JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Semicarbazide Formation in Flour and Bread

GREGORY O. NOONAN,* TIMOTHY H. BEGLEY, AND GREGORY W. DIACHENKO

Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, 5100 Paint Branch Parkway, College Park, Maryland 20740

Azodicarbonamide, an approved food additive, is commonly used as a flour additive and dough conditioner in the United States and Canada. A number of researchers have clearly established a link between the use of azodicarbonamide and semicarbazide contamination in commercial bread products. However, all of these studies have primarily focused on the final baked product and have not extensively investigated the processing and conditions that affect the final semicarbazide levels. In this study, a previously developed method for measuring free semicarbazide in bread was applied to dough samples during the mixing and kneading process. Additionally, flour and bread samples were spiked with biurea or azodicarbonamide to help elucidate semicarbazide formation pathways. The results showed that semicarbazide was not formed as a byproduct of azodicarbonamide decomposition to biurea, which occurs upon the addition of water. Indeed, semicarbazide was not detected after room temperature or elevated temperature dough maturation, but only after baking. It was concluded that although azodicarbonamide is the initial starting material, semicarbazide formation in bread occurs through a stable intermediate, biurea.

KEYWORDS: Azodicarbonamide; semicarbazide; bread additive; formation

INTRODUCTION

In October 2003, two reports by the European Food Safety Authority (*1, 2*) implicated foamed polyvinylchloride (PVC) cap liners, manufactured with azodicarbonamide, as the source of semicarbazide contamination found in a variety of jarred foods. These reports prompted work by Stadler et al. (*3*), which established the PVC seals as a source of semicarbazide contamination by showing that semicarbazide was a byproduct of the thermal decomposition of azodicarbonamide. This work also reported that structurally related precursors, biurea and urazole, formed semicarbazide during thermal decomposition, but at very low yields (≤0.01%). Stadler's work was quickly followed by studies (*4, 5*) which reported the detection of semicarbazide in bread and flour and established that the use of azodicarbonamide, an approved food additive (*6, 7*), was the source of the semicarbazide. Later studies (*8, 9*) confirmed the link between azodicarbonamide use and semicarbazide in bread and reported detectable levels of semicarbazide in a variety of commercially available bread products.

During the development of an analytical method to extract and quantify only free semicarbazide from bread (*8*), an apparent inconsistency was noted between the initial azodicarbonamide characterization work reported by Joiner et al. (*10*) and the clear link between azodicarbonamide and semicarbazide contamination in bread (*5, 8, 9*). Joiner and co-workers (*10*) established that azodicarbonamide is stable in dry flour, but is reduced to biurea during oxidation of sulfhydryl groups upon the addition of water. They reported that at room temperature, 45 min after the addition of water, there was no azodicarbonamide detected in the dough (LOD = 0.1 mg/kg). However, numerous studies have established that the presence of semicarbazide in bread is a result of the addition of azodicarbonamide. One possible source of the semicarbazide contamination is from the thermal decomposition of biurea formed during dough mixing and kneading. However, even if semicarbazide yields are 10-fold higher than those reported by Stadler et al. (*3*), semicarbazide concentrations would not be expected to exceed low micrograms per kilogram levels in bread. Semicarbazide concentrations as high as 400 μg/kg have been reported in bread products prepared with 45 mg/kg of azodicarbonamide and as high as 1.2 mg/kg in commercial bread products (*8*). Pereira et al. (*4*) suggested a mechanism of biurea hydrolysis, but did not present any data supporting the reaction pathway. Other possible sources of semicarbazide, such as the use of azodicarbonamide-treated flour for dusting during baking (*5*), can be excluded as the primary source of contamination, because breads that had not been dusted prior to baking also showed significant semicarbazide concentrations (400 μg/kg) (*8, 9*).

The objective of this work was to gain further understanding into possible semicarbazide formation pathways, specifically, determining if semicarbazide is formed upon the addition of water and during the decomposition of azodicarbonamide to biurea during dough maturation or if semicarbazide formation occurs only at elevated temperatures. A thorough understanding of the formation pathway would be beneficial in identifying processing changes to reduce semicarbazide concentrations in the final product.

* Corresponding author (e-mail: gregory.noonan@fda.hhs.gov).

10.1021/jf073198g
Published on Web 02/27/2008

MATERIALS AND METHODS

Chemicals. Semicarbazide (99%) and *o*-nitrobenzaldehyde (98%) were obtained from Aldrich Chemical (St. Louis, MO) and used as received. Acetonitrile (J. T. Baker), ethyl acetate (Burdick and Jackson), and methanol (EMD) were all of HPLC grade or better and were used without further purification. Water (18 MΩ) was obtained from an Aqua Solutions (Jasper, GA) water purification system. Isotopically labeled semicarbazide, (^{13}C, $^{15}N_2$), with a chemical purity of 99% and an isotopic purity of 97%, was purchased from Witega Laboratory (Berlin, Germany). Biurea (Pfaltz & Bauer, Waterbury, CT) and azodicarbonamide (Aldrich Chemical, St. Louis, MO) were recrystallized from hot water three and two times, respectively, prior to use. Analysis of recrystallized biurea and azodicarbonamide for semicarbazide, using the direct method reported by Stadler et al. (*3*), showed no detectable semicarbazide contamination (<100 ng/mg).

Flour and Bread Samples. All of the ingredients were purchased at a local grocery store and stored under the manufacturer's recommended conditions. Dough and bread loaves were prepared using a commercially available bread machine (Welbilt, Garden City, NY) and the standard white bread recipe (flour, water, yeast, butter, sugar, nonfat dry milk, salt). To produce spiked flour samples, recrystallized azodicarbonamide or recrystallized biurea was added to flour and mixed on an end-over-end mechanical mixer (Blend Master, East Strousburg, PA) for at least 1 h to homogenize the sample. The azodicarbonamide-spiked flour was used in flour heating studies and to make azodicarbonamide-spiked bread. The biurea flour was used for wet and dry flour heating studies. For bread studies, biurea was dissolved in the water used for bread production prior to addition to the bread machine. Loaves without any additional additives were prepared and used as blanks.

Equipment and Materials. Solid phase extraction columns (Bond Elut, 3 mL/500 mg C18) were purchased from Varian (Lake Forest, CA) and rinsed with 3 mL of methanol followed by 3 mL of water prior to use. Polypropylene centrifuge tubes obtained from Corning (Corning, NY) were used for all extractions, whereas glass scintillation vials, wrapped in foil, were used for semicarbazide derivatization reactions. Syringe filters, used to filter bread extract (0.45 μm, Nylon) and final extract (0.20 μm, Nylon), were purchased from Titan (Wilmington, NC). Heating experiments were performed in a convection oven (Linberg/Blue M, Ashville, NC) at 200 °C. A Glas-Col end-over-end tissue culture rotator with a 39 cm diameter turning wheel obtained from VWR International (Bridgeport, NJ) was used for sample extraction. Centrifugation was performed on a Marathon 21000R centrifuge from Fischer Scientific (Pittsburgh, PA).

Instrumental Conditions. Sample extracts (20 μL) were analyzed using an Agilent 1100 LC-MSD (Agilent Technologies, Palo Alto, CA) with a Zorbax Extend C18 column (3.0 × 250 mm, 5 μm, Agilent Technologies). The mobile phase consisted of water with 0.025% acetic acid (A) and acetonitrile (B) at a constant flow rate of 0.3 mL/min. The gradient profile started with 25% acetonitrile and increased to 50% acetonitrile over 5 min. This mobile phase was maintained for 5 min and then increased to 75% acetonitrile for another 3 min. This gradient profile gave acceptable resolution and reduced sample carry-over. The column temperature was maintained at 30 °C throughout the analysis.

Atmospheric pressure chemical ionization (APCI) in positive ion mode was used as the ionization source. Drying gas (N_2) flow and temperature were 4 L/min and 275 °C, respectively. Vaporizer gas (N_2) pressure and temperature were 60 psi and 350 °C, respectively. Selected ion monitoring (SIM) at *m*/*z* 209 for the $(M + H)^+$ of semicarbazone and *m*/*z* 212 for the isotopically labeled carbazone was used. Also collected were a SIM of *m*/*z* 192, a $[(M + H) - NH_3]^+$ fragment, a UV response ($\lambda = 270$ nm), and a total ion scan (*m*/*z* 50–500).

Calibration Standards. A stock solution of (^{13}C, $^{15}N_2$)-semicarbazide was prepared by dissolving 4 mg in 100 mL of water. A 5.0 mL aliquot was diluted to 50 mL to prepare a working stock of 4 μg/mL. An unlabeled semicarbazide stock solution in water was prepared at 0.05 mg/mL. A 1.0 mL aliquot was diluted to 50 mL to prepare a working stock solution of 1 μg/mL. Calibration standards were prepared by spiking semicarbazide (0−100 ng/mL) and (^{13}C, $^{15}N_2$)-semicarbazide (25 ng/mL) into water and performing the *o*-nitrobenzaldehyde derivatization. Calibration curves (area ratio vs mass ratio) were prepared and used to determine the relative response factor and to quantitate the semicarbazide concentration in unknowns.

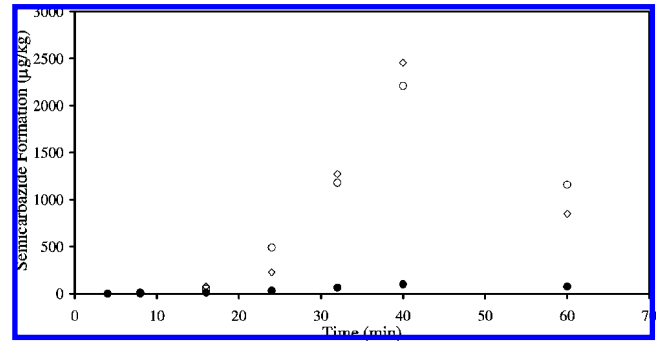

Figure 1. Measurement of semicarbazide concentrations in spiked flour heated at 200 °C: dry azodicarbonamide-spiked flour (◇); dry biurea-spiked flour (●); wet biurea-spiked flour (○). Points represent an average of three repetitions, with RSDs of ≤10%.

Semicarbazide Extraction Method. Flour, dough, and bread were processed using a procedure similar to the method previously described (*8*). Briefly, samples (2.5 g) were placed in 50 mL centrifuge tubes and spiked with (^{13}C, $^{15}N_2$)-semicarbazide (200 μL of 4.0 μg/mL) prior to the addition of water (25 mL). The samples were extracted for 0.5 h at room temperature by constant end-over-end mixing (40 rpm or 0.03 G force). Following extraction, the samples were centrifuged at 4000 rcf for 5–10 min, and an aliquot (~13 mL) of the supernatant was decanted, filtered (0.45 μm, nylon), and passed through a C18 SPE cartridge. The first 2 mL was discarded and the remaining eluate captured. The eluate (10 mL) was diluted with 10 mL of phosphate buffer (0.2 M, pH 3.5), and 200 μL of *o*-nitrobenzaldehyde (3 mg/mL in methanol) was added. The reaction mixture was protected from light and heated at 37 °C for 1 h. The derivative was loaded onto a SPE cartridge, rinsed with 3 mL of water, and eluted with 2 mL of ethyl acetate. The ethyl acetate was blown to dryness under helium, and the residue was reconstituted in 0.5 mL of acetonitrile and 1.5 mL of water before filtration (0.2 μm, nylon) into amber vials. Final extracts were refrigerated (≤4 °C) until analysis.

RESULTS AND DISCUSSION

Semicarbazide Formation during Heating. Biurea- and azodicarbonamide-spiked and unspiked (blank) flours were placed in test tubes and heated at 200 °C in a convection oven. A beaker filled with sand was used to hold the test tubes, ensure good thermal contact, and minimize temperature fluctuations during sample loading and removal. Test tubes were removed from the oven at various times and immediately immersed in an ice bath. Once cooled, the samples were extracted and analyzed using the procedure described above.

None of the blank flour samples showed detectable levels of semicarbazide (data not shown). Flours spiked with azodicarbonamide (◇) and biurea (●) initially showed an increase in semicarbazide with increasing time (**Figure 1**). However, semicarbazide concentration decreased from the 40 to 60 min heating times. The decrease is attributed to the decomposition of semicarbazide at long heating times, and a similar trend was reported by Stadler et al. (*3*) in their analysis of semicarbazide formation from thermal decomposition of azodicarbonamide, biurea, and urazole. Becalski et al. (*5*) also reported lower semicarbazide concentrations at higher temperatures (>200 °C) for azodicarbonamide-spiked flour.

The concentration of semicarbazide formed by heating flour for 40 min (2500 mg/kg) is significantly higher than the semicarbazide concentrations previously reported for breads (*8*) or heated flour (*5*). However, the semicarbazide concentrations reported at longer times are consistent with previously reported

Table 1. Semicarbazide Concentration Determined in Dough and Bread during Processing and Baking at 200 °C

	semicarbazide conc (μg/kg of bread)						
bread	mix	rise 1	rise 2	bread	RSD ($n = 5$) (%)	yield (%)	yield[a] (% in oil)
azodicarbonamide (45 mg/kg of flour)	<5	<5	<5	400	6	1.4	0.08
biurea (45 mg/kg of flour)	<5	<5	<5	300	10	1.1	0.01
blank	<5	<5	<5	<5		NA	NA

[a] Calculated from experimental parameters and results in Stadler et al. (*3*).

results. Although the semicarbazide formation trends are comparable between the azodicarbonamide- and biurea-spiked flours, the amount of semicarbazide formed under dry flour conditions is significantly different between the two additives. The azodicarbonamide-spiked flour showed semicarbazide concentrations approximately 25 times higher than the levels formed in biurea-spiked flour (100 μg/kg). Both flours were spiked at 45 mg/kg with the respective additive. The difference in semicarbazide formation is consistent, but larger, than the differences reported by Stadler et al. (*3*). They reported a >6-fold difference in semicarbazide formation between azodicarbonamide and biurea. Those experiments were performed with 50 mg of material suspended in vegetable oil (no water), so a direct quantitative comparison is not appropriate.

Along with dry flour heating experiments, water (14.7 mL) was added to biurea -spiked flour (20 g), and the wet flour was loaded into test tubes and heated at 200 °C. The flour to water ratio used was similar to the ratio used when making bread. The wet biurea-spiked flours (○) showed the same time-dependent semicarbazide formation trend noted for the dry flours (**Figure 1**) and showed semicarbazide formation levels (2200 μg/kg) comparable to those shown by azodicarbonamide-spiked flour. Again, these concentrations in wet biurea-spiked flours were approximately 22 times greater than those in the dry biurea-spiked flour. The high yield of semicarbazide from wet biurea-spiked flour reconcile the findings of Stadler et al. (*3*) and the clear link between azodicarbonamide use and semicarbazide concentrations in bread. Additionally, these results are consistent with the theory, postulated by Pereira et al. (*4*), that semicarbazide can form through the hydrolysis of biurea.

Semicarbazide Formation during Bread Processing. To further elucidate the pathway of semicarbazide formation, bread was made using blank, biurea-spiked (45 mg/kg), and azodicarbonamide-spiked (45 mg/kg) flour. Samples collected after initial mixing, first rise, second rise, and baking were analyzed for semicarbazide (**Table 1**). The first rise occurs at room temperature, whereas the second rise, just prior to baking, uses slightly elevated temperatures (50 °C) to condition the dough. Consistent with previous findings, the blank bread did not show detectable levels of semicarbazide at any of the sampling points. Bread dough produced with azodicarbonamide- and biurea-spiked flour showed comparable results, with no detectable semicarbazide after the initial mixing or after either the first or second rise. These results for bread dough are comparable to other azodicarbonamide-spiked flour results previously reported. Pereira et al. (*4*) and Becalski et al. (*5*) report low concentrations (<5 μg/kg) of semicarbazide in raw (unheated) azodicarbonamide-spiked flour. Periera et al. and Becalski et al. used HCl as the extraction solvent, leading to the detection of bound and free semicarbazide. However, we used only water in our extraction procedure, limiting our recovery to free semicarbazide and avoiding the potential production of semicarbazide from structurally related precursors. Additionally, we took great care in the purification of our azodicarbonamide and biurea, using up to three recrystallizations from hot water and finding no detectable levels of semicarbazide in our purified reagents. Becalski et al. (*5*) provided unheated flour results that are from commercial flours with azodicarbonamide added, and it is unclear if the semicarbazide reported is a contaminant of azodicarbonamide used in flour treatment. In either case, the amount of semicarbazide detected is extremely small compared to values reported for finished breads (*8, 9*). On the basis of our mixing and dough rise data, semicarbazide is not produced in significant quantities by the reduction of azodicarbonamide through the addition of water.

Bread baked with spiked flour containing biurea or azodicarbonamide showed comparable levels of semicarbazide after baking (**Table 1**). The semicarbazide concentrations for both breads are comparable to the levels previously reported for bread machine produced breads (*8*) and within the range of semicarbazide concentrations reported for commercial bread products (*5, 8*). Any small differences in the semicarbazide concentrations between the azodicarbonamide- and biurea-spiked flours were attributed to differences in bread morphology noted between azodicarbonamide- and biurea-spiked flour. Breads produced from azodicarbonamide-spiked flour consistently showed greater loaf volumes (approximately 30%) and more consistent morphology than those produced from biurea-spiked flour. Indeed, all of the breads containing azodicarbonamide contacted the lid of the bread machine during rising and baking., whereas those made with biurea flour gave denser loaves and remained within the confines of the bread machine.

Factors Leading to Semicarbazide Formation. The elucidation of the semicarbazide formation pathway presented above was the first step in determining which, if any, processing conditions could be altered to help reduce the amount of semicarbazide in the final product. Canas et al. (*11*) have reported that the ethyl carbamate concentrations are greatly influenced by the level of azodicarbonamide used in bread production. Indeed, they have shown that ethyl carbamate concentrations decrease sharply if <20 mg/kg of azodicarbonamide is used in flour treatment. On the basis of these results we prepared bread using flour spiked with different concentrations of azodicarbonamide (0−45 mg/kg), and the final products were analyzed for semicarbazide (**Figure 2**). Even at our lowest spiking level (5 mg/kg), semicarbazide was detected in the final product. In fact, there was a direct and linear correlation ($r^2 = 0.997$) between the level of azodicarbonamide used in flour and the semicarbazide concentrations determined in the bread. Although there is not a concentration of azodicarbonamide that can be used without producing semicarbazide, these data establish that semicarbazide levels can be reduced by reducing the azodicarbonamide starting concentration. In addition to azodicarbonamide starting concentration, the affect of baking time (0–30 min) on semicarbazide formation was briefly evaluated. Similar to the wet and dry flour experiments, an increase in semicarbazide concentrations with increasing baking times was noted from 0 to 30 min. Further studies were not performed, because it was noted that baking times had a dramatic impact on bread quality. Therefore, changes in the

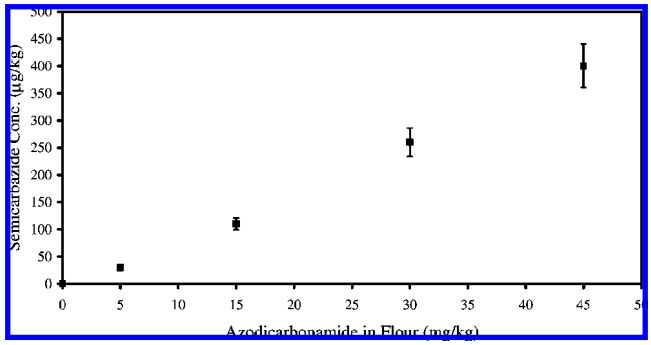

Figure 2. Measurement of semicarbazide formed in bread baked from flour treated with increasing concentrations of azodicarbonamide.

baking time would not be a viable option for reducing semicarbazide formation.

It is clear from our findings that semicarbazide is formed during the high-temperature baking process and not as a byproduct in the decomposition of azodicarbonamide to biurea upon the addition of water. The results also show that in the high-moisture−high-temperature environment necessary for baking bread, biurea decomposes to semicarbazide at similar yields noted for azodicarbonamide under dry conditions. It can be concluded, on the basis of the yield of semicarbazide from biurea and earlier work by Joiner et al. (*10*) showing the decomposition of azodicarbonamide upon the addition of water, that although azodicarbonamide is the initial starting material, semicarbazide formation occurs through a stable intermediate, biurea. These findings reconcile the apparent inconsistency between the low semicarbazide yields from the thermal decomposition of biurea reported by Stadler et al. (*3*) and the work by Joiner et al. (*10*) showing the decomposition of azodicarbonamide upon the addition of water. Finally, the results show that lowering azodicarbonamide concentrations in flour will reduce the concentrations of semicarbazide in the final product. A reduction to <20 mg/kg would be especially beneficial, because ethyl carbamate formation (*11*) is also reduced at this concentration. The addition of other processing aids (e.g., ascorbic acid) and the use of various baking processes have not been studied but may also affect the amount of semicarbazide formed during baking and warrant further investigation.

LITERATURE CITED

(1) European Food Safety Authority Document, "Additional advice on semicarbazide, in particular related to baby food ad hoc expert group meeting 9 October 2003" http://www.efsa.eu.int/science/afc/afc_documents/catindex_en.html, accessed Oct 14, 2003.

(2) European Food Safety Authority Document, "Advice of the ad hoc expert group set up to advise the EFSA on the possible occurrence of semicarbazide in packaged foods - 28 July 2003" http://www.efsa.eu.int/science/afc/afc_documents/catindex_en.html, accessed Aug 15, 2003.

(3) Stadler, R. H.; Mottier, P.; Guy, P.; Gremaud, E.; Varga, N.; Lalljie, S.; Whitaker, R.; Kintscher, J.; Dudler, V.; Read, W. A.; Castle, L. Semicarbazide is a minor thermal decomposition product of azodicarbonamide used in the gaskets of certain food jars. *Analyst* **2004**, *129*, 276–281.

(4) Pereira, A. S.; Donato, J. L.; DeNucci, G. Implications of the use of semicarbazide as a metabolic target of nitrofurazone contaminants in coated products. *Food Addit. Contam.* **2004**, *21*, 63–69.

(5) Becalski, A.; Lau, B. P.-Y.; Lewis, D.; Seaman, S. W. Semicarbazide formation in azodicarbonamide-treated flour: A model study. *J. Agric. Food Chem.* **2004**, *52*, 5730–5734.

(6) Requirements for Specific Standardized Bakery Products, Code of Federal Regulations, Part 136.110, Title 21, 2004.

(7) Cereal Flours and Related Products, Code of Federal Regulations, Part 137.105 and 137.200, Title 21, 2004.

(8) Noonan, G. O.; Warner, C. R.; Hsu, W.; Begley, T. H.; Perfetti, G. A.; Diachenko, G. W. The determination of semicarbazide (*N*-aminourea) in commercial bread products by liquid chromatography−mass spectrometry. *J. Agric. Food Chem.* **2005**, *53*, 4680–4685.

(9) Becalski, A.; Lau, B. P.-Y.; Lewis, D.; Seaman, S. Semicarbazide in Canadian bakery products. *Food Addit. Contam.* **2006**, *23*, 107–109.

(10) Joiner, R. R.; Vidal, F. D.; Marks, H. C. A new powdered agent for flour maturing. *Cereal Chem.* **1963**, *40*, 539–553.

(11) Cañas, B. J.; Diachenko, G. W.; Nyman, P. J. Ethyl carbamate levels resulting from azodicarbonamide use in bread. *Food Addit. Contam.* **1997**, *14*, 89–94.

Received for review November 1, 2007. Revised manuscript received January 17, 2008. Accepted January 18, 2008.

JF073198G