

# Effect of Added Asparagine and Glycine on Acrylamide Content in Yeast-Leavened Bread

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## ABSTRACT

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Effect of added asparagine and glycine on acrylamide content in yeast-leavened bread was studied in a designed experiment. Added asparagine strongly increased acrylamide content in the breads, while added glycine decreased the content. The more asparagine in the dough, the stronger

was the reducing effect of glycine. When glycine was applied on the surface of the fermented dough, there was also a significant reduction of acrylamide content in the bread. Addition of glycine but not asparagine caused an increased browning reaction during baking.

The formation of suspected carcinogenic acrylamide in heated starch-rich foods as a product of the Maillard reaction has been widely reported (Tareke et al 2002; Taeymans et al 2004). The side chain of asparagine delivers the backbone of the acrylamide molecule (Stadler et al 2002). Reducing sugars are not incorporated into the molecule but they are needed for formation of the Schiff base of asparagine. This Schiff base is transformed into a decarboxylated Amadori product known to be an intermediate product on the reported pathway to acrylamide. Free asparagine has been shown to be a limiting precursor for acrylamide formation in yeast-leavened bread (Surdyk et al 2004). White wheat flour has a very low content of free asparagine (Fredriksson et al 2004), while certain ingredients used in baking like germ, bran, and certain carrot products contain significant amounts.

A large addition ( $\leq 10$  g/kg of dough) of amino acids other than asparagine, such as glycine, leads to a reduction in the formation of acrylamide in bread and potato products (Rydberg et al 2003; Amrein et al 2004; Bråthen et al 2005). This reducing effect may be due to a competitive consumption of reactive carbonyls between asparagine and glycine. Another reason for this effect might be an elimination of acrylamide formed by a reaction with glycine because acrylamide reacts with the  $\text{NH}_2$  group of glycine (Friedman 2003).

Yeast-leavened bread represents a very important part of the human diet (Svensson et al 2003). Due to the high intake of this type of bread, efforts must be made to reduce the acrylamide contents of this food group. The main aim of this study was to investigate, in a designed experiment, whether there is an interaction between asparagine and glycine content in the dough on acrylamide content in yeast-leavened bread. A novel application technique for glycine was also studied.

## MATERIALS AND METHODS

### Baking

Dough was prepared according to the method established by Surdyk et al (2004). Ingredients were wheat flour (200 g, Nordmills, Uppsala, Sweden, 89.7% dry matter, 11.3% protein, 0.53% ash, and Falling Number  $>250$  sec), dry yeast (2.2 g, Kronjäst Original, Jästbolaget, Sollentuna, Sweden), sodium chloride (1.9 g, Falksalt, Halmstad, Sweden), and tap water (104 g, 48°C). Flour and yeast were mixed together for 1 min with the highest speed (62 rpm on the slow paddle and 93 rpm on the fast paddle) in a

farinograph (Brabender, Duisburg, Germany). Then, solutions of the salt, L-asparagine-monohydrate (for biochemistry, Merck, Darmstadt, Germany) and glycine (p.a., Merck, Darmstadt, Germany) in parts of the preheated tap water were added to the flour-yeast-mixture as well as the remaining water. All ingredients were mixed for 10 min with the highest speed in the farinograph. The dough was left in a leavening cupboard (34°C, 60% rh) for 60 min. Then the dough was divided with a plastic knife into three equal pieces of 100 g each. The pieces were molded, put into preoiled baking tins, and fermented (60 min) in a second leavening cupboard (39°C, 85% rh). One of the three dough pieces was frozen ( $-20^\circ\text{C}$ ) immediately after the second fermentation. The other two pieces were baked at 270°C for 15 min in a rotating laboratory oven (Simon, Stockport, UK). The breads were frozen after 1 hr of cooling at room temperature. Frozen dough and bread samples were freeze-dried, crushed, and ground in an ultracentrifuge mill (type ZM1, Retsch, Haan, Germany) to pass a 0.5-mm screen.

To reveal the distribution of acrylamide, bread was prepared following the general baking recipe described above. L-Asparagine-monohydrate (0.74 g), but no glycine, was added to the flour mixture. After 1 hr of cooling, the three breads were divided with a sharp knife into top cap (the part of the bread that was not covered by the baking tin) and the rest of the bread body. The breads were freeze-dried and milled as described above.

### Design of Experiment with Added Asparagine and Glycine

A randomized circumscribed central composite design of 22 experiments was made, including two blocks each with three repetitions of the central point and no repetition of cube and satellite points (Table I). Logarithmic scales were used to describe a wide range of amino acid levels. Factor levels were 0.08–1.80 g (0.50–12.02 mmol) of L-asparagine-monohydrate per 100 g of wheat flour and 0.04–1.57 g (0.50–20.89 mmol) of L-glycine per 100 g of wheat flour.

### Dough Analysis

Dough analysis was performed on one of the blocks of the designed experiment. The content of free asparagine and glycine in the fermented dough was analyzed as described by Davies (2002). Free amino acids were extracted with sulfosalicylic acid. Norleucine, a synthetic amino acid, was added as an internal standard. Amino acids were separated by cation exchange chromatography and quantified by postcolumn derivatization with ninhydrine (Biochrom Analysator, Pharmacia Biotech, Uppsala, Sweden).

The content of reducing sugars in the dough samples was determined as described by Hostettler et al (1951). Freeze-dried and milled dough (1.0 g) was extracted with 10 mL of water for 3.5 min on a roller mixer (Swelab, Årsta, Sweden). Then the sample was centrifuged (10 min at  $1,000 \times g$ ) and 1.0 mL of supernatant was mixed with 2.0 mL of Sumner's reagent containing 1% (w/v) 3,5-dinitrosalicylic acid in water (Merck, Darmstadt, Germany).

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The solution was heated for 5 min in a boiling water bath, cooled in running water, and diluted with water to 25 mL in a volumetric flask. Absorption was measured in a spectrophotometer (Shimadzu, Kyoto, Japan) at 530 nm. A standard curve was made with glucose solutions and molar content of reducing sugars was calculated. All samples were analyzed in triplicate.

### Bread Analysis

Once the breads were out of the oven, the central crumb temperature was measured using a thermocouple digital thermometer (Surdyk et al 2004). After 1 hr of cooling at room temperature, the fresh weight, volume by displacement with sago sand, and porosity according to the Dallman scale were recorded. Thereafter, breads were frozen, freeze-dried, crushed, and milled as described above.

The color of each milled bread was measured three times with a chromameter (Minolta, Osaka, Japan) and samples were thoroughly mixed between measurements (Mustafa et al 2005). The color difference from the absolute black ( $\Delta E^*$ ) was calculated from the  $L^*$ ,  $a^*$ , and  $b^*$  values.

The analysis of acrylamide in the milled samples followed the method recently published by Fohgelberg et al (2005). Deuterium-labelled acrylamide was added as an internal standard and acrylamide was extracted with water. The solutions were purified on two different solid-phase extraction columns and analyzed in duplicate by liquid chromatography tandem mass spectrometry.

### Glycine Spraying Experiment

Three batches of dough, with asparagine-monohydrate (0.55 g) but no glycine added, were prepared according to the general baking recipe described above. After the second fermentation, a glycine solution (1.34M in water) was sprayed on the surface of the top cap of the fermented dough in the baking tin by using a plastic spray bottle fixed on a tripod. The distance between the nozzle of the spray bottle and the top surface of the dough was 15 cm. Three doughs were sprayed one time each with the glycine solution by pressing the lever of the bottle once; three doughs were sprayed eight times (1 sec between repeated sprays) with the glycine solution; and three doughs were sprayed eight times with water (control). The repeatability of the spraying procedure was

evaluated by spraying water on filter papers of the same size as the baking tin opening and determining the increase in weight. Breads were baked directly after spraying and the content of acrylamide in breads was measured as described above.

### Statistics

The experiment with added asparagine and glycine was designed and evaluated by regression analysis using Minitab software (v.14, Minitab, State Collage, PA).

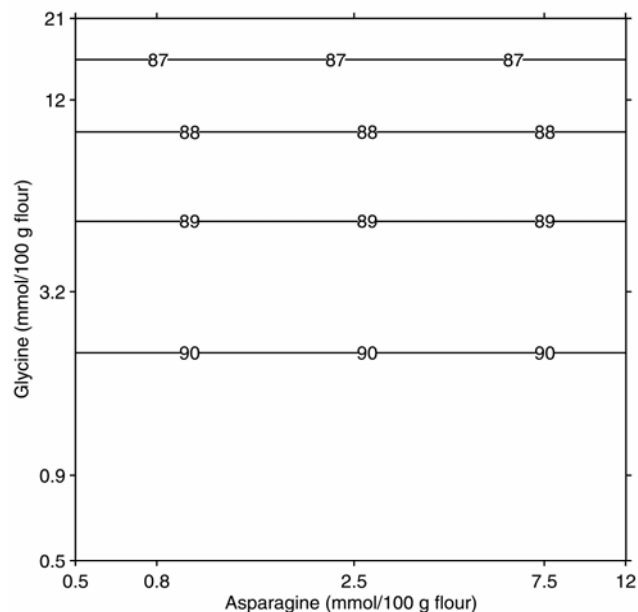


Fig. 1. Response surface showing effect of added glycine and asparagine on color ( $\Delta E^*$  value) of milled yeast-leavened bread. Design levels are indicated on logarithmic scales.

TABLE I  
Experimental Design, Content of Free Asparagine, Free Glycine, and Reducing Ends in Dough, and Color Difference ( $\Delta E^*$ ) and Acrylamide Content in Bread

Block	Added Asn (mmol/100 g of flour)	Added Gly (mmol/100 g of flour)	Asn After Fermentation (mmol/100 g of dough) (n = 1)	Gly After Fermentation (mmol/100 g dough) (n = 1)	Reducing Sugars (mmol/100 g of dough) (n = 3)	Color Difference ( $\Delta E^*$ ) (n = 3)	Acrylamide ( $\mu\text{g}/\text{kg}$ of bread)
1	0.50	3.24	— <sup>a</sup>	—	—	89.0 ± 0.1 <sup>b</sup>	161
1	0.80	0.87	—	—	—	90.4 ± 0.2	197
1	0.80	12.10	—	—	—	87.7 ± 0.2	145
1	2.45	0.50	—	—	—	90.9 ± 0.1	1,090
1	2.45	3.24	—	—	—	88.2 ± 0.1	1,020
1	2.45	3.24	—	—	—	90.3 ± 0.4	804
1	2.45	3.24	—	—	—	89.5 ± 0.1	805
1	2.45	20.89	—	—	—	86.9 ± 0.3	347
1	7.55	0.87	—	—	—	90.8 ± 0.1	2,990
1	7.55	12.10	—	—	—	88.6 ± 0.9	1,617
1	12.02	3.24	—	—	—	90.3 ± 0.0	3,640
2	0.50	3.24	0.37	3.41	8.3 ± 0.2	89.8 ± 0.2	122
2	0.80	0.87	0.54	0.96	5.8 ± 0.2	90.3 ± 0.2	231
2	0.80	12.10	0.83	12.77	9.5 ± 0.1	86.8 ± 0.2	135
2	2.45	0.50	2.24	0.61	5.5 ± 0.4	90.7 ± 0.1	946
2	2.45	3.24	2.32	3.16	5.8 ± 0.2	89.0 ± 0.2	719
2	2.45	3.24	2.36	3.21	6.2 ± 0.5	89.2 ± 0.3	809
2	2.45	3.24	2.34	3.44	7.4 ± 0.1	90.0 ± 0.4	723
2	2.45	20.89	2.6	21.95	8.0 ± 0.2	85.5 ± 0.7	357
2	7.55	0.87	7.92	0.96	7.7 ± 0.2	88.8 ± 0.2	3,160
2	7.55	12.10	7.76	13.05	8.0 ± 0.3	87.3 ± 0.3	1,710
2	12.02	3.24	12.82	3.5	6.1 ± 0.9	91.2 ± 0.1	2,830

<sup>a</sup> Dough analyses performed only on samples from block 2.

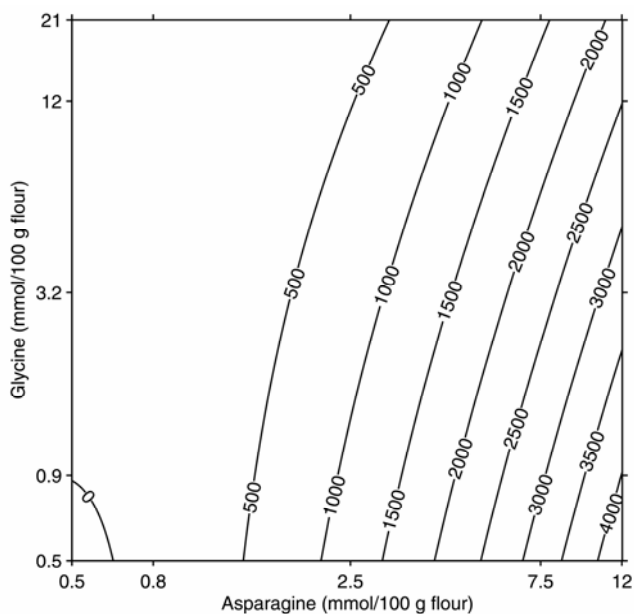
<sup>b</sup> Results are given as mean ± standard deviation.

**TABLE II**  
**P Values for Effect of Added Asparagine and Glycine and Their Interaction on Content of Free Asparagine, Free Glycine, and Reducing Ends in Dough, and Color Difference ( $\Delta E^*$ ) and Acrylamide Content in Bread from Response Surface Regression**

Factors	$\Delta E^*$	Asparagine	Glycine	Reducing Sugars	Acrylamide
Block	0.277	— <sup>a</sup>	—	—	0.214
Asn	0.214	<0.001	0.888	0.303	<0.001
Gly	<0.001	0.697	<0.001	0.024	<0.001
Asn × Asn	0.357	<0.001	0.979	0.314	<0.001
Gly × Gly	0.037	0.930	<0.001	0.651	0.431
Asn × Gly	0.276	0.698	0.898	0.097	<0.001
$R^2$ (%) <sup>b</sup>	73.7	98.1	97.7	54.4	97.4

<sup>a</sup> Dough samples from only one block were analysed.

<sup>b</sup> Explained variances by the model are given as  $R^2$ .



**Fig. 2.** Response surface showing effect of added asparagine and glycine on acrylamide content ( $\mu\text{g}/\text{kg}$  of bread) in yeast-leavened bread. Design levels are indicated on logarithmic scales.

## RESULTS AND DISCUSSION

### Experiment with Added Asparagine and Glycine

Central crumb temperature, volume, weight, and crumb porosity of the yeast-leavened breads did not significantly change when different amounts of amino acids were added to the dough. The central crumb temperature in the breads was  $99 \pm 0.4^\circ\text{C}$ , the bread volume was  $275 \pm 19$  mL, the weight of the fresh bread was  $85.0 \pm 0.5$  g; all breads had fully developed crumbs with porosity six on the Dallman scale.

The color difference ( $\Delta E^*$ ) of the milled breads varied between 85.5 and 91.2 (Table I). Added glycine significantly increased the color (reduced  $\Delta E^*$  values) of the milled breads, but added asparagine had no significant effect (Table II). The response surface model (Fig. 1) explained 73.7% of the variation in color. These results are in agreement with previous findings because glycine readily reacted with  $\alpha$ -dicarbonyls (Piloty et al 1979) and strongly enhanced browning (Ashoor et al 1984), while in a previous study the addition of asparagine to dough did not enhance browning of the bread crust with yeast-leavened wheat bread (Surdyk et al 2004).

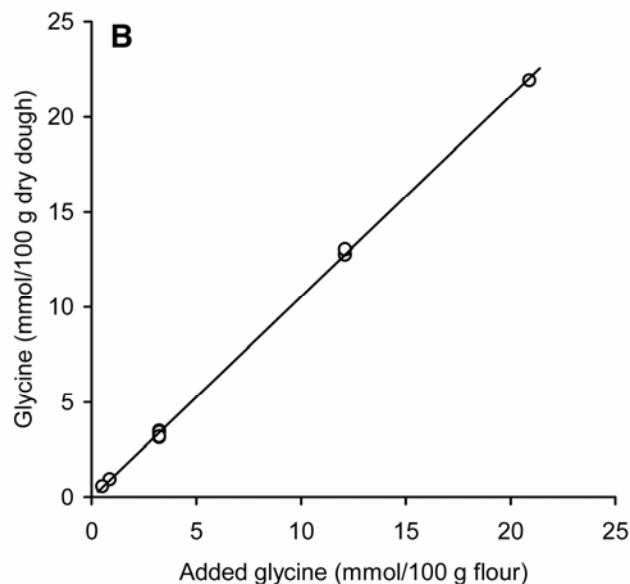
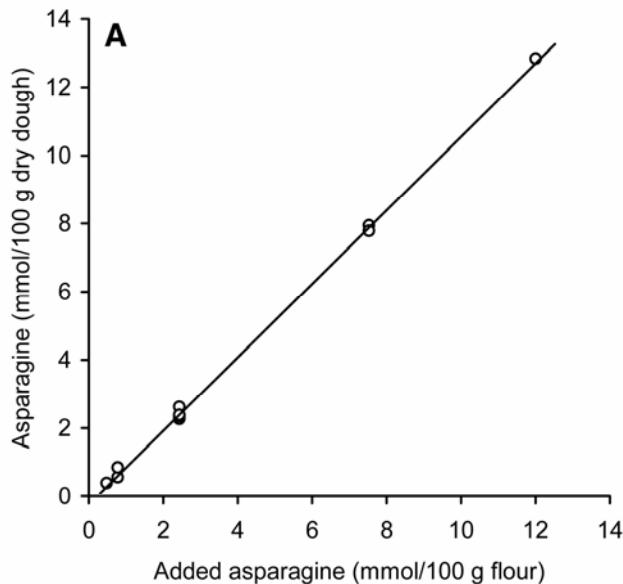
The acrylamide content in the breads was 122–3,640  $\mu\text{g}/\text{kg}$  of dry bread (Table I). Added asparagine strongly increased the content of acrylamide of the breads and added glycine decreased the content (Table II, Fig. 2). The higher the amount of asparagine added, the higher the reducing effect of added glycine on the content of acrylamide in the breads ( $P < 0.001$ ). Such an inter-

action effect has not been shown before. The levels of acrylamide in the breads are high compared with average levels (50  $\mu\text{g}/\text{kg}$  of bread) found in Swedish breads (Svensson et al 2003). This is due to the strong effect of added asparagine on the acrylamide content in bread as shown by Surdyk et al (2004), while the reducing effect of added glycine seems to be more moderate. In a very recent study, however, a stronger reducing effect (up to >90%) of added glycine on acrylamide content in bread crust of yeast-leavened bread was reported (Bråthen et al 2005). Other levels of amino acids in the dough and experimental conditions may be the reason for the difference in effect.

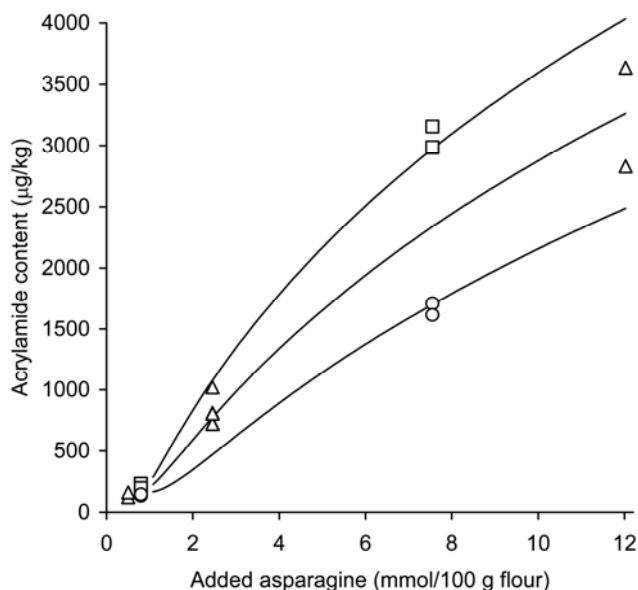
Because there were no significant differences between the two blocks in any of the parameters measured in the breads, amino acids and reducing sugars in the doughs were only measured in one of the blocks (Table I). A linear relationship with a slope close to one between added asparagine and analyzed asparagine content in fermented dough was found ( $y = 1.07x - 0.24$ ,  $R^2 = 1.00$ ) (Fig. 3). This result shows that the fermentation capacity of asparagine in the dough was limited and could highly reduce the content (original and added) only when the addition level was low. A similar relationship was found between added and analyzed glycine content in the fermented dough ( $y = 1.05x + 0.0004$ ,  $R^2 = 1.00$ ), showing that the effect of yeast fermentation on free glycine was very limited. Fredriksson et al (2004) found a strong and significant decrease in asparagine content during yeast fermentation, but in that case, the level of asparagine in the dough was lower. Benedito de Barber et al (1989) found that the content of free glycine in dough did not significantly change during the first 2.5 hr of yeast fermentation, which seems to be in line with our results.

The content of reducing sugars in fermented dough samples varied between 5.5 and 9.5 mmol/100 g (Table I). The mean level ( $7.1 \pm 1.3$  mmol/100 g of dough) was  $\approx 3\times$  higher than the analyzed content of reducing sugars in the flour (2.4 mmol/100 g). Addition of asparagine to the dough did not significantly influence the amount of reducing sugars in the fermented doughs, but addition of glycine to the dough significantly increased the amount of reducing sugars (Table II). One possible explanation could be that a high addition of glycine could lead to reduced yeast fermentation, consuming less reducing sugars. Surdyk et al (2004) suggested in a recent study, using the same basic recipe for the breads as in this experiment, that the molar concentration of reducing sugars in the dough, mostly formed during fermentation, strongly exceeds that of free asparagine. Thus, asparagine was considered to be the limiting factor for the formation of acrylamide. In this model study, however, reducing sugars were not always in excess, and the molar ratio between free amino acids (asparagine and glycine) and reducing sugars in the doughs varied greatly from sample to sample. Reducing sugars can also be formed in the crust from oligosaccharides and polysaccharides during heat treatment in the oven (Theander and Westerlund 1988).

Acrylamide content in bread as a function of added asparagine in the dough at three different levels of added glycine was computed from the response surface model (Fig. 4). Measured values are indicated in the figure and are generally found in close



**Fig. 3.** Correlation between added and determined asparagine (A) and glycine (B) in fermented dough.



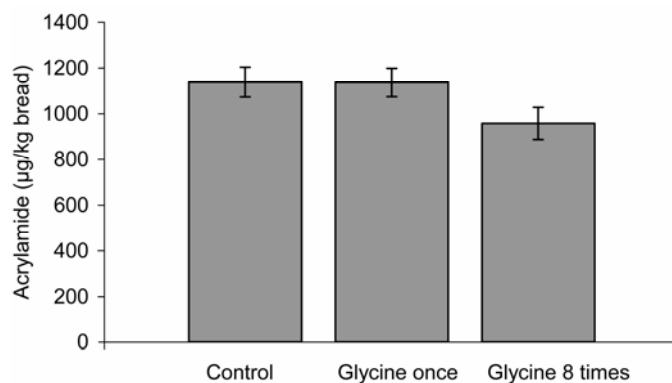
**Fig. 4.** Acrylamide content in bread as a function of added asparagine at three different glycine levels ( $\square$  0.87;  $\Delta$  3.24;  $\circ$  12.1 mmol of glycine/100 g of flour). Solid lines are computed from response surface model.

vicinity to the computed lines showing that added glycine reduced the acrylamide content in the bread at a certain level of added asparagine.

This reduction in acrylamide content could be explained by both a competition between the amino acids for the reducing sugars in the Maillard reaction or a further reaction of formed acrylamide with the added glycine as previously suggested (Rydberg et al 2003; Bråthen et al 2005). However, further studies are necessary to reveal the actual mechanisms.

#### Glycine Spraying

A previous publication showed that all acrylamide was formed in the crust (Surdyk et al 2004). It may thus be possible to reduce the application rate by applying glycine only to the surface of the fermented dough, thereby reducing the acrylamide content in the bread. Breads baked with added asparagine were divided into top



**Fig. 5.** Acrylamide content in yeast-leavened breads charged with asparagine after spraying the top surface with water (control) or a solution of glycine (1.34M) once or eight times. Error bars represent standard deviations.

cap ( $17.1 \pm 2.4$  g of dry matter) and bread body ( $40.7 \pm 2.4$  g). Average content of acrylamide in the top cap was  $1,560 \pm 206$  and in bread body only  $632 \pm 158$   $\mu\text{g}$  of acrylamide/kg of dry bread. This means that  $>50\%$  of the acrylamide was present in the top cap of the bread.

Spraying water or spraying an aqueous solution of glycine was repeatable (CV 12%) with each pressing of the lever delivering 0.2 g of solution. The correlation between number of sprayings ( $n = 1-8$ ) and measured amount of applied solution was very good ( $R^2 = 0.99$ ).

In the control breads, when only water was applied on the top of the dough, there was  $1,140 \pm 65$   $\mu\text{g}$  of acrylamide/kg of bread (Fig. 5). This content was not significantly changed when glycine was applied once on the top of the dough, but it was significantly reduced ( $P < 0.03$ ) to  $958 \pm 71$   $\mu\text{g}/\text{kg}$  of bread (16% reduction) when glycine was applied eight times. Thus, an application of glycine on the surface of the dough could be a method for lowering the content of acrylamide in commercial breads

An addition of glycine during dough making or spraying of glycine on the surface of fermented dough can lead to a significant reduction of acrylamide content in yeast-leavened breads. The more asparagine present in the dough, the stronger the reducing effect of added glycine. The color-enhancing effect of glycine needs to be considered for application in bakeries.

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