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Assessment of the Determination of Azodicarbonamide and Its Decomposition Product Semicarbazide: Investigation of Variation in Flour and Flour Products

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ABSTRACT: Azodicarbonamide, as a bleaching agent and improving agent, is a permitted food additive in certain countries and can be determined by high-performance liquid chromatography. However, it partially degrades with the heat of processing to form trace amounts of semicarbazide, which shows carcinogenicity and also has been shown to cause tumors. The concentration of semicarbazide in azodicarbonamide-treated flour was determined by isotope dilution (^{13}C, $^{15}N_2$-semicarbazide) liquid chromatography electrospray tandem mass spectrometry (LC-MS/MS). The quantification was obtained utilizing the homologous internal standard. The limits of detection were 1 mg/kg for azodicarbonamide and 0.5×10^{-3} mg/kg for semicarbazide. The rates of recovery were 82.3−103.1% for azodicarbonamide and 72.4−116.5% for semicarbazide. This study prepared four different types of flour products to investigate the variation of semicarbazide. The concentration of semicarbazide in all types of flour products is higher than that in flour, and the concentration of semicarbazide in outside of flour products is slightly higher than that in the inside. As the problem of food safety hazard aggravates daily, we should be more concerned about food security and human health.

KEYWORDS: Azodicarbonamide, semicarbazide, flour products, HPLC, LC/MS-MS

INTRODUCTION

Azodicarbonamide (ADA) previously was used as a blowing agent in rubber products and foamed polyethylene that were permitted in other food packaging applications.[1] In October 2003, two reports by the European Food Safety Authority[2] implicated that foamed polyvinylchloride (PVC) cap liners, manufactured with ADA, as the source of semicarbazide (SEM) contamination, were found in a variety of jarred foods.

In certain countries, ADA is also approved as a food additive[3] for use as a bleaching agent in cereal flour and as a dough conditioner. It is commonly used as a flour additive up to a maximum of 45 mg/kg in flour in the United States, Canada, and Asia.[3] However, ADA as a food additive is banned in Australia and Europe. In Singapore, use of ADA can result in up to 15 years imprisonment and a fine of $450 000.

As a flour bleaching agent and improving agent, ADA may cause an allergic reaction in those sensitive to other azo compounds.[4] The consumption of ADA may also heighten an allergic reaction to other ingredients in a food. ADA is stable in dry flour, but it reacts with moist flour as an oxidizing agent. The main reaction product is biurea (not urea), which is not stable during heating. Subsidiary reaction products include SEM and urazole at very low yields.[5,6] Namely, ADA partially degrades with the heat of processing to form trace amounts of SEM (Figure 1). Besides, SEM shows limited genotoxicity in vitro and carcinogenicity and also has been shown to cause tumors.[4]

SEM is a known metabolite of the veterinary drug nitrofurazone, and it is used as an indicator for the use of the drug in food of animal origin. Nitrofurazone and its metabolite SEM have strong carcinogenicity, teratogenicity, mutagenicity, and other side effects.[7] Many countries have promulgated bans prohibiting the use of nitrofurazone.[7] As SEM is not absorbed in the UV range of the spectrum,[8] the levels of SEM in ADA-treated flour are determined by isotope dilution (^{13}C, $^{15}N_2$-SEM)[9] liquid chromatography electrospray tandem mass spectrometry (LC-MS/MS).[10]

The molecular weight of SEM is 75 g/mol, and it has very serious matrix interference and lower sensitivity with ESI+ ion mode detection. After it is derived with 2-nitrobenzaldehyde, the efficiency of the ionization of metabolites is increased. Nucleophiles R-NH_2 acid catalyze environment free quickly and has a chemical reaction with the carbon acyl of 2-nitrobenzaldehyde[11] (Figure 2). In this way, the total contents of SEM can be analyzed.

With the development of science and technology, many researchers have discussed the way of the formation of SEM. One possible source of the SEM contamination is from the thermal decomposition of biurea formed during dough mixing and kneading.[1,12] Pereira et al.[13] proposed the use of a mechanism of biurea hydrolysis but did not present corresponding data supporting the reaction pathway. Gregory et al.[1] concluded that biurea decomposed to SEM at similar yields noted for ADA under dry conditions in the high moisture−high temperature environment necessary for baking bread.

People usually absorb many flour products in three meals per day, especially North America and Europe. Because ADA is allowed to be added to food in many countries and it can degrade to form trace amounts of SEM, it is essential to reduce the harm of flour products to human health. The objective of this study was

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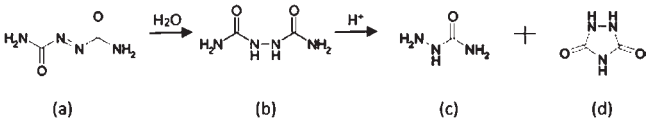

Figure 1. Mechanism of formation between ADA and SEM. (a) ADA, (b) biurea, (c) SEM, and (d) urazole.

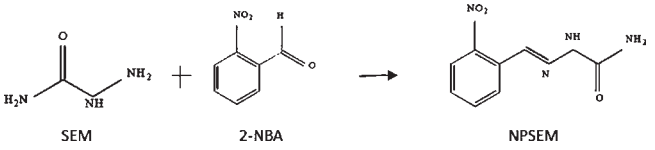

Figure 2. Reaction of hydrolysis and derivation of SEM.

to analyze the determination of ADA and its decomposition product SEM by corresponding instruments and to investigate the variation of SEM in flour and flour products by preparing four types of flour products. In addition, it is necessary to gain further understanding of possible SEM formation pathways. A thorough understanding of the formation of SEM would be beneficial in identifying processing changes to reduce SEM concentrations in the final product.

MATERIALS AND METHODS

Chemicals. ADA (99%), SEM (99%), and 2-nitrobenzaldehyde (99%) were obtained from Sigma Corp. (St. Louis, MO) and used as received. NaOH, HCl, and dipotassium hydrogen phosphate were obtained from Kermel (Tianjin, China). Acetonitrile, ethyl acetate, acetone, hexane, ammonium acetate, methane acid, and methanol from Kermel were all of high-performance liquid chromatography (HPLC) grade or better and were used without further purification. Water (18.2 MΩ.cm) was obtained from water purification system. Isotopically labeled SEM (^{13}C, $^{15}N_2$), with a chemical purity of 99% and an isotopic purity of 98%, was purchased from Sigma.

Flour and Products Samples. All of the ingredients were purchased at a local grocery store and stored under the manufacturer's recommended conditions. Flour products were prepared using a commercially available heating machine (Beijing, China) and the standard flour products recipe.[14−17]

Baked bread was made of 200 g of flour, 3.6 g of yeast, 12 g of sugar, 8 g of nonfat dry milk, 3 g of salt, 6 g of shortening, 0.008−0.01 g of ascorbic acid, and an appropriate amount of water, mixed, fermented for 90 min at 30 °C ± 1, and baked 20 min at 215 °C.

Steamed bread was made of 200 g of flour, 2 g of yeast, and an appropriate amount of water, fermented for 1 h at 38 °C, and steamed 20 min. The method of fried food was the same as steamed bread, and it was fried 3−5 min. The noodle was only made of 150 g of flour and an appropriate amount of water and boiled at 100 °C.

Equipment and Materials. The model Waters-TQD consisted of an autosampler, binary pump, degasser, and column oven (Waters, United States) with a MassLynx version 4.1 data system. Solid-phase extraction columns (Thermo, C18, 60 mg, 3 mL) were purchased from Thermo Electron Corp. and rinsed with 3 mL of methanol followed by 3 mL of water prior to use. Polypropylene centrifuge tubes obtained from Anpel (Shanghai, China) were used for all extractions, whereas glass scintillation vials, wrapped in foil, were used for SEM derivatization reactions. Syringe filters, used to filter flour and flour products extract (0.2 μm, Nylon), were purchased from Waters Corp. Autodispensers (Eppendorf) were used for accurately taken reagent. Centrifugation was performed on 20000 rpm centrifuge from Eppendorf.

Table 1. Gradient Elution of Acetonitrile and Water with 0.1% Methane Acid[a]

time (s)	A	B	flow (mL/min)
0.00	20.0	80.0	0.20
5.00	40.0	60.0	0.20
9.00	80.0	20.0	0.20
9.50	20.0	80.0	0.20
15.00	20.0	80.0	0.20

[a] A, acetonitrile; and B, water with 0.1 v/v methane acid.

Table 2. Different Levels of Labeling of ADA in Flour and Their Recoveries (Expressed with Values ± SDs)

	recovery (%)	
spike level ADA (mg/kg)	intra-assay	interassay
1	87.97 ± 4.99	92.37 ± 4.32
	92.53 ± 3.34	
	96.60 ± 1.61	
2	91.27 ± 3.40	95.87 ± 5.13
	94.93 ± 4.58	
	101.40 ± 1.61	
10	99.67 ± 2.71	99.92 ± 0.39
	100.37 ± 1.30	
	99.73 ± 1.27	

Instrumental Conditions. Sample extracts (30 μL) were separated in a Thermo C18 column (2.1 mm × 150 mm, 5 μ). The mobile phase consisted of acetonitrile (A) and water with 0.1 v/v methane acid (B) at a constant flow rate of 0.2 mL/min, gradient elution (Table 1). The column temperature was maintained at 35 °C throughout the analysis.

Ionization mode, positive ion electrospray; desolvation gas temperature, 450 °C; source temperature, 120 °C; desolvation gas flow, 800 L/h; cone gas flow, 50 L/h; collision gas, 0.14 mL/min; and collision gas pressure, 2.6 × 10^{-3} mbar (argon). Precursor ion → product ion transitions in multiple reaction monitoring (MRM), labeled SEM derivative m/z 212.1 → 192.1 (collision energy, 10 eV); native SEM derivative m/z 209.1 → 192.1 (12 eV); m/z 209.1 → 166.1 (12 eV); the cone voltage was 30 V for all MRM transitions; retention time for each MRM transition, 0.217 s; and interchannel delay, 0.1 s.

ADA Method. Flour and flour products (respectively, 1 g) were placed in 50 mL centrifuge tubes and were extracted with 10 mL of acetone. The reaction mixtures were protected on a rotary shaker at room temperature for 15 min, and the samples were centrifuged at 5000 rpm for 5 min. The 5 mL extract on upper was then concentrated under nitrogen at 40 °C and diluted to 1 mL with 20 mmol/L ammonium acetate aqueous solution.[18] After the lipid by hexane (1 mL) was removed, the extract was analyzed by HPLC from Agilent (Palo Alto, CA) with external standard method.[18] The samples were analyzed using a 2.1 mm × 150 mm C18 column, and the mobile phase consisted of methanol (A) and ammonium acetate aqueous solution with 0.1% methane acid (B) (A:B = 2:8) at a constant flow rate of 0.8 mL/min (λ = 283 nm). The column temperature was maintained at 30 °C throughout the analysis.

SEM Extraction Method. Flour and flour products (respectively, 2 g) were placed in 50 mL centrifuge tubes and spiked with 50 μL of (^{13}C, $^{15}N_2$)-SEM (0.05 mg/mL), 10 mL of hydrochloric acid (0.2 mol/L, pH 3.5), and 100 μL of 2-nitrobenzaldehyde (20 mg/mL, 20 mg in 1 mL of

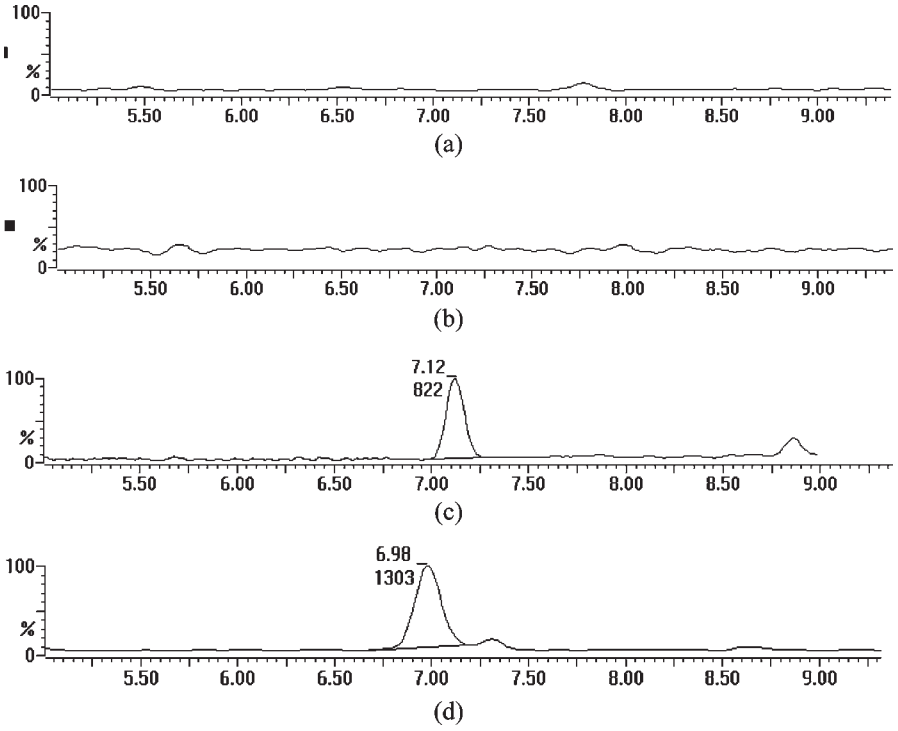

Figure 3. Concentration of SEM: (a) blank flour samples, (b) flour samples that had no treatment, (c) flour samples that were heated, and (d) flour samples that were wet.

dimethyl sulfoxide).[13] The reaction mixtures were protected from light and heated on a rotary shaker at 37 °C[19] for 16 h. After they were cooled to room temperature, the mixtures were adjusted to pH 7.2−7.3 with NaOH (1 and 4 M), HCl (0.2 and 1 M), and dipotassium hydrogen phosphate (1 M). The samples were centrifuged at 3000 rpm for 10 min and then passed through the C18 SPE cartridge (centrifuge tubes and spiked rinsed with 10 mL of 20% methanol−water), on the solid-phase extraction device (Agilent). The derivatives were loaded onto the SPE cartridge and rinsed with 10 mL of ethyl acetate. The ethyl acetate was blown to dryness under nitrogen at 40 °C, and the residue was reconstituted in 1.0 mL of 10% acetonitrile before filtration (0.2 μm, nylon) into amber vials. The final extracts were refrigerated (−4 °C) until analysis by LC-MS/MS.

RESULTS AND DISCUSSION

Selection of ADA Extraction. In the study of HPLC methods for analysis of ADA, a suitable extraction solvent should be chosen appropriately. ADA has strong polarity, which dissolves in dimethyl sulfoxide and *N*,*N*-dimethylformamide, and is very soluble in water, alcohol, acetone, etc.[18] Although dimethyl sulfoxide and *N*,*N*-dimethylformamide have high solubility, their retention times are similar with ADA and lead to produce interference.[18] In addition, it is uneasy to evaporate and remove, so it is not suitable to be used as extraction agent. If it was extracted by water, water-soluble components of flour would also interfere with the analysis of ADA. While methanol and acetonitrile have more water-soluble impurities as an extraction solvent, they are also not suitable for being an extraction agent. By comparison, acetone has been selected as an extraction solvent.

Optimization of Condition in HPLC and LC-MS. As compared to two kinds of mobile phase (methanol and ammonium acetate aqueous solution and acetonitrile and ammonium acetate aqueous solution) in HPLC, the results show that methanol and ammonium acetate aqueous solution with a proportion of 2:8 is more appropriate than the latter. While the concentration of the injected sample is 20 mg/kg, the area of peak is 33.22 mAU s. In addition, the disturbance that was caused by the mobile phase of methanol and ammonium acetate aqueous solution is smaller than others; thus, the former is selected as the mobile phase.

The ADA standard solution, with 0.2 μm pore size membrane filter treatment, was scanned in the range of 200−400 nm. According to the light of UV absorption spectra, the absorption peak of ADA in 283 nm was significant. Thus, it can be confirmed that the detection wavelength of ADA is 283 nm.

The column temperature has a great impact on the separation. In the context of effective separation of the components, it should be held at a lower column temperature, but it should be an appropriate retention time and not be trailing. In this study, the column temperature was maintained at 35 °C throughout the analysis. It can increase the distribution coefficient, increase selectivity, lower Dg, reduce the loss of fixative, extend column life, and reduce the detection of background.

For the same molecular structure of liquid chromatography, the size in length and filler size usually have many forms available. With the same packing, the longer of the length, the higher of plate number, but the column pressure is increased, and analysis time is extended. With the case of the same length, the smaller of the filler size, the higher of plate number, and the better of analysis. Thus, the sample extracts are separated in a Thermo C18 column (2.1 mm × 150 mm, 5 μ).

Limit of Detection and Recovery in HPLC. The method of HPLC for analysis of ADA has a good linearity in the range of 0.5−10 mg/L (r = 0.9991) with a quantification limit of 1.0 mg/kg. The recoveries of ADA from flour spiked at three levels of 1.0, 2.0, and 10.0 mg/kg were in the range of 82.3−103.1% with the relative standard deviations less than 5% (Table 2).

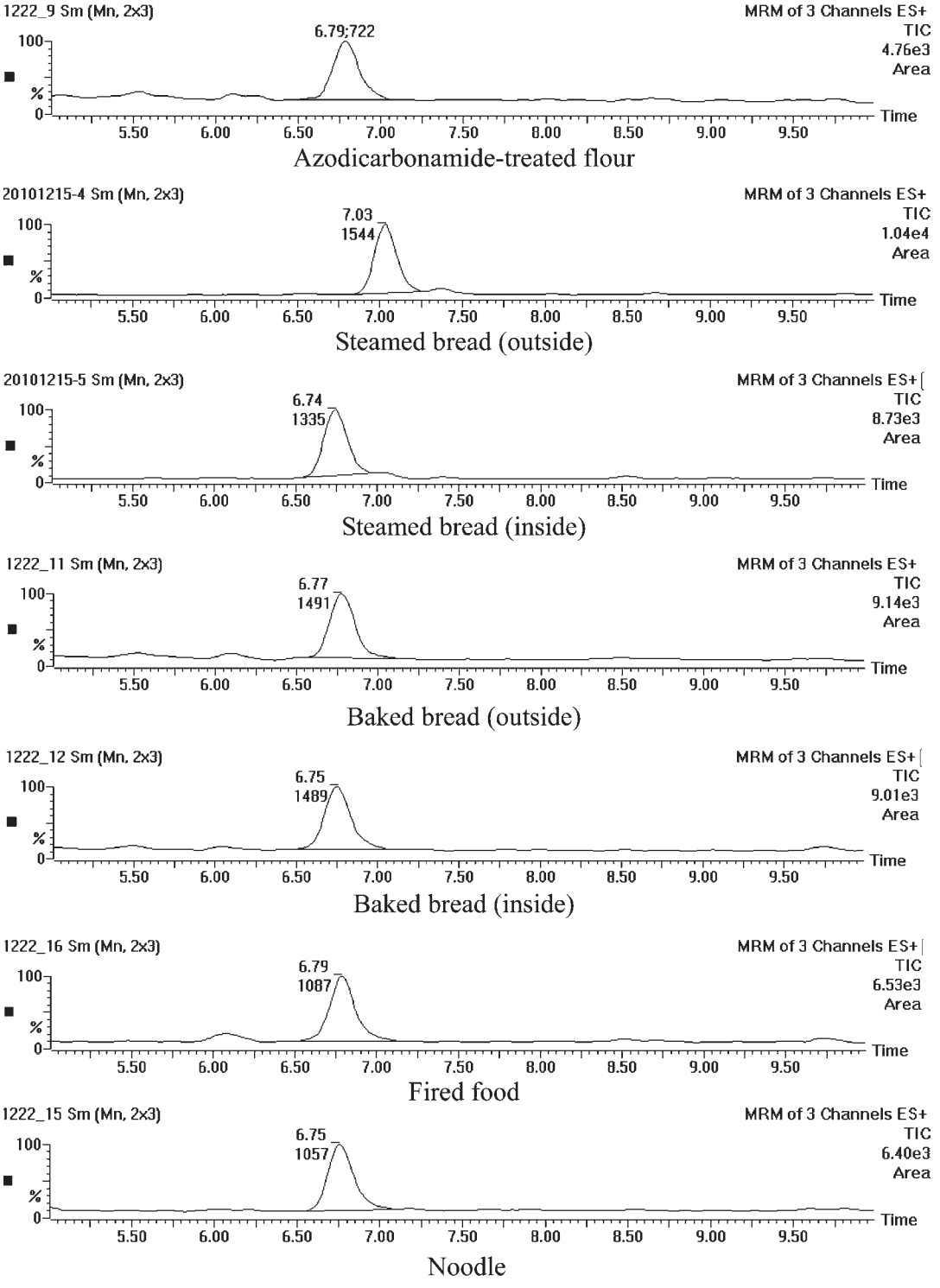

Figure 4. Concentration of SEM of flour and all kinds of flour products.

Formation Conditions of SEM. To test the hypothesis that the ADA present in the flour might decompose to SEM, four different group samples should be prepared. Group one: Do not add ADA to flour. Group two: Add ADA to flour but no treatment. Group three: Add ADA to flour, which has already been heated (60−70 °C). Group four: Add ADA to flour, which has already been wetted.

Statistical analysis of the data indicated that none of the blank flour samples and flour samples that did not dispose showed detectable levels of SEM (data are not shown), but flour samples that were heated and that were moist showed detectable levels of SEM (Figure 3), while it is clear from our findings that SEM is formed during the high-temperature heating process and wetting process.

Table 3. Different Levels of Labeling of SEM of All Kinds of Products and Recovery

				CV (%)	
flour products	spike level SEM (ng/mL)	concentration (ng/mL)	recovery (%)	intra-assay	interassay
steamed bread (outside)	2.5	20.6	85.50 ± 8.38	9.09	9.80
		20.2		11.02	
		20.9		9.50	
	5.0	22.1	78.37 ± 7.37	9.68	9.40
		21.9		10.17	
		22.6		8.51	
	7.5	23.9	77.77 ± 6.56	8.78	8.44
		23.8		8.95	
		24.7		7.69	
steamed bread (inside)	2.5	9.9	79.03 ± 6.01	8.29	7.60
		10.1		7.45	
		10.2		7.14	
	5.0	12.8	91.10 ± 4.19	4.42	4.60
		12.4		4.84	
		12.7		4.55	
	7.5	14.7	96.07 ± 8.00	9.08	8.33
		15.9		7.69	
		15.3		8.33	
baked bread (outside)	2.5	7.2	109.50 ± 8.31	7.15	7.59
		6.8		8.29	
		7.1		7.41	
	5.0	8.5	86.53 ± 3.09	3.66	3.57
		8.8		3.43	
		8.5		3.64	
	7.5	11.0	93.00 ± 4.03	4.52	4.34
		11.6		4.15	
		11.2		4.37	
baked bread (inside)	2.5	3.3	83.07 ± 6.41	7.63	7.72
		3.1		8.42	
		3.4		7.21	
	5.0	7.0	105.33 ± 10.91	9.36	10.36
		6.4		10.41	
		5.9		11.52	
	7.5	9.6	101.00 ± 10.07	8.96	9.97
		8.5		10.35	
		8.2		10.80	
fired food	2.5	7.9	104.47 ± 8.30	7.37	7.95
		7.5		8.64	
		7.7		7.94	
	5.0	9.1	80.97 ± 6.72	8.37	8.31
		9.5		7.64	
		8.8		9.01	
	7.5	11.8	82.23 ± 7.31	8.19	8.89
		11.3		8.84	
		10.7		9.79	
noodle	2.5	4.4	100.70 ± 10.39	9.93	10.32
		4		11.69	
		4.5		9.58	
	5.0	6.9	97.77 ± 8.21	8.04	8.40
		6.2		9.30	
		6.9		7.98	
	7.5	9.8	102.23 ± 4.72	4.21	4.61
		9.1		4.85	
		9.6		4.59	

It can be concluded, on the basis of the yield of SEM from biurea and earlier work showing the decomposition of ADA, that although ADA is the initial starting material, SEM formation occurs through a stable intermediate, biurea.[1] Namely, SEM is not formed as a byproduct of ADA decomposition to biurea, but it forms by the decomposition of biurea hydrolysis. Indeed, SEM is detected only after a high-temperature heating process and wetting process. In addition, when the concentration of the injected ADA is 20 mg/kg, the concentration of SEM is 0.012 mg/kg. While the concentration of the injected ADA is 30 mg/kg, the concentration of SEM is 0.022 mg/kg. Thus, the results show that the concentrations of SEM in the final product will be reduced if there are lower ADA concentrations in flour.

Variation of SEM in Flour and Flour Products. The method of LC-MS/MS for analysis of SEM developed in this research was patterned after earlier work in which the determination of total (bound and free) SEM was accomplished after acid hydrolysis

Table 4. Different Levels of SEM of All Kinds of Products That Do Not Spike

	steam bread		bake bread			
flour products	outside	inside	outside	inside	fired	noodle
level (ng/mL)	18.3	8.1	4.3	1.2	1.8	5.1

and derived by 2-nitrobenzaldehyde. The analysis was done by isotope dilution using (^{13}C, $^{15}N_2$)-SEM to increase the accuracy. The standard curve was linear in the range of 0−10 ng/mL with the quantification limit of 0.5 μg/kg (correlation coefficients >0.99).

According to the different processing techniques, flour had been processed into different flour products, including baked bread, steamed bread, fried food, and noodle. Different concentrations of standard solutions were added into the different flour samples and operated in accordance with the above method. It could be found that ADA in all four types of flour products converted to SEM due to high temperature and moisture. The concentration of SEM in all types of flour products is higher than that in flour, and the concentration of SEM in outside of flour products is slightly higher than that on the inside. The recovery rate was 72.4−116.5% with the relative standard deviations less than 11% (Figure 4 and Table 3). The nonspike sample for each case is shown in Table 4.

Further studies aimed at clarifying whether SEM is formed from ADA are needed, and the variations in flour and flour products have the regular pattern. In addition, more information on exposure to SEM from all sources would help to define the nature of what is now considered to be a low risk to human health.

The formation of SEM results from the decomposition of ADA. Therefore, the amount of ADA added in the flour can be monitored by measuring the concentration of SEM. The method of LC-MS is fast, simple, qualitative, quantitative, accurate, and sensitive. In addition, it suits the detection of SEM in flour and its products in the laboratory.

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