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The Determination of Semicarbazide (N-Aminourea) in Commercial Bread Products by Liquid Chromatography−Mass Spectrometry

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Recently, semicarbazide has been found in food in jars sealed with cap liners that were manufactured using azodicarbonamide as a blowing agent. These reports raised the concern that the use of azodicarbonamide—an approved dough conditioner—may result in semicarbazide residues in bread. To answer this question, a method based upon the previously reported liquid chromatography/tandem mass spectrometry determination of the semicarbazone of *o*-nitrobenzaldehyde was utilized. The method adopted for this work includes an extensive cleanup and reaction with *o*-nitrobenzaldehyde at pH 3.5, rather than with the widely used 0.1 M HCl, to form the semicarbazone derivative. A stable isotope dilution assay was used to determine the free semicarbazide present in the bread products. Levels of semicarbazide ranged from 10 to 1200 ppb in commercial bread products with azodicarbonamide listed among their ingredients.

KEYWORDS: Azodicarbonamide; semicarbazide; method development; bread additive; LC-MS

INTRODUCTION

In the United States, azodicarbonamide is permitted as a flour additive (*1*) and dough conditioner (*2*), as well as a blowing agent in the production of foamed polymer gaskets for food contact materials (*3*−*5*). Recently, foamed poly(vinyl chloride) (PVC) cap liners manufactured with azodicarbonamide have been implicated as the source of semicarbazide found in food in jars sealed with these liners (*6*, *7*). It was shown that semicarbazide is one of the products formed by the thermal decomposition of azodicarbonamide (*8*). These studies have raised international concerns about the use of azodicarbonamide in food contact materials because semicarbazide has been reported to be a weak animal carcinogen (*9*−*11*). Effective August 2005, the European Union will no longer permit the use of azodicarbonamide as a blowing agent in food contact materials (*12*). This work on jar lid gaskets also raised questions about the formation of semicarbazide residues in commercial bread products manufactured with azodicarbonamide, which is the focus of this paper.

Leitner et al. (*13*) previously developed a method for analyzing semicarbazide in food producing animals for the purpose of obtaining evidence of illegal usage of nitrofuran antibiotics. In the Leitner et al. (*13*) method, the sample is treated with 0.1 M HCl at 37 °C for 16 h with added *o*-nitrobenzaldehyde. The lengthy acid extraction step is used to hydrolyze tissue-bound semicarbazide, while the added *o*-nitrobenzaldehyde displaces the equilibria by capturing the semicarbazide as the semicarbazone product (*14*). This derivatization improves chromatographic separation and detection of semicarbazide (*14*, *15*).

Recently, Pereira et al. (*16*) and Becalski et al. (*17*), in studies limited to bench scale experimentation, applied the principles of the Leitner et al. (*13*) method to determine the levels of semicarbazide produced from azodicarbonamide-treated flour. Pereira et al. (*16*) have found semicarbazide in azodicarbonamide-treated flour used for breading chicken and showed that spiking flour with azodicarbonamide led to the production of semicarbazide in flour samples. Becalski et al. (*17*) reported that semicarbazide was found in heated dry and wet azodicarbonamide-treated flour and in bread that they prepared with azodicarbonamide-treated flour. Using methods similar to the Leitner et al. (*13*) method, Pereira et al. (*16*) and Becalski et al. (*17*) obtained results that reflect a combination of "free semicarbazide", hydrolyzed "bound semicarbazide", and possibly semicarbazide formed during extraction from structurally related precursors (other azodicarbonamide decomposition products) (*8*, *16*).

Our objective was to determine the concentration of semicarbazide in commercial bread products as they are offered for consumption. This was the first step in a risk analysis relevant to the use of azodicarbonamide in bread manufacture. Because little is known about the toxic effects or bioavailability of chemically combined forms of semicarbazide, we chose to limit this work to the quantitation of free semicarbazide in bread products rather than obtain results that were the sum of free and chemically combined forms of semicarbazide. Free semi-

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carbazide in the sample was determined by liquid chromatography−mass spectrometry (LC-MS) with stable isotope dilution analysis (SIDA) using (^{13}C,$^{15}N_2$)-semicarbazide. To minimize the hydrolysis of "bound semicarbazide" and to avoid the possible formation of semicarbazide from structurally related precursors, the extraction procedure utilized only deionized water. The water extraction was studied to ensure that the added isotope [(^{13}C,$^{15}N_2$)-semicarbazide] and the semicarbazide in the sample freely exchanged, i.e., the ratio of endogenous semicarbazide to (^{13}C,$^{15}N_2$)-semicarbazide quickly reached a constant value for each sample. Derivative formation of semicarbazide with *o*-nitrobenzaldehyde was carried out at pH 3.5 at 37 °C for 1 h. The extraction and derivative formation were performed under comparatively mild conditions, when contrasted with the conditions used in previous semicarbazide studies (*16*, *17*) that involved treatment of the test portion for 16 h at 37 °C with 0.1 M HCl. The procedure was used to analyze a number of commercial products as well as bread baked in our laboratory.

MATERIALS AND METHODS

Chemicals. Semicarbazide (99%), azodicarbonamide (97%), and *o*-nitrobenzaldehyde (98%) were obtained from Aldrich Chemical (St. Louis, MO) and used as received. Analysis of azodicarbonamide for semicarbazide, using the direct method (*8*), showed no detectable semicarbazide contamination (<100 ng semicarbazide/mg azodicarbonamide). Acetonitrile (JT Baker), ethyl acetate (Burdick and Jackson), and methanol (EMD) were all high-performance liquid chromatography 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).

Food Samples. A variety of commercial products were purchased from local food stores. The products included white, wheat, and multigrain breads as well as white sandwich rolls and hamburger buns. All of the breads were presliced, and all but two of the products listed azodicarbonamide as an ingredient. In addition to commercial products, loaves of white bread were prepared using a commercially available bread machine (Welbilt, Garden City, NY). One loaf with azodicarbonamide (45 mg/kg flour) and one with semicarbazide (5.1 mg/kg bread) were prepared using the standard white bread recipe (flour, water, yeast, butter, sugar, and nonfat dry milk). To ensure uniform distribution, azodicarbonamide was added as a finely divided suspension in water and the hydrochloride salt of semicarbazide was dissolved in water prior to addition to the other ingredients. A loaf without any additional additives was prepared and used as a blank.

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, while 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 (0.22 μm, PTFE), were purchased from Titan (Wilmington, NC) and Pall (East Hills, NY), respectively.

Instrumental Conditions. Bread extracts (20 μL) were analyzed using an Agilent 1100 LC-MSD with a Zorbax Extend C18 column (3.0 mm × 250 mm, 5 μm, Agilent). 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 carryover. The column temperature was maintained at 30 °C throughout the analysis.

Atmospheric pressure chemical ionization in the negative ion mode was used as the ionization source. The drying gas (N_2) flow and temperature were 4 L/min and 275 °C, respectively. The vaporizer gas (N_2) pressure and temperature were 60 psig and 350 °C. Selected ion monitoring (SIM) at *m*/*z* 209 for the M − H of semicarbazone and *m*/*z* 212 for the isotopically labeled carbazone were used. Also collected were a SIM of *m*/*z* 192, a M − NH_3 fragment, a UV response (λ = 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. Four sets of calibration standards were prepared by different analysts 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 concentrations in unknowns. At least one of four sets of calibration standards was analyzed with each run to give over 20 sets of calibration data. The overall regression line for all of the calibration data gave a relative response factor of 1.02 and a standard error of 5%.

Semicarbazide Extraction Method. Bread slices, rolls, and buns were cut into small pieces and then reduced to a free flowing coarse meal with a food processor. Water (40 mL) and (^{13}C,$^{15}N_2$)-semicarbazide (200 μL of 4.0 μg/mL) were mixed with ground bread (4 g) in a 50 mL centrifuge tube. The bread was extracted for 0.5 h at room temperature by constant end over end mixing (40 rpm). Following mixing, the samples were centrifuged at 4000 rcf for 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 milliliter was discarded, and the remaining eluate was 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, PTFE) into amber vials. The final extracts were stored at <4 °C until analysis.

HCl Extraction. The bread was spiked with internal standard and extracted with water as described above in Semicarbazide Extraction Method. The filtered supernatant (13 mL) was then acidified with concentrated HCl (130 μL) and heated to 37 °C for 16 h. After it was heated, the sample was allowed to cool and the pH was adjusted to between 4 and 6 with NaOH. The sample was then carried through the derivatization and SPE procedure described above. This HCl extraction method was compared, using four commercial bread products, with an overnight extraction/derivatization similar to that reported by Leitner et al. (*13*) and found to be equivalent.

RESULTS AND DISCUSSION

Evaluation of *o*-Nitrobenzaldehyde Reaction Conditions. Johansen and Edlund (*18*) reported that the rate of semicarbazone formation of pyridoxal-5′-phosphate reached a maximum at ca. pH 3.5. Upon the basis of this finding, we compared the formation of semicarbazone from the reaction of semicarbazide (200 μg/L) and *o*-nitrobenzaldehyde (3 mg/mL) in phosphate buffer (0.1 M) at pH 6.0, phosphate buffer at pH 3.5, and HCl (0.1 M, pH 1.2). **Figure 1** shows the LC-MS peak areas (normalized) for semicarbazone in the different media as a function of time. The reaction is fastest at pH 3.5, reaching completion in 0.5 h. The reaction in HCl reaches completion in approximately 1 h, while in phosphate buffer at pH 6.0 it is slower still, reaching completion in ≥19 h. Our results for the effect of pH on the rate of semicarbazone formation are consistent with those of Johansen and Edlund (*18*) and Talwar et al. (*19*).

At 19 h, the reactions run in phosphate buffers (pH 3.5 and 6.0) show comparable peak areas, which are nearly double the

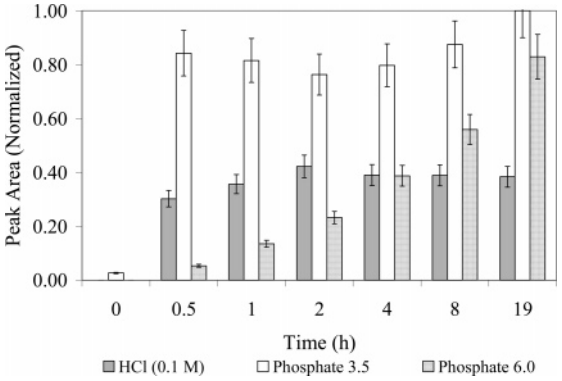

Figure 1. Derivatization of semicarbazide with *o*-nitrobenzaldehyde under different reaction conditions. Error bars represent the 95% confidence interval determined by Student's *t*-test.

peak area for the reaction run in HCl. This increased product yield is consistent with the results reported by Talwar et al. (*19*), who observed a maximum yield when the pH of the reaction mixture was held between 2 and 4. We have considered and evaluated two possibilities for the lower semicarbazone yield in HCl.

Conceivably, the lower response in HCl was simply an LC-MS ionization artifact due to matrix effects and not truly indicative of a lower yield. To address this possibility, four solutions (HCl, phosphate at pH 3.5 and 6.0, and water) were prepared with identical concentrations of the semicarbazone of *o*-nitrobenzaldehyde. All of the solutions gave equivalent results, with a relative standard deviation (RSD) of 1.0%, demonstrating that the composition of the derivatizing solution did not affect MS response.

We also considered the possibility that semicarbazide decomposes in the presence of HCl at 37 °C. A solution of semicarbazide in HCl was prepared and an aliquot immediately diluted 10-fold in phosphate buffer (pH 3.5). The remaining HCl solution was held at 37 °C for 1 h. Two separate aliquots of the remaining HCl solution were treated as follows: One was diluted 10-fold with phosphate buffer (pH 3.5), and the second one was diluted 10-fold with HCl (0.1 M). A control solution of semicarbazide in phosphate buffer (pH 3.5) was also prepared. *o*-Nitrobenzaldehyde was added to all four solutions, and the reaction was carried out at 37 °C for 1 h. The three solutions that had been derivatized in phosphate buffer (pH 3.5) showed comparable peak areas (3.35×10^5, 3.06×10^5, and 3.13×10^5): therefore, the heating in HCl had no effect on the final yield of semicarbazone. However, the sample that was derivatized in HCl showed a peak area of roughly half (1.35×10^5) the other samples. These data are consistent with our initial results and establish that the lower peak area is not due to degradation of semicarbazide but to a decreased yield of semicarbazone under acidic conditions. These results demonstrate that the production of semicarbazone is more favorable using a phosphate buffer at pH 3.5 than using 0.1 M HCl as the reaction medium. On the basis of these studies, it was concluded that the best estimate of semicarbazide concentration would be obtained by using a phosphate buffer at pH 3.5 as the *o*-nitrobenzaldehyde reaction medium.

Evaluation of Extraction Conditions. Four commercial bread products with azodicarbonamide listed as an ingredient and a negative control bread (bread machine) were extracted using water (Semicarbazide Extraction Method) and HCl (HCl Extraction Method). For the HCl extractions, the samples were extracted at 37 °C for 16, 24, and 46 h, with one bread also being extracted for 96 h. The semicarbazide concentrations

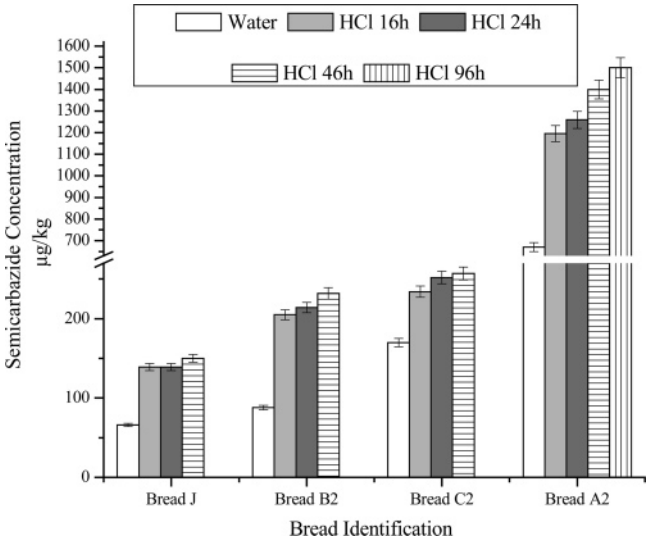

Figure 2. Long-term HCl extraction of semicarbazide from bread. Error bars represent the 95% confidence interval determined by Student's *t*-test.

measured in the four commercial products at each extraction condition are shown in **Figure 2**. No detectable levels of semicarbazide were observed for the control bread under any of the extraction conditions (data not shown). For all of the commercial products, the HCl extraction yielded higher semicarbazide concentrations than the water extraction. Additionally, **Figure 2** also shows that an increased HCl extraction time resulted in a statistically significant increase in semicarbazide concentration. These results are consistent with data previously reported for the analysis of tissue samples for metabolites from furazolidone residues (*14*, *20*, *21*) and led us to conclude that our observed increase in semicarbazide concentration with the addition of HCl arises from the hydrolysis of bound semicarbazide moieties.

Extraction Efficiency. SIDA is an excellent technique that compensates for analyte losses due to side reactions and adsorption of the analyte on the food matrix or glassware; however, the accuracy of the method requires that the endogenous semicarbazide and the added ($^{13}C,^{15}N_2$)-semicarbazide exhibit identical partitioning between the added solvent and the food matrix. Water was selected as the extracting solvent to minimize the hydrolysis of bound semicarbazide. With a pK_a of 3.53 (*22*), semicarbazide would be present predominantly as the ionic conjugate acid at the pH values of aqueous bread slurries; therefore, equilibration of the partitioning between the aqueous extract and the insoluble bread components should be readily achieved.

The efficiency of a single 0.5 h water extraction was tested by performing a "double extraction" on three commercial bread products with manufacturing-incurred semicarbazide residues. The products were extracted with added ($^{13}C,^{15}N_2$)-semicarbazide exactly as described in the Semicarbazide Extraction Method. After centrifugation, the supernatant was decanted and the wetted bread pellet was retained. The decanted water was replaced with deionized water, which contained the internal standard, and the extraction procedure was repeated. The first and second extracts were then processed and analyzed separately. The results shown in **Table 1** demonstrate that for the products tested nearly all (≥97%) of the free semicarbazide is captured with a single 0.5 h water extraction.

Extraction efficiencies were studied further with a loaf of bread that was baked with semicarbazide as an added ingredient, which did not include azodicarbonamide as a dough conditioner.

Table 1. Evaluation of the Efficiency of a Single 0.5 h Aqueous Extraction

sample	semicarbazide in 1st extraction (μg)	semicarbazide in 2nd extract[a] (μg)	% in 1st extraction
C2	0.61	<0.04	>99
D	0.96	<0.04	>99
A1	7.0	0.18	97

[a] Corrected for semicarbazide remaining in retained water in the bread following the first extraction.

Table 2. Comparison of Multiple Methods for the Extraction of Semicarbazide from Semicarbazide Spiked Bread

extraction method	semicarbazide spike (mg/kg)	semicarbazide measured (mg/kg)	recovery (%)
0.5 h at room temp	5.1	4.0	79
sonication	5.1	3.8	75
0.5 h at 37 °C	5.1	4.2	82
1 h at 37 °C	5.1	4.0	79
2 h at 37 °C	5.1	4.1	80
0.1 M HCl for 16 h	5.1	5.3	104

This provided a product that mimics manufacturing-incurred semicarbazide but with a known upper limit of semicarbazide (5.1 mg/kg). The semicarbazide-containing bread was extracted with water under various conditions: 0.5 h, 0.5 h with sonication, and heating to 37 °C for 0.5, 1, and 2 h. For comparison, HCl extractions for 16 h were also performed. All water extractions showed comparable results, yielding a value of approximately 80% of the semicarbazide added before baking (**Table 2**). These results establish that sonication, heating, or extended extraction times are not required to achieve total partitioning of added ($^{13}C,^{15}N_2$)-semicarbazide and endogenous semicarbazide. The ratio of endogenous semicarbazide to ($^{13}C,^{15}N_2$)-semicarbazide would be expected to increase with time, temperature, and sonication if steadily increasing amounts of encapsulated free semicarbazide partitioned into the aqueous phase. The absence of an increase with these more rigorous extraction conditions indicates that the isolation of some portion of the endogenous semicarbazide from the extracting solvent by encapsulation in the bread matrix is not a significant factor and that a simple 0.5 h water extraction is adequate. The HCl extraction method showed an increase in the recovery of the added semicarbazide (100%, within error) over the water extraction. As stated previously, the higher values found with the HCl extraction are attributed to the hydrolysis of bound semicarbazide moieties. These results are consistent with the bread extraction data presented above and with studies performed on animal tissue samples (*14*, *20*, *21*).

Spike Recovery. Commercial bread "I", which did not have azodicarbonamide listed as an ingredient, was used as the matrix for semicarbazide recovery experiments. Semicarbazide was spiked into 4 g samples of bread at three different levels (**Table 3**). Water (40 mL) and ($^{13}C,^{15}N_2$)-semicarbazide (0.99 μg) were added, and the samples were extracted and derivatized using the semicarbazide extraction method described previously. **Table 3** shows the average percent recovery and standard deviation ($n = 4$) for all spike levels. Each of the levels shows excellent recovery and good precision (RSD < 4%). This degree of accuracy and precision is expected when using SIDA and also establishes that the sample processing and instrumental conditions are suitable for bread matrices. There were also no coeluting contaminants with ions at *m*/*z* 209 or 212, which would have affected the recovery accuracy.

Table 3. Recovery Studies Using Semicarbazide Extraction Method

spike level (μg/kg)	average % recovery; $n = 4$	RSD (%)
blank	NA	NA
50	99	0.8
100	100	3.7
500	102	2.7

Table 4. Reproducibility of Bread Extraction and Analysis

sample	analyses	semicarbazide (μg/kg)	RSD (%)
I	5	<10	
C3	5	63	6.8
J	5	66	5.4
azodicarbonamide[a]	6	450	3.3
A2	5	670	7.9

[a] This bread was prepared in a bread machine in our laboratories with added azodicarbonamide at 45 mg azodicarbonamide/kg flour.

Reproducibility. Five bread products, ranging from <10 to 670 μg/kg, were extracted and analyzed five or more times (**Table 4**). Each replicate represents a new portion of ground bread, carried through the entire extraction procedure. Additionally, the analysis was performed over a period of 6 months and semicarbazide concentrations were determined using three different sets of calibration standards. Replicates for all of the products showed good reproducibility, with RSDs below 8% for all of the samples. Along with establishing the reproducible performance of the analytical method, these results also establish that the level of semicarbazide found in breads does not change significantly with time.

Application to Bread Products. Using the semicarbazide extraction method, semicarbazide concentrations were measured in 20 commercial products and two bread machine breads (**Table 5**). The 18 commercial products, listing azodicarbonamide as an ingredient, showed semicarbazide concentrations ranging from 10 to 1200 μg semicarbazide/kg of bread. In the two remaining commercial products, which did not have azodicarbonamide listed as an ingredient (breads I and J), one showed no detectable levels of semicarbazide and the other showed semicarbazide concentrations of 68 μg/kg. The azodicarbonamide (45 mg/kg flour)-spiked bread machine bread showed semicarbazide concentrations of 450 μg/kg, while semicarbazide was not detected in the unspiked (blank) bread machine bread. The mean semicarbazide concentration for all of the commercial products with detectable levels of semicarbazide was 260 μg/kg, and the median was 180 μg/kg. The values obtained using the semicarbazide extraction method represent the concentration of "free semicarbazide" in bread.

In addition to analyzing semicarbazide in homogenized bread samples, we also compared the semicarbazide concentrations in crusts with concentrations in samples taken from the center of the loaf (**Table 6**). For these analyses, the crusts and the interior of the loaf were ground separately. Determining confidence limits ($\pm 2\sigma$) based on an 8% RSD, only breads F and C1 showed a statistically significant, but minor, difference between their centers and their crusts, with the crusts showing higher values for both products. None of the breads showed significant differences between the semicarbazide concentration in the crust and in the homogenized bread sample. Our data show that semicarbazide is distributed fairly evenly throughout

Table 5. Semicarbazide Concentrations in Commercial Products

sample ID[a]	sample type	semicarbazide concn (μg/kg) with extraction condition: water (0.5 h) extraction	semicarbazide concn (μg/kg) with extraction condition: HCl (16 h) extraction
A1	kaiser roll	1200	2000
A2	white roll	670	1200
A3	white bun	130	
A4	white bread	270	
B1	white bread	410	
B2	potato bread	100	200
B3	white bread	320	
B4	multigrain bread	210	
C1	butter white bread	140	
C2	white bread	180	230
C3	potato bun	63	170
C4	Italian white bread	140	
C5	wheat bread	410	
D	Italian rolls	240	
E	white bread	10	
F	multigrain bread	92	
G	potato rolls	25	
H	French white bread	180	
I[b]	white bread	<10	
J[b]	white bread	68	140
blank[c]	white bread	<10	<10
azodicarbonamide[c]	white bread	450	850

[a] Each letter represents a product from a different manufacturer. [b] Azodicarbonamide was not listed as an ingredient. [c] These breads were prepared in bread machine in our laboratories one without and one with added azodicarbonamide at 45 mg azodicarbonamide/kg flour.

Table 6. Comparison of Semicarbazide Concentrations in Crust and Center of Breads

sample ID	bread type	semicarbazide (μg/kg): whole	semicarbazide (μg/kg): center	semicarbazide (μg/kg): crust
F	white bread	92	39	92
C1	butter white bread	140	83	150
B4	multigrain bread	210	170	200
A4	white bread	270	210	270
B3	white bread	320	300	380

the product for a variety of commercially available bread products. These findings are in contrast to the >10-fold difference between the crust and the bread center values reported by Becalski et al. (*17*). However, in that study, azodicarbonamide-treated flour was intentionally sprinkled on the outside of the bread prior to baking. For our commercial products, we found no indication that azodicarbonamide-treated flour had been sprinkled on the outside of the breads.

Although we tested a variety of bread types (white, wheat, and multigrain), the data do not show a correlation between the type of bread and the level of semicarbazide measured. Additionally, different forms (breads, rolls) of products were evaluated, but as with the type of product, none showed consistently higher levels of semicarbazide. Finally, multiple products from three manufacturers were analyzed and the levels were compared. Although manufacturer A showed the greatest range of semicarbazide levels (130−1200 μg/kg), none of the products from any one manufacturer showed consistently higher semicarbazide levels than products from the other manufacturers (**Table 5**).

As a further evaluation of our method and to offer some comparison to the more commonly used 16 h HCl extraction/derivatization procedure, several samples were evaluated using the HCl extraction method described previously. Comparison of semicarbazide levels in seven products plus a blank using both the semicarbazide extraction method and HCl extraction method is shown in **Table 5**. For each product, a single aliquot of bread was extracted in water and centrifuged and the supernatant was filtered. The filtrate was separated into two aliquots: one which was processed using the semicarbazide extraction method and the other was carried through the HCl extraction method. For all products, the HCl extraction method yielded higher levels of semicarbazide than the semicarbazide extraction method. The increase was due to the use of HCl ranges from 1.3 to 2.7 times that of water, with an average 1.9-fold increase. These results are consistent with our results of semicarbazide-spiked bread and with previous studies involving food animals (*20*, *21*). The HCl extraction method values (16 h) represent a combination of free and hydrolyzed bound semicarbazide and may also contain semicarbazide produced from structurally related precursors (other azodicarbonamide decomposition products) (*8*, *16*).

It is clear from our findings that, similar to foamed PVC, commercial bread products contain semicarbazide residues as a result of the use of azodicarbonamide. We have also shown that the use of acid extraction conditions leads to an increase in the measured semicarbazide concentrations over concentrations determined using more neutral conditions. This additional semicarbazide results from the hydrolysis of bound moieties and possibly structural precursors derived from decomposition of azodicarbonamide. Our method, which limits hydrolysis, yields an accurate measure of semicarbazide levels in bread as it is offered to the consumer. The results given can be used to make a preliminary estimate of consumer exposure to semicarbazide resulting from the use of azodicarbonamide in bread manufacture.

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