Simultaneous analysis of Nε-(carboxymethyl)lysine, reducing sugars, and lysine during the dairy thermal process

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ABSTRACT

A new analytical method allowing the simultaneous quantification of Nε-(carboxymethyl)lysine (CML), lysine, and reducing sugars (glucose, lactose, and galactose) is described. It is based on high performance anion-exchange chromatography with pulsed amperometric electrochemical detection. This method demonstrated a low limit of quantification (0.385 to 0.866 mg/L), excellent linear correlation ($R^2 > 0.997$), and desired calibration range (3.125 to 25 mg/L). In addition, lactose-lysine solutions containing sulfite (4 to 400 mmol/L) were heated at 110°C for 2 h. The results showed that sulfite inhibited the formation of CML and promoted the consumption of reducing sugars and lysine in the Maillard reaction model. The method proved to be useful for simultaneous analysis of CML, lysine, and reducing sugars (glucose, galactose, and lactose) in the Maillard reaction system. Moreover, sulfite was an effective inhibitor of CML formation.

Key words: Maillard reaction, Nε-(carboxymethyl)lysine, high performance anion-exchange chromatography with pulsed amperometric electrochemical detection (HPAEC-PAD), advanced glycation end product

INTRODUCTION

Maillard reactions (MR) are always prone to take place between reducing sugars (lactose and galactose, among others) and amino acids or milk proteins during the milk heating process (Guan et al., 2012). In general, MR are described in 3 reaction stages, including the early stage, the intermediate stage, and the advanced or final stage (Rufián-Henares et al., 2008). After a complex reaction, a wide range of products, including compounds termed advanced glycation end products (AGE), are produced during the advanced MR stage (Tauer et al., 2003; Rufián-Henares et al., 2004). The AGE (endogenous and exogenous) might contribute to

the complications of diabetes (Monnier et al., 1996) and uremia (Uribarri et al., 2003). A recent report showed that the amount of AGE ingested with heated milk was 25 to 75 mg, which was higher than the total amount of AGE in plasma and tissues (Henle, 2003).

Nε-(Carboxymethyl)lysine (CML), as an indicator of MR in foods and biological systems (Tauer et al., 1999), is one of the best characterized AGE identified in food (including milk and milk products; Hartkopf et al., 1994). The presence of AGE as contaminants in milk is undesirable and their removal is difficult. Pashikanti et al. (2010) and Silván et al. (2011) used environmentally friendly compounds to selectively inhibit AGE formation. Sulfite, an inhibitor of browning reactions in MR, had been confirmed to inhibit the formation of the brown polymer in the final MR stage (Guan et al., 2011). However, whether sulfite groups could inhibit the formation of CML in the final MR stage is still unclear. Therefore, understanding the interaction between CML and sulfite groups during dairy thermal treatment might provide further insight into reducing the amount of CML in food.

Methods have been developed for the determination of CML. Büser et al. (1987) determined CML by gas-liquid chromatography. Birlouez-Aragon et al. (2004) assessed CML and oxalic acid monoalkylamide by ELISA. Hegele et al. (2008) quantified free and protein-bound forms of AGE in dairy products by liquid chromatography with tandem mass spectrometry (LC-MS/MS) using a stable isotope dilution assay. Assar et al. (2009) used ultra performance liquid chromatography-mass spectrometry for the determination of CML in food systems. Their methods were stable, precise, and accurate for the quantitative analysis of CML. Unfortunately, a method allowing simultaneous analysis of CML, amino acids, and reducing sugars is still absent, but desirable, for expediently collecting quantitative data, which is the basis for the control of CML formation in MR or reaction modeling. The analysis method of amino acids and sugars has been improved by high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD), which is sensitive and highly selective (Clarke et al.,

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1999; Cataldi et al., 2000) but it has not yet been used for determination of CML. Theoretically, CML, a type of anionic compound because of its 2 carboxyl groups, should be offered excellent resolution by high-performance anion-exchange chromatography at alkaline pH. Therefore, the simultaneous analysis of sugars, lysine, and CML in MR using the HPAEC-PAD method could be appropriate.

This study aimed to develop an accurate and stable means for the simultaneous analysis of CML, lysine, and reducing sugars in model MR. Meanwhile, the effect of sulfite on CML formation in a model system was investigated.

MATERIALS AND METHODS

Chemicals

Lysine, glucose, lactose, galactose, and sodium sulfite and sodium hydroxide (50%) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). N ϵ -(Carboxymethyl)lysine was purchased from NeoMPS (Strasbourg, France). The mixed standard stock solutions of analytes (250 mg/L) were prepared by dissolving the analytes into deionized water.

Model MR

Lactose (40 mmol/L) and lysine (40 mmol/L) were dissolved in sodium phosphate buffer (0.2 mol/L, pH 7.4) and sodium sulfite (4, 40 or 400 mmol/L) was added. A control was made up containing no sodium sulfite. Aliquots (4 mL) of each mixed solution were put into screw-cap tubes and heated in an oil bath at 110°C for 2 h. After the samples were cooled to room temperature in iced water, the reaction samples were diluted with deionized water 25 fold and stored at 4°C for further analysis.

Analysis of CML, Lysine, and Reducing Sugars

High-performance anion-exchange chromatography with pulsed integrated amperometric electrochemical detection was used for the determination and quantitation of CML, lysine, and reducing sugars. The reaction mixtures were filtered through a Millex-HN nylon clarification kit of 0.45- μ m pore size (Millipore Corp., Bedford, MA), and analyzed on a DX 5000 Dionex system (Dionex Corp., Sunnyvale, CA), a gradient pump (model EG40) with online degassing, and a pulsed integrated amperometric electrochemical detector (ED40; Dionex Corp.). Separation was accomplished on a CarboPac PA1 anion-exchange column (250 $\times$ 4 mm; Dionex Corp.) and a CarboPac PA1 guard column (50

Table 1. Waveform of electrochemistry determination for N ϵ -(carboxymethyl)lysine, lysine, and reducing sugars¹

Time (s)	Potential (V) vs. pH	Integration
0.000	+0.13	Begin
0.040	+0.13	
0.050	+0.33	
0.210	+0.33	
0.220	+0.55	
0.460	+0.55	End
0.470	+0.33	
0.560	+0.33	
0.570	-1.67	
0.580	-1.67	
0.590	+0.93	
0.600	+0.13	

¹The waveform was used on a pulsed integrated amperometric electrochemical detector (PAD, ED40; Dionex Corp., Sunnyvale, CA) equipped with a pH reference electrode (part no. 061879; Dionex Corp.) and a gold work electrode (part no. 061875; Dionex Corp.).

$\times$ 4 mm; Dionex Corp.) using the gradient as follows: solvent A was water and solvent B was NaOH (500 mmol/L). Gradient elutions were started at 5% solvent B, which was maintained for 10 min, followed by a linear gradient of 5 to 0.5% solvent B in 10 min, followed by a linear gradient of 0.5 to 20% solvent B for 20 min, and then a hold at 70% solvent B for 20 min. All tests were conducted at a constant flow rate of 1 mL/min. The injection volume was 20 μ L. The column temperature was set at 30°C. The pH reference electrode (part no. 061879; Dionex Corp.) and gold work electrode (part no. 061875; Dionex Corp.) were used in HPAEC-PAD analysis. The waveforms used for the detection of CML, lysine, and reducing sugars are shown in Table 1.

Statistical Analysis

Means and standard deviations of the data were calculated for each treatment. Analysis of variance was performed to establish any significant differences ($P < 0.05$) among the applied treatments using the SPSS software package (SPSS 10.0 for Windows; IBM Corp., Armonk, NY).

RESULTS AND DISCUSSION

Separation of CML, Lysine, and Reducing Sugars

Figure 1 shows the chromatograms of CML, lysine, and reducing sugars ranging from 3.125 to 25 mg/L, separated by a high-performance anion-exchange column (CarboPac PA1). Peak 1 was lysine [retention time (RT) = 7.68 min], peak 2 was galactose (RT = 13.98 min), peak 3 was glucose (RT = 15.50 min), peak 4 was lactose (RT = 38.25min), and peak 5 was CML

Table 2. Linear calibration ranges, regression equations, and detection limits of analytes as measured by high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD)¹

Analyte	Calibration range (mg/L)	Regression equation ²	R ²	Relative SD (%; n = 6)	Method detection limit ³ (mg/L)	Quantification limit ⁴ (mg/L)
Lysine	3.125–25.000	$y = 1.4128x + 2.2316$	0.9987	1.89	0.212	0.708
Galactose	3.125–25.000	$y = 2.222x + 0.4931$	0.9988	2.53	0.135	0.450
Glucose	3.125–25.000	$y = 2.5982x + 0.1544$	0.9993	2.07	0.115	0.385
Lactose	3.125–25.000	$y = 2.2568x + 0.6931$	0.9992	2.67	0.133	0.443
CML ⁵	3.125–25.000	$y = 1.1543x + 2.2277$	0.9976	1.96	0.260	0.866

¹No sulfite in standard solutions and sodium hydroxide as gradient solvent.

²y = peak area of analytes (nC·min); x = concentration of analytes (mg/L).

³Signal-to-noise ratio (S/N) = 3 (per 20- μ L injection volume).

⁴S/N = 10 (per 20- μ L injection volume).

⁵N ϵ -(Carboxymethyl)lysine.

(RT = 56.40 min). All peaks were symmetrical and had no tail peak. The resolution (RES) of all analytes was higher than 1.5. The data stated above meant that using the CarboPac PA1 column was suitable for the simultaneous separation of CML, lysine and reducing sugars.

Performance Evaluation of the HPAEC-PAD Method

The HPAEC-PAD method performance was evaluated against quality parameters such as linearity and calibration, coefficient of determination, detection limit, accuracy, and recovery. The limit of quantification (LOQ) was based on signal-to-noise ratios of 10. As shown in Table 2, the lowest LOQ was glucose (0.385 mg/L), and the highest LOQ was CML (0.866 mg/L).

The method detection limit was based on signal-to-noise ratios of 3. The calculated method detection limit (0.133 to 0.260 mg/L) was comparable to the predetermined LOQ. Calibration equations were generated by calibration standards (3.125 to 25 mg/L). Calibration curves had excellent linearity with the coefficient of determination ($R^2 > 0.997$). Recovery was evaluated by spiking target analytes at 3 concentrations (3.125, 5.000, and 6.250 mg/L; n = 6) into the reaction mixture with 40 mmol of sulfite/L. The recovery was calculated with the following equation: percentage recovery = (total amount – spiked amount)/spiked amount $\times$ 100%. The results are shown in Table 3. Observed recoveries ranged from 90.3 (CML, 3.125 mg/L spiked) to 99.7% (galactose, 5.000 mg/L spiked).

Change in Reducing Sugars and Lysine

In this experiment, glucose and galactose degraded from lactose were found in all model systems. In addition, the amount of galactose was higher than that of glucose in each model system (Figure 2). Many studies had reported that the degradation of oligosaccharide would release its monosaccharide moiety in caramelization or MR (Hollnagel and Kroh, 2000; Kroh and Schulz, 2001). Glucose and galactose were formed due to the degradation of lactose in the MR model. However, the amount of galactose and glucose was different due to the degradation of lactose via the “peeling off mechanism” proposed by Hollnagel and Kroh (2000) and Morita et al. (1983). The mechanism was described as follows: the degradation of the oligo- or polysaccharide started from the reducing end of differently linked homoglucans in alkaline solution and further eliminated the monosaccharide moiety via enolization and rearrangement reactions. Specifically, in the lactose-lysine model system, the reducing carbonyl group of lactose was first attacked by the amino group of lysine (i.e., the glucose moiety of lactose was consumed) to subsequently form a Schiff base. After a series of enoliza-

Figure 1. The chromatograms of samples (25-fold dilution) and standard measured by high-performance anion-exchange chromatography with pulsed integrated amperometric electrochemical detection. 1 = lysine; 2 = galactose; 3 = glucose; 4 = lactose; 5 = N ϵ -(carboxymethyl)lysine. A, B, C, D, and E are the chromatograms of the 25 mg/L standard solution and of the model reaction mixtures (110°C for 2 h), containing 0, 4, 40, and 400 mmol of sulfite/L, respectively.

Table 3. Recoveries of analytes from the reaction mixture with 40 mmol of sulfite/L by the high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) method¹

Parameter	Lysine (mg/L)				Galactose (mg/L)				Glucose (mg/L)				Lactose (mg/L)				CML ² (mg/L)			
	3.125	5.000	6.250	6.250	3.125	5.000	6.250	6.250	3.125	5.000	6.250	6.250	3.125	5.000	6.250	6.250	3.125	5.000	6.250	6.250
Found ³ (mg/L)	2.893	4.675	5.783	5.783	3.007	4.983	6.084	6.011	2.993	4.669	6.011	6.011	2.913	4.683	5.943	5.939	2.821	4.797	5.939	5.939
Recovery (%)	92.6	93.5	92.5	92.5	96.2	99.7	97.3	96.2	95.8	93.4	96.2	96.2	93.2	93.7	95.1	95.0	90.3	95.9	95.0	95.0
RSD ⁴ (%)	2.31	1.29	1.97	1.97	2.68	1.53	1.35	2.78	2.43	1.27	2.78	2.78	2.18	3.01	2.54	1.64	2.96	1.85	1.85	1.64

¹Reaction mixtures with 40 mmol of sulfite/L were spiked with 3.125, 5.000, or 6.250 mg of the analyte.

²Nε-(Carboxymethyl)lysine.

³The total measured concentration minus the initial measured concentration.

⁴Relative SD (mean value of 6 determinations).

Figure 2. Change in lysine and reducing sugars measured by high-performance anion-exchange chromatography with pulsed integrated amperometric electrochemical detection as a function of sulfite concentration in the Maillard model system heated at 110°C for 2 h. Results are expressed as the mean ± SD of 3 experiments.

tion and rearrangement reactions, the Schiff base was rearranged into the 2,3-enediol structure, which further eliminated the glycosidic residue at C₄ to release the galactose moiety. Therefore, the peeling-off degradation of lactose contributed to galactose release and glucose consumption in the MR system.

Furthermore, the effects of sulfite on lysine and reducing sugars in the MR system are shown in Figure 3. Sulfite significantly promoted the consumption of lysine and lactose but decreased the formation of glucose and galactose in the reaction solutions ($P < 0.05$). A proposed explanation was made as follows (Figure 4): in the early stage of the MR, a nucleophilic reaction

Figure 3. Change in Nε-(carboxymethyl)lysine (CML) measured by high-performance anion-exchange chromatography with pulsed integrated amperometric electrochemical detection as a function of sulfite concentration in the Maillard model system heated at 110°C for 2 h. Results are expressed as the mean ± SD of 3 experiments.

Figure 4. Proposed mechanisms of lactose degradation and N ϵ -(carboxymethyl)lysine (CML) formation in a model system containing sulfite.

was carried out between sulfite and the carbon atom of the carbonyl group of open-chain lactose, which led to the formation of compound 1. Compound 1 was electrophilic and susceptible to producing compound 2 due to the attack from the amino group of lysine. However, compound 2 was inert to transform into a Schiff base or rearrange into compound 3 (i.e., the Amadori rearrangement products). Consequently, the degradation of compound 2, whose formation contributed to the consumption of lysine and lactose in the early stage

of the MR, was blocked and resulted in releasing less galactose and glucose. In addition, the formation of monosaccharide-sulfonate could also contribute to the consumption of galactose and glucose. But further research should be carried out to confirm this.

Effect of Sulfite on the Formation of CML

The effect of sulfite on the formation of CML is shown in Figure 3. N ϵ -(Carboxymethyl)lysine was generated

in the model reaction systems without or with sulfite (189.7, 175.4, 29.7, and 28.7 mg/L for the control and 4, 40, and 400 mmol of sulfite/L, respectively). The results suggest that sulfite significantly inhibited the formation of CML with respect to the control.

N ϵ -(Carboxymethyl)lysine can be formed by many pathways in food systems (Roux et al., 2009). It might be formed by the oxidation of fructoselysine (Ahmed et al., 1986) or generated during oxidation of ascorbic acid (Dunn et al., 1990). In addition, it could be directly derived from the nucleophilic reaction between glyoxal and the ϵ -amino group of lysine (Glomb and Monnier, 1995). In this experiment, the inhibiting effect of sulfite on CML formation can be explained as follows: as shown in Figure 4, added sulfite, an effective inhibitor of the peeling-off degradation of lactose, induced the formation of compound 2 but blocked the formation of compound 3, which then led to the reduction of the α -dicarbonyl compounds (including glyoxal) and the consumption of lysine. Adding sulfite also nucleophilically attacked glyoxal, resulting in the formation of compound 4, which could block the nucleophilic reaction between lysine and glyoxal. Consequently, the decrease in lysine and glyoxal contributed to the reduction in CML. Therefore, the inhibition of CML formation was a dose response to sulfite.

CONCLUSIONS

The HPAEC-PAD method is suitable for simultaneous quantitative analysis of CML, lysine, and reducing sugars (glucose, galactose, and lactose) in model MR systems. It promoted the early MR, yet it exerted an inhibitory effect on CML formation.

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