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Acrylamide in relation to asparagine in baked and toasted wheat and rye bread.

Abstract

Acrylamide in baked and toasted wheat and rye bread was studied in relation to the contents of asparagine in flour, dough, bread and toasts. The asparagine was consumed during bread preparation resulting in a reduced acrylamide contents in the products. In wheat bread 12% of the asparagine initially present in the flour (0.14g kg⁻¹) was left after yeast fermentation and baking, while for rye bread 82% of the asparagine was left after sourdough fermentation and baking. The asparagine present in the untoasted wheat bread had all reacted after hard toasting. Toasted wheat and rye bread slices contained 11-161 µg kg⁻¹ and 27-205 µg kg⁻¹ acrylamide compared to untoasted wheat and rye bread containing <5 µg kg⁻¹ and 7-23µg kg⁻¹ acrylamide. The dietary intake of acrylamide from bread (untoasted) of 2 µg/day is relatively low, however the acrylamide exposure from bread increases several times for people eating toasted bread.

Keywords: *Acrylamide, asparagine, bread, rye, wheat, toast, dietary, intake*

Introduction

The presence of acrylamide in heat treated food was recognized in 2002 (Tareke et al. 2002) and the same year it was clear that acrylamide is formed mainly from asparagine and a carbonyl compound through Maillard browning reactions during food processing (Mottram et al. 2002; Stadler et al. 2002). Several pathways and intermediates for acrylamide formation have been suggested including the intermediates: Schiff's bases, decarboxylated Amadori products (Yaylayan 2003), Strecker aldehydes (Mottram et al. 2002) and the deamination product 3-aminopropionamide which also generate acrylamide under aqueous conditions (Granvogl and Schieberle, 2006). Besides the main reaction routes through the Maillard reactions, acrylamide may be formed in bread after protein

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pyrolysis of added gluten (Claus et al. 2006). Also addition of cheese to bread has been found to increase the acrylamide formation, probably formed with 3-aminopropionamide as an intermediate in the cheese, which has been found to generate acrylamide (Granvogl and Schieberle 2006, Hedegaard et al. 2008).



Acrylamide is known to be carcinogenic in rodents and is classified by the International Agency for Research on Cancer as probably carcinogenic to humans (IARC 1994). The dietary acrylamide intake have been estimated to e.g. $0.3\text{--}0.8\mu\text{g (kg bw)}^{-1}\text{ day}^{-1}$ (WHO 2002); appr. $0.5\mu\text{g (kg bw)}^{-1}\text{ day}^{-1}$ (Svensson et al 2003). The dietary intake of acrylamide from bread is about 11% of the total dietary acrylamide intake of $31\mu\text{g day}^{-1}$ as estimated for the Swedish population. A similar contribution from bread related foods such as crisp bread and biscuits are estimated (Svensson et al. 2003). The acrylamide level in baked bread is relatively low, but due to high consumption of bread the contribution of acrylamide from bread is still important.

Toasting the bread, which is frequently used e.g. for breakfast meals, increases acrylamide significantly. While untoasted bread contains $<50\mu\text{g kg}^{-1}$ the acrylamide content may increase to a few to several hundreds micrograms per kilo bread after toasting (Konings et al. 2003; Becalski et al. 2003; Ahn et al. 2002). The acrylamide levels have been found to increase with increasing toasting time and whole meal rye bread was found to form more acrylamide than white wheat bread (Ahn et al. 2002).

The occurrence of acrylamide in bread may depend on several factors. Surdyk et al. (2004) showed that acrylamide in baked wheat bread correlated with the amount of asparagine added to dough, while the added reducing sugar fructose did not show a similar correlation, hence the asparagine was a limiting substrate for the acrylamide formation. The clear relationship between asparagine

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2
3 48 content in the dough and acrylamide formation has also been demonstrated in other wheat and rye
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5 49 bread model systems (Elmore et al. 2005; Bråthen et al. 2005).
6
7 50 One of the strategies to reduce acrylamide in cereal products may be to choose raw materials low in
8
9 51 asparagine by selecting optimum cultivars and growth conditions. However, variations in free
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11 52 asparagine in cereals are modestly researched. Claus et al. 2006 investigated agronomic factors
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13 53 related to the acrylamide formation in cereal products. They showed that nitrogen fertilization
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15 54 resulted in elevated amino acid levels resulting in increased acrylamide levels. The asparagine
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17 55 contents of selected European wheat varieties ranged from 0.07-0.66 g kg⁻¹, that is a factor of five
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19 56 between varieties and a factor of two within varieties (Taeymans et al. 2004).
20
21 57 Milling will differentiate the asparagine levels in the different milling fractions. Fredriksson et al.
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23 58 (2004) found that the contents of free asparagine are lower in sifted wheat flour i.e. 0.14- 0.17 g kg⁻¹
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25 59 (dw) compared to whole grain wheat flour i.e. 0.5 g kg⁻¹(dw) or wheat bran. Rye generally contains
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27 60 more asparagine: sifted rye flour 0.6 g kg⁻¹(dw) and whole grain rye flour: 1.1 g kg⁻¹(dw). Not only
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29 61 milling has been found to influence the levels of the limiting substrate asparagine. It was found that
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31 62 yeast fermentation of wheat dough depleted a major part of the asparagine (>80%), while
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33 63 sourdough fermentation of rye dough reduced the asparagine content to a smaller extent
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35 64 (Fredriksson et al. 2004). During baking, parameters such as water activity, heating time and
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37 65 temperature, surface to volume ratio, the presence of additives and pH also influence acrylamide
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39 66 levels (Surdyk et al. 2004; Rydberg et al. 2003). Acrylamide formation may increase when
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41 67 increasing pH e.g. by using baking soda or ammonium carbonate for bread leavening (Amrein et al.
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43 68 2005) as it will decrease when lowering pH (Jung et al. 2003; Pedreschi et al. 2004, 2005).
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45 69 Fermentation will cause pH in dough to drop due to release of carbon dioxide (Raccach et al. 2004)
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47 70 and it will reach pH~5 using yeast and pH~4.4-4.8 using sourdough (Suhr et al. 2004).
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Deleted: Favorable light and temperature conditions one year gave  acrylamide in bread compared to  year probably by enhancing amino acid and protein contents. Flour derived from sulphur deprived wheat was also found to contain high levels of asparagine (Muttucumaru et al. 2006, Granvogl & Schieberle 2007), so it is important that the cereals are grown at a sufficient sulfur level

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The aim of the present study was to find the relationship between asparagine levels and acrylamide formation in wheat bread and rye bread during baking and toasting in order to be able to reduce the acrylamide levels. Furthermore to assess the acrylamide levels in bread on the Danish market and to estimate the dietary intake of acrylamide from wheat and rye bread including toasted bread.

Materials and methods

Baking wheat bread and rye bread with asparagine additions

An industrial bakery (*Lantmännen Schulstad A/S, Hvidovre, Denmark*) made the wheat and rye breads in their laboratory bakery with different amounts of asparagine added (in August 2004). The experimental design appears from Figure 1. Four batches of wheat dough were prepared and two breads selected from each batch: One batch of dough was made without adding asparagine representing the natural background asparagine level and three batches were added asparagine at concentration levels of 0.05, 0.13 and 0.27 g kg⁻¹ dough. As the dough contains 60% wheat flour, the added amounts of asparagine based on flour were 0.09, 0.22 and 0.44 g kg⁻¹ corresponding to addition at approximately 0.6 - 3 times the actual background level of asparagine. The recipe for a wheat bread (appr. 800 g dough) included sifted wheat flour, yeast, salt, sugar vegetable oil and water. Kneading time was 7 min., holding time 20 min. and resting time at 32°C was 45 min. Baking was performed initially at 230°C for 2 min. and subsequently at 200°C for 30 min. Rye breads were made using the same experimental design. The asparagine was added to the dough at concentrations based on rye flour of 0.17, 0.43 and 1.75 g kg⁻¹ respectively corresponding to addition at 0.2 - 2 times the natural background level. The rye bread (based on appr. 900 g dough) was made from half and half mixing of sourdough and rye flour and addition of yeast, salt and water. Kneading time was 10 min, holding time 20 min, and resting time at 37°C was 77 min.

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3 95 Baking was performed initially at 280°C for 5 min., followed by 210°C for 35 min. and 190°C for
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9 98 *Toasting wheat bread and rye bread with asparagine additions*

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11 99 Toasting of the bread slices was done in a pushdown toaster (Tefal Delfini Compact Toaster 5396,
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13 100 800 W) with room for two slices. The four batches of bread (background level and the three
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15 101 addition levels) were analyzed either untoasted or after medium or hard toasting, for each level
16
17 102 using three slices from each of two breads (n=18). Medium toasting of wheat bread slices of 13 mm
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19 103 was performed 1.9 min. and hard toasting 2.8 min. Medium toasting of the rye bread slices of 8
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21 104 mm was done 2.8 min. and the hard toasting 3.1 min. All the toasted bread slices were medium to
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23 105 dark brown and were considered eatable. [The thicknesses of the bread slices were chosen from what](#)
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25 106 [is common practice among bakers and consumers.](#)

26 107
27 108 [Figure 1](#)
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33 112 *Chemicals*

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35 113 Acrylamide (>99.5%) was purchased from Sigma-Aldrich (St. Louis, MO, USA), asparagine
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37 114 monohydrate (>99%) from Acros Organics (Geel, Belgium), D₃-acrylamide (>98%) from Polymer
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39 115 Source (Dorval, Quebec, Canada), ¹⁵N₂-asparagine monohydrate (>98%) from Cambridge Isotope
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41 116 Labs. (MA, USA), methanol (>98%), formic acid (>98%) and acetonitrile (HPLC-grade) from
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43 117 Rathburn Chemicals (Walkerburn, UK).
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46 119 *Chemical analysis*
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The industrial bakery produced small wheat breads (baguettes) in their laboratory bakery. The recipe for the wheat breads (appr. 3000 g dough ~ 60 breads) included sifted wheat flour, yeast, salt, sucrose, margarine and water. Kneading time was 3+5 min., holding time 20+10 min., and resting time at 37°C was 50 min. Baking was performed at 220°C for 10 min. or 15 min. in a ventilated oven and with water spray added for 30 s..

The chemical analyses were performed using a recently developed LC-MS/MS method for simultaneous analysis of acrylamide, asparagine and reducing sugars (Nielsen et al. 2005) and in addition some of the acrylamide analyses were made with another LC-MS/MS method as described in Pedreschi et al. (2005).

Sliced toasted or untoasted wheat or rye bread was weighed, dried in an oven at 50°C over night and homogenized using a Braun handheld mixer (type 4169) fitted with a blender like sample compartment (type 4297) (Braun AG, Frankfurt/m, Germany). The drying temperature was set relatively low in order to avoid acrylamide formation during the drying process. The wet (semidry) and dry weight was determined from weighing before and after drying of the baked and toasted bread slices. Aliquots of 4 g of homogenate was extracted with MilliQ water after adding internal standards: 200 µL 10 µg mL⁻¹ D₃-acrylamide, 100 µL 880 µg mL⁻¹ ¹⁵N₂-asparagine. The sample was extracted by an Ultra Turrax mixer. After centrifuging, SPE cleanup was performed by an automated sampler using LiChroLut RP-C18 SPE-cartridges LC-MS/MS detection was performed on a triple quadrupole instrument (Micromass Quattro Ultima) after separation on a hypercarb LC-column (dimensions 2.1 mm x 100 mm, particle size 5 µm) eluted with 0.1% aqueous formic acid at 0.2 mL min⁻¹. The mass spectrometer was operated in ESI⁺ for acrylamide and saccharides and in ESI⁻ for asparagine. Capillary voltages of 3 kV (ESI⁺) and 2 kV (ESI⁻) were applied but for simultaneous detection in positive and negative mode a lower capillary voltage, e.g. 1 kV is recommended to prevent sparking from the capillary.

Some of the acrylamide analysis was performed after the methods were optimized to detect low concentrations (e.g. by increasing the HPLC injection volume and by optimizing the MS performance before analysing the bread samples), so the method was able to quantify down to 5 µg kg⁻¹. The repeatability and reproducibility of the acrylamide analyses at 50 and 250 µg kg⁻¹ (n=24) were 6-17% and 9-29% respectively. The laboratory has participated in proficiency tests for

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3 144 acrylamide in food and all results obtained have been with satisfactory test scores, including the
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5 145 results obtained in [an](#) EU validation study on acrylamide analyses in food with 22 samples (11
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7 146 matrices including bread and toasted bread: assigned value 38µg kg⁻¹, obtained results 41 and 44µg
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9 147 kg⁻¹) (Wenzl et al. 2006). [The detection limit of the asparagine analyses was 0.002 g kg⁻¹, the](#)
10
11 148 [repeatability and reproducibility measured at 0.02, 0.1 and 0.4 g kg⁻¹ 0-5% and 10-18% respectively.](#)
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13 149
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15 150 [Estimation of dietary acrylamide intakes](#)
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17 151 [Exposure estimates were accomplished using data from the Danish Nationwide Dietary Survey](#)
18
19 152 [made from 2000-2002 by the National Food Institute \(Lyhne et al. 2005\). Information on food](#)
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21 153 [consumption was collected from a representative sample of 4120 individuals aged 4-75 years. The](#)
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23 154 [participants in the survey kept a food record for seven consecutive days. The monitoring results of](#)
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25 155 [acrylamide in Danish foods \(appr. 330 samples\) were made 2002-2005 by the National Food](#)
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27 156 [Institute \(data not published elsewhere\).](#)

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30 158 **Results and discussion**

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34 160 The levels of acrylamide and asparagine in wheat and rye bread slices after baking and medium or
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36 161 hard toasting appear from Table I. The results presented are all means of six bread slices, each
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38 162 wheat bread slice weighing 34±2 g and each rye bread slice weighing 32±1 g.
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42 164 *Asparagine in flour and bread slices after asparagine additions to dough and subsequent*
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44 165 *fermentation, baking and toasting*
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46 166 The background content of asparagine in sifted wheat flour was 0.14 ± 0.004 g kg⁻¹ (based on three
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48 167 determinations and with [the precision measured as](#) 95% confidence levels) and in whole grain rye
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flour $0.76 \pm 0.02 \text{ g kg}^{-1}$, figures comparable to those reported by Frederiksson et al. (2004) of $0.14\text{--}0.17 \text{ g kg}^{-1}$ (dw) in sifted wheat flour and 1.1 g kg^{-1} (dw) in whole grain rye flour.

The levels of asparagine added to the wheat dough were 0.6, 1.6 and 3.1 times the natural background level and to the rye dough 0.2, 0.6 and 2.3 times the natural background level. The highest concentration after addition correspond to 0.6 g kg^{-1} asparagine in wheat flour and 2.5 g kg^{-1} asparagine in rye flour; hence levels likely to be present in cereal products used for bread production.

In Table II the reacted amounts of asparagine in the batches with different asparagine levels upon fermentation, baking and toasting are presented. Some asparagine had reacted during wheat bread production as can be seen from data determined on a dry weight basis (Table I), where differences from evaporation of water during toasting does not have to be considered. In the wheat batch with a natural background level of asparagine 12% was left after fermentation and baking. Upon hard toasting all asparagine in the wheat bread slices with the natural background content and the lowest addition level of asparagine had reacted.

[Table I](#)

[Table II](#)

In rye bread the relative reduction in asparagine during sourdough fermentation and baking was less than for the yeast fermented wheat bread, e.g. 18% at the natural background level and 9% for the highest addition level of asparagine. As the results are based on subtracting two large percentages, they may be more uncertain. The results are in accordance with those of Frederiksson et al. (2004), who also found sourdough fermentation to reduce asparagine less than yeast fermentation and at about the same rate, i.e. >80% for yeast fermentation of wheat bread and 17% for sourdough fermentation of rye.

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Deleted: were depleted in asparagine. The findings provide evidence for asparagine as the limiting factor in acrylamide formation in wheat bread, especially in toasted wheat bread.

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3 193 A general conclusion may be that rye flour contains more asparagine than wheat flour and
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5 194 sourdough fermentation and baking of rye dough reduces asparagine to a smaller extent than yeast
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7 195 fermentation and baking of wheat dough. Prolonging or optimizing the fermentation process,
8
9 196 especially the yeast fermentation of wheat bread may be an efficient tool to reduce the acrylamide
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11 197 formation in bread products.

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13 198 |
14 199
15 200 *Acrylamide in baked and toasted bread in relation to asparagine*
16 201 Baked wheat bread slices contained <5 µg kg⁻¹ acrylamide (<LOD), medium toasted 11-130 µg kg⁻¹
17
18 202 acrylamide and hard toasted 15-161 µg kg⁻¹ acrylamide for asparagine levels in flour of 0.14-0.60 g
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20 203 kg⁻¹ (natural background content to content after highest asparagine addition level). The
21
22 204 corresponding figures for the rye bread slices were: baked 7-23 µg kg⁻¹ acrylamide, medium toasted
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24 205 27-155 µg kg⁻¹ acrylamide; hard toasted 68-205 µg kg⁻¹ acrylamide for asparagine levels in flour of
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26 206 0.76-2.5 g kg⁻¹.

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28 207 In Figure 2, acrylamide concentrations in wheat and rye bread, initially baked with different
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30 208 asparagine addition levels, and after baking toasted to medium or hard level are plotted and marked
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32 209 with correlation coefficients. Due to the low acrylamide contents in the baked wheat bread slices no
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34 210 correlation was found between asparagine content in dough and acrylamide content in baked wheat
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36 211 bread. However, acrylamide contents in wheat bread correlated well with the asparagine levels
37 212 added to the dough for medium ($r^2 = 0.86$) and hard toasted bread slices ($r^2 = 0.87$). As smaller
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39 213 proportions of asparagine were consumed during yeast fermentation and baking for the higher
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41 214 asparagine additions the acrylamide contents increased exponentially with increasing asparagine
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43 215 levels (quadratic equations).

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45 216 The untoasted rye bread slices contained naturally 0.62 g kg⁻¹ (dw) asparagine, about 36 times
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47 217 more than the wheat bread slices. As for the wheat bread quadratic relationships between the

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content of asparagine in the rye flour, and acrylamide content in bread after medium ($r^2 = 0.86$) or hard ($r^2 = 0.88$) toasting appear (Figure 2b).

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Deleted: In the initial setup of the experiment, the natural asparagine level in rye was estimated too low, why the two lowest addition levels are close to the background level i.e. at only 1.4 and 1.6 times the actual asparagine background level in flour. For untoasted rye bread the asparagine additions all showed significantly higher acrylamide content compared to the background ($p < 0.05$) but the correlation between asparagine addition and acrylamide in baked bread is weaker ($r^2 = 0.45$). The medium and hard toasted rye bread were significantly higher in acrylamide for all asparagine additions ($p < 0.005$).

In the present study, a major part of the asparagine added to the wheat dough had reacted during preparation of the bread. It is known, that especially yeast fermentation promote elimination of asparagine, and that the reduction depends on e.g. time of fermentation, the amount of yeast added and pH (Frederiksson et al. 2004). When comparing the asparagine levels left in baked bread before toasting with the acrylamide content, in toasted bread slices good linear relationships are present for both medium toasted ($r^2 = 0.87$) and hard toasted ($r^2 = 0.94$) wheat bread and for asparagine levels of 0.017-0.29 g kg⁻¹ (dw) bread. The asparagine levels in baked rye bread also showed good linear correlations with acrylamide formed after medium toasting ($r^2 = 0.90$) and hard toasting ($r^2 = 0.76$).

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Deleted: In wheat and rye bread toasted for 3.1 min., representing the hard toasted wheat bread and the medium toasted rye bread, the ratio of acrylamide units in the toasted bread per unit asparagine in the untoasted bread was found to be higher in wheat bread (6.7×10^{-4}) compared to rye bread (0.89×10^{-4}) that is the ratio of acrylamide formation in wheat bread was seven times higher than in rye bread.

Figure 2

Deleted: Lindenmeier & Hofmann (2004) showed that due to their lower pH value (~4.0) sourdough fermented breads contained higher amounts of the key antioxidant in bread crust pronyl-L-lysine than breads that have been yeast-fermented only. Hence besides the lower acrylamide formation rate at lower pH, the increased antioxidant content in the crust of rye bread may further reduce acrylamide formation, as some antioxidants have an inhibitory effect on the acrylamide formation. Addition of the lysine-rich casein (lysine reduces the acrylamide yield) to the bread recipe also increased the amounts of antioxidant (Lindenmeier & Hofmann 2004, Claeys et al 2005). Hence the effect of lysine may be from increasing the amounts of antioxidants with inhibitory effects. ¶

Although it is not possible directly to compare between acrylamide formation in wheat and rye bread having different slice thicknesses, water contents and asparagine levels it seems as the rate of acrylamide formation is somewhat higher in wheat bread compared to rye bread. This may be explained by lower pH conditions in sourdough fermented rye bread compared to yeast fermented wheat bread (pH appr. 0.5 units lower in sourdough). A better retention of water in the bread when toasting rye bread compared to wheat bread may also reduce the acrylamide formation. The average water content in hard, medium and untoasted rye bread slices were 24±2%, 26±2% and 41±1% while the average water content in hard, medium and untoasted wheat bread slices were somewhat

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3 242 lower: 17±2%, 19±1% and 32±1%, respectively. However, the actual water contents at the toasted
4 surfaces were probably somewhat lower. Hedegaard et al. (2007) found that at high water activity
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7 244 the rate determining step of acrylamide formation from the Schiff's base is the elimination of water
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9 245 from an intermediate, while at lower water activity, water elimination becomes easier and
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11 246 decarboxylation of a reaction intermediate leading to acrylamide becomes the rate determining step.
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13 247 Hence the acrylamide formation rate increases when the water activity decreases. Hence if different
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15 248 reactions are rate determining, the overall reaction rate may differ for different water activities,
16
17 249 which may explain the exponential increase in acrylamide contents (as the water content are
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19 250 reduced) generally found during thermally processing of food at a fixed temperature. It was also
20 found in other model systems that acrylamide formation increased when lowering the water content
21
22 252 (Schieberle et al. 2005, Blank et al. 2005).
23
24 253 The proportions of asparagine in wheat bread reacting to give acrylamide were also estimated
25
26 254 (Table III). Generally the ratio is higher in medium toasted bread suggesting that at higher
27
28 255 temperatures and/or longer duration of toasting precursors are depleted faster, reacting faster in
29
30 256 competing reactions and/or acrylamide is eliminated to a larger extent by subsequent reaction. For
31
32 257 the hard toasted bread is seen, that as depletion of the asparagine pool approaches, the formation of
33
34 258 acrylamide diminishes, resulting in lower ratios at lower asparagine addition levels. At the low
35 asparagine levels in wheat bread asparagine was depleted after hard toasting while at higher
36 259 addition levels some asparagine was left. This may be interpreted as if asparagine was the limiting
37 precursor at low asparagine levels while at higher levels other factors also influenced the
38 260 acrylamide formation. However the other precursor the reducing sugars was not depleted (data not
39 shown).
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Deleted: Because much smaller proportions of the asparagine had reacted upon toasting of the rye bread the fractions reacted were more uncertain and are therefore not measured. ¶

Table III.

Acrylamide contents in wheat breads and rye breads produced in Denmark and comparison with other surveys.

In order to estimate the level of acrylamide in rye bread, in 2004 13 sifted and whole meal rye breads produced for or sold on the Danish market were sampled and analysed. They contained $12 \pm 5 \mu\text{g kg}^{-1}$ acrylamide (range <5 - $19 \mu\text{g kg}^{-1}$, $n=13$).

In 2005 ten samples of wheat breads, produced for or sold on the Danish market were analysed, containing $10 \pm 6 \mu\text{g kg}^{-1}$ acrylamide (range <5 - $22 \mu\text{g kg}^{-1}$).

The contents of acrylamide in bread found in the present study are comparable with the results found in other studies. Becalski et al. (2003) found $290 \mu\text{g kg}^{-1}$ of acrylamide in toasted wheat bread compared to $19 \mu\text{g kg}^{-1}$ in untoasted wheat bread ($n=2$). Light toasted rye bread contained $28 \mu\text{g kg}^{-1}$ acrylamide and untoasted rye bread $17 \mu\text{g kg}^{-1}$. Eerola et al. (2007) found that toasted wheat bread contained $111 \pm 10 \mu\text{g kg}^{-1}$ acrylamide ($n=5$) and untoasted wheat bread $<68 \mu\text{g kg}^{-1}$ acrylamide, which was the limit of detection ($n=5$). Ahn et al. (2002) found that acrylamide increased with longer toasting time. After 3.1 min. of toasting when the toasts were still considered eatable wheat bread contained appr. $60 \mu\text{g kg}^{-1}$ and rye bread contained appr. $80 \mu\text{g kg}^{-1}$ acrylamide. After 6 minutes wheat bread contained appr. $206 \mu\text{g kg}^{-1}$ and rye bread contained appr. $220 \mu\text{g kg}^{-1}$ acrylamide. Konings et al. (2003) reported acrylamide contents in toasted bread of $183 \pm 336 \mu\text{g kg}^{-1}$ (range <30 - $1430 \mu\text{g kg}^{-1}$, $n=17$). The contents in dark rye bread were $44 \pm 13 \mu\text{g kg}^{-1}$ (range 30 - $60 \mu\text{g kg}^{-1}$, $n=5$) and in wheat bread $<30 \mu\text{g kg}^{-1}$ ($n=6$).

Dietary acrylamide intake from bread

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The average dietary intake of wheat bread in Denmark is 125 g day⁻¹ and in addition Danes has a tradition for eating whole meal sourdough fermented rye bread as part of nearly every lunch meal comprising an average daily intake of 59 g rye bread day⁻¹ (Lyhne et al. 2005). In case all bread is consumed without toasting and the mean figures for acrylamide in bread is used: 10 µg kg⁻¹ in wheat bread and 12 µg kg⁻¹ in rye bread, the daily intake comprises 1.3 µg from wheat bread and 0.7 µg from rye bread, respectively. The daily intake of acrylamide from wheat and rye bread comprising 2.0 µg is about 8 % of the average daily acrylamide intake of appr. 25 µg day⁻¹ estimated for Danes (Figure 4). The acrylamide intake from bread is relatively low compared to other surveys, estimating e.g. 3.4 µg (Svensson et al. 2003) and 3.6 µg (Matthys et al. 2005). An explanation for the low Danish dietary acrylamide intake from bread may be that most Danish bread are produced using either yeast fermentation or sourdough fermentation, hence the contents of the precursor asparagine are reduced during preparation of the breads. Another explanation for the low acrylamide intake from bread may be that in previous studies on acrylamide in bread the limit of detection (LOD) was higher and the levels in bread often below the LOD. Hence in many cases LOD was used as the acrylamide level in bread. Finally in the present estimate, as the Danish Nationwide dietary survey do not have information on how frequently people eat toasted bread, the acrylamide intake from toasted bread is not included in the dietary acrylamide intake from bread. However, in case the average daily bread intake includes two hard toasted wheat bread slices (acrylamide 161 µg kg⁻¹ (ww) each of 28 g and with the high pre-processing asparagine level of 0.6 g kg⁻¹ (dw) which is about the concentration found in wholegrain wheat flour i.e. 0.5 g kg⁻¹ (Frederiksson et al. 2004), the daily acrylamide intake is estimated to 9 µg (36 % of the average Danish acrylamide intake). If a consumer eats an average amount of bread of which two slices of 25 g rye bread are hard toasted with asparagine and acrylamide concentrations of 1.2 g kg⁻¹ and 123 µg kg⁻¹ respectively, the daily acrylamide intake from bread comprises 7 µg (28 % of the average

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Deleted: Exposure estimates were accomplished using consumption data from the Danish Nationwide dietary survey made from 2000-2002 by the National Food Institute (Lyhne et al. 2005). Information on food consumption was collected from a representative sample of 4120 individuals aged 4-75 years. The participants in the survey kept a food record for seven consecutive days

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[Danish acrylamide intake](#)). Hence dietary habits with consumption of toasted bread, which are common throughout at least Europe, may increase the acrylamide intake from bread considerable. Especially intake of toasted wholemeal wheat bread and bread produced without leavening by fermentation may further increase the acrylamide exposure.

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[Figure 4.](#)

Conclusions

The contents of acrylamide in baked rye bread and in toasted wheat and rye bread are correlated to the contents of asparagine in the cereal product used for the bread production and at asparagine concentration ranges that may occur in grain products for bread production. The content of asparagine in the dough for wheat bread preparation is reduced considerably during bread production. After 65 min. raising time and subsequent baking, the asparagine content in the baked wheat bread was reduced to 12% compared to the content in the flour before yeast fermentation. The sourdough fermentation of rye bread showed a modest asparagine reduction to 82% of the content in the flour. This is probably due to the more acidic conditions in sourdough fermented rye bread compared to yeast fermented wheat dough. The contents of acrylamide formed in toasted wheat bread [slices](#) and toasted rye bread [slices](#) are proportional to the asparagine concentration of the untoasted bread. The dietary intake of acrylamide from untoasted bread of $2.0 \mu\text{g day}^{-1}$ is relatively low but the dietary acrylamide intake increases somewhat for people eating toasted bread.

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Acknowledgement

This research was supported by the Danish Ministry of Food, Agriculture and Fisheries as a part of the project “Reduction of the formation and the occurrence of acrylamide in food” [and The Nordic Innovation Centre Project NORDACRYL \(Acrylamide precursors; Limiting substrates and in vivo effects\)](#). Lone Hertz is acknowledged for performing parts of the chemical analyses.

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Table I. Levels of asparagine added to a) wheat dough and b) rye dough, asparagine content in wheat and rye bread slices and the acrylamide contents after medium or hard toasting of the bread slices (mean \pm 2sd).

Table II. Asparagine left after fermentation and baking, and subsequent medium or hard toasting of wheat and rye bread (in percentage of asparagine in the flour).

Table III. Amount of acrylamide formed and present [μ g] from asparagine reacted [mg] when toasting wheat bread (ratios calculated by subtracting analyte concentrations determined in medium or hard toasted bread from analyte concentrations determined in untoasted bread).

Figure 1. Experimental design for baking wheat and rye bread with addition of asparagine and subsequent medium or hard toasting of bread slices. All bread slices were analysed for acrylamide and asparagine.

Figure 2. Correlation between naturally occurring and added asparagine in flour and acrylamide in baked and toasted bread slices of a) wheat or b) rye.

Figure 3. Correlation between asparagine in untoasted bread and acrylamide in toasted bread slices of a) wheat or b) rye.

Figure 4. The proportions from different foods to the daily mean acrylamide intake for Danish adults of appr. 25 μ g.

Table I
a)

Wheat	Asparagine in flour (background + added level) [g kg ⁻¹ (dw)]	Asparagine in bread [g kg ⁻¹ (dw)]	Asparagine in bread [g kg ⁻¹ (ww)]	Acrylamide in bread [μg kg ⁻¹ (ww)]
Untoasted	0.14 (no addition)	0.017 ± 0.01	0.012 ± 0.01	<5*
	0.22 (+0.09)	0.037 ± 0.01	0.025 ± 0.01	<5*
	0.37 (+0.22)	0.11 ± 0.01	0.074 ± 0.01	<5*
	0.60 (+0.44)	0.29 ± 0.02	0.20 ± 0.01	<5*
Medium toasted	0.14 (no addition)	0.015 ± 0.02	0.012 ± 0.01	11 ± 4
	0.22 (+0.09)	0.032 ± 0.01	0.026 ± 0.01	26 ± 9
	0.37 (+0.22)	0.092 ± 0.01	0.075 ± 0.01	46 ± 9
	0.60 (+0.44)	0.24 ± 0.01	0.19 ± 0.01	130 ± 23
Hard toasted	0.14 (no addition)	<0.002*	<0.002*	15 ± 6
	0.22 (+0.09)	<0.002*	<0.002*	34 ± 9
	0.37 (+0.22)	0.014 ± 0.03	0.011 ± 0.02	65 ± 6
	0.60 (+0.44)	0.20 ± 0.01	0.17 ± 0.01	161 ± 15

b)

Rye	Asparagine in flour (total of background and added level) [g kg ⁻¹ (dw)]	Asparagine in bread [g kg ⁻¹ (dw)]	Asparagine in bread [g kg ⁻¹ (ww)]	Acrylamide in bread [μg kg ⁻¹ (ww)]
Untoasted	0.76 (no add.)	0.62 ± 0.02	0.36 ± 0.02	12 ± 3
	0.93 (+0.17)	0.78 ± 0.03	0.45 ± 0.01	7 ± 4
	1.19 (+0.43)	0.99 ± 0.04	0.58 ± 0.03	17 ± 2
	2.49 (+1.7)	2.3 ± 0.1	1.3 ± 0.1	23 ± 8
Medium toasted	0.76 (no add.)	0.72 ± 0.03	0.53 ± 0.02	27 ± 3
	0.93 (+0.17)	0.82 ± 0.03	0.62 ± 0.02	54 ± 9
	1.19 (+0.43)	1.08 ± 0.03	0.80 ± 0.03	65 ± 17
	2.49 (+1.7)	2.2 ± 0.2	1.6 ± 0.1	155 ± 14
Hard toasted	0.76 (no add.)	0.64 ± 0.04	0.48 ± 0.02	68 ± 4
	0.93 (+0.17)	0.75 ± 0.03	0.57 ± 0.02	100 ± 8
	1.19 (+0.43)	1.01 ± 0.03	0.79 ± 0.04	123 ± 12
	2.49 (+1.7)	2.2 ± 0.1	1.6 ± 0.04	205 ± 41

* Limit of detection (LOD)

Table II

Asparagine in flour [g kg ⁻¹]	Asparagine after fermentation and baking %	Asparagine after fermentation, baking and medium toasting %	Asparagine after fermentation, baking, and hard toasting %
WHEAT			
0.14	12%	11%	0%
0.22	17%	14%	0%
0.37	30%	25%	4%
0.60	49%	39%	33%
RYE			
0.76	82%	95%	84%
0.93	83%	88%	80%
1.19	83%	91%	85%
2.49	91%	89%	86%

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Table III.

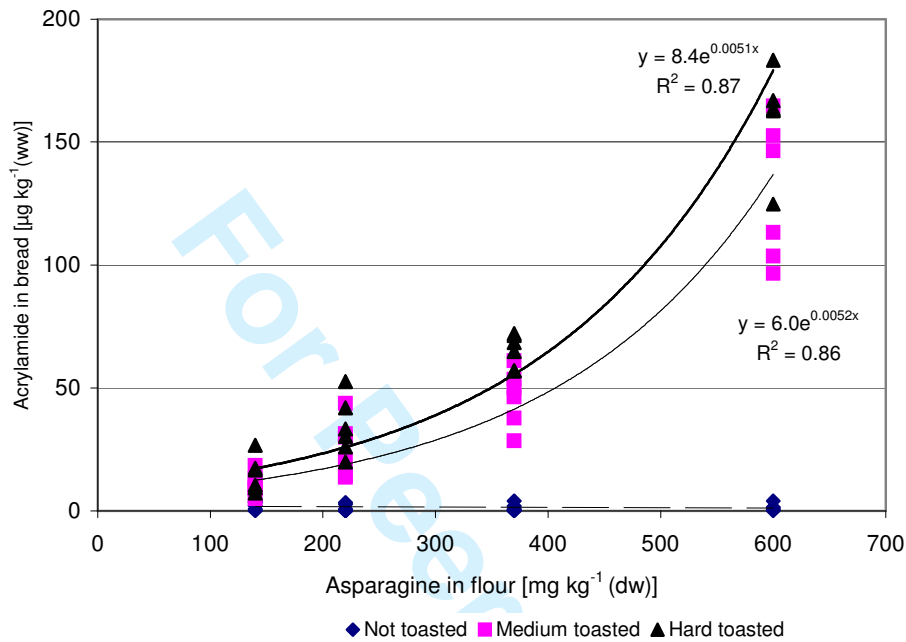
Asparagine level in dough g kg ⁻¹ (dw)	Medium toasted [µg acrylamide formed per mg asparagine reacted upon toasting]	Hard toasted [µg acrylamide formed per mg asparagine reacted upon toasting]
0.14 ± 0.01	5 ± 2	1 ± 0.4
0.22 ± 0.01	8 ± 4	1 ± 0.3
0.37 ± 0.01	4 ± 1	1 ± 1.0
0.60 ± 0.01	3 ± 1	2 ± 0.3

TYPE OF BREAD:	Rye bread Wheat bread
LEVEL OF ASPARAGINE ADDITION:	0. Natural asparagine level 1. Low asparagine addition 2. Medium asparagine addition 3. High asparagine addition
LEVEL OF TOASTING:	Untoasted slices (n=6) Medium toasted slices (n=6) Hard toasted slices (n=6)

Fig. 1

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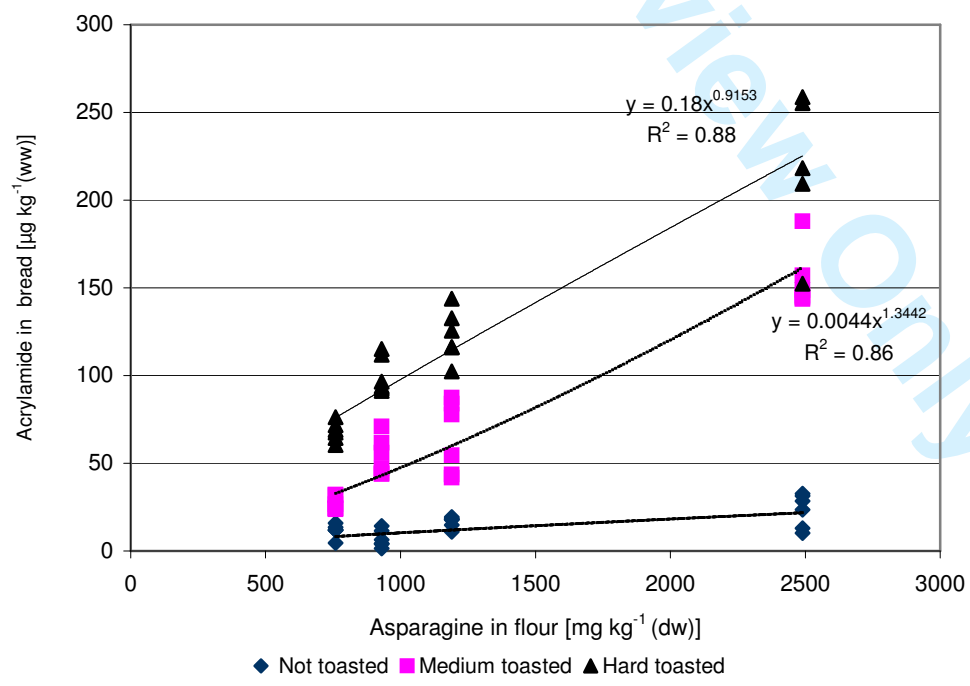


Fig 2

For Peer Review Only

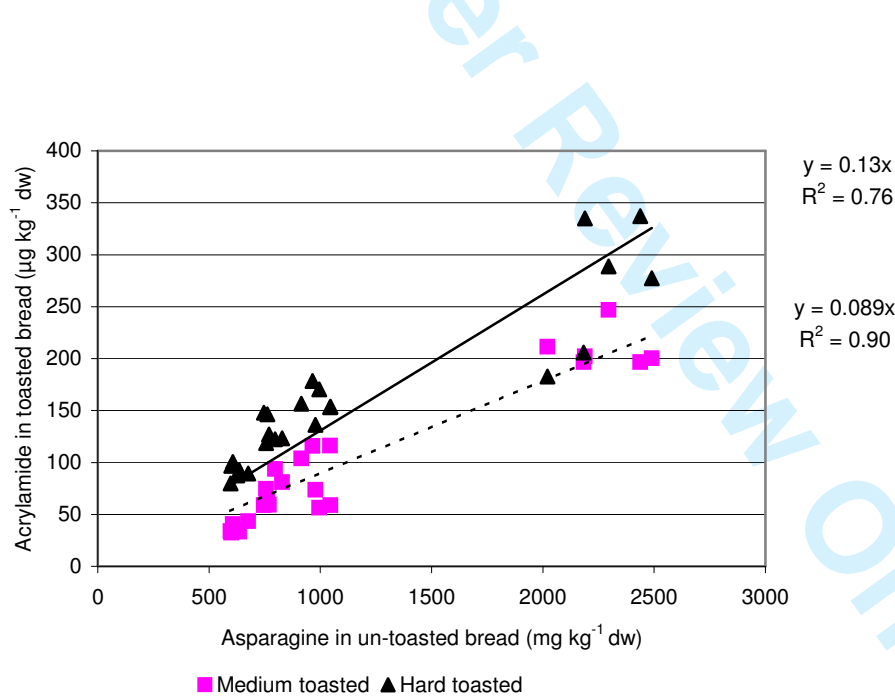
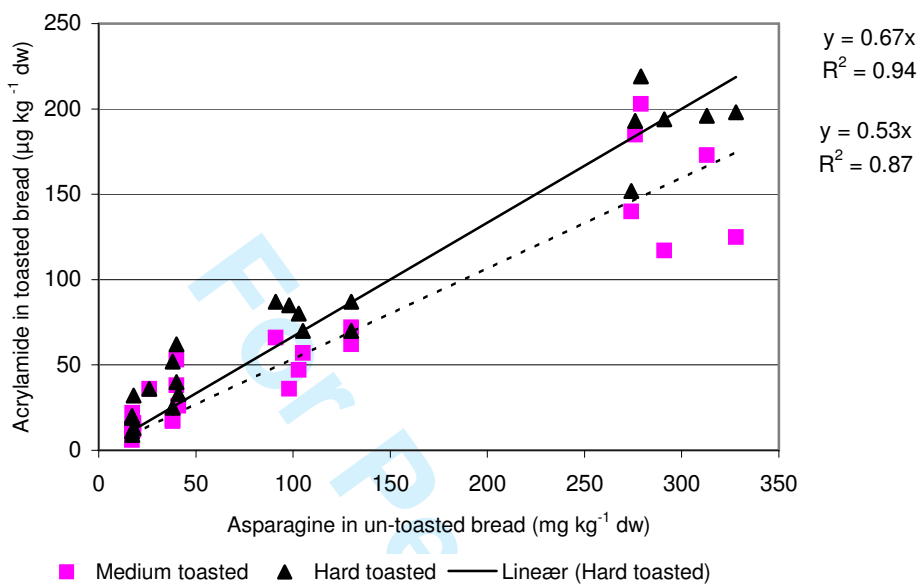


Fig. 3.

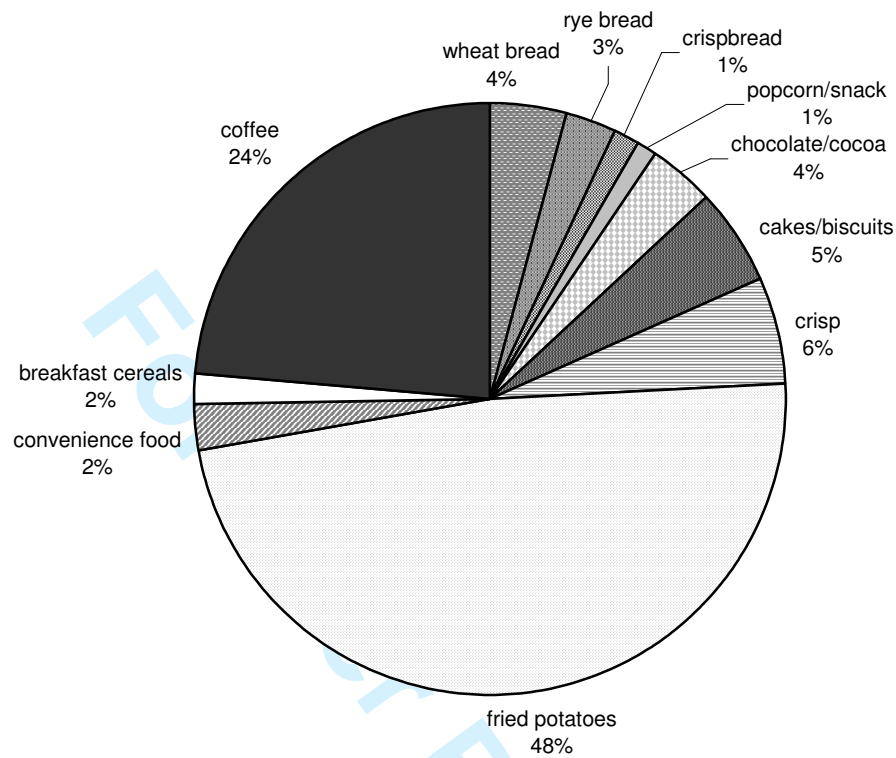


Fig. 4

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Acrylamide in relation to asparagine in baked and toasted wheat and rye bread.

Abstract

Acrylamide in baked and toasted wheat and rye bread was studied in relation to the content of asparagine in flour, dough, bread and toasted bread. Asparagine was consumed during bread preparation resulting in a reduced acrylamide contents in the products. In wheat bread 12% of the asparagine initially present in the flour (0.14 g kg⁻¹) was left after yeast fermentation and baking, while for rye bread 82% of the asparagine was left after sourdough fermentation and baking. The asparagine present in the untoasted wheat bread had all reacted after hard toasting. Toasted wheat and rye bread slices contained 11-161 µg kg⁻¹ and 27-205 µg kg⁻¹ acrylamide compared to untoasted wheat and rye bread containing <5 µg kg⁻¹ and 7-23 µg kg⁻¹ acrylamide. The dietary intake of acrylamide from bread (untoasted) of 2 µg/day is relatively low, however the acrylamide exposure from bread increases several times for people eating toasted bread.

Keywords: *Acrylamide, asparagine, bread, rye, wheat, toast, dietary, intake*

Introduction

The presence of acrylamide in heat-treated food was recognized in 2002 (Tareke et al. 2002) and the same year it was clear that acrylamide is formed mainly from asparagine and a carbonyl compound through Maillard browning reactions during food processing (Mottram et al. 2002; Stadler et al. 2002). Several pathways and intermediates for acrylamide formation have been suggested including the intermediates: Schiff's bases, decarboxylated Amadori products (Yaylayan 2003), Strecker aldehydes (Mottram et al. 2002) and the deamination product 3-aminopropionamide which also generate acrylamide under aqueous conditions (Granvogl and Schieberle, 2006). Besides

the main reaction routes through the Maillard reactions, acrylamide may be formed in bread after protein pyrolysis of added gluten (Claus et al. 2006). Also addition of cheese to bread has been found to increase the acrylamide formation, probably formed with 3-aminopropionamide as an intermediate in the cheese, which has been found to generate acrylamide (Granvogl and Schieberle 2006, Hedegaard et al. 2008).

Acrylamide is known to be carcinogenic in rodents and is classified by the International Agency for Research on Cancer as probably carcinogenic to humans (IARC 1994). The dietary acrylamide intake have been estimated to e.g. $0.3\text{--}0.8\text{ }\mu\text{g (kg bw)}^{-1}\text{ day}^{-1}$ (WHO 2002); appr. $0.5\text{ }\mu\text{g (kg bw)}^{-1}\text{ day}^{-1}$ (Svensson et al 2003). The dietary intake of acrylamide from bread is about 11% of the total dietary acrylamide intake of $31\text{ }\mu\text{g day}^{-1}$ as estimated for the Swedish population. A similar contribution from bread related foods such as crisp bread and biscuits are estimated (Svensson et al. 2003). The acrylamide level in baked bread is relatively low, but due to high consumption of bread the contribution of acrylamide from bread is still important.

Toasting the bread, which is frequently used e.g. for breakfast meals, increases acrylamide significantly. While untoasted bread contains $<50\text{ }\mu\text{g kg}^{-1}$ the acrylamide content may increase to a few to several hundreds micrograms per kilo bread after toasting (Konings et al. 2003; Becalski et al. 2003; Ahn et al. 2002). The acrylamide levels have been found to increase with increasing toasting time and whole meal rye bread was found to form more acrylamide than white wheat bread (Ahn et al. 2002).

The occurrence of acrylamide in bread may depend on several factors. Surdyk et al. (2004) showed that acrylamide in baked wheat bread correlated with the amount of asparagine added to dough, while the added reducing sugar fructose did not show a similar correlation, hence the asparagine

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3 49 was a limiting substrate for the acrylamide formation. The clear relationship between asparagine
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5 50 content in the dough and acrylamide formation has also been demonstrated in other wheat and rye
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7 51 bread model systems (Elmore et al. 2005; Bråthen et al. 2005). One of the strategies to reduce
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9 52 acrylamide in cereal products may be to choose raw materials low in asparagine by selecting
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11 53 optimum cultivars and growth conditions. However, variations in free asparagine in cereals are
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13 54 modestly researched. Claus et al. 2006 investigated agronomic factors related to the acrylamide
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15 55 formation in cereal products. They showed that nitrogen fertilization resulted in elevated amino acid
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17 56 levels resulting in increased acrylamide levels. [The asparagine contents of selected European wheat
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19 57 varieties ranged from 0.07-0.66 g kg⁻¹, that is a factor of five between varieties and a factor of two
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21 58 within varieties (Taeymans et al. 2004).
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23 59

Comment [k1]: Left out to shorten the introduction

24 60 Milling will differentiate the asparagine levels in the different milling fractions. Fredriksson et al.
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26 61 (2004) found that the contents of free asparagine are lower in sifted wheat flour i.e. 0.14- 0.17 g kg⁻¹
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28 62 ¹(dw) compared to whole grain wheat flour i.e. 0.5 g kg⁻¹(dw) or wheat bran. Rye generally contains
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30 63 more asparagine: sifted rye flour 0.6 g kg⁻¹(dw) and whole grain rye flour: 1.1 g kg⁻¹(dw). Not only
31
32 64 milling has been found to influence the levels of the limiting substrate asparagine. It was found that
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34 65 yeast fermentation of wheat dough depleted a major part of the asparagine (>80%), while
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36 66 sourdough fermentation of rye dough reduced the asparagine content to a smaller extent
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38 67 (Fredriksson et al. 2004). During baking, parameters such as water activity, heating time and
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40 68 temperature, surface to volume ratio, the presence of additives and pH also influence acrylamide
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42 69 levels (Surdyk et al. 2004; Rydberg et al. 2003). Acrylamide formation may increase when
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44 70 increasing pH e.g. by using baking soda or ammonium carbonate for bread leavening (Amrein et al.
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46 71 2005) as it will decrease when lowering pH (Jung et al. 2003; Pedreschi et al. 2004, 2005).
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Fermentation will cause pH in dough to drop due to release of carbon dioxide (Raccach et al. 2004) and it will reach pH~5 using yeast and pH~4.4-4.8 using sourdough (Suhr et al. 2004).

The aim of the present study was to find the relationship between asparagine levels and acrylamide formation in wheat bread and rye bread during baking and toasting in order to be able to reduce the acrylamide levels. Furthermore to assess the acrylamide levels in bread on the Danish market and to estimate the dietary intake of acrylamide from wheat and rye bread including toasted bread.

Materials and methods

Baking wheat bread and rye bread with asparagine additions

An industrial bakery (*Lantmännen Schulstad A/S, Hvidovre, Denmark*) made the wheat and rye breads in their laboratory bakery with different amounts of asparagine added (in August 2004). The experimental design appears from Figure 1. Four batches of wheat dough were prepared and two breads selected from each batch: One batch of dough was made without adding asparagine representing the natural background asparagine level and three batches were added asparagine at concentration levels of 0.05, 0.13 and 0.27 g kg⁻¹ dough. As the dough contains 60% wheat flour, the added amounts of asparagine based on flour were 0.09, 0.22 and 0.44 g kg⁻¹ corresponding to addition at approximately 0.6 - 3 times the actual background level of asparagine. The recipe for a wheat bread (appr. 800 g dough) included sifted wheat flour, yeast, salt, sugar vegetable oil and water. Kneading time was 7 min., holding time 20 min. and resting time at 32°C was 45 min. Baking was performed initially at 230°C for 2 min. and subsequently at 200°C for 30 min.

Rye breads were made using the same experimental design. The asparagine was added to the dough at concentrations based on rye flour of 0.17, 0.43 and 1.75 g kg⁻¹ respectively corresponding to

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3 96 addition at 0.2 - 2 times the natural background level. The rye bread (based on appr. 900 g dough)
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5 97 was made from half and half mixing of sourdough and rye flour and addition of yeast, salt and
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7 98 water. Kneading time was 10 min, holding time 20 min, and resting time at 37°C was 77 min.
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9 99 Baking was performed initially at 280°C for 5 min., followed by 210°C for 35 min. and 190°C for
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11 100 12 min.

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15 02 *Toasting wheat bread and rye bread with asparagine additions*

16
17 03 Toasting of the bread slices was done in a pushdown toaster (Tefal Delfini Compact Toaster 5396,
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19 04 800 W) with room for two slices. The four batches of bread (background level and the three
20
21 05 addition levels) were analyzed either untoasted or after medium or hard toasting, for each level
22
23 06 using three slices from each of two breads (n=18). Medium toasting of wheat bread slices of 13 mm
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25 07 was performed 1.9 min. and hard toasting 2.8 min.. Medium toasting of the rye bread slices of 8
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27 08 mm was done 2.8 min. and the hard toasting 3.1 min.. All the toasted bread slices were medium to
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29 09 dark brown and were considered eatable. The thicknesses of the bread slices were chosen from what
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31 10 is common practice among bakers and consumers.

32 11
33 12 Figure 1
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37 15 *Chemicals*

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39 16 Acrylamide (>99.5%) was purchased from Sigma-Aldrich (St. Louis, MO, USA), asparagine
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41 17 monohydrate (>99%) from Acros Organics (Geel, Belgium), D₃-acrylamide (>98%) from Polymer
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43 18 Source (Dorval, Quebec, Canada), ¹⁵N₂-asparagine monohydrate (>98%) from Cambridge Isotope
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45 19 Labs. (MA, USA), methanol (>98%), formic acid (>98%) and acetonitrile (HPLC-grade) from
46
47 20 Rathburn Chemicals (Walkerburn, UK).

Chemical analysis

The chemical analyses were performed using a recently developed LC-MS/MS method for simultaneous analysis of acrylamide, asparagine and reducing sugars (Nielsen et al. 2005) and in addition some of the acrylamide analyses were made with another LC-MS/MS method as described in Pedreschi et al. (2005).

Sliced toasted or untoasted wheat or rye bread was weighed, dried in an oven at 50°C over night and homogenized using a Braun handheld mixer (type 4169) fitted with a blender like sample compartment (type 4297) (Braun AG, Frankfurt/m, Germany). The drying temperature was set relatively low in order to avoid acrylamide formation during the drying process. The wet (semidry) and dry weight was determined from weighing before and after drying of the baked and toasted bread slices. Aliquots of 4 g of homogenate was extracted with MilliQ water after adding internal standards: 200 µL 10 µg mL⁻¹ D₃-acrylamide, 100 µL 880 µg mL⁻¹ ¹⁵N₂-asparagine. The sample was extracted by an Ultra Turrax mixer. After centrifuging, SPE cleanup was performed by an automated sampler using LiChroLut RP-C18 SPE-cartridges LC-MS/MS detection was performed on a triple quadrupole instrument (Micromass Quattro Ultima) after separation on a hypercarb LC-column (dimensions 2.1 mm x 100 mm, particle size 5 µm) eluted with 0.1% aqueous formic acid at 0.2 mL min⁻¹. The mass spectrometer was operated in ESI⁺ for acrylamide and saccharides and in ESI⁻ for asparagine. Capillary voltages of 3 kV (ESI⁺) and 2 kV (ESI⁻) were applied but for simultaneous detection in positive and negative mode a lower capillary voltage, e.g. 1 kV is recommended to prevent sparking from the capillary.

Some of the acrylamide analysis was performed after the methods were optimized to detect low concentrations (e.g. by increasing the HPLC injection volume and by optimizing the MS performance before analysing the bread samples), so the method was able to quantify down to 5 µg

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3 146 kg⁻¹. The repeatability and reproducibility of the acrylamide analyses at 50 and 250 µg kg⁻¹ (n=24)
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5 147 were 6-17% and 9-29% respectively. The laboratory has participated in proficiency tests for
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7 148 acrylamide in food and all results obtained have been with satisfactory test scores, including the
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9 149 results obtained in an EU validation study on acrylamide analyses in food with 22 samples (11
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11 150 matrices including bread and toasted bread: assigned value 38µg kg⁻¹, obtained results 41 and 44µg
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13 151 kg⁻¹) (Wenzl et al. 2006). The detection limit of the asparagine analyses was 0.002 g kg⁻¹, the
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15 152 repeatability and reproducibility measured at 0.02, 0.1 and 0.4 g kg⁻¹ 0-5% and 10-18% repectively.
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18 154 *Estimation of dietary acrylamide intakes*

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20 155 Exposure estimates were accomplished using data from the Danish Nationwide Dietary Survey
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22 156 made from 2000-2002 by the National Food Institute (Lyhne et al. 2005). Information on food
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24 157 consumption was collected from a representative sample of 4120 individuals aged 4-75 years. The
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26 158 participants in the survey kept a food record for seven consecutive days. The monitoring results of
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28 159 acrylamide in Danish foods (appr. 330 samples) were made 2002-2005 by the National Food
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30 160 Institute (data not published elsewhere).
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32 161

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34 162 **Results and discussion**

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36 163 The levels of acrylamide and asparagine in wheat and rye bread slices after baking and medium or
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38 164 hard toasting appear from Table I. The results presented are all means of six bread slices, each
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40 165 wheat bread slice weighing 34±2 g and each rye bread slice weighing 32±1 g.
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44 167 *Asparagine in flour and bread slices after asparagine additions to dough and subsequent*
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46 168 *fermentation, baking and toasting*

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The background content of asparagine in sifted wheat flour was $0.14 \pm 0.004 \text{ g kg}^{-1}$ (based on three determinations and with the precision measured as 95% confidence levels) and in whole grain rye flour $0.76 \pm 0.02 \text{ g kg}^{-1}$, figures comparable to those reported by Frederiksson et al. (2004) of 0.14–0.17 g kg^{-1} (dw) in sifted wheat flour and 1.1 g kg^{-1} (dw) in whole grain rye flour.

The levels of asparagine added to the wheat dough were 0.6, 1.6 and 3.1 times the natural background level and to the rye dough 0.2, 0.6 and 2.3 times the natural background level. The highest concentration after addition correspond to 0.6 g kg^{-1} asparagine in wheat flour and 2.5 g kg^{-1} asparagine in rye flour; hence levels likely to be present in cereal products used for bread production.

In Table II the reacted amounts of asparagine in the batches with different asparagine levels upon fermentation, baking and toasting are presented. Some asparagine had reacted during wheat bread production as can be seen from data determined on a dry weight basis (Table I), where differences from evaporation of water during toasting does not have to be considered. In the wheat batch with a natural background level of asparagine 12% was left after fermentation and baking. Upon hard toasting all asparagine in the wheat bread slices with the natural background content and the lowest addition level of asparagine had reacted.

Table I

Table II

In rye bread the relative reduction in asparagine during sourdough fermentation and baking was less than for the yeast fermented wheat bread, e.g. 18% at the natural background level and 9% for the highest addition level of asparagine. As the results are based on subtracting two large percentages, they may be more uncertain. The results are in accordance with those of Frederiksson et al. (2004), who also found sourdough fermentation to reduce asparagine less than yeast fermentation and at

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3 194 about the same rate i.e. >80% for yeast fermentation of wheat bread and 17% for sourdough
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5 195 fermentation of rye.
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9 197 A general conclusion may be that rye flour contains more asparagine than wheat flour and
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11 198 sourdough fermentation and baking of rye dough reduces asparagine to a smaller extent than yeast
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13 199 fermentation and baking of wheat dough. Prolonging or optimizing the fermentation process,
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15 200 especially the yeast fermentation of wheat bread may be an efficient tool to reduce the acrylamide
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17 201 formation in bread products.

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20 204 *Acrylamide in baked and toasted bread in relation to asparagine*
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22 205 Baked wheat bread slices contained <5 µg kg⁻¹ acrylamide (<LOD), medium toasted 11-130 µg kg⁻¹
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24 206 acrylamide and hard toasted 15-161 µg kg⁻¹ acrylamide for asparagine levels in flour of 0.14-0.60 g
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26 207 kg⁻¹ (natural background content to content after highest asparagine addition level). The
27
28 208 corresponding figures for the rye bread slices were: baked 7-23 µg kg⁻¹ acrylamide, medium toasted
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30 209 27-155 µg kg⁻¹ acrylamide; hard toasted 68-205 µg kg⁻¹ acrylamide for asparagine levels in flour of
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32 210 0.76-2.5 g kg⁻¹.

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35 212 In Figure 2 acrylamide concentrations in wheat and rye bread, initially baked with different
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37 213 asparagine addition levels, and after baking toasted to medium or hard level are plotted and marked
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39 214 with correlation coefficients. Due to the low acrylamide contents in the baked wheat bread slices no
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41 215 correlation was found between asparagine content in dough and acrylamide content in baked wheat
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43 216 bread. However, acrylamide contents in wheat bread correlated well with the asparagine levels
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45 217 added to the dough for medium ($r^2 = 0.86$) and hard toasted bread slices ($r^2 = 0.87$). As smaller
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47 218 proportions of asparagine were consumed during yeast fermentation and baking for the higher
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asparagine additions the acrylamide contents increased exponentially with increasing asparagine levels (quadratic equations).

The untoasted rye bread slices contained naturally 0.62 g kg^{-1} (dw) asparagine, about 36 times more than the wheat bread slices. As for the wheat bread quadratic relationships between the content of asparagine in the rye flour and acrylamide content in bread after medium ($r^2 = 0.86$) or hard ($r^2 = 0.88$) toasting appear (Figure 2b).

In the present study, a major part of the asparagine added to the wheat dough had reacted during preparation of the bread. It is known, that especially yeast fermentation promote elimination of asparagine, and that the reduction depends on e.g. time of fermentation, the amount of yeast added and pH (Frederiksson et al. 2004). When comparing the asparagine levels left in baked bread before toasting with the acrylamide content in toasted bread slices good linear relationships are present for both medium toasted ($r^2 = 0.87$) and hard toasted ($r^2 = 0.94$) wheat bread and for asparagine levels of $0.017\text{--}0.29 \text{ g kg}^{-1}$ (dw) bread. The asparagine levels in baked rye bread also showed good linear correlations with acrylamide formed after medium toasting ($r^2 = 0.90$) and hard toasting ($r^2 = 0.76$).

Figure 2

Although it is not possible directly to compare between acrylamide formation in wheat and rye bread having different slice thicknesses, water contents and asparagine levels it seems as the rate of acrylamide formation is somewhat higher in wheat bread compared to rye bread. This may be explained by lower pH conditions in sourdough fermented rye bread compared to yeast fermented wheat bread (pH appr. 0.5 units lower in sourdough). A better retention of water in the bread when

1
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3 243 toasting rye bread compared to wheat bread may also reduce the acrylamide formation. The average
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5 244 water content in hard, medium and untoasted rye bread slices were $24\pm2\%$, $26\pm2\%$ and $41\pm1\%$
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7 245 while the average water content in hard, medium and untoasted wheat bread slices were somewhat
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9 246 lower: $17\pm2\%$, $19\pm1\%$ and $32\pm1\%$, respectively. However, the actual water contents at the toasted
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11 247 surfaces were probably somewhat lower. Hedegaard et al. (2007) found that at high water activity
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13 248 the rate determining step of acrylamide formation from the Schiff's base is the elimination of water
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15 249 from an intermediate, while at lower water activity, water elimination becomes easier and
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17 250 decarboxylation of a reaction intermediate leading to acrylamide becomes the rate determining step.
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19 251 Hence the acrylamide formation rate increases when the water activity decreases. Hence if different
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21 252 reactions are rate determining, the overall reaction rate may differ for different water activities,
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23 253 which may explain the exponential increase in acrylamide contents (as the water content are
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25 254 reduced) generally found during thermally processing of food at a fixed temperature. It was also
26
27 255 found in other model systems that acrylamide formation increased when lowering the water content
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29 256 (Schieberle et al. 2005, Blank et al. 2005) .
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31 257
32 258 The proportions of asparagine in wheat bread reacting to give acrylamide were also estimated
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34 259 (Table III). Generally the ratio is higher in medium toasted bread suggesting that at higher
35
36 260 temperatures and/or longer duration of toasting precursors are depleted faster, reacting faster in
37
38 261 competing reactions and/or acrylamide is eliminated to a larger extent by subsequent reaction. For
39
40 262 the hard toasted bread is seen, that as depletion of the asparagine pool approaches, the formation of
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42 263 acrylamide diminishes, resulting in lower ratios at lower asparagine addition levels. At the low
43
44 264 asparagine levels in wheat bread asparagine was depleted after hard toasting while at higher
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46 265 addition levels some asparagine was left. This may be interpreted as if asparagine was the limiting
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48 266 precursor at low asparagine levels while at higher levels other factors also influenced the
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acrylamide formation. However the other precursor the reducing sugars was not depleted (data not shown).

Figure 3

Table III.

Acrylamide contents in wheat breads and rye breads produced in Denmark and comparison with other surveys.

In 2004, in order to estimate the level of acrylamide in rye bread, 13 sifted and whole meal rye breads produced for or sold on the Danish market were sampled and analysed. They contained $12 \pm 5 \mu\text{g kg}^{-1}$ acrylamide (range <5 - $19 \mu\text{g kg}^{-1}$, $n=13$). In 2005, ten samples of wheat breads, produced for or sold on the Danish market were analysed, containing $10 \pm 6 \mu\text{g kg}^{-1}$ acrylamide (range <5 - $22 \mu\text{g kg}^{-1}$). The content of acrylamide in bread found in the present study are comparable with the results found in other studies. Becalski et al. (2003) found $290 \mu\text{g kg}^{-1}$ of acrylamide in toasted wheat bread compared to $19 \mu\text{g kg}^{-1}$ in untoasted wheat bread ($n=2$). Light toasted rye bread contained $28 \mu\text{g kg}^{-1}$ acrylamide and untoasted rye bread $17 \mu\text{g kg}^{-1}$. Eerola et al. (2007) found that toasted wheat bread contained $111 \pm 10 \mu\text{g kg}^{-1}$ acrylamide ($n=5$) and untoasted wheat bread $<68 \mu\text{g kg}^{-1}$ acrylamide, which was the limit of detection ($n=5$). Ahn et al. (2002) found that acrylamide increased with longer toasting time. After 3.1 min. of toasting when the toasts were still considered eatable wheat bread contained appr. $60 \mu\text{g kg}^{-1}$ and rye bread contained appr. $80 \mu\text{g kg}^{-1}$ acrylamide. After 6 minutes wheat bread contained appr. $206 \mu\text{g kg}^{-1}$ and rye bread contained appr. $220 \mu\text{g kg}^{-1}$ acrylamide. Konings et al. (2003) reported acrylamide contents in toasted bread of $183 \pm 336 \mu\text{g kg}^{-1}$ (range <30 - $1430 \mu\text{g kg}^{-1}$, $n=17$). The contents in dark rye bread were $44 \pm 13 \mu\text{g kg}^{-1}$ (range 30 - $60 \mu\text{g kg}^{-1}$, $n=5$) and in wheat bread $<30 \mu\text{g kg}^{-1}$ ($n=6$).

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5 294 *Dietary acrylamide intake from bread*
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7 295 The average dietary intake of wheat bread in Denmark is 125 g day⁻¹ and in addition Danes has a
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9 296 tradition for eating whole meal sourdough fermented rye bread as part of nearly every lunch meal
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11 297 comprising an average daily intake of 59 g rye bread day⁻¹ (Lyhne et al. 2005). In case all bread is
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13 298 consumed without toasting and the mean figures for acrylamide in bread is used: 10 µg kg⁻¹ in
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15 299 wheat bread and 12 µg kg⁻¹ in rye bread, the daily intake comprises 1.3 µg from wheat bread and
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17 300 0.7 µg from rye bread, respectively. The daily intake of acrylamide from wheat and rye bread
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19 301 comprising 2.0 µg is about 8 % of the average daily acrylamide intake of appr. 25 µg day⁻¹
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21 302 estimated for Danes (Figure 4). The acrylamide intake from bread is relatively low compared to
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23 303 other surveys, estimating e.g. 3.4 µg (Svensson et al. 2003) and 3.6 µg (Matthys et al. 2005). An
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25 304 explanation for the low Danish dietary acrylamide intake from bread may be that most Danish bread
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27 305 are produced using either yeast fermentation or sourdough fermentation, hence the contents of the
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29 306 precursor asparagine are reduced during preparation of the breads. Another explanation for the low
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31 307 acrylamide intake from bread may be that in previous studies on acrylamide in bread the limit of
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33 308 detection (LOD) was higher and the levels in bread often below the LOD. Hence in many cases
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35 309 LOD was used as the acrylamide level in bread. Finally in the present estimate, as the Danish
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37 310 Nationwide dietary survey do not have information on how frequently people eat toasted bread, the
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39 311 acrylamide intake from toasted bread is not included in the dietary acrylamide intake from bread.
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41 312 However, in case the average daily bread intake includes two hard toasted wheat bread slices
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43 313 (acrylamide 161 µg kg⁻¹(ww)) each of 28 g and with the high pre-processing asparagine level of 0.6
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45 314 g kg⁻¹ (dw) which is about the concentration found in wholegrain wheat flour i.e. 0.5 g kg⁻¹
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47 315 (Frederiksson et al. 2004), the daily acrylamide intake is estimated to 9 µg (36 % of the average
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49 316 Danish acrylamide intake). If a consumer eats an average amount of bread of which two slices of 25

Comment [k2]: the first paragraph moved to *materials and methods*

g rye bread are hard toasted with asparagine and acrylamide concentrations of 1.2 g kg^{-1} and $123 \mu\text{g kg}^{-1}$ respectively, the daily acrylamide intake from bread comprises $7 \mu\text{g}$ (28 % of the average Danish acrylamide intake). Hence dietary habits with consumption of toasted bread, which are common throughout at least Europe, may increase the acrylamide intake from bread considerable. Especially intake of toasted wholemeal wheat bread and bread produced without leavening by fermentation may further increase the acrylamide exposure.

Figure 4.

Conclusions

The contents of acrylamide in baked rye bread and in toasted wheat and rye bread are correlated to the contents of asparagine in the cereal product used for the bread production and at asparagine concentration ranges that may occur in grain products for bread production. The content of asparagine in the dough for wheat bread preparation is reduced considerably during bread production. After 65 min. raising time and subsequent baking, the asparagine content in the baked wheat bread was reduced to 12% compared to the content in the flour before yeast fermentation. The sourdough fermentation of rye bread showed a modest asparagine reduction to 82% of the content in the flour. This is probably due to the more acidic conditions in sourdough fermented rye bread compared to yeast fermented wheat dough. The contents of acrylamide formed in toasted wheat bread slices and toasted rye bread slices are proportional to the asparagine concentration of the untoasted bread. The dietary intake of acrylamide from untoasted bread of $2.0 \mu\text{g day}^{-1}$ is relatively low but the dietary acrylamide intake increases somewhat for people eating toasted bread.

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6
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8
9 344 effects). Lone Hertz is acknowledged for performing parts of the chemical analyses.

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36 427
37 428
38 429 Table I. Levels of asparagine added to a) wheat dough and b) rye dough, asparagine content in
39 430 wheat and rye bread slices and the acrylamide contents after medium or hard toasting of the bread
40 431 slices (mean±2sd).
41 432
42 433 Table II. Asparagine left after fermentation and baking, and subsequent medium or hard toasting of
43 434 wheat and rye bread (in percentage of asparagine in the flour).

Table III. Amount of acrylamide formed and present [μg] from asparagine reacted [mg] when toasting wheat bread (ratios calculated by subtracting analyte concentrations determined in medium or hard toasted bread from analyte concentrations determined in untoasted bread).

Figure 1. Experimental design for baking wheat and rye bread with addition of asparagine and subsequent medium or hard toasting of bread slices. All bread slices were analysed for acrylamide and asparagine.

Figure 2. Correlation between naturally occurring and added asparagine in flour and acrylamide in baked and toasted bread slices of a) wheat or b) rye.

Figure 3. Correlation between asparagine in untoasted bread and acrylamide in toasted bread slices of a) wheat or b) rye.

Figure 4. The proportions from different foods to the daily mean acrylamide intake for Danish adults of appr. 25 μg .