JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Moisture Enhances Acrylamide Reduction during Storage in Model Studies of Rye Crispbread

ARWA MUSTAFA,*,† ROGER ANDERSSON,† KARL-ERIK HELLENÄS,‡ PER ÅMAN,† AND AFAF KAMAL-ELDIN†

Department of Food Science, Swedish University of Agricultural Sciences (SLU), P.O. Box 7051, S-750 07 Uppsala, Sweden, and Swedish National Food Administration, Uppsala, Sweden

The effect of storage conditions on the residual acrylamide content of unfermented rye crispbread was studied in a model system. When milled samples were stored at −80 to 6 °C for up to 224 days in double sealed plastic bags, no change in acrylamide content was observed. However, when the milled samples were stored under warmer conditions (20 and 40 °C), a notable reduction in acrylamide was noted (22% and 29%, respectively). When stored at 40 °C for 70 days in glass tubes, acrylamide content in the samples decreased by 37% in the capped samples, while the decrease in the uncapped samples was in the order of 15%. Finally, a notable reduction of 80% was found when samples were stored at increased moisture level at 40 °C for 70 days in capped glass containers. These results highlight that moisture content seems to be of importance for reduction of acrylamide content during storage of food and analytical samples.

KEYWORDS: Acrylamide; moisture content; rye crispbread; storage conditions

INTRODUCTION

Understanding the formation and reduction of acrylamide (AA) in foods is of crucial importance because its presence in food is a possible health concern. To date, a large number of studies on AA have been published and summarized in reviews (*1*−*7*). It is now established that AA is found in a wide range of foods, mainly carbohydrate-rich, and that its content depends on the raw materials used and processing conditions. The main proposed mechanism for the formation of AA is via a reaction between free asparagine (Asn) and carbonyl compounds, mainly reducing sugars, leading to the formation of Schiff's base that can form AA by two different pathways, one involving 3-aminopropionamide (*8*−*11*). Minor routes for the formation of AA include decarboxylation of Asn with decarboxylase (*12, 13*) and pyrolytic reactions of wheat gluten (*14*).

It has been found that the levels of AA in crispbread and coffee decrease upon storage (*15, 16*). For example, a 45−65% decrease was found in roasted and ground coffee after storage in sealed containers at room temperature for 6 months but not after storage at −40 °C for 8 months (*15*). We have found that AA content decreased by 40% in whole grain rye crispbread stored for 20 months (*17*). Despite this confirmed reduction of acrylamide content in coffee and crispbread during storage, the factors involved in this reduction remain mysterious. The aim of this study was, therefore, to investigate the reduction of AA during storage of crispbread and to identify the factor(s) involved in this reduction.

MATERIALS AND METHODS

Bread and Milled Bread Samples. Crispbread used in this study was whole grain rye crispbread (Delikatess, Wasa AB, Filipstad, Sweden), which was produced without yeast fermentation and obtained as freshly baked. We have compared the AA content in rye crispbread samples that were stored as (i) whole bread pieces (∼12 × 6 cm) and milled directly before extraction for AA, (ii) coarsely milled to pass a 2 mm sieve then extracted in that state, or (iii) finely milled to pass a 0.5 mm sieve before storage. Duplicate samples (10 g), used for these comparisons, were stored in double sealed plastic bags for 70 days at 40 °C and were analyzed in two replicates. Because no differences were obtained in this experiment, milled samples were used as a model in the incubation experiments. Thus, crispbread samples were milled directly before incubation to pass a 0.5 mm sieve using an ultracentrifuge mill type ZM (Retsch, Hann, Germany). All samples were incubated in dark closed cabinets.

Effect of Storage Temperature. Milled samples (10 g) were stored in double sealed plastic bags (6 × 8 cm) at −80, −20, 6, 20, or 40 °C. AA content was analyzed in the fresh samples and at fixed time intervals up to 224 days of storage. Samples were stored and analyzed in two replicates.

Effect of Storage Containers' Capping. Five sets of 4 g of samples, milled to pass a 0.5 mm sieve, were stored at 40 °C for 35 or 70 days in vertical glass tubes (30 mL, ∼11 cm long). One set was capped and the second set was uncapped throughout the storage periods. A third set was stored capped for the first half of the storage period and uncapped for the second half, while the fourth set was stored uncapped for the first half of the storage period and then capped for the second half. The fifth set was stored capped, but was opened for durations of ∼20 s and stirred using a vortex twice a week throughout the storage

* Corresponding author. Phone: + 46(0) 18 67 2048. Fax: + 46(0) 18 67 2995. E-mail: arwa.mustafa@lmv.slu.se.
† Swedish University of Agricultural Sciences.
‡ Swedish National Food Administration.

10.1021/jf801405q CCC: $40.75
Published on Web 11/11/2008

periods. These samples were stored and analyzed in three replicates. A further study on the effect of surface area was carried out by storing milled samples (4 g) in horizontal glass tubes (30 mL) either open or capped for 70 days at 40 °C. These samples were stored and analyzed in duplicates.

Effect of Gas Phase. The effect of gases was studied in samples that were incubated in the presence of air, or oxygen-free argon, or nitrogen at 40 °C for 70 days. Milled samples of 4 g in 30 mL sealed tubes were stored and analyzed in three replicates, except for nitrogen where samples were placed in glass containers (50 mL, ~13 g) that are first flushed with the required gas and then stored under constant pressure maintained by a nitrogen-filled balloon. The samples were incubated in duplicates and analyzed as three analytical replicates.

Effect of Moisture. Milled samples (4 g) were stored in capped glass tubes (30 mL). Small glass tubes containing 3 mL of water were placed inside the larger tubes, and samples were stored for 70 days at 40 °C. The experiment was carried out with fresh samples and samples that were vacuum-dried overnight at 30 °C. Fresh samples, with and without water, were stored and analyzed in three replicates, while dried samples, with and without water, were stored and analyzed in duplicates.

Extraction and Analysis of AA. Milled samples were extracted with water after the addition of deuterium-labeled AA as internal standard exactly as described before (*18, 19*). Briefly, the extracts were centrifuged and the supernatant was exposed to ion-exchange solid-phase extraction in two phases for cleanup. The determination of AA content in the extracts was performed using liquid chromatography−tandem mass spectrometry with electrospray ionization. Results of AA analysis are expressed as μg/kg of the original crispbread samples.

Statistics. Results were evaluated by analysis of variance using the software Minitab (Minitab Inc., State Collage, PA).

RESULTS AND DISCUSSION

Use of Milled Sample as a Model System. The effect of particle size on the level of AA in stored whole grain rye crispbread was investigated by milling the samples to different sizes (0.5 and 2 mm) before storage as compared to storing whole pieces of crispbread and then performing the milling just before extraction for AA. There was no significant difference in the levels of AA when samples were stored as wholes pieces or as a milled product at 40 °C for 70 days (results not shown). This made it suitable to use finely milled samples in the following experiments on the effects of different factors on the reduction in AA levels during storage of rye crispbread. The use of milled samples offered the advantage of having more representative samples that were directly analyzed for AA instead of using whole bread pieces that needed to be milled before analysis.

Effect of Storage Temperature. The effect of storage temperature on AA content was studied by storing milled samples in double sealed plastic bags for up to 224 days in different temperatures ranging from −80 to 40 °C. The higher temperatures resulted in a significant decrease ($p < 0.001$) in the AA content. When samples were stored at 20 °C, the first significant decrease (13%) was observed after 56 days with a total decrease of 22% observed after 224 days of storage. On the other hand, samples stored at 40 °C showed a significant decrease (17%) after 14 days of storage with a total decrease in the order of 29% in the 224 days. The decrease of AA at 40 °C was faster at the first stage of storage as compared to that at 20 °C and seems to level out after 56 days of storage (**Figure 1**). In the lower storage temperatures (−80, −20, and 6 °C), the level of AA varied within a limited range, indicating that AA in rye crispbread was relatively stable under cold storage conditions. Comparing the reduction in AA content to that reported recently in rye crispbread that was in the order of 40% when stored for 20 months (*17*), it could be deduced that the major decrease in that study probably took place in the period when the samples were kept at room temperature. The samples from the previous study were not made of the same recipe as was used here and were subjected to different treatments before and during storage.

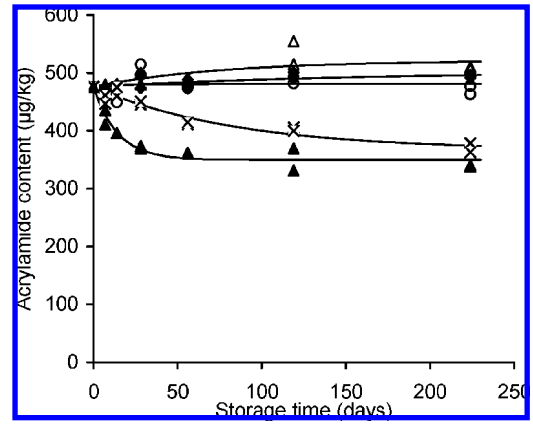

Figure 1. Levels of acrylamide content in milled rye crispbread stored at different temperatures for different time intervals: ◆, −80 °C; △, −20 °C; ○, 6 °C; ×, 20 °C; ▲, 40 °C. Incubations were performed in duplicates, and all data points are included in this figure.

When the reduction in AA content during storage at 20 °C was tested for its kinetic order, no good fit was found for the first or the second order (**Figure 2a** and **b**). These results show that the kinetics for AA reduction is complicated and cannot be explained by a simple model. The elimination of AA is usually studied associated with formation at high temperature treatments in model systems. Studies on the kinetics of AA have previously reported the process of formation and elimination as complicated (*20*). In this model study of crispbread, we are studying reduction in residual AA that was taking place at much lower temperatures (<40 °C) as compared to the previous studies (>150 °C); therefore, it would not be possible to make a valid comparison. From this study, the level of AA reached a steady state between 50−100 days of storage at 40 °C. Therefore, the following experiments were performed up to 35 and 70 days.

Effect of Capping of Storage Containers. In this experiment, samples were stored for 35 or 70 days intervals in glass tubes that were subjected to different modes of capping and uncapping (**Figure 3**). The level of reduction in AA content during storage for 70 days was higher in samples that were capped (37%) as compared to those uncapped (15%) throughout storage. The decrease was slower in the uncapped samples as revealed by a significant interaction between mode of capping and the duration of storage (**Table 1**). When the modes of capping were compared, it was shown that there was no significant difference between samples stored uncapped and those stored uncapped in the first half of the storage period and then capped in the second half. These results indicate that most of the capping impact in the reduction took place in the first half of the storage time. Moreover, stirring samples for about 20 s twice a week throughout the storage time did not facilitate further significant reduction as compared to samples stored as capped. This implies that exposing new surface area during storage was not of importance.

The effect of surface area was studied by storing samples in a horizontal and vertical position, as capped or uncapped. Results showed that there was no significant effect of the tube position and thus surface area. These results agree with the above experiment showing that a higher reduction in AA content was

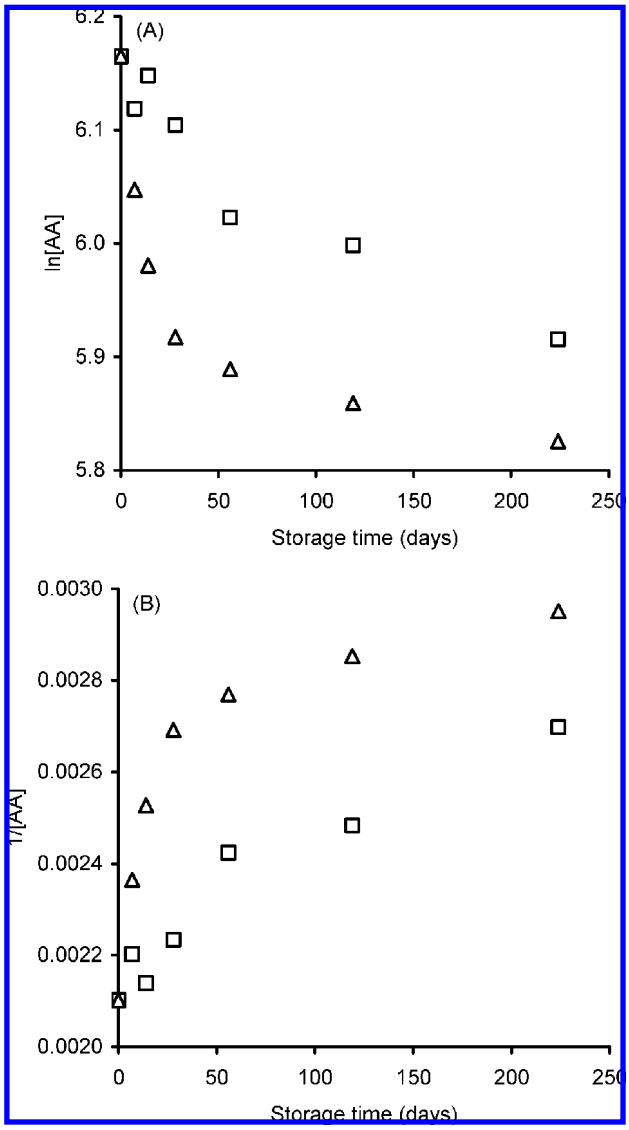

Figure 2. Testing the reaction kinetics for acrylamide reduction: (**A**) logarithm of the reactant concentrations versus time (first order) and (**B**) reciprocal of the reactant concentrations versus time (second order); □, whole grain rye crispbread stored at 20 °C; △, whole grain rye crispbread stored at 40 °C. Points are presented as means of duplicate incubations.

achieved when samples were stored in capped containers. Results from the above experiments suggested that some compound(s) in the headspace of the storage tubes (gas phase or moisture) are affecting the reduction in AA level in capped tubes.

Effect of Gases. To test for the effect of the type of gas phase, samples were incubated with air, argon, or nitrogen at 40 °C for 70 days. The degree of reduction in the level of AA was not different between the samples, indicating that the observed reduction in AA content was not related to reaction with oxygen (**Figure 4**).

Effect of Moisture. Because the type of gas had no effect on the reduction of AA, an experiment was carried out where the moisture content was varied during storage at 40 °C to test if water could be the volatile compound of importance for AA reduction. When testing for the effect of moisture content on the reduction of AA, the amount was significantly lower ($p < 0.001$) when samples were stored with their initial water content as compared to when stored after vacuum-drying (**Figure 5**). Furthermore, the incubation of the original crispbread powder

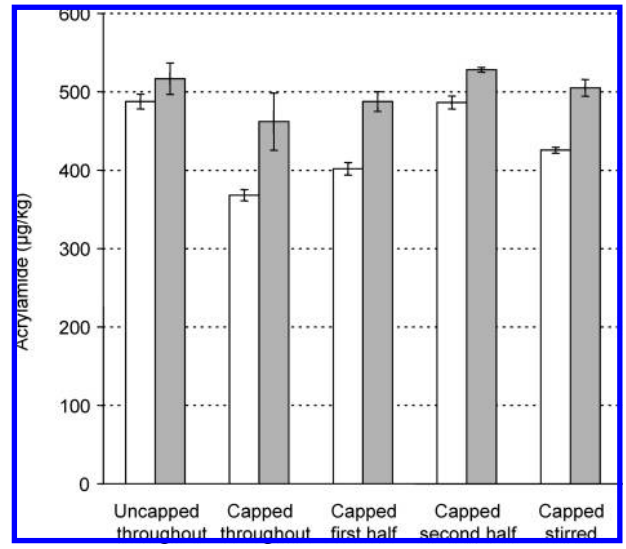

Figure 3. Effect of capping during storage (40 °C, glass tubes) of milled rye crispbread on the content of acrylamide. Samples were stored for 35 (white) or 70 (gray) days. The capped stirred samples were opened for 20 s and stirred twice a week throughout storage. The content of acrylamide in the crispbread before storage was 580 μg/kg.

Table 1. *P* Values for the Effects of Capping and Time of Storage on the Content of Acrylamide

	capping regime[a]		
factor	throughout	first half	second half
capped	<0.001	<0.001	n.s.[b]
storage time	0.001	<0.001	0.001
interaction capped × time	0.032	0.006	n.s.[b]

[a] Each column shows one two-way ANOVA analyzing the effects of capping, storage time, and their interaction. Samples capped throughout, during first half, and during second half of the storage were compared to samples uncapped throughout (see **Figure 3**). [b] Not significant.

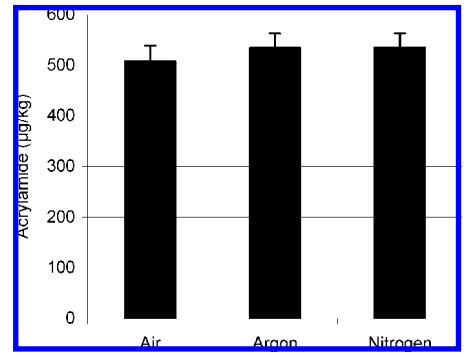

Figure 4. Effect of gases on the reduction of acrylamide during warm storage (40 °C for 70 days) of milled crispbread in closed glass tubes ($n = 3$).

with water, in an inserted glass tube, led to about a 70% decrease in AA levels in the native and vacuum-dried crispbread powder samples. These results show that the water content is of major importance for AA reduction during the storage of crispbread with a long shelf life, up to 24 months. Crispbread (typical moisture content 3−5%) is hygroscopic and can easily take up water, leading to variable effects on acrylamide levels depending on the storage conditions.

The influence of water on AA reduction during storage may be one reason behind the complicated kinetic profiles in the experiments presented in **Figure 2a** and **b**. At the end of storage for 250 days, the moisture content of samples stored at 20 °C

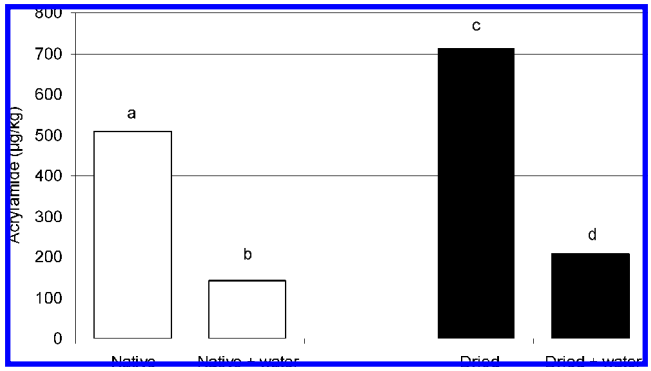

Figure 5. Effect of water on the reduction of acrylamide during storage of milled rye crispbread at 40 °C in closed glass tubes for 70 days. Results are expressed as μg acrylamide/kg of original crispbread material. Different letters indicate statistically different values (Tukey's pairwise comparison, $n = 3$, $p < 0.001$).

was 6%, whereas that of samples stored at +40 °C was 3%, showing that the moisture content was not stable during storage at +40 °C. Much more studies are needed to propose and investigate the possible mechanisms behind this effect of water. For example, although visual signs were not detected in our experiments, effects of moisture on microbial activities cannot be excluded, as it was shown by Skanker et al. (*21*) that AA is degraded by soil microbes. In addition, water might have a role as a medium or a catalyst in unknown AA degradation reaction(s). Naturally, future kinetic experiments need to be conducted under strictly controlled humidity before we can extrapolate these effects to other temperatures.

In summary, AA content in crispbread was relatively stable after storage for 224 days at temperatures between −80 and 6 °C. This result is of practical relevance for analytical samples that need to be stored over longer periods of time. Higher temperatures (20 and 40 °C) during storage resulted in a significant decrease in AA content in the crispbread powder especially during the early stages of storage.

The reduction of AA was greater in capped as compared to uncapped tubes. Moisture was identified as an important factor enhancing AA reduction in this model system of crispbread during storage at 40 °C. These results may be of importance for the content of AA in crispbreads stored for longer periods of time as well as for explaining the reduction of AA content in food stored around room temperature as observed in several model experiments. The mechanism of action of water leading to enhancement of AA reduction is not known, and further studies dedicated to its exploration are essential before this phenomenon can be exploited for practical applications.

ACKNOWLEDGMENT

We thank Wasabröd AB for supplying the crispbread samples.

NOTE ADDED AFTER ASAP PUBLICATION

There was an error in Figure 3 in the version published ASAP November 11, 2008; the corrected version was reposted December 3, 2008.

LITERATURE CITED

(1) Claus, A.; Carle, R.; Schieber, A. Acrylamide in cereal products: A review. *J. Cereal Sci.* **2008**, *51*, 18–133.
(2) Friedman, M. Chemistry, biochemistry, and safety of acrylamide. A review. *J. Agric. Food Chem.* **2003**, *51*, 4504–4526.
(3) Konings, E. J. M.; Ashby, P.; Hamlet, C. G.; Thompson, G. A. K. Acrylamide in cereal and cereal products: A review on progress in level reduction. *Food Addit. Contam.* **2007**, *24*, 47–59.
(4) Lingnert, H.; Grivas, S.; Jägerstad, M.; Skog, K.; Törnqvist, M.; Åman, P. Acrylamide in food: mechanisms of formation and influencing factors during heating of foods. *Scand. J. Food Nutr.* **2002**, *46*, 159–172.
(5) Stadler, R. H.; Scholz, G. Acrylamide: An update on current knowledge in analysis, levels in food, mechanisms of formation, and potential strategies of control. *Nutr. Rev.* **2004**, *62*, 449–467.
(6) Taeymans, D.; Wood, J.; Ashby, P.; Blank, I.; Studer, A.; Stadler, R. H.; Gonde, P.; Van Eijck, P.; Lalljie, S.; Lingnert, H.; Lindblom, M.; Matissek, R.; Muller, D.; Tallmadge, D.; O'Brien, J.; Thompson, S.; Silvani, D.; Whitmore, T. A review of acrylamide: An industry perspective on research, analysis, formation and control. *Crit. Rev. Food Sci.* **2004**, *44*, 323–347.
(7) Zhang, Y.; Zhang, Y. Formation and reduction of acrylamide in Maillard reaction: A review based on the current state of knowledge. *Crit. Rev. Food Sci.* **2007**, *47*, 521–542.
(8) Mottram, D. S.; Wedzicha, B. L.; Dodson, A. T. Acrylamide is formed in the Maillard reaction. *Nature* **2002**, *419*, 448–449.
(9) Stadler, R. H.; Blank, I.; Varga, N.; Robert, F.; Hau, J.; Guy, P. A.; Robert, M. C.; Riediker, S. Acrylamide from Maillard reaction products. *Nature* **2002**, *419*, 449–450.
(10) Yaylayan, V. A.; Wnorowski, A.; Locas, C. P. Why asparagine needs carbohydrates to generate acrylamide. *J. Agric. Food Chem.* **2003**, *51*, 1753–1757.
(11) Zyzak, D. V.; Sanders, R. A.; Stojanovic, M.; Tallmadge, D. H.; Eberhart, B. L.; Ewald, D. K.; Gruber, D. C.; Morsch, T. R.; Strothers, M. A.; Rizzi, G. P.; Villagran, M. D. Acrylamide formation mechanism in heated foods. *J. Agric. Food Chem.* **2003**, *51*, 4782–4787.
(12) Granvogl, M.; Jezussek, M.; Koehler, P.; Schieberle, P. Quantitation of 3-aminopropionamide in potatoes - A minor but potent precursor in acrylamide formation. *J. Agric. Food Chem.* **2004**, *52*, 4751–4757.
(13) Granvogl, M.; Schieberle, P. Thermally generated 3-aminopropionamide as a transient intermediate in the formation of acrylamide. *J. Agric. Food Chem.* **2006**, *54*, 5933–5938.
(14) Claus, A.; Weisz, G. M.; Schieber, A.; Carle, R. Pyrolytic acrylamide formation from purified wheat gluten and gluten-supplemented wheat bread rolls. *Mol. Nutr. Food Res.* **2006**, *50*, 87–93.
(15) Andrzejewski, D.; Roach, J. A. G.; Gay, M. L.; Musser, S. M. Analysis of coffee for the presence of acrylamide by LC-MS/MS. *J. Agric. Food Chem.* **2004**, *52*, 1996–2002.
(16) Hoenicke, K.; Gatermann, R. Studies on the stability of acrylamide in food during storage. *J. AOAC Int.* **2005**, *88*, 268–273.
(17) Mustafa, A.; Kamal-Eldin, A.; Petersson, E. V.; Andersson, R.; Åman, P. Effect of extraction pH on acrylamide content in fresh and stored rye crisp bread. *J. Food Compos. Anal.* **2008**, *21*, 351–355.
(18) Petersson, E. V.; Rosén, J.; Turner, C.; Danielsson, R.; Hellenäs, K. E. Critical factors and pitfalls affecting the extraction of acrylamide from foods: An optimization study. *Anal. Chim. Acta* **2006**, *557*, 287–295.
(19) Rosén, J.; Nyman, A.; Hellenäs, K. E. Retention studies of acrylamide for the design of a robust liquid chromatography-tandem mass spectrometry method. *J. Chromatogr., A* **2007**, *1172*, 19–24.
(20) Claeys, W. L.; De Vleeschouwer, K.; Hendrickx, M. E. Kinetics of acrylamide formation and elimination during heating of an asparagine-sugar model system. *J. Agric. Food Chem.* **2005**, *53*, 9999–10005.
(21) Shanker, R.; Ramakrishna, C.; Seth, P. K. Microbial degradation of acrylamide monomer. *Arch. Microbiol.* **1990**, *154*, 192–198.

Received for review May 6, 2008. Revised manuscript received September 16, 2008. Accepted October 5, 2008.

JF801405Q