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Effects of Asparagine, Fructose, and Baking Conditions on Acrylamide Content in Yeast-Leavened Wheat Bread

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A repeatable procedure for studying the effects of internal and external factors on acrylamide content in yeast-leavened wheat bread has been developed. The dough contained wheat endosperm flour with a low content of precursors for acrylamide formation (asparagine and reducing sugars), dry yeast, salt, and water. The effects of asparagine and fructose, added to the dough, were studied in an experiment with a full factorial design. More than 99% of the acrylamide was found in the crust. Added asparagine dramatically increased the content of acrylamide in crusts dry matter (from about 80 μg/kg to between 600 and 6000 μg/kg) while added fructose did not influence the content. The effects of temperature and time of baking were studied in another experiment using a circumscribed central composite design. Mainly temperature (above 200 °C) but also time increased the acrylamide content in crust dry matter (from below 10 to 1900 μg/kg), and a significant interaction was found between these two factors. When baked at different conditions with the same ingredients, a highly significant relationship ($P < 0.001$) between color and acrylamide content in crust was found. Added asparagine, however, did not increase color, showing that mainly other amino compounds are involved in the browning reactions.

KEYWORDS: **Acrylamide; bread; asparagine; fructose; temperature; time; baking**

INTRODUCTION

In April 2002, a group of Swedish researchers reported that some heat-treated starch rich foods contained high levels of acrylamide (*1*). Later, a link between acrylamide formation and the Maillard reaction was suggested (*2*−*5*). Acrylamide is known to be a neurotoxin, a carcinogen in animals, and a probable carcinogen in humans (*6*, *7*).

After the initial finding, acrylamide has been found in many foods of our everyday life, and heat-treated potato and cereal products and coffee are major sources of intake (*8*). The chemistry, biochemistry, analysis, occurrence, metabolism, and toxicology of acrylamide have recently been reviewed (*7*, *9*). Thus, it is now well-established that free amino acids, mainly asparagine, and reducing sugars are important precursors for acrylamide in foods and that processing conditions, such as time, temperature, water activity, and matrix, will influence its formation and degradation. Very little designed research has so far been done on how these factors will influence the acrylamide content in bread.

The aims of this research were to establish a model for studies of internal and external factors of importance for the acrylamide content in yeast-leavened wheat bread and to investigate, in designed experiments, the effects of added asparagine and fructose on acrylamide content in the bread as well as the importance of temperature and time of baking.

MATERIALS AND METHODS

Baking. A dough was prepared with wheat flour (200 g, 18% protein, 0.45% ash, 0.017% asparagines, 0.013% glucose, and 0.005% fructose, Bagarns Bästa, Nordmills, Uppsala, Sweden), dry yeast (2.0 g, Kronjäst Orginal, Jästbolaget, Sollentuna, Sweden), sodium chloride (1.9 g), and water (125 mL). The flour and yeast were mixed together for 1 min in a Farinograph (Brabender, Duisburg, Germany). Then, preheated solutions (48 °C) of 115 mL of tap water and 10 mL of water containing varying amounts of asparagin and fructose and the sodium chloride were added. The selected level of water was established by adding water to the flour in the Farinograph until a mixing curve peak of 500 Brabender Units was reached. The ingredients were mixed for 10 min with the highest speed in the Farinograph, and the formed dough was left in a leavening cupboard (34 °C, 60% RH) for 60 min. The dough was then divided into three pieces of 100 g each with a plastic knife. These pieces were molded and placed in preoiled baking tins, which were placed in a second leavening cupboard (39 °C, 85% RH) for 60 min. In our standard procedure, the fermented dough was baked at 270 °C for 15 min in a rotating laboratory oven (Simon, Greenfield, England).

Asparagine/Fructose Experiment. Three different equimolar concentrations of asparagine and fructose were added to the dough (0.1, 0.4, and 0.7 g for asparagin and 0.07, 0.29, and 0.51 g for fructose to 100 g of flour). A full factorial design was used as outlined in **Figure 1**. Each dough was prepared and baked (270 °C for 15 min) twice except for the central point (0.40 g of asparagin and 0.29 g of fructose),

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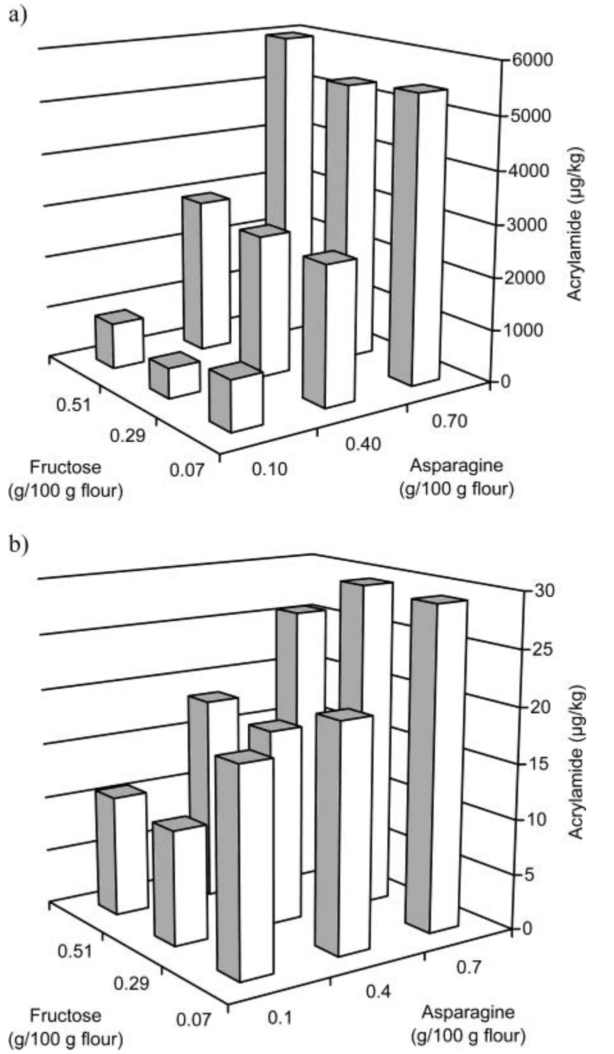

Figure 1. Effect of added asparagine and fructose on acrylamide content (μg per kg dm) in crust (**a**) and crumb (**b**) of yeast-leavened wheat bread. All breads were baked at 270 °C for 15 min.

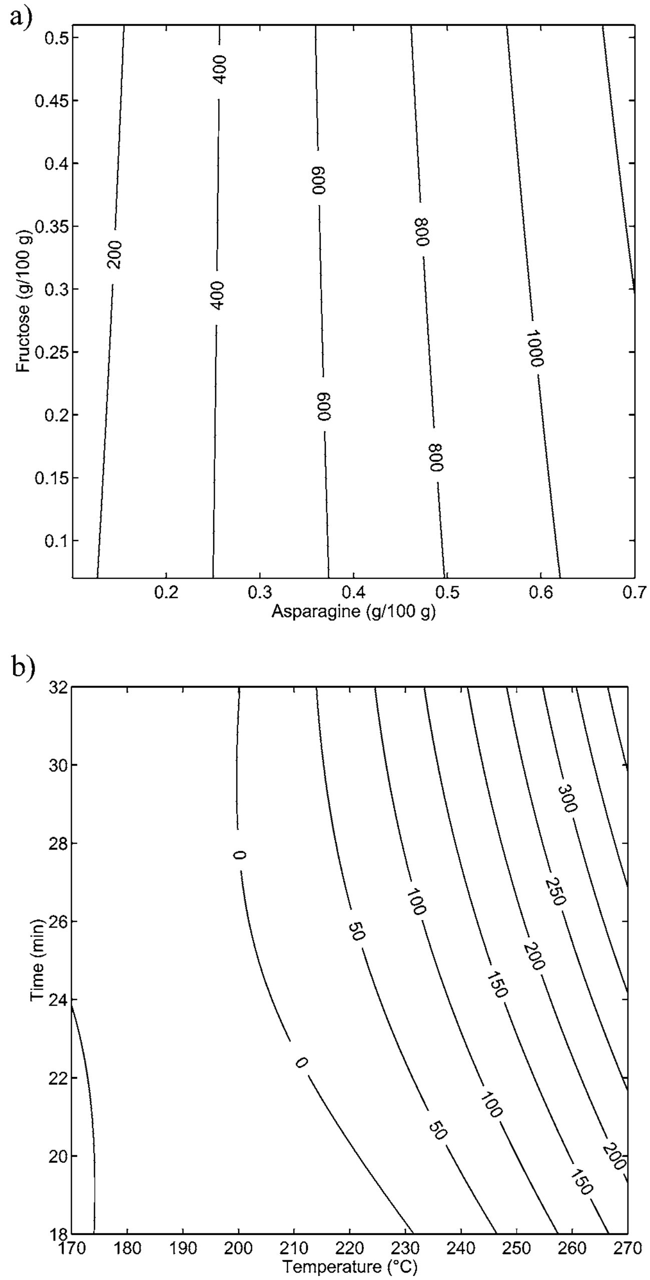

Figure 2. Graphs of response surface models of acrylamide content (μg per kg fresh bread) in yeast-leavened wheat breads baked at 270 °C for 15 min from the asparagine/fructose experiment (**a**) and the temperature/time experiment (**b**).

which was prepared and baked four times. Experiments were carried out in random order to avoid systematic errors.

Time/Temperature Experiment. During this baking experiment, five different temperatures (150, 170, 220, 270, and 290 °C) and five different times (15, 17, 25, 32, and 35 min) were used in a circumscribed central composite design as outlined in **Figure 3**. To each dough, 0.10 g of asparagine per 100 g of flour was added but no fructose. Each dough was prepared and baked twice except for the central point (220 °C and 25 min), which was prepared and baked four times. Also in this case, experiments were made in a random order.

Analyses of Bread. Once the bread was out of the oven, the central crumb temperature was measured with a thermocouple digital thermometer (3 mm diameter). After about 1 h of cooling at room temperature, the fresh weight, volume by displacement with pearl sagosand, and porosity according to the Dallman scale were recorded.

The breads were thereafter placed in a homemade template and divided with a knife into four parts of equal size and shape. One part was divided into crust (varied in thickness from about 5 mm for underbaked bread with light crust to about 11 mm for overbaked bread with dark crust) and crumb with a thin knife. The crumbs and crusts from the three breads of each dough were combined, weighed, freeze-dried, weighed again to determine yield and moisture content, and then disintegrated in a food mixer.

The color of the crust was measured with a Chroma Meter (Minolta, Milton Keynes, England) as described in the manual. Three measures were taken for each bread to make an average. The ΔE^* value was calculated from the L^*, a^*, and b^* values in order to represent the total color difference; the formula is $\Delta E^* = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{1/2}$.

Analysis of acrylamide in crusts and crumbs followed essentially the procedure of Rosén and Hellenäs (*10*). The freeze-dried and disintegrated sample was mixed with water, and deuterium-labeled acrylamide was added as an internal standard. The sample was homogenized, and acrylamide was extracted. The extract was purified by column chromatography, filtered, and analyzed two times by LC-MS-MS. Positive electrospray and multiple reaction monitoring were used.

Statistics. The designed experiments were evaluated by multiple regression analysis in Microsoft Excel.

RESULTS AND DISCUSSION

Baking. The wheat flour used in this study was selected to contain a low content of asparagine and reducing sugars (*11*) in order to restrict the acrylamide formation during baking as

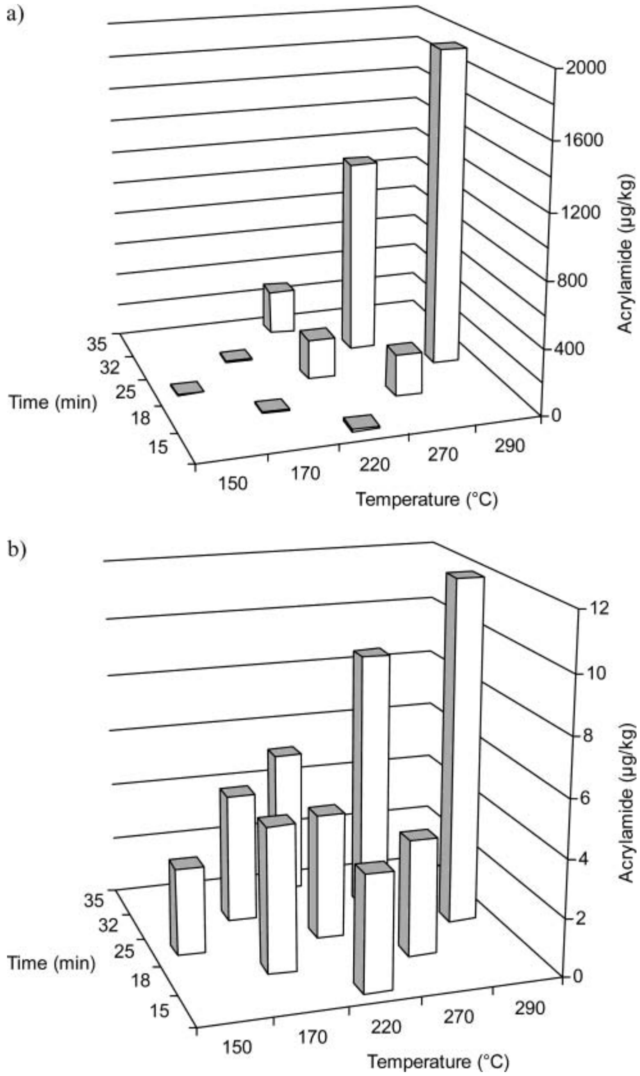

Figure 3. Effect of time and temperature of baking on acrylamide content (μg per kg dm) in crust (**a**) and crumb (**b**) of yeast-leavened wheat bread. A 0.1 g amount of asparagine was added to 100 g of flour.

much as possible when no precursors were added. During the standard conditions for baking without added precursors, about 80 μg of acrylamide per kg of dry crust was detected. The measured central temperature of the breads varied between 97 and 101 °C showing that all breads had a fully developed crumb, which was classified with porosity six on the Dallman scale for all breads. All breads were of acceptable eating quality, but crust color varied from rather light to dark in the time/temperature experiment. Bread fresh weight (76.5−94.9 g) and volume (190−240 mL) were dependent on baking temperature and time but not on the amounts of added asparagine and fructose.

Repeatability Study. To study the repeatability of the whole procedure, doughs were made at different days without any added asparagine and fructose and breads were baked at 270 °C for 15 or 20 min. Ten samples of crust were prepared for each baking time (15 and 20 min), and the analyzed contents of acrylamide in these samples were 85.0 ± 10.5 and 154.1 ± 15.9 μg per kg dry matter, respectively. The corresponding coefficients of variation were 12.3 and 10. 3%, respectively, which is very satisfactory since the LC-MS-MS method used for acrylamide detection by itself has a reported coefficient of variation of about 6% at these ranges of acrylamide content (*10*).

Table 1. Coefficients and *P* Values for Effects of Asparagine, Fructose, and Their Interaction on Acrylamide Content in Crust, Crumb, and Fresh Bread from Regression Analyses of the Response Surface Models of the Asparagine/Fructose Experiment

factor	coefficient	*P* value
	dry crust	
asparagine	2400	<0.001
fructose	NS[a]	NS
interaction	NS	NS
	dry crumb	
asparagine	7.2	<0.005
fructose	NS	NS
interaction	NS	NS
	fresh bread	
asparagine	540	<0.001
fructose	NS	NS
interaction	NS	NS

[a] Not significant.

Asparagine/Fructose Experiment. The amount of asparagine and reducing sugars added to the dough in this experiment was relatively high as compared to the natural content in wheat (*12*, *13*). The highest asparagine concentration is not likely to be found in wheat flour but could possibly arise from other ingredients in consumer bread products. In this study, equimolar levels of fructose and asparagine were included in the design since it has been reported that the highest acrylamide formation occurred when this proportion was used in a test tube (*2*). Fructose was chosen as the reducing sugar since it is known that added fructose increased acrylamide content more than added glucose in heat-treated potato products (*14*). In this experiment, the yield of crust was 33% (range of 32−34%) of the dry matter for all breads and the yield of crumb was 67% (range of 66−68%).

Added asparagine had a very strong influence on the acrylamide content in the crust of yeast-leavened wheat bread (**Figure 1a**). At the highest level of added asparagine, up to 6000 μg of acrylamide per kg of dry crust was found. On the other hand, fructose did not seem to influence the content. Regression analysis of the response surface model showed a highly significant effect for added asparagine but nonsignificant effects for added fructose or their interaction (**Table 1**). It is interesting that fructose did not have an effect since, as mentioned above, added fructose has been reported to greatly increase the acrylamide content in heat-treated potato products. Some of the crusts were therefore analyzed for their content of reducing sugars by GLC as trimethylsilyl-ethers. High contents of reducing sugars were detected in the crusts (0.40−0.64 g of fructose and 0.25−0.35 g of glucose per 100 g of dry crust for the samples from the addition levels of 0.10 and 0.70 g of asparagin per 100 g of flour), indicating that free sugars were formed during baking and consequently reducing sugars are not a limiting factor for acrylamide formation in yeast-leavened wheat bread. High contents of reducing sugars (mainly maltose) have previously been found in bread fractions (*15*).

Asparagine added to the dough also increased the level of acrylamide in the isolated crumb of yeast-leavened wheat bread, although to a much lower level (**Figure 1b**). Also in this case, regression analysis of the response surface model showed a significant effect for added asparagin and nonsignificant effects for added fructose or their interaction (**Table 1**). In this study, more than 99% of the acrylamide in the bread was detected in the crust and it has been reported previously from in vitro studies that acrylamide is not formed from asparagin and reducing

Table 2. Coefficients and *P* Values for Effects of Asparagine, Fructose, and Their Interaction on Acrylamide Content in Crust, Crumb, and Fresh Bread from Regression Analyses of the Response Surface Models of the Temperature/Time Experiment

factor	coefficient	*P* value
	dry crust	
temperature	530	<0.001
time	150	<0.05
interaction	210	<0.05
temperature × temperature	340	<0.001
time × time	NS[a]	NS
	dry crumb	
temperature	1.8	<0.01
time	NS	NS
interaction	NS	NS
temperature × temperature	1.6	<0.05
time × time	NS	NS
	fresh bread	
temperature	160	<0.001
time	51	<0.01
interaction	81	<0.01
temperature × temperature	116	<0.001
time × time	NS	NS

[a] Not significant.

sugars at temperatures below 100 °C (*3*). Because the inner temperature of the crumb generally does not exceed 100 °C, it is very likely that the detected acrylamide in the crumb originated from crust parts remaining in the crumb due to incomplete separation of the crust.

As the proportion of crumb and crust was known, acrylamide content in the fresh yeast-leavened wheat breads could be calculated. Added asparagine increased the acrylamide content in the breads in a very similar pattern as in the crusts (not shown). Regression analysis of the response surface model showed also in this case a highly significant effect for added asparagin (**Table 1**). Within the range of asparagine added, the response surface model revealed an almost linear increase from 200 to about 1200 μg per kg fresh bread (**Figure 2a**). The levels of acrylamide found in this experiment are higher than what is generally reported for wheat bread, even at the lowest level of asparagine added (*16*). The content found in this study for breads without added asparagine and fructose (17 μg/kg dry bread) was, however, in the same range as previously reported.

Time/Temperature Experiment. In this experiment, a low amount of asparagine but no fructose was added in order to ensure that significant amounts of acrylamide were formed in the yeast-leavened wheat breads. A circumscribed central composite design with five different baking temperatures (150−290 °C) and five different baking times (15−35 min) was used. This design and these conditions were used in order to ensure that all breads had a fully baked crumb and a well-defined and not too overbaked crust. The proportion of crust in the dry breads increased with temperature and time of baking, from 30% when baked at 170 °C for 17 min to 45% when baked at 270 °C for 32 min. Both baking temperature and baking time increased the content of acrylamide in the crust (**Figure 3a**). At lower temperatures and times, little acrylamide (<20 μg per kg dry crust) was detected in the breads and the highest content (1800 μg of acrylamide per kg of dry crust) was detected when the breads were baked at 290 °C for 25 min. Regression analysis of the response surface model showed significant effects for both temperature and time as well as for their interaction (**Table 2**). The effect of temperature was most pronounced as revealed by the highest coefficient and the lowest *P* value. In the crumbs,

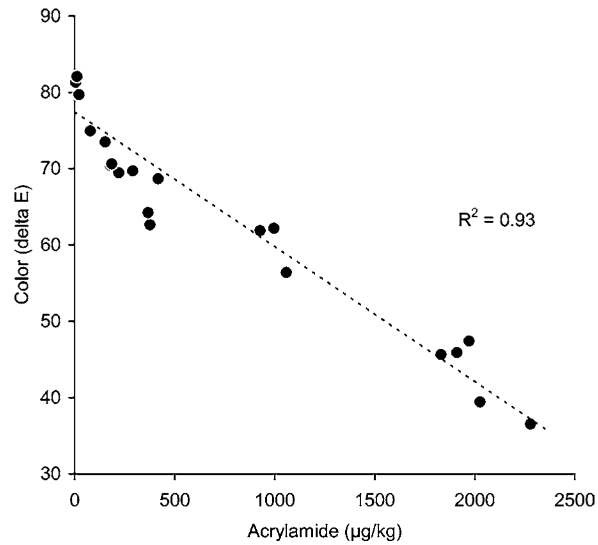

Figure 4. Relationship between color (ΔE^*) and acrylamide content (μg/kg dry crust) in breads from the temperature/time experiment using the same recipe with 0.1 g of asparagine added to 100 g of flour. A 0 represents black, and 100 represents white.

a similar pattern for acrylamide content as in the crusts could be observed but with much lower values (**Figure 3b**). Also in this case, it is very likely that the low amounts of acrylamide found in the crumbs mainly originated from incomplete separation of the crust. The regression analysis of the response surface model showed only a significant effect for temperature. The content of acrylamide in the fresh yeast-leavened breads was calculated and revealed a very similar pattern as for the content in the crusts, containing more than 99% of the acrylamide present in the breads (not shown). Significance levels for effects in the regression analysis showed very similar values as for corresponding effects in the crust**.** The graphic representation of the response surface model indicated that significantly increased amounts of acrylamide were present in the breads baked at 18 min when the temperature exceeded 230 °C but when baked at 32 min already at 200 °C (**Figure 2b**). The increase in acrylamide content with temperature was progressive.

The color of the crust of breads in the time/temperature experiment was measured with a Minolta system. The color varied from almost white to dark brown. A highly significant correlation ($P < 0.001$) was found between color and acrylamide content in these crusts when the breads were baked with the same recipe (**Figure 4**). However, when the breads were baked at 270 °C for 15 min with higher levels of added fructose and asparagine (up to 0.51 g of fructose and 0.70 g of asparagin per 100 g of flour) in the asparagine/fructose experiment, the color did not change significantly (ΔE^* varied between 54 and 60) while the acrylamide content increased dramatically from about 700 to 7000 μg per kg of dry crust, as has been discussed above. These results clearly showed that other amino compounds other than asparagin and/or reducing sugars are mainly involved in the browning reactions of the crust.

CONCLUSIONS

A repeatable procedure for studying the effects of internal and external factors on acrylamide content in yeast-leavened wheat bread has been developed. In this procedure, a flour with a low content of known acrylamide precursors (free asparagin and reducing sugars), standardized dough making and baking conditions, and a sensitive quality-assured LC-MS-MS method for acrylamide determination in food were used.

Different amounts of asparagine and fructose were added to the flour in a designed experiment, and baked breads were fractionated into crust and crumb. In this experiment, as well as the other experiments carried out in this study, more than 99% of the acrylamide in the breads was found in the crusts. Added asparagine dramatically increased arylamide content in the breads while added fructose did not influence the content. These results clearly show that low acrylamide yeast-leavened breads should be baked with ingredients with a low level of free asparagine or a process that lowers the content of free asparagine before it is converted to acrylamide.

Different baking temperatures and baking times but the same ingredients were use in another designed experiment. Especially baking temperatures above 200 °C but also baking times increased the acrylamide content in the breads, and a significant interaction between these two factors was also found. Consequently, also, the baking conditions are of great importance for the acrylamide content in yeast-leavened bread.

When baked with the same recipe but with different baking temperatures and times, a strong correlation between color and acrylamide content in crust was found. When asparagine was added to the flour, which was baked at the same conditions, however, crust color did not change significantly while acrylamide increased dramatically. These results indicate that baking with ingredients with low levels of free asparagine could result in breads with appropriate crust color.

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NOTE ADDED AFTER ASAP

In the original ASAP Posting on March 10, 2004, several instances occurred when μg was incorrectly written as mg. This was corrected on March 12, 2004.

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