phase A was 0.1% formic acid and Phase B was acetonitrile, the CML total ion flow chromatogram of the C_{18} column (A) and HILIC column (B). Phase A was water and Phase B was methanol, the CML total ion flow chromatogram of the HILIC column (C). Phase A was water and Phase B was acetonitrile, the CML total ion flow chromatogram using the HILIC column (D). Phase A was water and Phase B was acetonitrile, the CML total ion flow chromatogram of the HILIC column (E). Phase A was 0.1% formic acid and 5.0 mmol/L ammonium acetate and Phase B was acetonitrile, the CML total ion flow chromatogram of the HILIC column (E). Phase A was 0.1% formic acid and Phase B was acetonitrile, the CML total ion flow chromatogram of the HILIC column (F) in the sample. CML, N^ε-(1-Carboxymethyl)-L-Lysine; HILIC, hydrophilic interaction chromatography.

The regression equation and correlation coefficient were calculated as follows: $Y = 644.732 \cdot X - 532.947$ and $R^2 = 0.9994$. The linear range of CML was 1.0–50.0 ng/mL. Taking the response value Y (CML peak area/CML- D_4 peak area) and the concentration ratio of CML and CML- D_4 X as the vertical and horizontal axes, respectively, the regression equation and correlation coefficient were as follows: $Y = 2.436 \cdot X - 0.118$, and $R^2 = 0.9989$. The internal

standard curve of CML is shown in Figure 4B. Breusch–Pagan test and ncvTest were used to verify the standard curve regression model. The results showed that the P -values were greater than 0.1. It indicated that the error variances of the regression model were not changed with the level of fitted values, and there was no significant heteroscedasticity. In the linear range, both standard curve regression models exhibit good accuracy. The scedasticity

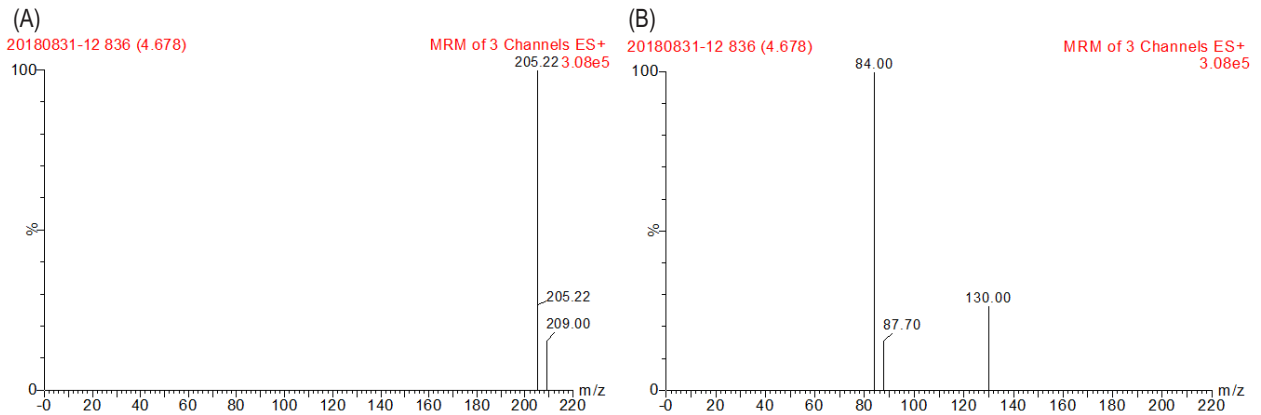


Figure 3. The primary mass chromatogram (A) and the secondary mass chromatogram (B) of CML and CML-D₄. CML, N^ε-(1-Carboxymethyl)-L-Lysine.

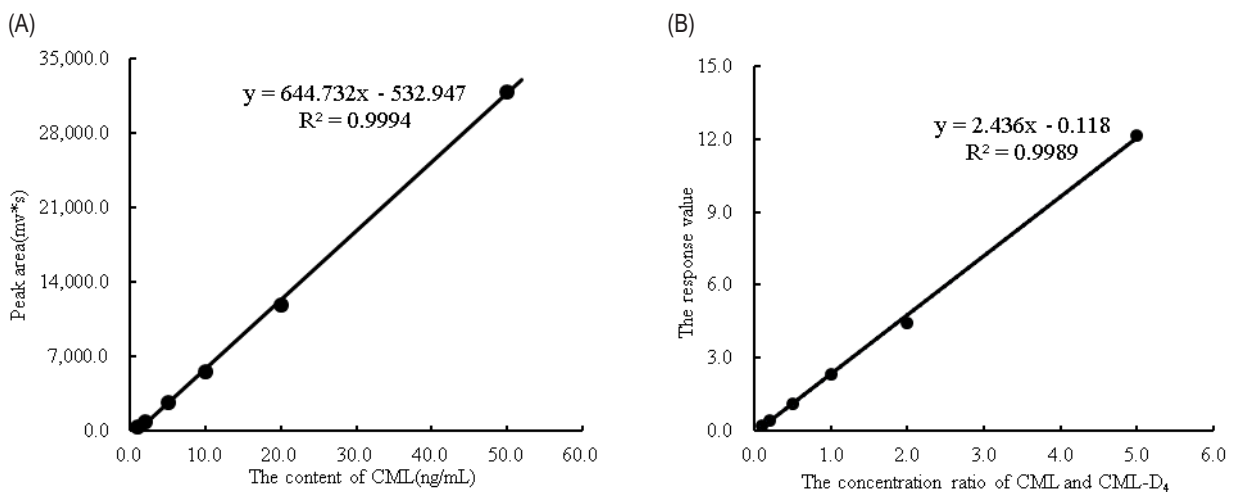


Figure 4. The external standard curve (A) the internal standard curve (B) of CML. CML, N^ε-(1-Carboxymethyl)-L-Lysine.

verification result is shown in Tables 1 and 2. Therefore, it was evident from the results that the method exhibited a wide linear range and excellent correlation. In the event that the CML content in the sample exceeded the boundaries of the linear range, the sample solution needed to be diluted appropriately to ensure that the concentration remained within the linear range of the standard curve.

The S/N (X) mean value was 78.83 of three 1.0 ng/mL CML parallel samples. According to $D = 3 C/X$ and $Q = 10 C/X$, the LOD and LOQ of CML via this detection method were approximately 0.038 ng/mL and 0.127 ng/mL, respectively. Therefore, it is evident that the LOD and LOQ are low, indicating the high sensitivity of this method.

Due to the high CML content in the brewing soy sauce samples, the sample was diluted before detection. To facilitate calculation and reduce deviations, the calculation results of the UPLC-MS/MS working software

Table 1. The scedasticity verification of the external standard curve.

Inspection methods	Statistical values	P
Breusch-Pagan	0.064	0.799
ncvTest	0.021	0.885

Table 2. The scedasticity verification of the internal standard curve.

Inspection methods	Statistical values	P
Breusch-Pagan	0.326	0.568
ncvTest	0.440	0.507

were represented in the sample concentration. The background value of the CML content was 15.4 ng/mL, and the adding standard recovery rate of CML ranged from 86.94 to 92.61%, while the RSD ranged from 3.69 to 6.18% as shown in Table 3. For accuracy in the methodological

Table 3. The adding standard recovery rate of CML.

Adding standard concentration (ng/mL)	Average recovery rate (%)	RSD (%)
10.0	92.28 ± 1.25	6.18
20.0	92.61 ± 1.64	3.69
30.0	86.94 ± 0.89	4.27

CML, N^ε-(1-Carboxymethyl)-L-Lysine; RSD, relative standard deviation.

validation, acceptable recovery rates generally ranged from 80.0 to 120.0% (Diaz *et al.*, 2004). Therefore, the recovery rate of this method was within the acceptable range rendering it highly accurate.

Due to the high CML content in the brewing soy sauce samples, the sample was diluted before detection. To facilitate calculation and reduce deviations, the calculation results of the UPLC-MS/MS working software were represented in the sample concentration. The same sample was injected and analyzed six times within a short time. The average content over six injections was 15.4 ng/mL. According to the formula, the RSD value of the repeatability test was 3.44%. The generally acceptable RSD range of precision is less than 10.0%, and the RSD of this method is 3.44%, which satisfies the precision requirements.

Due to the high CML content in the brewing soy sauce samples, the sample was diluted before detection. To facilitate calculation and reduce deviations, the calculation results of the UPLC-MS/MS working software were represented in the sample concentration. Six parallel samples were injected and analyzed. The average content over six injections was 15.1 ng/mL. According to the formula, the RSD value of the parallel test was 6.56%. The generally acceptable RSD range of precision is less than 10.0%. The RSD of this method is 6.56%, which satisfies the precision requirements.

Determination of the CML content in the brewing soy sauce samples

The CML content in the eight brewing soy sauce samples was determined with the UPLC-MS/MS and the isotope method of internal standards. Figure 2F is the CML total ion flow chromatogram in the sample. It can be seen that the sample has the same retention time as the CML standard, and the baseline noise is low, with fewer impurities. The method established can be used to detect the content of CML in the brewing soy sauce. The detection results are shown in Table 4 and indicate that the content range of CML in the brewing soy sauce samples is 3.16 ± 0.18 – 15.10 ± 1.19 µg/mL. According to the data in Table 4, the CML content in different brewing soy sauce samples is

Table 4. The CML content of brewing soy sauce samples.

Sample source	Ordinal number	CML content(µg/mL)
A	A ₁	8.30 ± 0.35
B	B ₁	6.36 ± 0.22
C	C ₁	10.51 ± 0.68
D	D ₁	15.10 ± 1.19
E	E ₁	10.38 ± 0.93
	E ₂	5.60 ± 0.21
F	F ₁	11.58 ± 0.74
	F ₂	3.16 ± 0.18

A, B, C D, E, and F represent different brands, and 1 and 2 represent different samples of the same brand, respectively. CML, N^ε-(1-Carboxymethyl)-L-Lysine.

different, which may be related to the raw material selection and fermentation process of soy sauce.

Conclusions

A method for the detection of CML in brewing soy sauce was obtained by optimizing the SPE conditions, selecting a chromatography column, as well as optimizing the liquid chromatography and mass spectrometry conditions during this experiment. This method was successfully applied for measuring the CML content in eight brewing soy sauce samples. This method can not only effectively remove interfering substances but also does not require derivatization in the sample pretreatment, with the characteristics of preventing contamination and prolonging the service life of the chromatography column. This method was proven to be simple, accurate, sensitive, and precise. The establishment of the detection method provides a basis for further studying the detection of CML content in different kinds of food, the affecting factors of the CML production in food processing, and guiding humans to improve food processing technology.

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Conflict of Interest

All authors declare that they have no conflict of interest.

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent

Not applicable.

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