Acrylamide in Bread

Precursors, Formation and Reduction

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Abstract

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Acrylamide is found at concentrations up to a few mg/kg in a wide range of foods, mainly carbohydrate-rich foods subjected to high thermal processing. Acrylamide is formed in foods *via* the Maillard reaction with free asparagine and reducing sugars as the precursors, with the former generally being limiting in cereal-based products. Bread is among the products that can contain high levels of acrylamide, so this thesis investigated factors affecting acrylamide formation in bread.

A robust extraction procedure coupled with the chromatographic method from Ez-Faast® technology was set up and validated to analyse free amino acids in cereals, cereal fractions and products. Free amino acids were found to be mostly concentrated in the bran fraction. Asparagine was a major free amino acids in the cereals analysed. A major decrease in asparagine was found to occur during yeast fermentation.

The content of acrylamide in rye crispbread was found to be mainly controlled by the time and temperature of baking and the level of asparagine present, but not by the fructose level. Studies on interactions between added asparagine, glycine and fermentation time on the formation of acrylamide in soft wheat bread showed that fermentation time has a reducing effect on acrylamide formation, which was governed by the level of asparagine present in the system. Glycine addition significantly decreased acrylamide depending on the initial levels of asparagine in the dough, and increased the colour intensity of the bread.

Tests on the effect of storage conditions on acrylamide content showed that storage temperature affects its disappearance from the bread. Acrylamide levels were more stable at cold temperatures up +6 °C. A significant decrease in acrylamide was found at warmer temperatures (+20 and +40 °C). Reduction of acrylamide was greater in closed containers, with most reduction taking place in the early stages of warm storage. Increased moisture content resulted in a major decrease in the acrylamide content during storage.

Keywords: acrylamide, , asparagine, bread, fermentation, free amino acids, glycine, moisture content, precursors, storage, time and temperature

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Populärvetenskaplig sammanfattning

Att äta en väl sammansatt kost är viktigt för ett långt och hälsosamt liv. Det kan dock vara svårt att veta vad som är hälsosamt och att mäta alla faktorer som är involverade i detta. En urgammal metod att bereda maten för att öka tillgängligheten av näringsämnen och hållbarheten är att värmebehandla den. Värmebehandlingen ger också maten smak, färg och en rad positiva ämnen genom en serie av kemiska reaktioner som benämns Maillardreaktionen. Tyvärr bildas även onyttiga ämnen vid dessa reaktioner, t ex akrylamid. En internationell cancerorganisation (IARC) har definierat akrylamid som en möjlig carcinogen för människa, och det är därför viktigt att behandla livsmedel på ett sådan sätt att bildningen av akrylamid och andra skadliga ämnen minimeras, samtidigt som de positiva effekterna av värmebehandlingen inte äventyras. Att akrylamid kan bildas vid upphettning av kolhydratrika livsmedel uppmärksammades under 2002 efter pionjärinsatser av svenska forskare. Efter detta har en betydande mängd forskning genomförts kring olika aspekter av akrylamid, till exempel att ta fram metoder för att minska innehållet i mat och att studera akrylamidens carcinogena effekter ytterligare.

I Sverige är de viktigaste källorna till akrylamidintag potatisbaserade produkter, som chips och pommes frites, spannmålsbaserade produkter, och kaffe. Eftersom bröd är ett baslivsmedel, och den kanske viktigaste spannmålsbaserade produkten, är det särskilt viktigt att minska innehållet i denna produktkategori så mycket som möjligt.

I vår forskning har vi identifierat olika faktorer som påverkar bildningen av akrylamid i bröd. Den fria aminosyran asparagin som finns i råvarorna var begränsande för bildningen av akrylamid i knäckebröd. Jästen minskade mängden fri asparagin i systemet under jäsningen och därmed bildningen av akrylamid vid gräddningen av bröden. Mängden asparagin i systemet påverkade däremot inte färgbildningen. Kortare tid och/eller lägre temperatur vid gräddningen minskade akrylamidhalten i mjukt bröd, men kunde resultera i produkter med för ljus skorpa. Tillsats av den fria aminosyran glycin minskade bildningen av akrylamid, speciellt vid högre halter asparagin i systemet, samtidigt som det bidrog till färgbildningen, vilket medförde att en minskad värmebehandling kan användas.

Vi studerade även i modellförsök hur lagring av knäckebröd påverkade innehållet av akrylamid. Resultaten visade att då knäckebröden lagrades torrt och kallt så påverkades inte innehållet av akrylamid. Vid temperaturer kring rumstemperatur och högre kunde emellertid innehållet minska drastiskt, särskilt vid en högre vattenhalt.

Popular science summary

Eating a balanced diet is one crucial aspect of leading a healthy life. This implies that the overall quality of food should be monitored. Heating is a very old measure to preserve food and make it readily available for eating. Another major advantage of heating food is that it adds taste and colour, making it more appetising. Colour and flavour compounds are produced during food processing, mainly *via* a series of reactions termed the Maillard reaction. Unfortunately, hazardous compounds such as acrylamide are also generated during these reactions. Controlling acrylamide formation during food processing is a critical step in ensuring that organoleptic qualities are not jeopardised. Since in 1994 the International Agency for Research on Cancer (IARC) defined acrylamide as a probable human carcinogen. However, acrylamide in food only began to attract attention in 2002, when this chemical was reported to have a dietary source. Research in acrylamide is particularly important as the Joint FAO/WHO Expert Committee on Food Additives (JECFA) considers its presence an indicator of human health concern.

In Sweden and other countries the major contributors to acrylamide intake are potato-based products, cereal-based products and coffee. Among the cereal products, bread makes the highest contribution to average daily intake. Thus, it is important to study bread in relation to acrylamide formation.

In this research, we tried to identify factors that affect acrylamide formation in bread and to introduce some mitigating measures that may control its formation. The presence of the free amino acid asparagine was found to be an enhancing factor for acrylamide formation. Yeast was found to consume asparagine during fermentation resulting in products with low levels of acrylamide. Some measures used to control acrylamide formation might simultaneously result in products that are insufficiently coloured. Our studies showed that adding the amino acid glycine enhances colour formation. Furthermore, if the formation of acrylamide is controlled through limiting asparagine content, no adverse effect on colour takes place. Another suggested measure to control acrylamide formation is to use moderate combinations of time and temperature of baking. However, this gives better efficacy when coupled with additional acrylamide-reducing measures.

In a model study with rye crispbread, we have shown that acrylamide content decreases during storage in warm temperatures particularly, when the moisture content is high. However, acrylamide content tend to be more stable when stored at cold temperatures.

Content

Appendix

The present thesis is based on the following papers, which are referred to in the text by their Roman numerals

Papers I – V

I. Mustafa, A., Åman, P., Andersson, R. & Kamal-Eldin, A. 2007. Analysis of free amino acids in cereal products. *Food Chemistry* 105, 317-324

II. Mustafa, A., Andersson, R., Rosén, J., Kamal-Eldin, A. & Åman, P. 2005. Factors influencing acrylamide content and color in rye crisp bread. *Journal of Agricultural and Food Chemistry* 53, 5985-5989.

III. Mustafa, A., Fink, M., Kamal-Eldin, A., Rosén, J., Andersson, R. & Åman, P. 2008. Interaction effects of fermentation time and added asparagine and glycine on acrylamide content in yeast-leavened bread. *Submitted.*

IV. Mustafa, A., Kamal-Eldin, A., Petersson, E.V., Andersson, R. & Åman, P. 2008. Effect of extraction pH on acrylamide content in fresh and stored rye crispbread. Accepted for publication by *Journal of Food Composition and Analysis*

V. Mustafa, A., Andersson, R., Hellenäs, K. E., Åman, P. & Kamal-Eldin, A. 2008. Moisture affects acrylamide reduction during storage in model studies of rye crispbread. *Manuscript*

Author's main contribution to the papers

Papers I, II, IV and V; Planed the study and performed the statistical evaluation of the data together with the co-authors. Took major responsibility of performing the laboratory work, writing up the papers and manuscripts. Took the responsibility of submitting the papers and acted as the corresponding author.

Paper III; Shared doing the laboratory work with Martin Fink. Performed the scientific and the statistical evaluation of the data together with the co-authors. Took the major responsibility of the writing up and submitting the paper and is the corresponding author.

List of abbreviations

AA	Acrylamide
3-APA	3-Aminopropionamide
a*-value	Degree of redness
Ala	Alanine
Asn	Asparagine
Asp	Aspartic acid
b*-value	Degree of yellowness
CV	Coefficient of variation
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration (USA)
GC	Gas chromatography
Glu	Glutamic acid
GRAS	Generally recognised as safe
Hb	Haemoglobin
HMF	Hydroxymethylfurfural
HPLC	High performance liquid chromatography
RI	Refractive index
IARC	International Agency for Research on Cancer
JECFA	Joint FAO/WHO Expert Committee on Food Additives
MCPDS	Monochloropropanediol isomer
MOE	Margin of exposure
MS	Mass spectrometry
NOEAL	No observed adverse effect level
OBC	Oat bran concentrate
Pro	Proline
Ser	Serine
SPE	Solid Phase Extraction
Trp	Triptophane
Val	Valine
WHO	World Health Organization

1. Introduction

1.1 Background

The discovery of acrylamide (AA) in foods dates back to 1997, when workers in the railway tunnel through the Hallandsås mountain range in south-west Sweden were found to have high exposure level of AA (0.07-17.7 nanomol/g Hb). These levels exceeded the No Observed Adverse Effect Level (NOAEL) and were related to leakage of AA from the tunnel during construction (Hagmar *et al.*, 2001). These findings required further investigation in relation to cancer, since AA is classified as a 'probable human carcinogen' (IARC, 1994) and is defined as a compound with the potential to cause a spectrum of toxic effects. Assessment of the uptake of AA and cancer risks for the exposed workers also revealed a possible cancer risk associated with the background exposure to AA (Törnqvist, 2005). It was calculated that the background levels corresponded to a daily intake of 100 µg of AA. Investigations on the origin of these background levels showed that it was not possible to correlate them to any possible known sources of human exposure to AA. This led to the assumption that food might be the source of AA exposure (Tareke *et al*., 2002; Granvogl *et al.*, 2007).

Tareke *et al.* (2000) showed that feeding rats with fried animal feed resulted in a ten-fold increase in haemoglobin (Hb) adduct levels of AA compared with controls. The content of AA in the heat-treated feed correlated with the measured increase in adduct levels of AA in the rats fed with fried feed (Tareke *et al.*, 2000). In 2002, the same research group showed that AA is found in a range of cooked foods, with the highest content and range in carbohydrate-rich foods, 50-4000 µg/kg, and a range of 5-50 µg/kg in protein-rich foods (Tareke *et al.*, 2002). These findings were followed by the press conference in April 2002 by the Swedish National Food Administration and Stockholm University reporting a dietary source for AA. This announcement led to world-wide attention to AA research in food due to its known toxic effect. The mutagenic and carcinogenic properties of AA are related to its metabolite, glycidamide. In further studies in 2002, the formation of AA in foods was demonstrated to take place during Maillard reactions involving free asparagine (Asn) and reducing sugars under high temperature (Mottram, Wedzicha & Dodson, 2002; Stadler *et al.*, 2002)

Following these events, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) undertook a comprehensive review of data on the occurrence of AA from many countries, mainly in Europe and North America. The outcome of this review was that the main AA contributing food groups were identified as potato-based products, cereal-based products and coffee (JECFA, 2005).

In Sweden, the total contributions from cereal-based products make up to 33% of the total AA intake (Hellenäs, 2008). Nonetheless, daily consumption of cereal-based products, particularly of whole grain type, is of great importance due to its beneficial and health-promoting effects. Bread is a widely consumed commodity, with 100% of the Swedish population regularly consuming soft bread and 77%

consuming crispbread. The content of AA in these two products is 30-160 and 30-1900 µg/kg, respectively (Svensson *et al.*, 2003). Wheat and rye are the major cereals used in the production of soft bread and crispbread. Rye contribution to dietary fibre intake may reach up to 40% (in Finland), which makes it an important component in the diet (Anon, 2003). It is especially important as it contains up to 15 g of fibre /100 g. These particulars made it crucial to investigate AA in bread, since it is important to achieve lower levels in the commonly consumed food groups.

1.2 Chemistry of acrylamide

AA is a small hydrophilic small molecule (Anon, 1991). It is an odourless solid and its colour ranges from colourless to white (**Table 1**). AA is generally formed from the hydration of acrylonitrile with sulphuric acid between 90-100 °C or by catalytic hydration using a copper catalyst. It is soluble in a number of polar solvents, *e.g.* acetone, acetonitrile and water. AA is susceptible to polymerisation during heating, which prevents the determination of boiling point at ambient pressure. At 3.34 kPa (25 mm Hg), it boils at 125 °C. It is regarded as a thermally unstable compound.

Table 1 *Physical parameters of acrylamide*

Parameter	**Specification**
Chemical formula	C_3H_5NO
Molecular weight	71.08 g /mol
Melting point	84 – 85 °C
Solubility	216g / 100 g water at 30 °C
Boiling point	125 °C at 3.34 kPa
Vapour pressure	0.007 mm Hg at 20°C
Vapour density (Air = 1)	2.4 at 175 °C
Specific gravity	1.1222 kg/dm^3 at 30 °C

AA (**Figure 1)** possesses two functional groups, an amide group and the electron-deficient vinylic double bond that makes it readily available for a wide range of reactions, including nucleophilic and Diel-Alder additions and radical reactions. These reactions are of importance in biological systems. Reactions of the amide residue include hydrolysis, dehydration, alcoholysis and condensation with aldehydes, while the vinylic double bond reacts with ammonia, aliphatic amines, phosphines, chlorine, bromine, bisulphite and dithiocarbamates, as well as proteins (Friedman, 2003; Girma *et al.*, 2005).

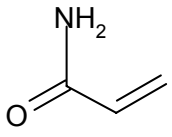

Figure 1. Chemical structure of the acrylamide molecule.

AA forms polymers and dimers and its polymers and copolymers are reported to have a wide range of applications, mainly in the agricultural and industrial sectors. AA is a biodegradable compound that is used for the purification of waste and drinking water. It is also used as a flocculent and in the synthesis of polymers and gels (Bologna *et al.*, 1999; Smith, Prues & Oehme, 1996). AA is used in research laboratories for the preparation of polyacrylamide for gels for electrophoresis. It was reported that laboratory workers using polyacrylamide gels for electrophoresis had Hb adducts for AA in higher levels compared with the control group, who were not expected to show Hb adducts for AA. The finding of Hb adducts for AA in the control group indicated another source for AA exposure since it was know to be found in tobacco smoke (Bergmark, 1997).

AA is a weakly acidic and basic conjugated amide. It has the ability to coordinate with metal ions (Girma *et al.*, 2005). This coordination occurs to free AA or to the amide residue of AA adduct. The reaction takes place either at the organic group or the amide residue. The presence of metal ions promotes Michael addition of AA. This reaction occurs *via* 1:1 complexes between AA and the metal ions in which the coordination of the metal ions enhances the electrophilicity of the AA and accelerates the reaction rate. AA chemically undergoes Michael addition type reactions to the vinylic double bond that make it reactive to nucleophiles, including amino and thiol groups in amino acids and proteins. This kind of reaction results in the formation of AA adducts with the N-terminal valine residue in haemoglobin. Such adducts are a useful biomarker for exposure to AA in both experimental animals and humans (Bergmark *et al.*, 1993; Rice, 2005; Tareke *et al.*, 2000).

1.3 Acrylamide in food

AA is found in a wide variety of food products, with the major contribution originating from cereal-based products, potato-products and coffee (**Table 2**). Among cereal products, the main contribution is from pastries and biscuits, processed cereal products and breads.

The contribution of each individual product varies between countries depending on food habits among many other factors (**Figure 2**). Coffee and green tea are among the products with a high AA content, as are cocoa products. Milk products, fish and seafood are examples of products that are found at the lower end of AA content range. The content in the different foods shows a wide range of variation. Consequently, the average exposure rate differs not only between countries but also among age groups (**Table 3**).

Table 2 Content of acrylamide in different food items according to JECFA 2005

Food Item	number of samples*	Mean content (µg/kg)	CV %
Cereals and cereals-based products (collective)	11 327	366	151
Breads and rolls	5 145	446	130
Pastry and biscuits	4 980	350	162
Breakfast cereals	1 130	96	131
Pizza	85	33	270
Fish and seafood	107	25	180
Meat and offal	325	19	174
Milk and milk products	147	5.8	119
Nuts and oilseeds	203	84	233
Potato Products (collective)	10 077	477	108
Potato baked	99	169	150
Potato crisps	3 555	752	73
Potato chips	6 309	334	128
Coffee and Tea	1 455	509	120
Coffee (ready to drink)	93	13	100
Coffee (ground, instant or roasted)	709	288	51
Coffee decaffeinated	34	688	169
Coffee extracts	119	1 100	93
Coffee substitutes	368	845	90
Green tea (roasted)	101	306	67
Cocoa products	23	220	111
Sugars and honey	133	24	87
Vegetables	193	17	206
Fruits dried and processed	49	131	125
Alcoholic beverages	99	6.6	147
Infant formula	117	< 5	82
Baby food (dry powder)	24	16	73
Baby food (biscuits, etc.)	32	181	106
Dried food	13	121	266

* The total numbers of individual samples, allowing for the number of samples blended into composites.

For example, in the Netherlands children and teenagers have a higher exposure rate. Fohgelberg *et al.*, (2005) estimated the AA intake for Swedish infants in their first year of life to be in the range 0.04-1.2 µg/kg bodyweight/day based on analyses of breast milk and infant formulae. In Germany, bread accounts for about 18-46% of AA intake due to the high consumption (Hilbig *et al.*, 2004). In the Netherlands, the mean acrylamide exposure is in the order of 0.48 µg/kg bodyweight /day (Konings *et al.*, 2003). In Sweden, the main source of AA intake

is potato-based products, accounting for 45%, while 22% comes from coffee intake and 33% from cereal-based products, where intake of bread (soft and crisp) contributes *ca* 22% (Hellenäs, 2008) (**Figure 3**).

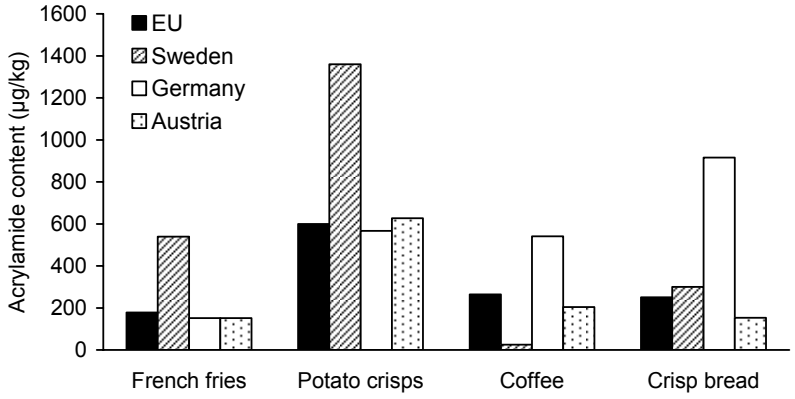

Figure 2. Acrylamide content in the most common food products in some European countries (JECFA 2005).

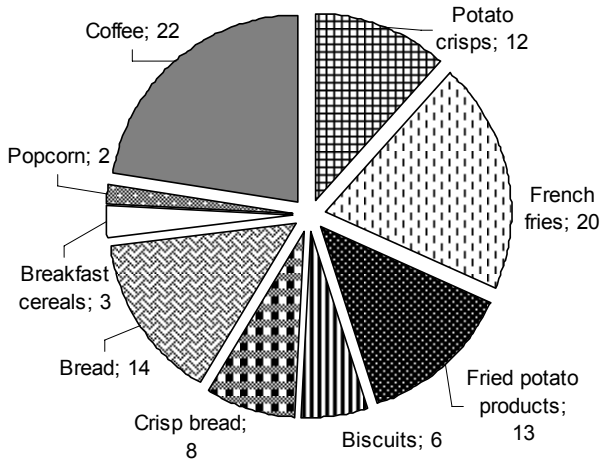

Figure 3. Percentage contribution of different products to acrylamide intake in Sweden. The average per capita intake is estimated at 26 µg/day (Hellenäs 2008).

In a recent report, JECFA identified average AA intake to be in the range 0.3-2.0 µg/kg bodyweight/day, with a mean of 1 µg/kg bodyweight/day for the general population and intakes in the range 0.6-5.51 µg/kg bodyweight/day, with a mean of 4 µg/kg bodyweight/day for the high level consumers (FAO/WHO, 2007). When calculating for the margin of exposure (MOE), JECFA concluded that AA in food is a human health concern and that the pivotal effects of AA for risk assessment are genotoxicity and carcinogenicity. The higher exposure level is reported to be in the order of 100 µg/person when including exposure to AA

originating from cosmetics and tobacco, using averages based on Hb adduct levels in the Swedish population (Tareke *et al.*, 2002).

Table 3 *Acrylamide exposure estimates (2002 - 2004).*

	Mean daily intake µg/kg - day	Age group	Source
Sweden	0.45	18 - 74	Svensson et al. (2003)
Norway	0.51	16 - 30	Dybing and Sanner (2003)
Netherlands	1.04	1 - 6	Konigs et al. (2003)
	0.71	7 - 18	
	0.48	1 - 97	
Germany	1.1	15 - 18	http://www.bfr.bund.de/cm/208/Absc haetzung_der_Acrylamid_Aufnahme _durch_hochbelastete_Nahrungsmitt el_in_Deutschland_Studie.pdf
Switzerland	0.28	16 - 57	http://www.bag.admin.ch/verbrau/akt uell/d/DDS%20acrylamide%20preli minary%20communication.pdf
France	1.4	2 - 14	http://www.afssa.fr/ftp/afssa/basedoc /acrylpoint2sansannex.pdf
European Union	0.2 - 0.4		http://europa.eu.int/comm/food/fs/sc/ scf/out131_en.pdf

1.4 Metabolism and toxicology

AA is biotransformed *in vivo* to its epoxide glycidamide, which has been shown to be genotoxic in a variety of *in vitro* and *in vivo* test systems (Rice, 2005). The AA metabolite glycidamide has the ability to form DNA adducts, which account for a genotoxic and cancer risk increasing agent (Törnqvist, 2005). It has been demonstrated that AA is transformed to its metabolite glycidamide through the Hb adducts in both animals and humans. It is the glycidamide that gives rise to detectable DNA adduct levels in rodents exposed to AA. The maximum tolerable level of AA is reported to be 0.5 µg AA/litre of water. AA is also found in tobacco smoke, resulting in smokers having higher exposure levels than non-smokers (Abramsson-Zetterberg, 2003; Bergmark, 1997; Jägerstad & Skog, 2005; WHO, 2003). An overall evaluation of the carcinogenicity of AA reported that 'AA and its metabolite glycidamide form covalent adducts with DNA in mice and rats; AA and glycidamide form covalent adducts with haemoglobin in exposed humans and rats; AA induces gene mutation and chromosomal aberration in germ cells of mice and rats and forms covalent adducts with protamines in germ cells of rodents *in vivo*; AA induces gene mutation and chromosomal aberrations in cultured cells *in vitro*; AA induces cell transformation in mouse cell lines' (IARC, 1994).

In studies on the cancer risk for the large bowel, bladder or kidney, no excess cancer risk could be related to dietary AA (Dybing & Sanner, 2003; Mucci *et al.*, 2003). On the other hand, an increased risk of postmenopausal endometrial and ovarian cancer has been observed with high levels of dietary AA, mainly among non-smokers, a kind of association not found with breast cancer (Hogervorst *et al.*, 2007). Olesen and co-workers recently reported a positive association between AA Hb levels and oestrogen receptor-positive breast cancer when adjustment was made for smoking behaviour (Olesen *et al.*, 2008).

1.5 Reaction pathways and kinetics

1.5.1 Maillard reaction

Amino-carbonyl interactions in foods compose a series of reactions between amines, amino acids, peptides and protein with reducing sugars, termed the Maillard reaction or non-enzymatic browning (Friedman, 1996). The reaction with amino compounds results in the formation of *N*-glycosides. Non-enzymatic glycosylation (glycation) is the covalent attachment of sugars to α- or ε- NH_2 groups of amino acids and proteins to form glycated proteins. The first glycation product (Schiff base) rearranges to a more stable ketoamine (Amadori product). The Amadori product can then form crosslinks with either amino groups or proteins.

In foods, the Maillard reaction results in formation of brown pigments *e.g.* melanoidins and production of volatile compounds that are potentially desirable aroma substances. However, the Maillard reaction can also result in off-flavour formation (Belitz & Grosch, 1999) and may also contribute to adverse effects on foods during storage and processing. These effects include loss of nutritional qualities, which is related to decreased digestibility and destruction of essential amino acids (Belitz & Grosch, 1999; Friedman, 2005). They may also result in toxic and antitoxic compounds. Maillard reaction products have antioxidative effects. An example of an antioxidant is that formed during the reaction between tryptophan and fructose or glucose. It is reported that the antioxidants formed in the advanced stages of the Maillard reaction are stronger than those produced in its early stages. It is known to produce antimutagenic products that are related to the inhibitory effect of the glucose-tryptophan reaction. Maillard reaction products are also known to produce mutagenic and carcinogenic products that include melanoidins, furans and other products.

1.5.2 Pathways of acrylamide formation

The first studies on the mechanistic pathway for the formation of AA in food proposed the Maillard reaction as the major pathway, mainly involving the free amino acid asparagine (Asn) and with reducing sugars as the major precursors in the reaction (Mottram, Wedzicha & Dodson, 2002; Stadler*, et al.*, 2002). Thereafter, several studies suggested different pathways within the Maillard reaction or alternative pathways (**Table 4**).

Some of the suggested pathways *via* the Maillard reaction are summarised in **Figure 4.** The first step is the reaction between free Asn and a carbonyl source (amino-carbonyl reaction), resulting in *N*-glycosyl asparagine, which in turn undergoes hydrolysis, producing the stable Shiff base (Blank *et al.*, 2005; Stadler *et al.*, 2002). The Shiff base further undergoes decarboxylation that results in a decarboxylated Schiff base, which after tautomerisation forms decarboxylated Amadori compound. From this intermediate AA is formed, along with an aminoketone *via* β-elimination reaction and cleavage of the carbon-nitrogen covalent bond (Becalski *et al.*, 2003; Mottram, Wedzicha & Dodson, 2002; Stadler *et al.*, 2004). The decarboxylated Shiff base may decarboxylate, forming 3-aminopropionamide (3-APA), which in turn forms AA with the elimination of ammonia (Granvogl *et al.*, 2004; Granvogl & Schieberle, 2006; Zyzak *et al.*, 2003). It has been suggested that 3-APA is formed during Asn thermal degradation and AA is formed thereafter. Granvogl *et al.* (2004) reported that reducing sugars are not always needed for the production of AA. At higher temperatures (100-180 °C) decarboxylation of Asn occurs, resulting in formation of 3-APA and thereafter AA. A further study in foods showed that 3-APA is a transient intermediate of AA formation during food processing (Granvogl & Schieberle, 2006).

An alternative route proposed is the formation of pyrolytic AA from wheat gluten, with protein-bound alanine as the key amino acid. In a model experiment, thermal treatment of gluten resulted in the formation of high amounts of AA (Claus *et al.*, 2006b). When gluten was added to the dough, the increase in AA content was in the order of 20%, with a high correlation to the amount of added gluten.

The Maillard reaction is also responsible for colour formation during food processing. This fact gave rise to the question of whether a correlation exists between AA content and colour intensity. Studies on the relationship between colour and AA content in bread crusts with different AA content showed that the colour varied from almost white to dark brown. A highly significant correlation ($P<0.001$) was found between colour and AA content in these crusts when the breads were baked with the same recipe (Surdyk *et al.*, 2004). In gingerbread, the AA concentration and browning intensity both increased with baking time and correlated with each other (Amrein *et al.*, 2004).

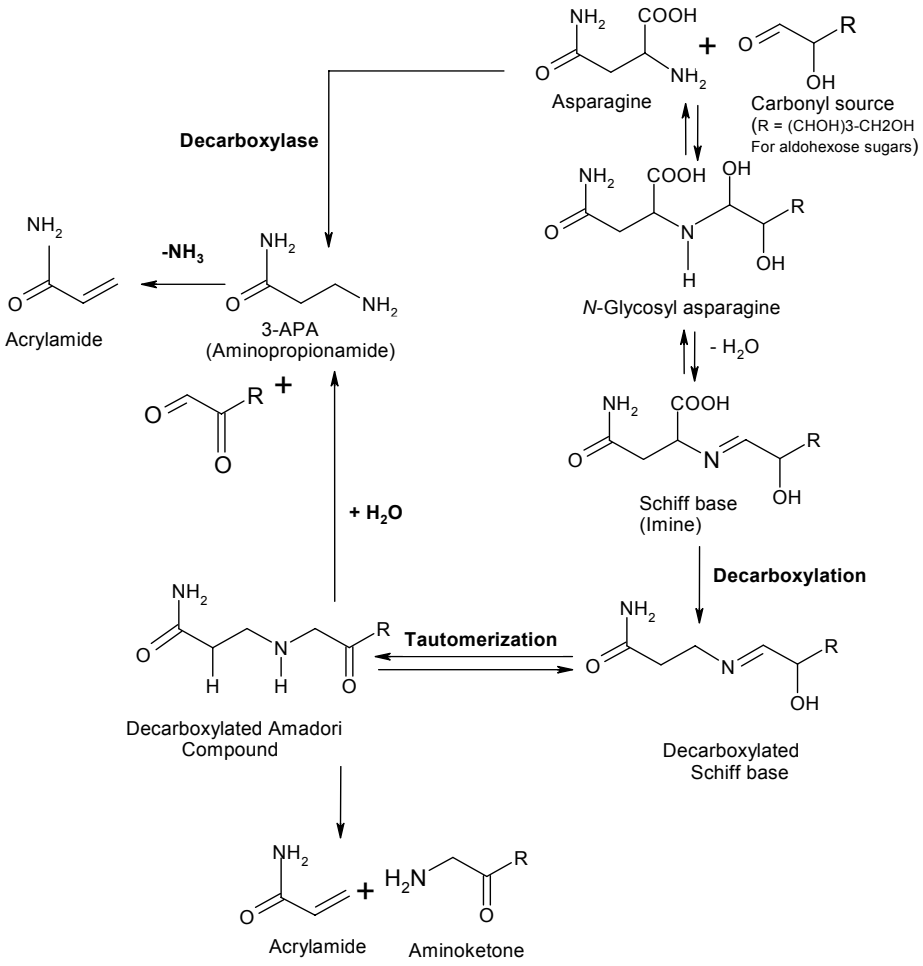

Figure 4. Some suggested pathways for acrylamide formation from asparagine, adapted from Blank et al. (2005), Stadler et al. (2004) and Granvogl et al. (2006).

Table 4 Suggested mechanistic pathways for acrylamide formation

Suggested intermediates and / or pathway	**References**
Decarboxylation of the Schiff base	Yaylayan *et al* 2003; Zyzak *et al* 2003; Stadler *et al* 2004
Decarboxylated Amadori product	Yaylayan *et al* 2003; Stadler *et al* 2004
3-Aminopropionamide	Granvogl *et at* 2004; Zyzak *et al* 2003
Wheat gluten	Claus *et al* 2006
Acrylic acid + NH_3 (ammonia from thermal degradation of amino acids)	Stadler *et al* 2003
Acrolein (from triolein) + Asn Acrolein + NH_3 Acrylic acid + NH_3 (amino dehydroxlation)	Yasuhara *et al* 2003
Acrylic acid from 2-propenal and subsequent reaction of acrylic acid with NH_3	Vattem *et al* 2003

1.5.3. Model studies in foods

In mechanistic model studies, factors affecting the formation of AA include heating time and temperature, pH, ratio of free amino acids to reducing sugars and moisture content (Friedman, 2003; Stadler, 2004; Stadler *et al.*, 2004; Zhang & Zhang, 2007a). In a food model study of the relationship between AA and its precursors (free Asn and reducing sugars), it was shown that large losses of Asn, water and total reducing sugars were accompanied by a large increase in AA (Elmore *et al.*, 2005). A linear relationship was obtained by plotting Asn loss against AA formation. In that study, 0.29% of the free Asn in potato was converted to AA, while 0.80 and 0.98% of that in rye and wheat products, respectively, was converted to AA.

Studies of the reactivity of free Asn and reducing sugars have shown that only a proportion of the two reactants results in AA formation. A reaction yield in the range of 0.1-0.3% of the initial Asn content was reported by some studies under optimal model reaction conditions (Becalski *et al.*, 2003; Mottram, Wedzicha & Dodson, 2002; Stadler *et al.*, 2002; Yasuhara *et al.*, 2003). Similar rates were

found in model food systems, <0.3% in bread (Surdyk *et al.*, 2004) and <1% in potato, wheat flour and corn starch (Biedermann *et al.*, 2003).

The rate limiting precursors for AA formation (free Asn or reducing sugars) depend mainly on its content in raw materials. Surdyk *et al.* (2004) showed that the content of Asn, but not fructose, added to dough was strongly correlated to AA content in bread. In contrast, in potatos, where Asn is in abundance, increasing the levels of reducing sugars during storage was translated into increased content of AA in the fried product (Becalski *et al.*, 2003). Similarly, when the content of Asn in potatoes was depleted by enzymatic digestion with asparaginase, a pronounced decrease in the AA formation was achieved (Zyzak *et al.*, 2003). Studies on cereal-based products have shown that free Asn is the limiting precursor for AA formation (Amrein *et al.*, 2004; Becalski *et al.*, 2003; Surdyk *et al.*, 2004).

1.5.4 Kinetic studies

The formation of AA is mainly formed *via* the Maillard reaction, which is highly complex and cannot be explained by simple reaction kinetics since more than one reactant needs to be followed and modelled by means of multi-response modelling (Martins, Jongen & van Boekel, 2000). Similarly, in order to predict and control the amount of AA formed, the formation mechanisms and kinetics of AA as a function of process and product variable need to be known (Claeys, De Vleeschouwer & Hendrickx, 2005a). AA formation is influenced by temperature of the reaction with the onset of its formation at ~120 °C (Becalski*, et al.*, 2003; Friedman, 2003; Mottram, Wedzicha & Dodson, 2002; Rydberg *et al.*, 2003; Tareke *et al.*, 2002). Generally, the onset of AA formation is at around 120 °C. In these studies, it has been shown that qualitative and quantitative (kinetic) data depend on the food model studied. In addition, the nature of the reactants, the molar ratio, the water activity (a_w), the matrix and the heating equipment are all reported to influence the formation of AA.

Factors affecting AA formation/elimination and the study of its kinetics are quite interrelated. Model system studies on the effect of amino acids on AA have shown that the formation and elimination kinetics could be modelled by two consecutive first-order reactions (Claeys, De Vleeschouwer & Hendrickx, 2005a). The ratio of elimination to formation rate constant depends on the amino acids (other than Asn) present. A further kinetic study in aqueous systems aimed at determining the importance of reducing sugars on AA formation has shown that the carbonyl group is essential, especially at high temperatures (Knol *et al.*, 2005). This study provided a multi-response model and suggested that AA is not an end product in the Maillard reaction but rather an intermediate.

The formation and the elimination of AA are highly controlled by the time and temperature of the reaction. Considerable amounts of AA are formed when foods are exposed to temperature above 120 °C, when the temperature exceeds 160 °C it was shown that the formed AA is eliminated and its residual amounts depend on temperature and exposure duration (Wedzicha *et al.*, 2005). In foods and model systems, the formation of AA follows a number of interactive pathways that

involve the synthesis and the degradation of intermediate compounds (Corradini & Peleg, 2006). In model studies, it has been assumed that all the intermediate reactions follow the first order reaction kinetics and that the temperature dependence of the rate constants follows the Arrhenius model (Claeys, De Vleeschouwer & Hendrickx, 2005a; Knol *et al.*, 2005). However, the reactants and the products of the Maillard reactions that are responsible for the AA formation in foods are contentiously changing (Corradini & Peleg, 2006). The rate constants in the food systems are not only temperature dependant because of the complex character and the interactive nature of the molecules. Thus, it is not possible to suggest that some of the intermediate reactions have the same energy of activation. In a model system studied by Corradini and co-workers (2006) it was reported that the level of AA production and rate are both stimulated by temperature elevation. On the other hand, the rate of the process leading to AA degradation also increases with temperature and their effect becomes noticeable after progressively short time as the temperature reaches a sufficiently high level. From this model, it could be shown that the process of formation and degradation of AA goes simultaneously at elevated temperatures. In their model, they suggest that the temporary rate of AA synthesis or degradation is the isothermal rate at the momentary temperature, at a time that corresponds to its momentary concentration.

1.6 Analytical methods

Studies in AA research involve a range of analytical techniques to determine the contents of free amino acids and reducing sugars in raw materials and ingredients, as well as methods for extraction and determination of AA content in the final products.

1.6.1 Amino acid analysis

A number of methods can be used for the analysis of free amino acids and these include either liquid or gas chromatography. A cation-exchange chromatography method has been used for analysis of amino acids (Davies, 2002). Samples are extracted using 5-sulphosalicylic acid with norleucine as internal standard. The method involves a post-column derivatisation of the amino acids with ninhydrin, which produces coloured amino acid derivatives that can be determined spectrophotometrically at 570 and 440 nm. Another method that involves an amino acid analyser uses buffer at acidic pH (Granvogl *et al.*, 2007).

Gas chromatography is also used after derivatisation of both functional groups in the amino acid to suitable volatile derivatives (Davies, 2002; Molnar-Perl, 2000). For this purpose, Husek recommended derivatisation of amino acids with propyl chloroformate (Husek, 1991; Husek, 1998). In 2001, Phenomenex released an analytical kit based on this method for analysing a range of free amino acids in physiological fluids. The extraction and clean-up involve a simple solid phase extraction (SPE) step, followed by a rapid derivatisation reaction and analysis by gas chromatography (GC) with internal standard (Farkas & Toulouee, 2003). This method has recently been applied to the analysis of amino acids in potato, wheat

and rye products, but no validation of the method for these types of matrices has been reported (Claus *et al.*, 2006a; Elmore *et al.*, 2005).

1.6.2 Reducing sugar analysis

A number of methods are used for analysis of reducing sugars. Liquid chromatography with refractive index detection (HPLC-RI) is used for the determination of reducing sugars in dough, involving aqueous-alcohol extraction. Fructose, glucose, sucrose and maltose are detected with refractive index detector (Claus *et al.*, 2006a). Ion exchange chromatography is another technique for the analysis of sugars. Doughs and flours are extracted using aqueous alcohol, and then analysed by Dionex ion chromatography (Elmore *et al.*, 2005). Recently, reducing sugars in fermented dough were analysed by a spectrophotometric method (Fink *et al.*, 2006; Hostettler, Borel & Deuel, 1951). Samples were extracted with water and then mixed with Sumner's reagent containing 1% (w/v) 3,5-dinitrosalicylic acid (Hostettler, Borel & Deuel, 1951). The solution was heated in a water bath, cooled and diluted. The absorption was measured at 530 nm.

1.6.3 Methods for acrylamide analysis

Water at neutral pH is the most common solvent for extraction before chromatographic analysis of AA (Wenzl *et al.*, 2003). However, it was shown that the level of analyzed AA increases by increasing the pH of extraction (Eriksson & Karlsson, 2006). This study indicates that increasing the pH of the extraction medium releases more AA depending on the matrix. In bread products, the ratio of increase between pH 12 and pH 6 is in the range 1.1-4.1. Since these studies were reported, some efforts have been made to test for the source of this extra AA, suspected as being trapped in the matrix where the alkaline medium facilitates its extraction. A recent study showed that this additional AA was not due to improvement of the alkaline extractability but was an extraction artefact (Goldmann *et al.*, 2006). Extraction at high pH seems to result in formation of AA from water soluble precursors during thermolysis.

The cleanup procedure after extraction for LC-MS methods includes SPE steps that involve two sets of columns: the Isolute Multimode column, which is mixed-phase, combining hydrophobic interaction containing silica-based C-18 groups with anion and cation exchangers (Petersson *et al.*, 2006; Rosén, Nyman & Hellenäs, 2007). This column is used as a chemical filter to retain the maximum possible amount of matrix component (non-polar compounds as well as anions and cations) without retaining AA. This column is then followed by an ENV+ column, which comprises a highly crossed linked polystyrene divinylbenzene resin with a very high surface area modified with non-ionisable hydroxide groups. It gives higher retention due to its polymeric nature with a graphite carbon phase. The phenolic group on ENV+ favours retention of AA through hydrogen bonding with the amide function.

Determination and detection of AA in heat-treated foods have been performed using a number of chromatographic methods. Their assays are based on mass spectrometry (MS) for the detection of AA, which is coupled with a chromatographic separation step using either high performance liquid chromatography (HPLC) or GC. These methods have been summarised in inclusive reviews (Friedman, 2003; Stadler & Scholz, 2004; Taeymans *et al.*, 2004; Wenzl, de la Calle & Anklam, 2003; Zhang, Zhang & Zhang, 2005). LC-MS methods for the analysis of AA depend mainly on the advantage of AA solubility in water and efficient extraction therein (Stadler *et al.*, 2004). AA is readily extractable from the food matrix, as protease and amylase digestion of crispbread samples prior to water extraction does not result in increased levels of AA (Jezussek & Schieberle, 2003). These results indicate that inclusion of AA in starch/protein gels is not very probable during bread making. The first LC-MS method for AA analysis in food was developed by Rosén & Hellenäs, (2002). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods for food analysis have recently been improved and closely explained (Petersson *et al.*, 2006; Rosén, Nyman & Hellenäs, 2007). The HPLC column used is a hypercarb column (graphitised carbon as stationary phase). In this assay water is used as the mobile phase since it provides the best retention of AA. The mobile phase used is 0.1% acetic acid since it improves ionisation, resulting in a stable MS response for AA. The mass detector used is a triple quadrupole mass spectrometer operating in positive electrospray and multiple reactions monitoring mode (MRM) (Petersson *et al.*, 2006; Rosén, Nyman & Hellenäs, 2007).

Furthermore, methods using GC-MS are either based on bromination of the analyte or direct analysis without derivatization are as well used for AA dtermination. The former method involves the bromination of AA to 2,3-dibromopropionamide. Bromination has the advantage that a more volatile compound is produced and the AA derivative is identified by its retention time and ratio of characteristic MS ions. An alternative procedure is silylation of the amide moiety, furnishing N,O-bis(trimethylsilyl)acrylamide. The GC-MS method without derivatisation employs liquid-liquid extraction of the analyte. The extraction solvents are primarily water or a mixture of water and an organic solvent. A major drawback of this method is the lack of characteristic ions in the mass spectrum of the non-derivatised AA, while co-extracted substances produce almost the same fragmentation pattern and may therefore interfere.

1.7 Factors affecting acrylamide formation

Many studies have investigated factors affecting its formation in foods and means to introduce mitigating measures. These studies are inclusively summarised in reviews (Claus, Carle & Schieber, 2007; Friedman, 2003; Konings *et al.*, 2007; Lingnert *et al.*, 2002; Taeymans *et al.*, 2004). The present study focuses mainly on measures related to cereal products, with the main emphasis on bread (**Table 5**).

1.7.1 Cultivars and growing conditions

The contents of AA precursors, namely free Asn and reducing sugars, are governed by both plant variety and cultivars. For example, in different wheat and spelt cultivars a wide range of Asn contents has been reported, but no great variation has been observed in rye (Claus*, et al.*, 2006a; Taeymans *et al.*, 2004). From a general point of view, the Asn content and the crude protein content are affected by harvest year, weather conditions and humidity. Sprouting gives rise to high protease activity, which results in high Asn levels in wheat. In one study, the content of free Asn in nine wheat cultivars ranged between 5-25 mg/100 g flour, while in two cultivars of rye it was 41 & 44 mg/100 g flour (Claus *et al.*, 2006a). However, the contents of reducing sugars in these cereals were much higher, ranging between 0.39-0.93 g/100 g flour in the nine wheat cultivars, while it was 1.1 & 1.2 g/100 g flour in the two rye cultivars (Claus *et al.*, 2006a).

It has been reported that Asn is formed in most parts of the plant and accumulates during physiological processes such as seed germination and nitrogen transport. Asn is also reported to accumulate during stress conditions of mineral deficiency, drought, salt, toxic metal exposure and pathogen attack (Lea *et al.*, 2007). A recent study showed that using nitrogen fertilisers during wheat cultivation results in increased levels of free amino acids and crude protein (Claus *et al.*, 2006a), which is crucial because it mops up some of the free amino acids. However, this will not take place if sulphur is in short supply, as sulphur deficiency results in decreased synthesis of the major seed storage proteins. Sulphur fertilisation is known to lower the amount of free amino acids in plants. It has recently been shown that growing wheat under sulphur-deficient condition results in an ample content of free Asn, which translates to a high concentration of AA and extremely high content of 3-APA (Granvogl *et al.*, 2007; Halford *et al.*, 2007). The latter is reported to generate AA without the need for carbohydrates (Granvogl & Schieberle, 2006; Granvogl & Schieberle, 2007). Growing wheat with a high content of sulphur resulted in a lower content of Asn, but no effect on reducing sugars was observed (Granvogl & Schieberle, 2007; Muttucumaru *et al.*, 2006). A study analysing the content of Asn in the germ and different extraction rates from wheat showed that the highest concentration of Asn in the wheat is found in the germ, where it is in the order of 5 g/kg flour compared with 1.5 and 0.5 g/kg in the bran and the whole grain flour respectively (Fredriksson *et al.*, 2004).

1.7.2 Raw ingredients and processing parameters

The baking agent NH_4HCO_3 has been found to enhance the formation of AA in bakery products in both model systems and practical production (Amrein *et al.*, 2004; 2005; Weisshaar, 2004). NH_4HCO_3 increases the formation of α-dicarbonyls from glucose and fructose. The generation of glyoxal from glucose or fructose is higher with NH_4HCO_3 compared with HCO^-_3 (Amrein *et al.*, 2006). Sugar fragments, such as glyoxal, glyceraldehydes and others, form much more AA in reaction with asparagine under mild conditions compared with glucose and fructose. When NH_4HCO_3 was totally replaced by $NaHCO_3$ in semi-finished

biscuits produced on an industrial scale, a 70% decrease in AA content was achieved (Amrein *et al.*, 2007; Graf *et al.*, 2006).

The types of sugars needed in the Maillard reaction for the formation of AA are reducing sugars (glucose and fructose). Amrein *et al.* (2004) suggested the replacement of reducing sugars with sucrose, since it lacks the carbonyl group. A major drawback of this reducing measure is that the final products tend to be insufficiently coloured, but it could be applied to products in which colour is not a crucial measure. Comparatively, when fructose was added to yeast-leavened wheat bread, it made no significant contribution to AA formation (Surdyk*, et al.*, 2004).

1.7.3 Organic acid addition and pH

Addition of citric acid in the order of 1% dough results in a decline in the pH and further reduction in the AA formed by a factor of 40 (Amrein *et al.*, 2004). However, this effect results in gingerbread with an acidic taste and insufficient leavening and browning, related to the forced protonation of NH_3, which in turn results in a reduced gas volume during baking. The reduction in AA content and colour intensity is due to the protonation of the α-amino group of Asn, which hinders the formation of the N-substituted glycosylamine. Similarly Cook & Taylor, (2005) reported high reduction in AA related to a decrease in pH. The presence of NaCl in a model food matrix of Asn and glucose was found to bring a decrease in AA content in the order of 32% when the salt was added in the range 0-1% (Kolek, Simko & Simon, 2006). In that study, differential scanning calorimetry measurement showed that the elimination of AA via polymerisation was strongly accelerated by the NaCl. A recent study on wheat bread showed that addition of NaCl plays an ambiguous role. At low doses (up to 2%), it lowered AA by inhibition of the enzyme activities, but higher additions markedly increased AA content due to growth inhibition of the yeast (Claus, Carle & Schieber, 2007).

A further suggestion for the reduction of AA involves the combined effect of added citric acid and soya protein hydrolysate. It has been suggested that addition of lysine might reduce AA via Michael addition reactions (Cook & Taylor, 2005). Furthermore, when other types of consumable acids (lactic, tartaric, citric and hydrochloric acids) were tested in semi-finished biscuits and biscuits models, these studies reported a decline in pH in the order of 30% and a varying degree of reduction in AA depending on the level of acid addition (Graf *et al.*, 2006; Levine & Smith, 2005; Taeymans *et al.*, 2004). The reduction in AA was attributed to hydrolysis leading to aspartic acid at the low pH. In addition, low pH results in moderate Maillard reactions and therefore lowers AA formation. However, these effects are usually accompanied by decreased browning intensity and affect flavour formation. Thus, it would best to evaluate the possibility of acid additions for each product individually.

1.7.4 Addition of ions

The effect of divalent cations on the formation of AA was studied in a model system by Gökmen & Senyuva (2007a). Divalent cations, *e.g.* Ca^{2+} and Mg^{2+}, were found to prevent AA formation completely, while monovalent cations, *e.g.* Na^{+} and K^{+}, reduced AA formation by almost half. Adding cations into the reaction mixture increased the rate of glucose decomposition while most Asn remained unreacted. The presence of Ca^{2+} prevented the formation of Schiff base and consequently hindered AA formation. In a further study from the same group, a similar reduction effect of added cations on AA formation was observed, but a side-effect to this measure was observed (Gökmen & Senyuva, 2007b). Increased formation of hydroxymethylfurfural (HMF) and furfural was observed during heating in relation to the addition of cations. HMF and its derivative are known to have cytotoxic, genotoxic and carcinogenic effects.

1.7.5 Antioxidant addition

Addition of antioxidants has also been addressed as a measure to control the formation of AA in foods. Examples of antioxidants used in a potato model system are lipophilic ascorbyl palmitate and hydrophilic sodium ascorbate, which showed no effect on the formation of AA (Rydberg *et al.*, 2003). When phenolic antioxidants from cranberry and oregano were used in deep-fried potato slices, only cranberry had an effect on AA formation (Vattem & Shetty, 2003). This suggests that formation of AA in this product is the result of non-oxidative processes. However, when potato chips were processed with olive oil and added rosemary, a 20% decrease in AA content was observed (Becalski *et al.*, 2003). A study on the effect of antioxidants from bamboo leaves and extract of green tea has shown a decrease in the order of 83 and 78%, respectively, in AA content of fried bread sticks (Zhang & Zhang, 2007b). Addition in the order of 1 g/kg of antioxidant from bamboo leaves and extract of green tea did not result in any significance difference in flavour and texture of the bread compared with the original recipe. A phenolic antioxidant, a dihydroxyphenolic compound from virgin olive oil, has recently been proposed as a reliable mitigation strategy for AA formation in fried crisps (Napolitano *et al.*, 2008).

A recent study on the effect of prooxidants and antioxidants on the formation of AA in bread showed that addition of 1% of rosemary extract resulted in a 57-67% reduction in AA levels depending on the nature of the extract (Hedegaard *et al.*, 2008). The flavonoids epicatechin and epigallocatechin gallate were also reported to reduce AA formation in a model system of glucose and Asn. That study suggests a role of free radicals in the formation of AA since a free radical was detected using Electron Spin Resonance (ESR). Furthermore, more AA formed in another model with water in oil emulsion containing oxidised rapeseed oil compared with a similar emulsion with non-oxidised oil. These results point to the impact of oxidative processes on the elimination of AA since oxidised vegetable oil has been shown to promote degradation of AA.

Table 5 Mitigation measures suggested in various studies for the reduction of acrylamide in cereal products.

Cultivars and growing conditions	Ingredients	Process formulation and additives	Technological aspects
* Choosing varities and cultivars low in asparagine	* Replacing reducing sugars with sucrose	* Using yeast fermentation	* Using steam baking
* Using sulphur containing fertilisers	* Replacing NH_4HCO_3 with $NaHCO_3$	* Using sour dough and lactic acid fermentation (favourable effect varies according the product and recipe)	* Using infrared radiation baking
	* Adding: NaCl Divalent cations (Ca^{2+} , Mg^{2+})	* Using pellet-to-flaking extrusion cooking rather than direct expansion extrusion cooking process	* Using circulating oven with radiofrequency assisted heating
	* Avoiding ingredients that are rich in free Asn, *e.g.* almonds, sesame and poppy seeds	* Using low temperature and long times of baking	* Using a moderate combination of time and temperature of baking
	* Avoiding ingredients that might include AA, *e.g.* heat-treated fibre products	* Using gradient time-temperature of backing rather than linear	
	* Using milling fraction that are low in asparagine	* Using asparaginase	
		* Adding amino acids (glycine, cysteine)	
		* Adding antioxidants	
		* Adding consumable acids to reduce pH	

1.7.6 Amino acid addition

In a food model system, the reaction rate of all amino acids relative to formation of AA in the Maillard reaction was found to be similar (Wedzicha *et al.*, 2005). This means that the rate of formation of AA from Asn is proportional to the concentration of each amino acid. The amino acids mop up the precursor and the amount of individual amino acids lost depends on the relative numbers of molecules of each present. These findings suggest that the competing reactions are all fast. Addition of cysteine to the dough resulted in significantly lower AA

content in the bread, with low addition levels showing greater reducing effect (Claeys, De Vleeschouwer & Hendrickx, 2005b). This was explained by its gluten weakening properties or by it reacting with AA to form cysteinyl-S-β-propionamide by Michael addition of the cysteine SH group to the vinylic double bond of AA (Rydberg *et al.*, 2003; Taeymans *et al.*, 2004). Addition of cysteine favours AA reduction in bakery products but care should be taken with this reduction measure since it can affect dough rheological and sensorial properties at elevated dosages (Claus, Carle & Schieber, 2007).

Furthermore, glycine (Gly) and glutamine used with wheat bread result in reduction of AA content depending on the levels of addition (Bråthen *et al.*, 2005; Fink *et al.*, 2006; Low *et al.*, 2006). The reducing ability of Gly could be attributed to competition with Asn for reducing sugars and/or reaction with the AA formed. It has also been proposed that a Michael addition reaction takes place with the amino group of Gly, resulting in AA binding (Rydberg *et al.*, 2003; Wedzicha *et al.*, 2005)

1.7.7 Enzyme addition

Addition of the enzyme asparaginase has been reported as a measure to reduce the formation of AA since it results in the hydrolysis of Asn to aspartic acid and ammonia (Zyzak *et al.*, 2003). This reducing effect of asparaginase was first reported in a potato model system that resulted in 88% reduction in Asn and a subsequent reduction of AA in the order of 99%. Similar results were also reported in a model system of wheat (Weisshaar, 2004). In an experiment with bakery products, asparaginase resulted in a 75% decrease in Asn content, which was reflected as 55% reduction in AA content (Amrein *et al.*, 2004). The incomplete hydrolysis of Asn was related to the limited mobility of the enzyme and substrate within the dough. Recently the effective dose for enzyme addition to AA formation was reported to be 200-1000 U/kg dough (Amrein *et al.*, 2007). Fermentation time was reported to have an effect only at low doses. The effect of asparaginase in reducing AA content seems to vary according to the product type and possible product formulation parameters. Amrein *et al.* (2007) reported no effect of asparaginase in hazelnut biscuits. A general conclusion from the experiments using asparaginase identifies it as a promising measure for the reduction of AA while still retaining the organoleptic qualities of products. Recently, the US FDA (Food and Drug Administration) has recognised asparaginase from *Asperiglus niger* as 'generally recognised as safe' (GRAS) (Kuilman & Wilms, 2007).

1.7.8 Fermentation

Studies of the effect of yeast fermentation on AA reduction in yeast-leavened wheat bread suggest that prolongation of the fermentation time results in a substantial reduction in AA content in bread and bread rolls (Fredriksson *et al.*, 2004). Yeast is reported to consume Asn during fermentation (Benedito De Barber, Prieto & Collar, 1989). Nevertheless, extensive yeast fermentation is not recommended since it may results in degradation of the protein network and

subsequent flattening of bread rolls and/or production of monochloropropanediol isomer (MCPDs), a compound defined as a potential genotoxic carcinogen (Claus, Carle & Schieber, 2007; Hamlet, Sadd & Gray, 2004).

The effect of sourdough (lactic acid bacteria and yeast) fermentation on AA content has been studied in breads with and without added free Asn (unpublished results). In dough made with added Asn, the AA content was reduced at higher levels of added sourdough. However, in the dough made without added free Asn, the content of AA increased when more sourdough was added. The bacteria might have hindered the ability of the yeast to consume Asn during fermentation. On the other hand, sourdough fermentation used in dough preparation of crispbread produced a 75% decrease in AA content, an effect that was attributed to a drop in the pH from 6.0 in the control to 3.7 (Claus, Carle & Schieber, 2007).

1.7.9 Processing and baking techniques

The effect of time and temperature of baking in cereal-based products has been investigated in numerous studies (Amrein *et al.*, 2004; Bråthen & Knutsen, 2005; Surdyk *et al.*, 2004). In soft wheat bread, > 99% of the AA content is found in the crust and the content of AA linearly increases with time and baking temperature, an effect that can be attributed to high rate of water loss from the surface of the bread.

The effect of extrusion technology in breakfast cereals in relation to AA content has also been addressed (CIAA, 2007; Claus, Carle & Schieber, 2007). Breakfast cereal made by extrusion puffing was found to have a low content of AA. Extrusion cooking gelatinises starch but results in a product with low water content due to evaporation and with very mild toasting and therefore very few Maillard products are formed.

Various baking technologies, including air jet impingement, infrared radiation baking, traditional baking and steam baking, have also been considered in relation to the control of AA formation. The main findings from these studies are that steam baking causes a 40% decrease in AA content, resulting in a product that has sensory properties similar to traditionally baked bread. With infrared radiation baking it was possible to reduce the AA content in flat bread cakes by 60% yet create a product with sensory properties similar to standards (Heatox, 2007). A recent study showed that baking in air-circulating oven with radiofrequency assisted heating resulted in a considerable decrease in AA content. This technology is said to be better suited to thin bakery products, as radiofrequency heating resulted in internal browning of thicker products (Anese, Sovrano & Bortolomeazzi, 2007).

1.8 Effect of storage on AA content

Some foods, *e.g*. biscuits and crispbread, are stored for considerable periods of time and it is therefore important to investigate the behaviour of AA during storage. The effect of storage on AA content has not been intensely studied, but some studies conducted on a range of products have found no common pattern in the degree of AA reduction (Hoenicke & Gatermann, 2005). The reduction of AA in different biscuits has been found to range from no decrease to a decrease in the order of 20%. Furthermore, Stadler, (2005) tested the stability of AA in some food products that were stored for different periods of time and found that AA in cereal products was stable for up to 12 months. In addition, a major decrease in AA was seen in coffee, up to 67% depending on the storage conditions and temperature. Another study on coffee reported a reduction in AA content ranging from 40-65% after 6 months of storage at room temperature, while in samples from the same batch that were stored at -40 °C in a sealed container no substantial decrease was observed (Andrzejewski *et al.*, 2004).

1.9 Objectives

The main aim of this thesis was to investigate some of the factors affecting the content of AA in bread in relation to precursor content, product formulation and storage conditions. The specific objectives of the research were to:

- Analyse the contents of free amino acids in cereals (rye, wheat, oats and barley), fractions and products (**Paper I**).
- Identify the main precursor for AA content in rye crispbread (**Paper II**).
- Investigate the effect of time and temperature of baking on AA content in rye crispbread (**Paper II**).
- Investigate the effect of fermentation on the content of AA in soft wheat bread (**Papers I & III**).
- Investigate the effect of additives (**Papers II & III**).
- Identify relationships between colour and AA content in bread (**Papers II & III**).
- Investigate the effect of extraction pH on AA content (**Paper IV**).
- Investigate the effect of different storage conditions on AA content (**Papers IV & V**).

2. Materials and Baking

2.1 Materials

Flour samples of rye bran, sifted rye flour, whole grain wheat, wheat bran, sifted wheat flour, low fibre oat flour, oat bran and oat groats were supplied by Lantmännen Mills (Uppsala, Sweden) and whole grain rye flour and rye crispbread (Delikatess) were obtained from Wasabröd AB (Filipstad, Sweden).

2.2 Whole grain rye crispbread baking

The recipe for baking rye crispbread was obtained from Wasabröd AB and modified for laboratory conditions. Whole grain rye flour, fresh yeast solution (100 mL of 15%, weight/volume, Original Kronjäst, Jästbolaget, Sweden), and tap water (350 mL) were mixed together in a kitchen dough maker (Electolux Assistant, Sweden) for 10 min. The dough was then transferred to a leavening cupboard and allowed to ferment at 30 °C and 85% relative humidity for 90 min. After this fermentation, the dough was kneaded and 90 g of the dough was spread out with a rolling pin on a silicone baking sheet (Silpat, Åsö AB, Åtvidaberg, Sweden) within a square frame (1.5 mm height) to set the thickness of the bread. A circular cutter (170 mm in diameter) was used to shape the bread and a spiked pattern rolling pin was used to give the crispbread its characteristic pattern. This bread was then taken for a second fermentation in the same leavening cupboard for 50 min. Bread was baked in a rotating laboratory oven (Simon, Greenfield, UK) at 250 °C for 8 min (standard procedure), except for the time-temperature experiment. After baking, the bread was dried in an upright position for 25 min at a temperature of 105 °C. (**Paper II**).

2.3 Soft wheat bread baking

The yeast-leavened wheat bread was baked according to the method used by Surdyk *et al*. (2004). The ingredients were wheat flour (Kungsörnen, Nordmills, Uppsala, Sweden), dry yeast (Kronjäst Original, Jästbolaget, Sollentuna, Sweden), salt and warm tap water. Flour and yeast were mixed in a farinograph (Brabender, Duisburg, Germany). Solutions of the salt and amino acids, L-asparagine monohydrate (for biochemistry, Merck, Darmstadt, Germany) and glycine (p.a., Merck, Darmstadt, Germany), were dissolved in parts of the water and added to the mixture of yeast and flour, and finally the remainder of the water was added. All ingredients were mixed and left to leaven, dough was then divided into portions of 100 g each. Thereafter, dough was moulded and placed in pre-oiled baking tins that were taken for a second fermentation period. A portion of the dough was frozen at -20 °C directly after the second fermentation for further analysis. Leavened portions were baked for 15 min at 270 °C. Breads were frozen after 1 h of cooling. Frozen dough and bread samples were freeze-dried for further analysis, crushed and milled in an ultra centrifuge mill (Retsch, Haan, Germany) to pass a 0.5 mm screen (**Paper III**).

3. Results and Discussion

3.1 Analysis of free amino acids in cereal fractions and products (Paper 1)

3.1.1 Method set-up and validation

The free amino acid contents in cereals (rye, wheat, barely and oats), fractions and products were analysed using alcohol extraction followed by the EZ-Faast method (**Paper I**). For extraction, 50% aqueous ethanol at 50 °C was used (**Figure 5**). The rationale behind this choice was to inactivate enzymes, prevent the extraction of polysaccharides and other viscous polymers, and avoid starch gelatinisation. The extracts from this procedure were non-viscous and readily applicable for SPE. Chloroformate derivatives of the free amino acids in the extracts were then analysed using GC-FID. All analysed amino acids were well separated within 8 min of run-time with a good consistency in the retention time (CV ≤0.2%) (Figure 1 in **Paper I**). The amount of the starting material for extraction ranged between 200-500 mg, depending on the content of free amino acids.

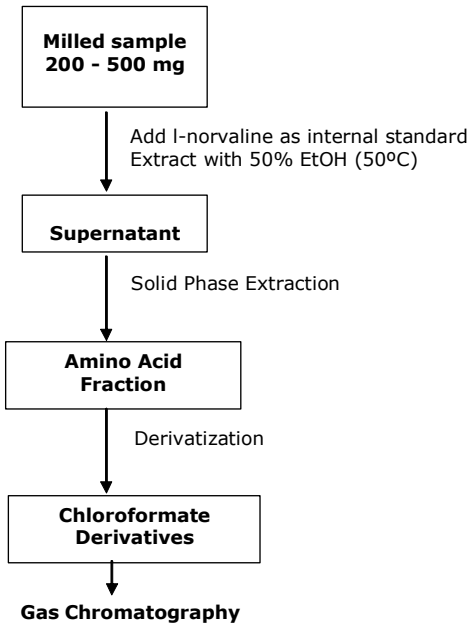

Figure 5. Schematic diagram for the analysis of free amino acids in cereal fractions and products.

This method was validated by spiking a range of samples (rye bran, sifted rye flour, rye crispbread and soft wheat bread) to test for recovery. The amino acids used in the recovery test were Asn, Gly, Val, Ser, Ala, Asp, Trp, and Glu. Most of those amino acids had a satisfactory recovery in the order of 100 ± 10%, except

for Trp and Glu. These problems in recovery were further investigated by spiking a sample of rye flour with different amounts of Glu and Trp. The recovery of Glu (130-185%) was found to increase with the amount of sample used, while Trp showed a low recovery (in the order of 75%), regardless of the sample amount. Since the recovery and repeatability tests were quite satisfactory (CV 5-10%) for most amino acids, this method was regarded as robust and able to quantify the content of free amino acids in different cereal fractions and products within 1 h of extraction and determination. However, values for Glu and Trp were removed from the results as we deemed the figures obtained unreliable.

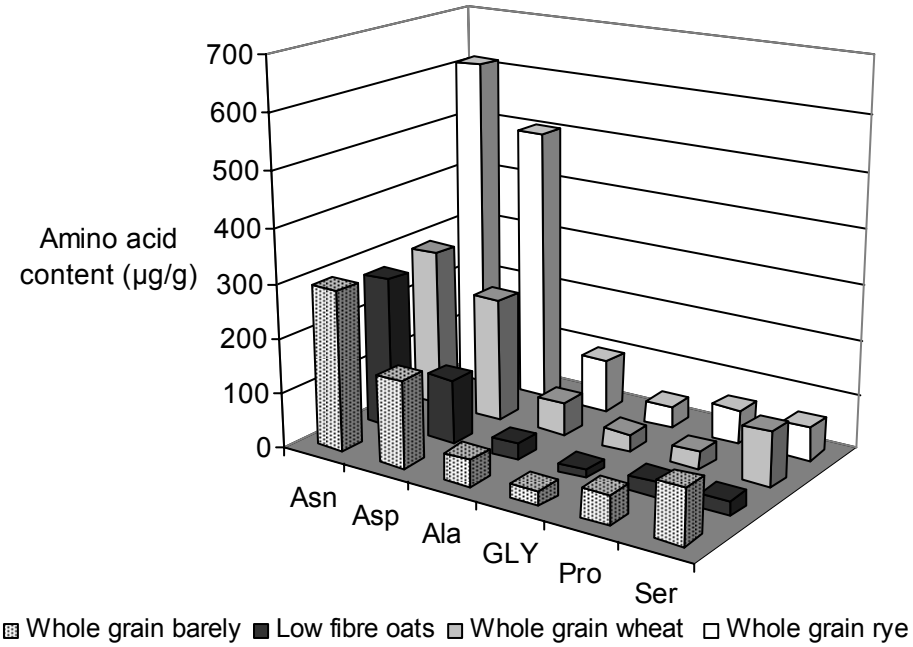

Figure 6. Content of major amino acids (µg/g) in flours from different cereals.

3.1.2 Free amino acid content in cereal fractions and products

Analysis of free amino acids in different cereals and cereal fractions showed that it was possible to analyse most of these, with their contents varying according to the type of cereal and the milling fraction (**Figure 6**). Asn and Asp were the dominant amino acids in the cereals analysed. Asn was the amino acid with the highest overall concentration, with its highest actual content found in rye. Details of all amino acid contents are given in Table 3 in **Paper 1**. It has been shown in other studies that the content of Asn in wheat has a wide range of variation depending on the cereal variety and growing conditions, but the variation in rye cultivars remains in a limited range (Claus*, et al.*, 2006a; Lea*, et al.*, 2007; Taeymans*, et al.*, 2004). Our results showed that the content of Asn and that of other free amino acids varied with the cereal milling fraction, with the highest concentration found in the bran and the lowest content in the sifted fraction (**Figure 7**). This

observation was valid for both rye and wheat. The pattern of amino acids in the different fractions was valid for all amino acids. In general, rye fractions were richer in all free amino acids compared with wheat fractions.

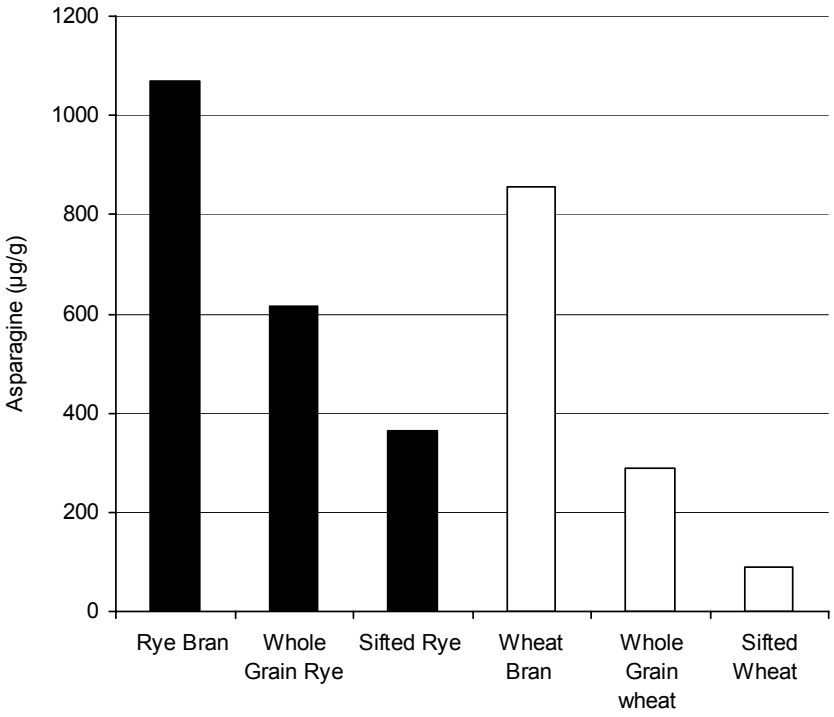

Figure 7. Asparagine content in different fractions of cereals.

3.2 Identifying AA precursors in rye crispbread (Paper II)

Formation of AA during bread making is the result of reactions between reducing sugars and Asn at high thermal treatment (Becalski *et al.*, 2003; Stadler, 2004; Stadler *et al.*, 2004; Zyzak *et al.*, 2003). To investigate the limiting precursor for AA formation in whole grain rye crispbread, a circumscribed central composite design (CCC) with different levels of added Asn and fructose was used. The content of Asn in these breads ranged 31-349 µg/kg bread, *i.e.* seven-fold higher than in breads baked with standard recipes without added Asn, where the AA content is in the order of 10 µg/kg. Response surface regression analysis showed that added Asn significantly increased AA formation ($p<0.001$), while fructose did not show any significant effect. **Figure 8** shows a strong correlation ($R^2 = 0.95$) between added Asn and AA formed in the bread, with no similar correlation obtained for added fructose. Similar results have been obtained in other cereal products, where the formation of AA corresponded to the addition of Asn and adding reducing sugars had no effect (Amrein *et al.*, 2004; Bråthen *et al.*, 2005; Bråthen & Knutsen, 2005; Surdyk *et al.*, 2004). It could be concluded that the Asn levels in the raw ingredients largely determines AA content and therefore

measures to decompose or limit its presence before baking would be expected to result in less formation of AA.

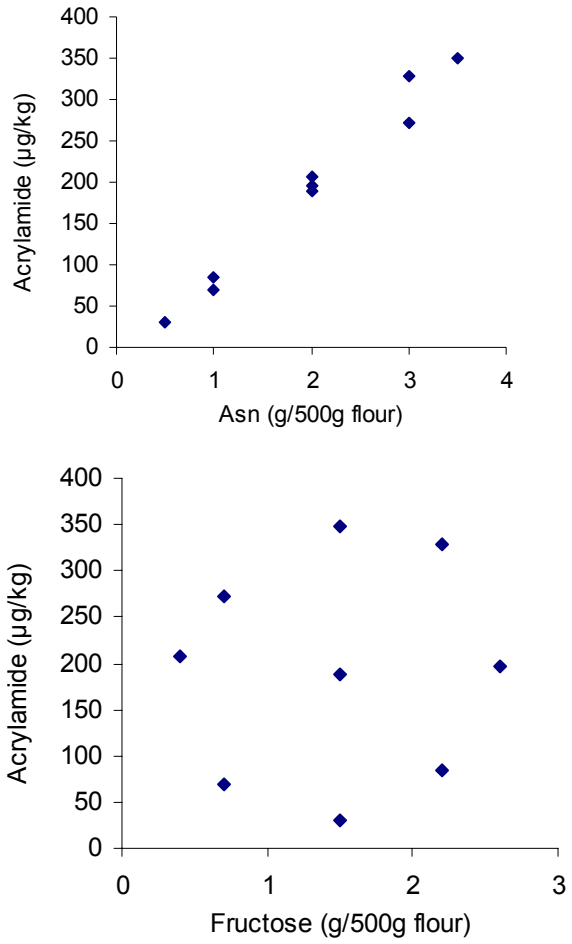

Figure 8. Relationship between added asparagine and fructose on acrylamide content in rye crispbread. Baked for 8 min at 250 °C.

3.3 Factors affecting AA content (Papers II & III)

3.3.1 Time and temperature of baking

AA is reported to be formed mainly in products subjected to high temperature treatments. The effect of time and temperature of baking on rye crispbread was studied in a randomised CCC design with standard baking recipe and various times and temperatures of baking (**Paper II**). The AA content ranged 8-31 µg/kg bread. The response surface regression analysis showed that time and temperature of baking, as well as the interaction between the two factors, had significant effects on the AA content, which increased with time and baking temperature in an accelerating slope. The AA content remained below 10 µg/kg of bread at a lower time-temperature combination, limited by 13 min at 230 °C and 7 min at 270 °C, according to the surface response model. These results suggest that low temperatures and long baking times can be measures for controlling AA during baking. Similar results have previously been shown when the time and temperature of baking were assessed for different types of products (Biedermann *et al.*, 2003; Bråthen & Knutsen, 2005; Elmore *et al.*, 2005; Rydberg *et al.*, 2003; Surdyk *et al.*, 2004). In a model of starch system with added AA precursors, it was shown that the content of AA increases with time and temperature of baking, irrespective of the precursor levels in the system (Bråthen & Knutsen, 2005). These results suggest that the content of AA could be controlled to a certain degree by controlling the time and temperature of baking.

3.3.2 Fermentation

Yeast fermentation has been reported as a means to reduce AA formation, since yeast consumes Asn as a nitrogen source for its metabolic activity (Benedito De Barber, Prieto & Collar, 1989; Fredriksson *et al.*, 2004). To test the effect of fermentation on Asn content in soft wheat bread, the flour, dough and bread were analysed for their content of Asn **(Paper I).** It was observed that during fermentation and baking the content of free Asn decreased considerably (by 85%) (**Figure 9**). A rapid degradation of Asn took place after fermentation, with a limited variation occurring during baking. Similar results were confirmed in the rye crispbread, where 90% of Asn disappeared during fermentation and baking.

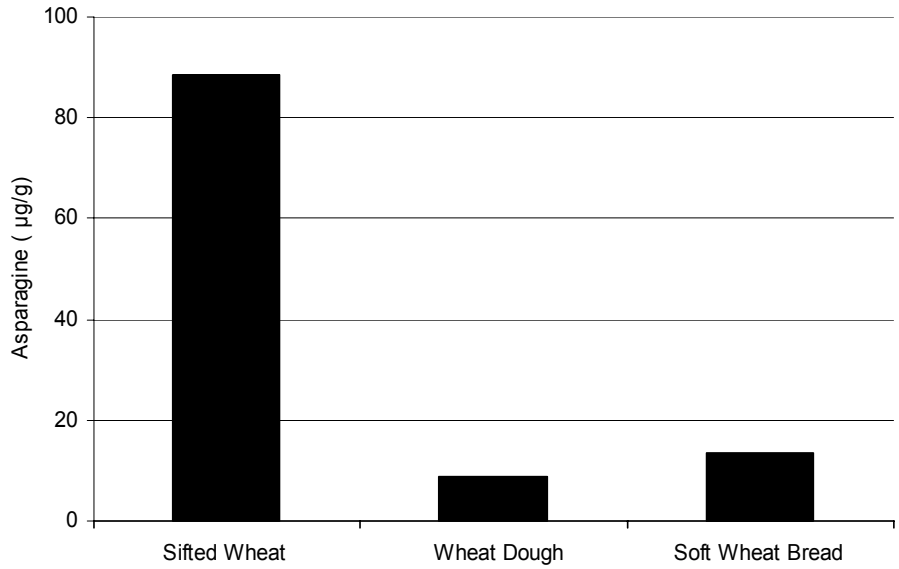

Figure 9. Content of asparagine in sifted flour, dough and bread of wheat, (contents are given on dry weight basis).

The interaction effects of fermentation time and added Asn on Asn content in the fermented dough on the content of AA in soft wheat bread was studied using a two-dimensional central composite design **(Paper III)**. The content of Asn decreased significantly with fermentation time ($p<0.001$). An interaction effect was observed between the levels of added Asn and fermentation time, indicating that the reducing effect of fermentation time depends on the level of Asn present in the system. This reduction in Asn along with the fermentation time was expressed as a significant decrease in AA content ($p=0.005$). When this effect was compared with similar breads made with higher additions of Asn, it was shown that the reducing effect of fermentation time in Asn was not expressed as reduced formation of AA. It seems that when there is ample Asn in the system, even long fermentation times do not limit its contribution to AA formation. There was a strong correlation between Asn content in dough and AA content in bread at all levels of added Asn, which further supports the finding that Asn is the critical precursor for AA content in bread reported earlier. Consequently, it could be deduced that the efficacy of yeast fermentation time is controlled by the levels of Asn present in the system. Therefore, ingredients that might enhance Asn content should be avoided in similar systems. In a pilot study by Claus *et al.* (2008), it was shown that minimum levels of AA in bread rolls were achieved after 60 minutes of fermentation, while longer fermentation tended to result in flattened bread (Claus *et al.*, 2008). It was suggested that extensive yeast fermentation is best avoided since it results in the formation of MCPDs, which are known genotoxic carcinogens (Hamlet, Sadd & Gray, 2004). Yeast fermentation could be used as an

efficient measure to control AA formation since it decomposes Asn, the major precursor for AA formation during fermentation.

3.3.3 Additives

Addition of Gly has been reported as a measure to control the formation of AA (Bråthen*, et al.*, 2005; Fink*, et al.*, 2006; Low*, et al.*, 2006). Our study examined the effect of added Gly on the content of AA along with added Asn and varying fermentation time in yeast-leavened wheat bread (**Paper III**). In this experiment an interaction effect between added Gly and Asn was found to have a reducing effect on AA, indicating that the reducing effect of added Gly depends on the level of Asn in the system (**Figure 10**). This effect might be due to competition with Asn for reducing sugars and/or to reaction(s) with the AA formed (Rydberg*, et al.*, 2003). In a further study with low levels of Asn, we did not observe the reducing effect of added Gly on AA formation. These results suggest that Gly might be used as an effective means to reduce AA content in systems where there is high abundance of Asn.

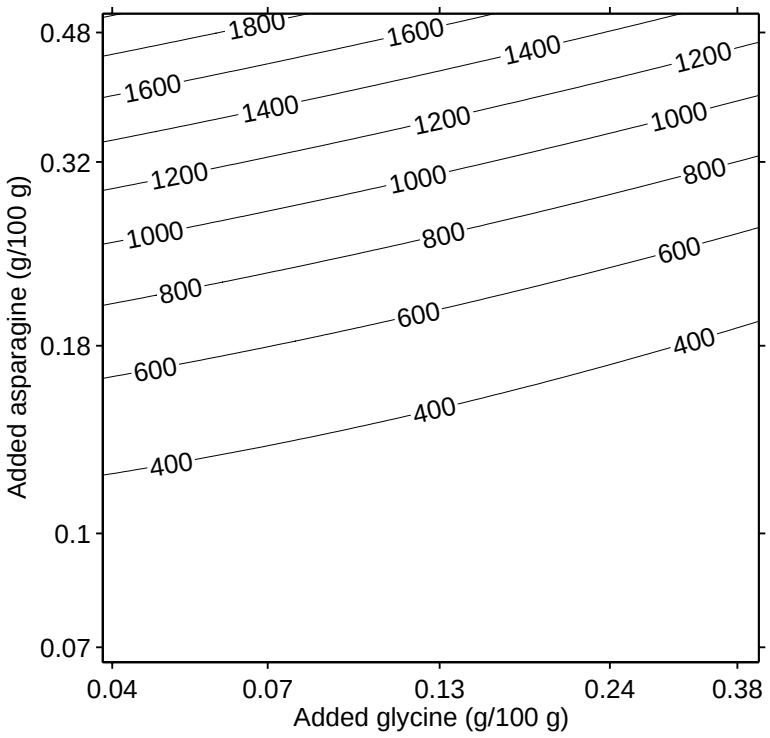

Figure 10. Effect of added glycine (Gly) and asparagine (Asn) on acrylamide content in wheat bread.

Earlier studies reported that water activity may be an important factor for AA formation (Friedman, 2003; Stadler *et al.*, 2004). This phenomenon was tested by adding oat bran concentrate (OBC) and lichenase to the dough. OBC was used for

its documented water binding capacity, and the enzyme lichenase was used to selectively degrade β-glucan in order to avoid its effect (Roubroeks *et al.*, 2000). Thus, using lichenase enabled us to separate the effect of β-glucan from the effect of other components of OBC. OBC is an alcohol-extracted product that does not contain free amino acids. It has also been reported that the addition of bran to biscuit formula increases the AA content compared with biscuits made from wheat flour (Taeymans *et al.*, 2004). In our experiment, added OBC did not have any effect on the formation of AA, which indicates that the level of dietary fibre can be increased by the addition of an ingredient such as OBC without influencing the AA content.

3.4 Relationship between AA formation and colour (Papers II & III)

It was important to investigate whether there is a relationship between colour and AA formation and to test whether the measures to reduce AA formation would jeopardise colour development. In a baking experiment on rye crispbread, colour was increased by the combined effect of time and temperature and the AA content had the same pattern of increase (**Paper II**). However, when the time and temperature of baking were kept constant and the levels of AA precursors (Asn and fructose) were changed, the perceived effect on colour was limited. These results indicate that time and temperature of baking had a higher effect than added fructose and Asn in the variation of colour intensity. Furthermore, the addition of Asn resulted in formation of more AA and limited variation in colour formation. This shows that Asn contributes to the pathway leading to AA, but its effect on colour formation is limited. Therefore, controlling AA through limiting the availability of Asn would not affect colour formation, although the quality of colour may be affected if time and temperature of baking are changed.

Gly is an amino acid that is reported to enhance colour formation in bakery products, whether added before dough fermentation or on the surface of the dough before baking (Bråthen *et al.*, 2005; Fink *et al.*, 2006; Low*, et al.*, 2006). The relationship between colour formation and fermentation time and added Gly and Asn was studied in soft wheat bread (**Paper III**). In this model the colour intensity was affected by Gly but not by Asn (**Figure 11**). A correlation was only obtained with Gly and the a*-value (degree of redness), and the observed deviation from the regression line could be explained by variation in the fermentation time. The correlation between Gly and colour indicates that unlike Asn, Gly takes part in the Maillard reaction(s), leading to colour formation. It was previously reported that Gly, when heated with reducing sugars, resulted in high browning intensity (Ashoor & Zent, 1984). In our study, there was an initial increase in the intensity of colour with Gly addition and fermentation time, where the highest colour intensity was achieved at intermediate fermentation times. The decrease in colour at longer fermentation times might be due to the consumption of other amino acids and sugars, *i.e.* other main precursors for colour formation. These results recommend the use of Gly along with AA reducing measures that tend to produce

insufficiently coloured products, *e.g.* when replacing reducing sugars with sucrose or when using lower temperatures for baking.

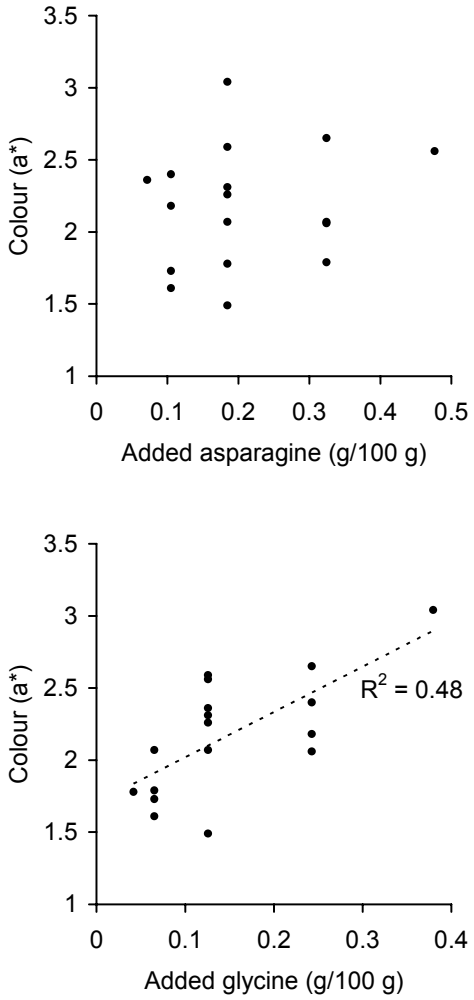

Figure 11. Correlation between colour and added asparagine and glycine.

3.5 Effect of extraction pH on AA content (Paper IV)

Water at neutral pH is regarded as the common extraction solvent for analysis of AA. However, it has been shown that the level of AA analysed is increased by increasing the pH of extraction, an effect attributed to matrix binding effects (Eriksson & Karlsson, 2006). We tested this effect using samples of whole grain rye crispbread with added AA precursors (Asn and fructose) (**Paper IV**). The average increase in AA yield in alkaline extraction compared with neutral extraction from freshly baked bread was 37%. These results suggest that added

precursors have a significant increasing effect on the level of extra AA obtained during alkaline extraction. The level of intermediate(s) involved in the generation of extra AA was dependent on the added Asn ($p = 0.028$). A strong correlation was also obtained between neutral and alkaline extraction ($R^2 = 0.99$).

The higher yield in AA obtained with alkaline extraction seems to be proportional to the content measured by neutral extraction, leading to the further suggestion that the formation of intermediate(s) is proportional to the formation of AA. The effect of extraction pH was also studied in commercial crispbread with no added precursors. This bread was stored for 8 months at 20 and 40 °C. The average increase at 40 °C in the extra AA yield was in the order of 16.5% ± 1.9, with a strong correlation between the two extraction pH values (**Figure 12**). In this experiment, the yield of extra AA at alkaline and neutral pH was correlated but not proportional. The average ratio between AA content at alkaline and neutral extraction at the different storage intervals was in the order of 1.19 ± 0.05, and this constant ratio might suggest their formation from a common intermediate, since in this experiment the same bread was subjected to similar conditions and treatments.

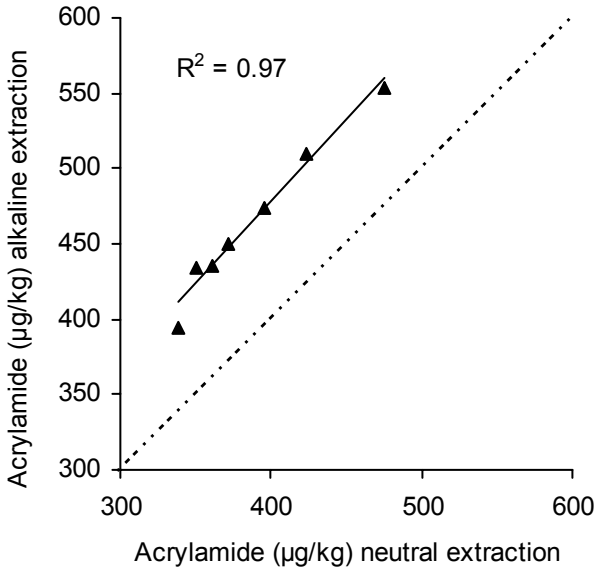

Figure 12. Correlation between neutral and alkaline extraction of acrylamide in samples stored for 8 months at 40 °C.

The effect of alkaline extraction on AA content was further investigated in a whole grain rye product that was baked with added labelled AA. Results showed 14% higher value for native AA when extracted under alkaline compared with neutral conditions. On the other hand, the alkaline extraction of the labelled AA revealed negligible change in content between the two extraction methods. These findings rule out the possibility that entrapped AA was released by extraction at alkaline pH. However, it could be regarded as an extraction artefact. This was in line with a suggestion that the extra AA present is formed during the alkaline

extraction process from AA intermediate(s) present in the matrix and can therefore be regarded as an artefact (Goldmann *et al.*, 2006). A recent study showed that the levels of AA Hb adduct (a biomarker for AA exposure) in mice blood were linearly correlated (R^2 = 0.98) with the AA content obtained by neutral but not alkaline extraction (Eriksson, 2005). This indicates that water extraction at neutral pH reveals an AA content relevant to food safety.

3.6 Effect of storage conditions on the reduction of AA content (Paper V)

The decrease in AA content during storage has been addressed in a few scattered studies (Andrzejewski *et al.*, 2004; Delatour *et al.*, 2004; Hoenicke & Gatermann, 2005; Stadler, 2005), The level of AA in some of the products was quite stable, while others showed a wide range of decrease. The aim of this study was to investigate the reduction in AA during storage of rye crispbread and how it is affected by storage conditions.

The effect of temperature was studied by storing milled samples of rye crispbread in double sealed plastic bags at different temperatures for up to 224 days. At the lower temperatures (-80 to +6 °C), the levels of AA was found to be stable throughout the storage experiment. On the other hand, a notable reduction was observed at higher temperatures (+ 20 and + 40 °C) (**Figure 13**). The rate of decrease was higher at 40 °C than at 20 °C.

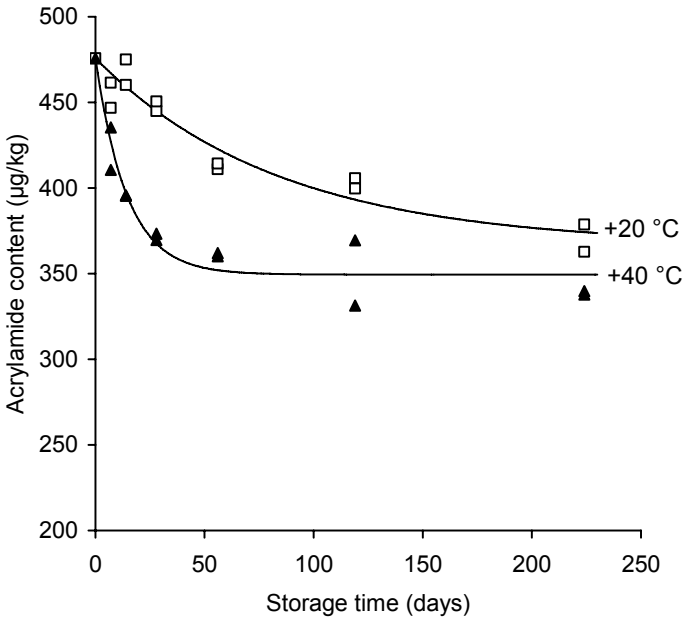

Figure 13. Levels of acrylamide content in rye crispbread stored at different temperatures [□] + 20°C; [▲] + 40°C.

The rate of reduction during storage was tested for the reaction order. At 20 °C a similar fit was found for first and second order kinetics. However, neither of these fits was obtained at the 40 °C storage (**Figure 14**). These results show that the kinetics for AA reduction are complicated and cannot be explained by a simple model. The elimination of AA is usually studied in association with formation and at high temperature treatments (> 120 °C) in model systems. Studies on the kinetics of AA have previously reported the process of formation and elimination as complicated (Claeys, De Vleeschouwer & Hendrickx, 2005b). The conditions for our study that showed reduction in AA in bread during storage at 40 °C are different to those present in literature that deals with AA formation and elimination at much higher temperatures. Thus, it might not be possible to make a valid comparison.

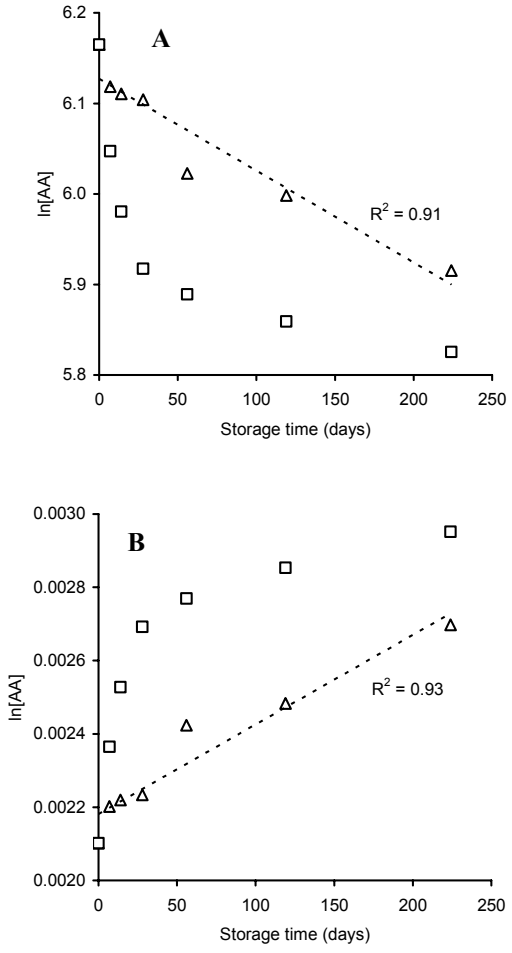

Figure 14. Measurement of the kinetic order A) logarithm of the reactant concentrations versus time (first order); B) reciprocal of the reactant concentrations versus time (second) in whole grain rye crispbread stored at [Δ] 20 °C; and at [□] 40 °C.

Since the reduction in AA was more pronounced at 40 °C, further studies were carried out at this temperature. Sets of samples of 4 g of rye crispbread were stored in 30 mL glass tubes for 70 days at 40 °C, with one set capped and another uncapped throughout the storage periods (**Figure 15**).

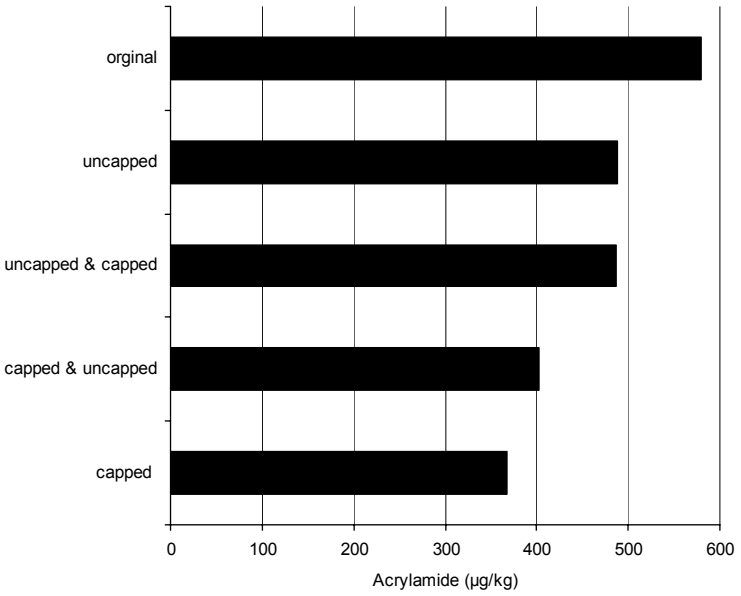

Figure 15. Effect of capping during storage of rye crispbread at 40 °C for 70 days on the acrylamide content.

A third set was stored capped for the first half of the storage period and uncapped for the second half, while the fourth set was stored uncapped for the first half of the storage period and then capped for the second half. The highest level of reduction was found in the samples that were stored capped throughout the storage period (37%), while the reduction in the uncapped samples accounted for 15%. It was also shown that when samples were initially stored uncapped, capping them in the second half of the storage period did not induce further reduction. Furthermore, uncapping the samples in the second half of the storage period did not retard the reduction. These results showed that the level of reduction during storage is higher in samples stored in capped tubes and that most reduction takes place at the early stages of storage.

An experiment was designed to control moisture content during storage. Samples of rye crispbread were stored in capped glass tubes. Small glass tubes containing of water were placed inside the larger tubes in half of the samples and the samples stored for 70 days at 40 °C. The experiment was carried out with milled fresh samples as well as with milled samples that were vacuum-dried overnight at 30 °C. The results showed a dramatic decrease in AA content at elevated moisture content, of 82 and 64% for native and vacuum-dried samples,

respectively after 70 days of storage (**Figure 16**). The AA content was significantly lower ($p<0.001$) when samples were stored with their initial water content compared with when stored after vacuum-drying. These results clearly show that water content is of major importance for AA reduction during storage.

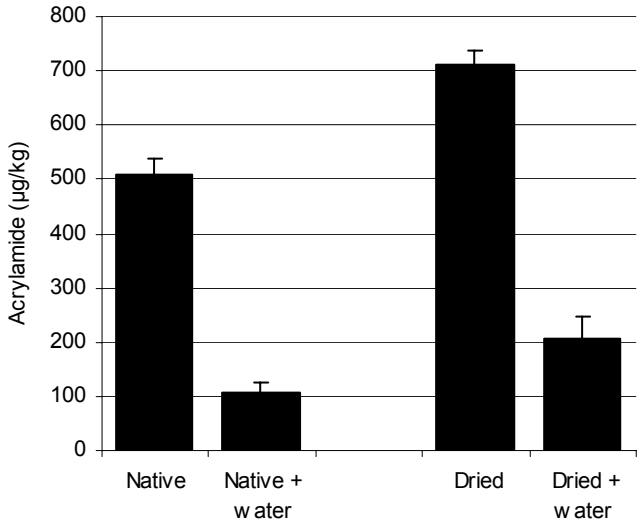

Figure 16. Effect of moisture content on the reduction of acrylamide during storage at 40 °C in closed glass tubes for 70 days (n = 3).

The importance of the water content in the sample for AA reduction during storage may also be used to explain the lack of consistent kinetics in the experiments presented in **Figure 14.** Samples stored for 250 days at -20 °C and +20 °C had a moisture content of 6%, whereas samples stored at +40 °C had a moisture content of 3%, showing that the moisture content was not stable during storage. These findings can explain why it was impossible to fit the reduction for storage at 40 °C with a simple kinetic model when the reaction rate constant(s) was not stable during the experiment.

4. Main Findings

- ★ A rapid method to analyse free amino acids in cereal fractions and products was set up and validated.
- ★ Generally rye is richer in free amino acids than wheat and Asn and aspartic acids are the dominant amino acids.
- ★ The content of free amino acids varies in the milling fractions. The highest concentrations of amino acids are found in the bran and the lowest in the sifted fraction, a pattern that was found to be similar in all the analysed amino acids.
- ★ Asn is the limiting precursor for AA formation in both whole grain rye crispbread and soft wheat bread.
- ★ Controlling the time and temperature of baking could be used as a means to limit the formation of AA.
- ★ Yeast fermentation can cause a large decrease in Asn resulting in a reduced AA content. The efficacy of fermentation time in controlling the content of AA depends on the levels of Asn present in the system.
- ★ Added Gly could be used as a measure to control AA content in systems where there are ample amounts of Asn present.
- ★ Gly contributes to colour formation therefore, it could also be used along with other AA reducing measures that might result in insufficiently coloured products.
- ★ Although Asn is an important precursor for AA formation, it does not contribute to colour formation. Thus measures to limit its availability would not directly affect the colour of the product.
- ★ Extracting AA with water during analysis at neutral pH gives the actual content and the extra AA found in alkaline extraction can be regarded as an artefact.
- ★ AA levels in rye crispbread are stable during storage at cold temperatures up to +6 °C. Warmer temperatures (20 and 40 °C) result in a reduction in AA content.
- ★ Increasing the moisture content during warm storage results in a large decrease in AA content.

5. Future Research & Recommendations

Acrylamide is a potential human carcinogen and therefore it is important to keep its content in foods as low as possible.

- ★ Yeast consumes asparagine for its metabolic activity during fermentation. Studies on bread have shown that it has an important role in the reduction of acrylamide, when the contents of free asparagine are low. Investigating the efficiency of different yeast strains would widen the scope of using yeast fermentation as an alternative option for acrylamide reduction.

- ★ Using asparaginase has been reported to result in products with lower content of acrylamide. Its efficiency varied according to the nature of the product. Testing its efficacy in different cereal products will be a useful tool to provide product specific recommendations.

- ★ Measures to reduce acrylamide formation have different extents of efficiency. Investigating the use of combined means of controlling the formation of acrylamide, *e.g.* the use of asparaginase along with other acrylamide reducing measure(s) might be a tool to reduce acrylamide contents to lower levels.

- ★ Our studies have shown that acrylamide content decreases during warm storage. Further studies are necessary to investigate on the mechanism of acrylamide reduction during warm storage and the possibility for applying this procedure to relevant products *e.g.* long-shelf life products.

- ★ It was reported that acrylamide could be formed *via* pathways other than the Maillard reaction *e.g.* from gluten. Further research on the formation of acrylamide from the suggested alternative pathways will promote the use of other means to reduce acrylamide formation at the lower levels.

6. References

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