Systematic studies on process optimization to minimize acrylamide contents in food

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Systematic Studies on Process Optimization to Minimize Acrylamide Contents in Food

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Doctor of Natural Sciences

presented by

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Zurich 2005
Ich danke härzlich...

Mine Eltere, mine Gschwächer und minere Fründin, wo mich immer unterstützt und in schwierige Momänt mittreid und ufgmuntered hend.

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Abbreviations

3-APA .............................................................. 3-Aminopropanamide
AA ........................................................................ Acrylamide
ABC ................................................................. Almond Board of California
ALARA ............................................................ As Low As Reasonably Achievable
Asn .................................................................. Asparagine
Asp .................................................................... Aspartic acid
BAG ................................................................... Bundesamt für Gesundheit
BW ................................................................. Body Weight
d ........................................................................ day
DNA ..................................................................... Deoxyribo Nucleic Acid
E.coli ................................................................. Escherichia coli
ECD ................................................................. Electron Capture Detector
EH ........................................................................ Epoxide Hydrolase
FiAL ................................................................. Fédération des Industries Alimentaires
Fru ...................................................................... Fructose
Glc ....................................................................... Glucose
Gln ....................................................................... Glutamine
Glu ....................................................................... Glutamic acid
GST ................................................................. Glutathione Transferase
HPLC ................................................................. High Performance Liquid Chromatography
IARC ................................................................. International Agency for Research on Cancer
KLZH .............................................................. Kantonales Labor Zürich
LC ...................................................................... Liquid Chromatography
LOD ..................................................................... Limit of Detection
LOQ ..................................................................... Limit of Quantification
m/z ..................................................................... mass over charge
Max .................................................................. Maximum
Min .................................................................... Minimum
min ...................................................................... minute(s)
MOE ................................................................ Margin Of Exposure
MRM ................................................................ Multi Reaction Monitoring
MS ...................................................................... Mass Spectrometry
n ........................................................................ number of samples
n.d. ...................................................................... not detected
NOAEL ........................................................ No Observed Adverse Effect Level
NOEL ........................................................ No Observable Effect Level
r .......................................................................... recovery
RSD ..................................................................... Relative Standard Deviation
SD ...................................................................... Standard Deviation
SIM ..................................................................... Single Ion Monitoring
s:n ...................................................................... signal to noise ratio
SPE ..................................................................... Solid Phase Extraction
SPME .............................................................. Solid Phase Micro Extraction
Suc ...................................................................... Sucrose
T .......................................................................... Temperature
TFAA .............................................................. Total Free Amino Acids
u ........................................................................ unit for relative atomic mass
UV ...................................................................... Ultra Violet (light)
WHO ............................................................... World Health Organization
Summary

Acrylamide is formed in the Maillard reaction from free asparagine and reducing sugars. It is found in numerous heated food products at concentrations sometimes exceeding 1000 µg/kg. Due to its neurotoxic and carcinogenic properties acrylamide presents a potential health hazard and its content in foods should be as low as reasonably achievable. The critical factors for acrylamide formation have to be identified in the different food products to reduce the dietary exposure. The aim of this thesis was to find ways to decrease the acrylamide content in heated potatoes, bakery products, and roasted almonds.

Two studies on the composition and acrylamide formation of Swiss potatoes showed that the content of reducing sugars in the raw potatoes determined the acrylamide formation. Neither the content of free asparagine nor the farming system influenced the acrylamide formation. Significant differences regarding reducing sugars and acrylamide were detected between different cultivars. However, the effect of the extreme climate in the summer of 2003 was even larger. The selection of cultivars with low content of reducing sugars and an appropriate storage are thus prerequisites to reduce the acrylamide content of fried potato products.

Gingerbread, crackers, and a semi-finished biscuit were investigated as bakery products. The baking agent NH₄HCO₃, reducing sugars, and the amount of free asparagine were found to be the key factors for acrylamide formation. Acrylamide formation in bakery was more complex than in potatoes and no general way for mitigation is at hand. The most feasible approaches are the use of NaHCO₃ instead of NH₄HCO₃, the replacement of reducing sugars by sucrose, minimization of free asparagine, and avoiding enhanced browning. The application of an asparaginase to hydrolyze the free asparagine in dough may present an elegant solution in the future when this enzyme becomes available at low costs.

Among the different almond products, roasted almonds contained most acrylamide. The critical factors for acrylamide formation were the concentration of free asparagine in the raw almonds, and the roasting temperature. The amount of acrylamide in roasted almonds can be decreased by selecting cultivars with low content of free asparagine, by lowering roasting temperatures, and by omitting dark roastings. Acrylamide was not stable in roasted almonds during storage at room temperature.
Zusammenfassung


1. Introduction

Acrylamide is a reactive molecule with a relative molecular mass of 71.08. It is synthesized since the mid-1950s and used as monomer for the production of polyacrylamide, accounting for about 60,000 tons worldwide. This polymer has numerous applications in industry such as wastewater treatment, mining, cosmetics, or paper industry and it is used in analytical laboratories for electrophoresis.

Due to its reactivity and its wide application numerous studies on the toxicology of acrylamide and on the occupational exposure to this chemical were performed since the 1980s. Heavily exposed workers showed neurotoxic effects and their blood contained elevated levels of acrylamide bound to hemoglobin. In vivo and in vitro studies showed that acrylamide is metabolized to a reactive epoxide, that both compounds bind to proteins and DNA, and that acrylamide induced tumors at several sites in rodents after chronic exposure. On the basis of these results the International Agency for Research on Cancer classified acrylamide as probably carcinogenic to humans.

The detection of acrylamide in food was reported by Swedish researchers in April 2002 for the first time. The presence of acrylamide in food caused a worldwide alert and immediately prompted national health authorities, food companies, and universities to start many research projects. These investigations focused on analysis, occurrence and formation in food as well as on toxicological aspects. The occurrence of acrylamide in heated food was quickly verified and the Maillard reaction was found to be the key for acrylamide formation. The widespread occurrence at concentrations sometimes exceeding 1000 µg/kg and its known toxicity made acrylamide a very important issue of food safety.

The present thesis started only six months after the first detection of acrylamide in food. At that time, information on the different factors influencing the formation of acrylamide was scarce. The aim of this investigation was thus to contribute to the understanding of acrylamide formation in food. As a first step, the analytical competence to determine acrylamide and its precursors in food had to be established. The determination of precursors in raw materials and the identification of critical factors for acrylamide formation were the guidelines to find ways to reduce the acrylamide content in different products. Investigated raw materials and food products were potatoes, bakery products, and roasted almonds. A close collaboration with industry was maintained and industrial production was followed as closely as possible to allow for a direct and fast transfer of the obtained results.

In order to share the gained knowledge as quickly as possible, the results were published in scientific journals right after completion of each sub-project. The doctoral thesis was thus carried out as a “paper thesis” and the chapters 3 to 9 represent the seven publications obtained during this thesis. These publications are preceded by a comprehensive literature review and followed by a general concluding discussion.
2. Literature review

The following review focuses on mechanism of formation and on occurrence of acrylamide in food with particular emphasis on potato products, bakery products, and roasted products. In addition the history of the detection of acrylamide in food as well as some analytical and toxicological aspects are addressed. The literature for this chapter covers the publications that were available until the end of July 2005.

2.1. Detection of acrylamide in food

Acrylamide is a well known chemical and widely used as cement binder and to produce a large range of polymers and copolymers. These polymers serve as gels for electrophoresis and size exclusion chromatography, in the paper and textile industry, as flocculants in the waste water treatment and the purification of drinking water, in cosmetics, in ore processing, and as soil conditioner [1]. The first reports on the analysis of acrylamide in food focused on tomatoes [2] and mushrooms [3] grown on polyacrylamide gels which contained some residual monomers. However, no acrylamide was found in both cases. Since no other sources than contamination were considered, the search for acrylamide in food products stopped at that time.

Human exposure to acrylamide was known to occur by direct contact when handling the chemical e.g. during industrial production, preparation of gels [4], through cigarette smoke [5-7], and by application of polyacrylamide containing cosmetics [8]. Because of the toxicity of acrylamide (see below) these exposures were monitored. Since ingested acrylamide forms hemoglobin adducts, the exposure to this chemical can be monitored by measuring these adducts in the blood. Thereby the different exposures and the individual metabolism are taken into account [4]. Highly exposed workers from a polymer factory in China had elevated levels of hemoglobin adducts of acrylamide and its metabolite [4, 9]. These results were confirmed with blood samples from smokers and from laboratory workers preparing polyacrylamide gels for electrophoresis. The highest adduct levels were detected in the smokers, followed by the persons who prepared gels and were non-smokers. Surprisingly, a high background level was observed in the control group, i.e. people who did not smoke nor prepared gels [10]. At that time, no other source for acrylamide exposure was known and thus no explanation for this background in the “unexposed” control group was at hand.

This led to further research to identify the unknown source of exposure. Since acrylamide was determined in cigarette smoke and high temperatures were known to enable the formation of toxic compounds such as polycyclic hydrocarbons [11, 12] and heterocyclic amines [13, 14], the formation of acrylamide in food during heating was assumed. The finding that wild animals, which feed on non-heated feed, have lower levels of hemoglobin adducts supported this theory. To corroborate this hypothesis rats were fed either with fried or unheated feed. The rats feeding the fried diet showed a 13 times higher level of acrylamide adducts in their blood as compared to the control group receiving the unheated feed [15]. This supported the assumption that acrylamide may be formed in food during heating. In fact, acrylamide
concentrations of about 200 µg/kg were determined in the fried feed which led to the conclusion that acrylamide might be a cooking carcinogen [15]. Although the results seemed clear and correct, these findings received only little attention by the media and caused no public concern in the year 2000.

It was in 2002 only, when two Swedish groups published the detection of acrylamide in a wide range of heated food at concentrations sometimes exceeding 1000 µg/kg [16, 17], a great public health concern emerged. The worldwide concern about acrylamide in food was pushed by the Swedish “acrylamide alarm” and its big echo in Swedish media [18]. The reason for the dramatic effect in Sweden was actually a coincidence: During the construction of a railway tunnel near Hallandsås in 1997, large amounts of acrylamide (originating from a sealant used in the construction) leaked out, poisoned workers [19], contaminated ground and open water, and killed or poisoned fish and cows [18, 20]. The medial dramatization of the accident made acrylamide a public health threat related to food. The consequences were panic reactions with food being withdrawn in the retail and population sensitized for this chemical [18, 21]. Therefore, the Swedish media and population had the view that acrylamide was poisonous and its presence in food was a scandal and both were highly sensitive regarding acrylamide from then on [18, 22]. When acrylamide was detected in many food products in 2002, this “old fear” was promptly aroused. Furthermore, the communication of the findings was confusing and poorly coordinated between the authorities and the researchers who had discovered acrylamide in food. This intensified the “alarming effect” on both media and scientific community [18, 22].

2.2. Physical and chemical properties of acrylamide

Acrylamide (= 2-propenamide, CAS-Nr. 79-06-1) is a small molecule with a relative molecular mass of 71.08. Its chemical structure is shown in Figure 1.

![Figure 1: Chemical structure of acrylamide.](image)

At room temperature acrylamide is a white, crystalline solid. It is highly soluble in water (215.5 g / 100 mL at 30 °C) and well soluble in polar organic solvents such as methanol, ethanol, and acetone, but it is almost insoluble in non-polar solvents like hexane and heptane. Acrylamide has a melting point of 84-85 °C and a boiling point of 146 °C (at 3.3 kPa). At temperatures above 84 °C spontaneous polymerization occurs [23]. Acrylamide is industrially produced either by hydration of acrylonitrile catalyzed by copper catalysts or by enzymatic hydration of acrylonitrile using microbial nitrile hydratase [1, 23]. Acrylamide is not known to occur as a natural compound, except in heated food. However, it can be produced by some microorganisms [1].
Due to its double bond in conjugation to the amide group, acrylamide reacts with nucleophilic SH- and NH$_2$-groups through a Michael type reaction. The thiol group of cysteine residues, ε-NH$_2$-group of lysine side chains and α-NH$_2$ groups of free and N-terminal amino acids are most important in this context [1]. These reactions are important for both detoxification, formation of hemoglobin adducts, and toxic effects (see below).

### 2.3. Toxicology of acrylamide

#### 2.3.1. Metabolism

Due to its polarity and small size acrylamide is readily absorbed and distributed in animals and humans as shown with $^{13}$C- and $^{14}$C-labeled acrylamide [24-27]. In mice the half-life time for acrylamide and glycidamide was about 1.5 h which points to a fast elimination [27]. Absorption in humans was more efficient after oral uptake compared to dermal administration [25]. Interactions of acrylamide with the food matrix such as dietary proteins are likely to influence its uptake. In model studies with Caco-2 cells acrylamide was highly bioavailable and passed the cell membranes via passive diffusion. However, proteins like chicken albumin reduced the uptake by the Caco-2 cells [28]. After ingestion, acrylamide is distributed through the bloodstream in the whole body. Administration of $^{14}$C-acrylamide to mice showed that acrylamide is found in the thymus, pancreas, heart, liver, kidney, blood, and brain. However, the distribution changed when the animals were infected simultaneously with a common virus indicating that metabolism might be altered upon infections [29]. Acrylamide was also found in human breast milk [30] although concentrations were usually very low (< 0.5 µg/kg) [31]. Acrylamide also penetrated the human placenta [30] and thus it was also found bound to hemoglobin in neonates [32]. Low amounts were also found in eggs of highly exposed quails, which indicates the possibility of a carry over from feed-borne acrylamide to eggs [33].

Conjugation with glutathione (GSH) [26] and epoxidation to glycidamide [34] present the major metabolic pathways of ingested acrylamide. Figure 2 gives an overview on these metabolic pathways. The conjugation to GSH is catalyzed by GSH-transferases (GST) [35] or takes places spontaneously [36]. In rodents the epoxidation is catalyzed by cytochrome P450 2E1 oxidase in the liver [34, 37], which is a saturable process [1, 38, 39]. Toxicokinetic studies revealed that at lower doses the enzymatic conversion of acrylamide to glycidamide becomes more important compared to other metabolic pathways [27, 40]. The epoxide group of glycidamide can be cleaved by an epoxide hydrolase (EH) leading to 2,3-dihydroxypropanamide [41]. The formation of glycidamide is considered as the critical step for genotoxic effects of acrylamide (see below) [42].

Acrylamide and glycidamide can react with macromolecules such as hemoglobin [4, 40], serum albumin [43], DNA [44, 45] and enzymes [1]. In vivo they bind to the α-amino group of the N-terminal valine in hemoglobin whereby stable adducts are formed which serve as indicators of exposure (biomarkers) [4, 46]. A linear dose response was observed for the concentration of hemoglobin adducts of both acrylamide and glycidamide in humans to which $^{13}$C$_3$-acrylamide had been orally
administered. The same study also revealed that humans converted acrylamide less efficiently to glycidamide than rats did [25].

The GSH-conjugates of acrylamide and glycidamide are converted to mercapturic acids and finally excreted in the urine whereby a transpeptidase, glutathionase, and acetyl-coenzyme A are involved [1, 25, 47]. In humans, the largest part of the urinary metabolites originated from the direct conjugation of native acrylamide with GSH. However, 2,3-dihydroxypropanamide, glycidamide and traces of its GSH-derivatives were also detected. In total, all urinary metabolites accounted for about one third of the administered dose. The rest of the dose formed adducts with proteins (e.g. with hemoglobin) and DNA or was not absorbed at all [25].

Figure 2: Metabolic pathways of acrylamide, adapted from reference [41].

2.3.2. Neurotoxicology

Exposure of laboratory animals and humans to acrylamide at high doses produced neurotoxicity characterized by ataxia and skeletal muscle weakness. Most data was obtained in animal experiments with very high doses in the range of 10 - 50 mg per kg body weight (BW) [48] which is far beyond any dietary exposure (see below) [49]. However, the neurotoxicity of acrylamide might be cumulative [48] which means that already a comparatively low exposure (e.g. through the diet) might not be negligible. But at present, studies on dietary exposure and neurotoxicological effects are not available. Thus, the neurotoxicological relevance of acrylamide in food cannot be assigned.
Neurotoxicity was observed in laboratory animals upon daily exposure in the range of 0.5 mg - 50 mg per kg BW [48]. Peripheral neuropathy was observed in humans upon occupational exposure and comprised impairment of vibration sensation, loss of ankle reflexes, weakness in legs, tremor, numbness and tingling in hands and feet, and polyneuropathy (heavy exposure) [9, 19, 50]. The clinical signs were positively correlated with the hemoglobin adducts and the concentration of mercapturic acids in the urine [9, 19]. In most cases these effects were reversible when the exposure ceased and was not too severe [19].

Currently, there are two main hypotheses about the mechanism of neurotoxic effects. The older one emphasizes axonopathic changes and inhibition of fast axonal transport in the central and peripheral nervous system [51-53]. However, new evidence suggests that acrylamide acts directly at nerve terminals causing presynaptic dysfunction and eventual degeneration [52, 54]. Damages in the nerve terminal in both the peripheral and central nervous system could account for the sensory, motor and autonomic deficits observed [48]. The acrylamide-induced synaptic dysfunction seems to be caused by the binding of acrylamide to SH-groups in presynaptic proteins leading to a reduced release of neurotransmitter [54]. A recent study also suggested that the neurotoxic activity might be related to an altered expression of nitric oxide synthases which may influence the release of neurotransmitters as well [55].

To date, the risk of neurotoxic effects caused by dietary acrylamide is considered to be very low [56], because the no observed adverse effect level (NOAEL) in animals is 0.5 mg/kg BW, which is unlikely to be achieved by dietary exposure [57].

2.3.3. Carcinogenicity

The International Agency for Research on Cancer (IARC) evaluated acrylamide in 1994 and classified it as “probably carcinogenic to humans” (Group 2A) [23]. This classification was based on positive results with bioassays in rodents and was supported by evidence that acrylamide is metabolized by mammalian tissues to its reactive epoxide glycidamide [23]. Acrylamide is a multiorgan carcinogen in both rats and mice which strongly implies that it presents a potential carcinogen to humans. Tumors were observed in the thyroid gland, testes, lung, mammary gland, clitoral glands, and brain [42]. In a two years study with Fischer 344 rats acrylamide was administered in the drinking water at doses of 0 to 2 mg/kg BW to assess the chronic toxicity and oncogenic potential. For the highest dose significant increases of tumors were found in thyroid gland, mammary gland, testes, central nervous system, oral cavity, uterus, and clitoral gland [58]. A second lifetime oncogenicity study confirmed the increase of thyroid, mammary and testicular tumors, but not of other tumors reported in the first study. The no observable effect level (NOEL) for scrotal mesotheliomas (i.e. tumor in testes) was determined as 0.5 mg/kg BW [59]. In contrast, a cohort study of 8500 occupationally exposed workers did not reveal any significant association between mortality from cancer and the exposure to acrylamide [60]. However, the exact exposure was not precisely known and was probably far below the doses in the animal studies. Nevertheless, some studies state that the cancer risk from acrylamide is overestimated because human exposure is too low and because thresholds for carcinogenesis are observed [61, 62]. Acrylamide and glycidamide are both reactive towards DNA and can form several adducts. The main adducts of acrylamide (N1-(2-carboxyethyl)-adenine) and
glycidamide (N7-(2-carbamoyl-2-hydroxyethyl)-guanine) are shown in Figure 3. However, due to its epoxide group glycidamide is much more reactive towards DNA than acrylamide [63-65]. While the formation of DNA adducts from acrylamide is a saturable process, glycidamide induces DNA adducts dose-dependently [63, 65]. Both compounds formed DNA adducts at specific sites within the genes tested [63]. Adducts of glycidamide to purine bases have been detected in liver, kidney and lungs of mice [66].

![N1-(2-carboxyethyl)-adenine](image)

![N7-(2-carbamoyl-2-hydroxyethyl)-guanine](image)

**Figure 3: Most frequent DNA-adducts of acrylamide (left) and glycidamide (right) [65].**

Acrylamide is not mutagenic in the Salmonella assay (with and without a metabolic activation system), but there is clear evidence that acrylamide and glycidamide are mutagenic and clastogenic in mammalian cells: Gene mutations and chromosomal aberrations were observed in germ cell and somatic cells of rodents [23, 42]. In male germ cells the effect of acrylamide is almost exclusively clastogenic, but data are hardly comparable to humans because very high doses were applied [67]. The genotoxic mechanism of acrylamide is not fully elucidated, but there is evidence that the alkylation of DNA by glycidamide is the critical step [42, 63, 68, 69]. Mice having no gene for Cytochrome P450 2E1 showed no reduced fertility after administration of acrylamide as observed in the wild type group [70]. Thus, germ cell mutagenicity is most likely glycidamide mediated. However, several studies have reported that glycidamide is not a strong mutagen and not very effective in DNA strand breaking as compared to other mutagens [68, 69, 71]. Beside the direct reaction with DNA as suspected cause for tumors, acrylamide might also increase DNA synthesis in target sites [72] and/or impair DNA repair [73]. Altogether, acrylamide is a multi-site carcinogen and it is genotoxic. The reactivity of glycidamide towards DNA leading to DNA adducts and clastogenic effects are considered as key processes for carcinogenicity. The mechanism is not fully characterized, and it cannot be excluded that there is no threshold for the induction of tumors in humans, and therefore, the dietary exposure to acrylamide should be as low as reasonably achievable (ALARA principle) [57, 74-76].
2.3.4. Human exposure to acrylamide

Although acrylamide was detected in cigarette smoke (about 1 - 2 µg per cigarette) long before it was detected in food [7, 77] it has not attracted as much attention as dietary acrylamide. This is surprising because several studies monitoring acrylamide exposure identified smoking as an important source. Bergmark showed in 1997 that smokers not working with acrylamide exhibited a two times higher level of hemoglobin adducts compared to nonsmoking people who prepared acrylamide gels. The adduct concentration in smokers was even four times larger compared to nonsmoking persons. A positive correlation was found between hemoglobin adducts and the number of cigarettes smoked per day [10]. Furthermore, smoking during pregnancy increased the hemoglobin adducts of neonates [32]. Thus smoking is a very important source for acrylamide as also shown in other reports [57, 78, 79].

Cosmetics are another non-food source for human exposure. Polyacrylamide is used in cosmetic formulations as a binder, thickener, film former, and hair fixative and 0.05 - 2.8% are added depending on the product. Polyacrylamide contains some residual monomers (about 0.2 - 0.3% of polymer) because of incomplete polymerization. Consequently, acrylamide concentrations in cosmetic products can range from 10 to 1000 µg/kg [8].

Acrylamide is found in a very broad range of food products. Without being a complete overview Table 1 shows some examples of food categories and the range of acrylamide contents.

<table>
<thead>
<tr>
<th>Category</th>
<th>Acrylamide content [µg/kg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato chips</td>
<td>100-3770</td>
</tr>
<tr>
<td>French fries</td>
<td>50-680</td>
</tr>
<tr>
<td>Fried meat</td>
<td>15-45</td>
</tr>
<tr>
<td>Fried noodles</td>
<td>10-580</td>
</tr>
<tr>
<td>Fried rice crackers</td>
<td>17-64</td>
</tr>
<tr>
<td>Bread</td>
<td>20-40</td>
</tr>
<tr>
<td>Crisp bread</td>
<td>25-2800</td>
</tr>
<tr>
<td>Gingerbread</td>
<td>80-7800</td>
</tr>
<tr>
<td>Fine bakery</td>
<td>60-3300</td>
</tr>
<tr>
<td>Breakfast cereals</td>
<td>25-850</td>
</tr>
<tr>
<td>Coffee (ground)</td>
<td>40-480</td>
</tr>
<tr>
<td>Roasted nuts</td>
<td>10-2000</td>
</tr>
<tr>
<td>Popcorn</td>
<td>160-180</td>
</tr>
<tr>
<td>Black olives</td>
<td>1960</td>
</tr>
<tr>
<td>Prune juice</td>
<td>270</td>
</tr>
</tbody>
</table>

The large variation in some categories reflects differences in raw material, composition, process conditions and/or cooking habits. Acrylamide is mostly found in
starch-rich food, whereas meat products contain only little. High acrylamide contents are found in products that are subjected to high temperatures in processes such as frying, baking, and roasting. These products have low water content or have at least a dry crust. Raw or boiled food usually contain no or very little acrylamide [16]. However, there are also remarkable exceptions: Black olives and prune juice contain acrylamide [80] although they are not processed at high temperatures and their water content is high.

The human exposure to acrylamide through the diet has been estimated for various countries. Table 2 gives an overview over some published values. These data indicate a mean daily intake of acrylamide of about 0.5 µg/kg BW. However, for unusual high intakes (95th percentile) an exposure of 1 - 3 µg/kg BW may result [57, 88-90].

Table 2: Overview of dietary exposure to acrylamide.

<table>
<thead>
<tr>
<th>Mean Exposure [µg/kg BW • d]</th>
<th>Group of Population</th>
<th>Country</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.57</td>
<td>General</td>
<td>Germany</td>
<td>[57]</td>
</tr>
<tr>
<td>0.30</td>
<td>Adolescents</td>
<td>Germany</td>
<td>[89]</td>
</tr>
<tr>
<td>0.43</td>
<td>Children</td>
<td>Germany</td>
<td>[89]</td>
</tr>
<tr>
<td>0.04 - 1.2</td>
<td>Babies</td>
<td>Sweden</td>
<td>[31]</td>
</tr>
<tr>
<td>0.5</td>
<td>General</td>
<td>Sweden</td>
<td>[90]</td>
</tr>
<tr>
<td>0.48</td>
<td>General</td>
<td>Netherlands</td>
<td>[91]</td>
</tr>
<tr>
<td>0.5</td>
<td>General</td>
<td>Netherlands</td>
<td>[88]</td>
</tr>
<tr>
<td>0.28</td>
<td>Adults</td>
<td>Switzerland</td>
<td>[92]</td>
</tr>
<tr>
<td>0.34</td>
<td>Children</td>
<td>Norway</td>
<td>[93]</td>
</tr>
<tr>
<td>0.34</td>
<td>Adults</td>
<td>Norway</td>
<td>[94]</td>
</tr>
<tr>
<td>0.5</td>
<td>Adults</td>
<td>France</td>
<td>[94]</td>
</tr>
<tr>
<td>0.51</td>
<td>Adolescents</td>
<td>Belgium</td>
<td>[95]</td>
</tr>
</tbody>
</table>

The estimated daily intake of 0.5 µg/kg BW is about three times the exposure that was calculated from the background level of hemoglobin adducts, i.e. 100 µg per day correspond to about 1.5 µg/kg for a 60 kg person [16]. These higher estimates based on biomarkers could indicate unknown sources of exposure or could be due to the small and thus not representative group which was investigated [96].

The most important food sources for dietary acrylamide are [88, 90-92, 96]:

- Fried potato products (chips, French fries)
- Coffee
- Breakfast cereals
- Bread and crisp bread
- Sweet bakery (gingerbread, biscuits, cookies)
The contribution of a particular product to the total exposure may vary considerably between different countries reflecting the different eating habits and cooking traditions [96]. For example, coffee was calculated to contribute 39% to the total exposure in Sweden [90], while it was only 13% in the Dutch population [91]. In the US 35% is attributed to fried potato products, but only 7% to coffee [96].

With the assumption of a conservative intake of 1 µg/kg BW the following margins of exposure (MOE) for acrylamide can be calculated [74]:

- Neurotoxic effects  MOE =  500
- Reproductive and developmental effects  MOE =  2,000
- Carcinogenic effects  MOE =  300

An MOE of 300 is low for a compound which is genotoxic and carcinogenic. This becomes particularly clear when the MOE for carcinogenic effects of other compounds in food are considered.

- Ethylcarbamate  MOE = 20,000
- Polycyclic aromatic hydrocarbons  MOE = 25,000

Thus a human health concern about acrylamide is indicated and has a much higher priority compared to other carcinogenic substances occurring in food [74]. The low MOE for acrylamide not only reflects its high concentrations in food, but also the high number of sources. This is an important difference to the two other carcinogens mentioned above for comparative purposes.

Two epidemiological studies focused on the relationship between cancer incidences and dietary exposure calculated from food questionnaires. The first one was published by Mucci et al. who reanalyzed a Swedish case-control study originally designed to investigate relations between cancers (large bowel and urinary tract) and heterocyclic amines in fried food. The authors did not detect an excess risk or trend to bowel, bladder or kidney cancer for people with moderate or high calculated exposure to acrylamide [97]. The second study was published by Pelucchi et al. and compared the consumption of fried potatoes with the risk of cancer at various sites. The authors stated that evidence for an important association between cancer risk and consumption of fried or baked potatoes was lacking [98]. However, both studies had considerable limitations:

- In the study of Pelucchi and coworkers only fried potato products were included, but other important sources for acrylamide such as coffee were omitted. The browning of the products was not considered, and no acrylamide contents were determined. These factors may lead to an underestimation of the exposure and of the association with a cancer risk [42, 99, 100].
- The food questionnaires used in the study of Mucci et al. lacked precision and the available data on acrylamide contents in food were scarce at that time. Some target organs for tumors were not considered (e.g. thymus). Furthermore, statistical power calculation showed that the size of the study was clearly too small to detect small increases in cancer risks related to acrylamide [100, 101].
However, the study by Mucci et al. showed at least that an excess risk of cancers of large bowel, kidney or bladder related to acrylamide does not exist. But since the cancer risk from dietary acrylamide is not considered to be particularly high compared to other risks (additional risk of 100 to 4,500 per million [42, 56]) this outcome was somehow to be expected.

2.4. Analysis of acrylamide

In the early 1990s and particularly after its detection in food numerous methods to determine acrylamide have been published. The aim of this chapter is not to give a comprehensive and in-depth overview on all methods available. For extensive reviews refer to Castle and Eriksson [102], Wenzl et al. [103], as well as Zhang and Zhang [104]. This chapter names the most important techniques, shows some differences between them, and some of the pitfalls observed so far. The method for acrylamide analysis used for this thesis is discussed separately.

2.4.1. Extraction, clean-up, and derivatization

Acrylamide is highly soluble in water which is therefore mostly the extraction solvent. Generally, the sample has to be homogenized in water which is done by mixing (Polytron), shaking or stirring. Some methods mention the advantage of enzymes, e.g. amylases, to degrade the food matrix [105, 106] while other groups report that they had no influence on the analytical result [107]. Swelling of the water-sample mixture in a warm water bath is also used to facilitate extraction [108].

After extraction into the water phase, the slurries are either centrifugated or directly subjected to a further clean-up. To eliminate the co-extractives different clean-up procedures and derivatizations are applied, e.g.

- Liquid-liquid extractions with organic solvents [107-111]
- Carrez clarification [109-111]
- Solid phase extraction (SPE) [17, 109] and solid phase micro extraction (SPME) from the headspace [112]
- Derivatization of acrylamide: Silylation [112]; trapping with L-valine [113], bromination [2, 3, 16, 86, 111, 114]; reaction with 2-mercaptobenzoic acid [107]
- Concentration by solvent evaporation [2, 16, 107-110]

While LC-based methods usually do not include a derivatization step, bromination of the analyte is the classical approach for the determination of acrylamide by GC [2, 3, 114] and is still used in recently developed methods [16, 86, 111]. Since acrylamide is a small and polar molecule, bromination offers several advantages.
The dibromo-derivative

- is much more volatile and less polar which facilitates GC-separation
- is readily soluble in organic solvents which enables liquid-liquid extraction from an aqueous phase
- has a higher mass giving more specificity for the detection by mass spectrometry
- exhibits the isotopic pattern of bromine ($^{79}\text{Br}/^{81}\text{Br}$) which makes identification in the mass spectrum easy and specific

However, this derivatization step has also some inherent drawbacks, as

- the bromination is time consuming (often carried out overnight)
- the toxic and corrosive bromine has to be handled
- a spontaneous loss of HBr in the hot GC-inlet, i.e. loss of analyte is observed [102]. This can be overcome by deliberate and complete conversion to 2-bromopropanamide with triethylamine prior to GC-analysis [111].

Coffee and roasted cacao have been reported to be particularly difficult regarding extraction and separation [109, 110]. Most of the problems can be overcome by a more complex clean-up and monitoring of ion transitions in the MS/MS mode for the detection.

A known pitfall during extraction is artificially formed acrylamide. Pedersen and Olson postulated that higher acrylamide contents were found if the sample was subjected to continuous Soxhlet extraction with methanol at 65 °C for several days [115]. However several groups argued that under these conditions acrylamide is formed from co-extracted precursors and thus Soxhlet extraction suffered from de novo formation of acrylamide [116-118].

Very recently, a study reported that a high pH for aqueous extraction as well as the application of digestive enzymes led to the determination of higher acrylamide contents in different food products. The authors speculated that their procedure allowed for the extraction of acrylamide "trapped" by the matrix [106]. However, there are considerable gaps in this study, e.g. the de novo formation of acrylamide during the extraction at high pH values has not been considered and therefore cannot be excluded. Since up to now the general consensus was that extraction of acrylamide was complete [103], these results will have to be confirmed or rejected.

2.4.2. Separation, detection, identification, and quantification

Gas chromatography with mass spectrometric detection (GC-MS) and high-performance liquid chromatography (HPLC) with tandem mass spectrometric detection (LC-MS/MS) are the most widely used techniques. GC is a high-resolution procedure and thus offers a better separation compared to LC. The advantages of the LC approach are that aqueous extracts and large volumes can be injected. Thus, the clean-up is often simpler and more straightforward in LC-methods and no derivatization is needed. However, due to the poor chromatographic retention of acrylamide, resolution may be poor on LC columns and co-elution of other compounds may cause false signals or ion-suppression [102]. Therefore, transitions from a precursor ion to a product ion are often monitored in MS/MS (multiple
reaction-monitoring mode, MRM). This gives a higher selectivity and at least partially
overcomes possible problems with co-eluting compounds [102].
Mass spectrometry is a very specific, selective and sensitive method for detection
and is most often used for GC- and LC-methods. However, attempts are made to
combine LC-separation with less expensive detectors. Recently, methods with UV- or
ECD- (Electron Capture Detection) detection have been published [119, 120].
However, UV- and ECD-detection never achieve the same selectivity as MS-
detection. The identification of the analyte is generally based on

- Retention time (as compared to the isotopic internal standard or to an external
  standard)
- Mass ($m/z$) of the molecular ion and of fragment ions
- Intensity of the ion transitions in MS/MS experiments.

Quantification is carried out by comparison to the internal standard whereby the
response factors of the analyte and the standards have to be taken into account. The
linearity of the method is determined for quality assurance and sometimes also used
for quantification with a calibration curve. The use of an internal standard is
imperative because it compensates for losses during clean-up and upon injection.

2.4.3. Method used in this thesis
Although acrylamide is not a natural candidate for GC/MS without derivatization
(soluble in polar solvents, high polarity, low volatility, small mass) several GC/MS
methods have been successfully applied. The method used in this thesis belongs to
this group and is shortly discussed below. It was developed by Biedermann et al.
from the Official Food Control Authority of the Canton of Zurich (KLZH), Switzerland
and was published in 2002 [108].
The homogenized sample is mixed with water (Polytron). After swelling in a water
bath at 70 °C, 1-propanol is added and the mixture is intensively shaken. The solvent
is evaporated and the residue dissolved in acetonitrile. After defatting twice with
hexane the sample is injected on-column onto a polar Carbowax capillary. The
method has the following advantages:

- The time consuming bromination step is omitted. This offers a higher sample
  throughput and reduces the use of corrosive and hazardous chemicals.
- The evaporation of the water-propanol overrides the difficulties in extracting
  acrylamide from an aqueous phase and allows for concentration of the
  analyte.
- Dissolution in acetonitrile enables a first clean-up (salts, proteins) which is
  followed by defatting twice with hexane to eliminate non-polar co-extractives
  (fats).

The on-column injector has to be held at a moderate temperature (e.g. 70 °C) to
prevent potential artificial formation of acrylamide from co-extracted precursors [102,
108]. Chemical ionization with methane (to avoid fragmentation in the ion source)
and single ion monitoring (SIM) are used to obtain higher specificity, better sensitivity
and lower detection limits [108]. The low mass (71 u), the rather unspecific
fragmentation pattern (loss of ammonia and water), and the poor abundance of
isotopic peaks are some minor disadvantages on the MS-side which can be overcome by the application of MS-MS. The use of two different internal standards, both added at the very beginning of the analytical procedure, offers several advantages.

- $^{13}$C$_3$-acrylamide exhibits the same physical (except the mass) and chemical properties as the analyte itself. It has the same elution time but a different mass ($m/z = 75$ versus $m/z = 72$ in positive chemical ionization). It is therefore an ideal internal standard for a mass spectrometric detection.
- Methacrylamide has similar properties, but a different mass ($m/z = 86$) as compared to acrylamide. It elutes shortly before acrylamide and therefore offers a second base for quantification if interference of co-eluting substances is encountered for the isotopic standard.

The recovery standard (butyramide, $m/z = 88$) added before injection allows to determine the amount of standard recovered during extraction and clean-up and serves thus for quality assurance. If more than 50% are lost, the analysis has to be repeated.

2.5. Formation of acrylamide: Precursors and mechanisms

When acrylamide was detected in food, the mechanism of acrylamide formation was completely unknown. However Tareke et al. already published some characteristics of the acrylamide formation [16]:

- Raw food contained no detectable amounts of acrylamide (< 5 µg/kg), which means that a heating process is required.
- Protein-rich food contained less acrylamide than starch-rich food.
- The highest concentrations were found in fried potato products, such as potato chips (UK: crisps) and French fries.
- The amounts in fried meat were much lower (< 50 µg/kg).
- The acrylamide formation strongly increased when most of the water in the sample had evaporated.
- Temperature had a strong influence on acrylamide formation which started at temperatures above 120 °C.

On the basis of their results Tareke et al. speculated that acrylamide is formed from reactive C$_3$-compounds (formed by sugar degradation) and ammonia as source for the nitrogen. Although they did not consider amino acid residues as possible backbones for the acrylamide molecule, they stated that the formation of acrylamide showed similarities with the Maillard reaction.

Several groups then found that acrylamide is indeed formed in the Maillard reaction. Mottram et al. reported the formation of acrylamide from asparagine heated with glucose or the dicarbonyl 2,3-butanedione. Their hypothesis was that acrylamide is formed in the Maillard reaction via the dicarbonyl-assisted Strecker degradation of asparagine [121].
At the same time Stadler et al. demonstrated that pyrolysis of asparagine with glucose (or other sugars) generated acrylamide [122]. 1700 times more acrylamide was formed compared to pyrolysis of asparagine alone. They showed with isotope labeled glucose and asparagine that the sugar backbone was not incorporated in the acrylamide molecule. Furthermore, the nitrogen atom of acrylamide originated from the amide group of asparagine, whereas the nitrogen of the α-NH₂ group was not incorporated. Stadler et al. also found that acrylamide is formed from early Maillard reaction products, e.g. the N-glucoside of asparagine which confirmed the key role of the Maillard reaction [122].

The identification of asparagine as a precursor for acrylamide was supported by several clues:

- The side chain of asparagine resembles the acrylamide molecule as can be seen in Figure 4. From a purely stoichiometric point of view, decarboxylation and deamination of asparagine would lead to acrylamide.
- The highest amounts of acrylamide were found in fried potato products. Potatoes are known to contain large amounts of free asparagine [123].
- Free asparagine is also found in other edible plants including cereals [124, 125] which could explain the widespread occurrence of acrylamide in heated food.

![Figure 4: Structural formulas of asparagine and acrylamide.](image)

The identification of free asparagine as precursor for acrylamide was published shortly later also by other groups [81, 126, 127]. Many publications also reported a minor acrylamide formation when asparagine was heated alone. Furthermore, aspartic acid, glutamine, methionine, cysteine, and lysine formed small amounts of acrylamide during pyrolysis with a reducing sugar [81, 121, 122, 126]. Small impurities (asparagine!) in the used amino acids might have caused this formation [81]. The formation of acrylamide from acrylic acid and ammonia and/or amines was yet another explanation (see below) [128].

Recently, a deeper insight in the mechanism of acrylamide formation was provided by Zyzak et al. [129], Yaylayan et al. [130], Stadler et al. [131], and Schieberle et al. [132]. Zyzak and coworkers gave clear evidence with isotope labeled compounds that the backbone of the acrylamide originates from the asparagine side chain. They also found that acrylamide is formed from 2-deoxyglucose and asparagine implying that
dicarbonyls and the Amadori rearrangement are not essential for acrylamide formation. 2-desoxyglucose cannot undergo this rearrangement because it lacks a hydroxyl group next to the carbonyl group [129]. This was supported by the findings that acrylamide can also be formed from asparagine and octanal [81]. Finally, the group proposed a mechanism for acrylamide formation via the direct decarboxylation of the Schiff base which can either directly generate acrylamide by elimination of an imine or hydrolyze to form 3-aminopropanamide which further degrades to acrylamide via elimination of ammonia. This proposed pathway is shown in Figure 5.

![Diagram of acrylamide formation](image)

**Figure 5: Mechanism of acrylamide formation postulated by Zyzak et al. [129].**

In the model system used, Zyzak and coworkers were able to monitor the consumption of asparagine and glucose, the appearance of the corresponding imine, and the formation of 3-aminopropanamide and acrylamide. In excess of glucose, they detected a peak corresponding to the decarboxylated imine. They suggested that the decarboxylation is the rate limiting reaction.
Another mechanism was postulated by Yaylayan and coworkers [130]. Firstly, they showed that maleimide was the primary product when asparagine was heated in absence of sugars. Secondly, they found that succinimide was formed in heated mixtures of asparagine and glucose via intramolecular cyclization of the Amadori product. They postulated that the cyclization of the Amadori product is competed by an intramolecular cyclization of the Schiff base yielding an oxazolidin-5-one intermediate which forms an azomethine ylide by decarboxylation. From this ylide the decarboxylated Amadori product can be formulated which cannot undergo cyclization due to the missing carboxyl group but leads to acrylamide via a retro Michael reaction as shown in Figure 6. The limiting step for this pathway is considered to be the cleavage of the carbon-nitrogen bond in the β-elimination reaction to acrylamide, and not the decarboxylation. In contrast to the pathway postulated by Zyzak et al. [129], the imine does not undergo direct decarboxylation but firstly forms the oxazolidin-5-one intermediate which facilitates the decarboxylation.

This pathway explains why asparagine needs a reducing sugar to form acrylamide: Without any carbonyl group no imine can be formed which can decarboxylate via an oxazolidin-5-one intermediate. In the absence of sugars, the carboxyl group of asparagine can only undergo the intramolecular cyclization leading to succinimide.

Stadler and coworkers [131] published an in-depth mechanistic study that provided further insight into the acrylamide formation. They have found that N-glucosyl asparagine generated more acrylamide than the synthesized Amadori product and more than binary mixtures of asparagine and dicarbonyls. Furthermore, the Strecker alcohol of asparagine, 3-hydroxypropanamide, generated less acrylamide than binary
mixtures of glucose and asparagine. Therefore, they concluded that a) the Strecker degradation of asparagine with dicarbonyls via the Strecker aldehyde (as suggested by Mottram et al. [121]) is only a marginal pathway, and b) that the pathway for acrylamide formation seemed to occur prior to the Amadori rearrangement which is in accordance with the two other studies [129, 130]. Furthermore, evidence was given for the \( \beta \)-elimination reaction of the decarboxylated Amadori product leading to the corresponding vinylogous compounds provided the presence of a \( \beta \)-proton (Figure 7) which was in line with the mechanism postulated by Yaylayan et al. [130]. The type of carbonyl had a strong influence on the amount of acrylamide generated upon heating with asparagine: Stadler and coworkers suggested that hydroxy carbonyls such as acetol (hydroxyacetone) generate more acrylamide than dicarbonyls because the hydroxyl group favors the formation of the decarboxylated Amadori product whereas a carbonyl group next to the nitrogen atom in the azomethine ylide may preferably lead to the corresponding imine [131]. They also speculated that the peak at \( m/z = 251 \) observed by Zyzak et al. may also be attributed to the decarboxylated Amadori product itself instead of the decarboxylated imine as supposed by Zyzak et al. [129].

![Figure 7: \( \beta \)-Elimination reaction of the decarboxylated Amadori product, adapted from reference [131].](image)

Schieberle and coworkers suggested that 3-aminopropanamide (3-APA) might be formed from asparagine either by thermal degradation of asparagine in the presence of \( \alpha \)-dicarbonyls (pathway A in Figure 8) or by the enzyme decarboxylase (pathway B in Figure 8) [132, 133]. The thermal formation follows the first steps of the Strecker degradation where three different tautomers can be postulated. The hydrolysis of one tautomer leads to 3-APA (“Strecker amine”) while another tautomer yields the Strecker aldehyde of asparagine. Free asparagine also formed 3-APA in the reaction with 2-oxopropionic acid (pyruvic acid). In addition, a hypothetical pathway for the formation of 3-APA from asparagine and a \( \alpha \)-hydroxycarbonyl compound (e.g. glucose) was postulated via the oxidation of the enamino which can be formulated from the imine of asparagine and the \( \alpha \)-hydroxycarbonyl compound [132]. Thus, free asparagine might be degraded to 3-APA during heating of food whereby a very potent precursor is formed.

Granvogl et al. showed that acrylamide can be formed in high amounts without the interaction of a reducing sugar with asparagine. Heating of 3-APA in aqueous and dry systems produced acrylamide at yields of 29 mol-% and 63 mol-%, respectively. This is beyond any yields reported for asparagine - carbonyl mixtures. The reaction is shown in Figure 8 (pathway B). Furthermore, 3-APA formed more acrylamide in the absence of glucose, and the formation started already at 100 °C [133]. Thus, 3-APA is a very potent precursor and it opens a way to acrylamide outside the Maillard
reaction. On the basis of the possible enzymatic formation, 3-APA might be present in raw food. In fact, they determined 3-APA in raw potatoes in the range of 130 to 290 µg/kg and observed its contents even increased during storage at elevated temperatures [133]. Although 3-APA is present in potatoes in much smaller amounts than free asparagine, it has to be taken into account as an additional precursor because 3-APA is much more effective in forming acrylamide. 3-APA alone cannot explain the acrylamide formation in heated potatoes and thus asparagine is still considered as a key precursor. However, 3-APA may be the key intermediate in the formation of acrylamide from asparagine and it might be formed during heating in other foods as well.

Figure 8: Formation of 3-aminopropanamide (3-APA) from asparagine. A: thermal generation via α-dicarbonyl assisted Strecker reaction. B: enzymatic generation. Adapted from references [132, 133].
In addition to the specific pathway via the sugar or dicarbonyl assisted decarboxylation of asparagine [129-132], a nonspecific pathway involving the initial formation of acrylic acid from different sources and its subsequent amidation was recently reported by Yaylayan et al. [128]. They have found that aspartic acid, β-alanine and carnosine formed acrylic acid and acrylamide in the presence and absence of a reducing sugar. Figure 9 shows the formation of acrylamide from β-alanine via acrylic acid.

![Diagram](image_url)

**Figure 9**: Formation of acrylic acid and acrylamide from β-alanine, adapted from reference [128].

The amidation of acrylic acid would produce acrylamide and N-alkylated derivatives. Yaylayan and coworkers postulated that creatine formed ammonia, methylamine, and dimethylamine upon pyrolysis, and they found that co-pyrolysis of creatine and carnosine led to formation of acrylamide, N-methacrylamide and N,N-dimethylacrylamide. N-methylacrylamide was also detected in cooked meat samples and thus the authors speculated that the small amounts of acrylamide in meat products might be due to the formation of N-methyl derivatives instead of acrylamide itself [128]. The formation of acrylic acid upon pyrolysis of aspartic acid-glucose mixtures was also reported earlier [134] and the group of Yasuhara demonstrated that acrylic acid and ammonia formed acrylamide during heating [135].

Apart from amino acids as precursors, acrolein and acrylic acid, formed during oxidation of frying oil, plus ammonia were suggested to generate acrylamide. However, experiments with ammonium salts, oils, and acrolein revealed that this might be irrelevant [81, 126, 136] although the formation of acrylamide from acrylic acid and ammonia has been demonstrated in a model system [135].

Weisshaar and Gutsche found that small amounts of acrylamide were formed when ascorbic acid was heated with ammonium acetate. This clearly pointed to yet another mechanism in addition to asparagine as precursor [126]. To date, no publications are available that would give further insight into this aspect.

In the context of acrylamide formation some more aspects are important as well.

- The formation of acrylamide occurs concurrently to its elimination. Acrylamide is reactive towards nucleophilic compounds such as thiols and primary amines and can polymerize above 85 °C [1]. The measured acrylamide content is always the difference of total formation minus elimination. The elimination becomes apparent when time-temperature experiments are performed with models and food. In the beginning acrylamide contents increase, while they decrease at long times or high temperatures [81, 121, 136-140].
Apart from time, temperature and the amount of precursors, the physical and physico-chemical properties of the system also play an important role. Robert and coworkers have shown that the molecular mobility of the precursors is a critical parameter in dry systems. This mobility is linked to the melting behaviour and the release of crystallization water. In liquid systems, the chemical reactivity as such becomes more important [141]. In addition, acrylamide was formed in higher amounts and at lower temperatures in amorphous systems compared to crystalline systems [142].

In analogy to the formation of acrylamide from asparagine, other vinylogous compounds are found, at least in model systems. Stadler et al. showed that pyrolysis of glucose and aspartic acid generated acrylic acid. Acrylic acid can form acrylamide if a donor for ammonia (e.g. glutamine; NH₃) is present [134, 135]. For glutamine, only very small amounts of 3-butenamide were found whereas large amounts of 2-pyrrolidinone were formed [134] which is also often observed in heated food [143].

Because acrylamide is formed in the Maillard reaction, reactions of other free amino acids as well as formation of melanoidins and flavor compounds (e.g. pyrazines) have to taken into account as well [144, 145].

In summary it can be stated, that acrylamide is predominantly formed from free asparagine and reactive carbonyls (e.g. reducing sugars) at a temperature of 120 °C or higher. The backbone of the acrylamide molecule originates solely from the side chain of asparagine. There is a general consensus that the first step is a nucleophilic attack of the α-NH₂ group to the carbonyl group leading to the imine and N-glycosides of asparagine, respectively. For the decarboxylation of the imine three different pathways are currently postulated. Acrylamide can additionally be formed in a nonspecific pathway via amidation of acrylic acid which can originate from different sources. 3-aminopropanamide is another and very potent precursor for acrylamide and might be a key intermediate formed from asparagine. Concurrently to its formation, acrylamide is also eliminated by reaction with matrix compounds or by polymerization.

2.6. Acrylamide in potato products

Potato products such as chips, French fries, hash browns, and baked potatoes can contain up to a few milligrams acrylamide per kg [16, 81, 127]. Due to their high contents and frequent consumption, potato products significantly contribute to the human exposure and therefore, they have received great attention. In contrast, raw, boiled, steamed, or mashed potatoes contain no or very little acrylamide. Obviously, temperatures above 120 °C and drying of the matrix are needed for acrylamide formation. In this chapter only chips and French fries are discussed. Most of the literature focuses on these two products, while much less publications deal with hash browns, baked potatoes, and croquettes [146-148]. The influence of the composition of the raw potatoes and the heating process are discussed and the main approaches to reduce the acrylamide concentrations are presented.
2.6.1. Influence of raw material

Potatoes have been known to contain glucose, fructose, sucrose, and asparagine as well as many other free amino acids for decades [149]. Two studies of this thesis addressed the composition of the raw potatoes and will be presented and discussed separately in the chapters 3 and 4 [150, 151]. Several studies showed that the contents of sugars and free asparagine can strongly vary between different batches [127, 152, 153]. Potatoes are particularly rich in free asparagine and its contents range from 2000 to 4000 mg/kg fresh weight [152]. This at least partly explains the high acrylamide contents found in fried potatoes. The amount of sugars and amino acids is influenced by different factors:

- **Cultivar:** The content of reducing sugars and free amino acids can differ significantly between potato cultivars [150-152].

- **Fertilizers:** Nitrogen fertilization can influence the composition of potato tubers [124, 127]. However, the influence of fertilization on acrylamide formation is still under debate and not all studies come to the same conclusion. A recent study reported that a moderate N-fertilization combined with a good provision of potassium resulted in the lowest contents of free asparagine and reducing sugars, while an excess of N-fertilization led to increased concentrations of these compounds. The results were also consistent with the acrylamide content of French fries prepared from those potatoes [154]. However, other factors such as climate and year seem to be more important [152, 155].

- **Climate:** Temperature, sunlight, and rainfall affect the metabolism in potato plants. Variations of sugars and amino acid contents are thus observed in potatoes of the same cultivar grown on the same field over different harvests [151, 152].

- **Storage:** Storage is very important for potato quality and many processing aspects. Storage below 8 °C leads to the accumulation of sugars ("low temperature sweetening") [156]. This can increase the acrylamide formation dramatically [127, 157-160]. Reconditioning of cold-stored potatoes at 15 °C can eliminate most but not all of the accumulated sugars [159]. The content of free amino acids can also change during storage [123].

- **Greening:** Exposure to daylight led to the formation of greenish potatoes which formed more acrylamide which was attributed to the elevated content of reducing sugars [127].

A convenient method to characterize potatoes in relation to acrylamide formation was developed by Biedermann et al. Raw potatoes are grated, spread on a metal grid and heated in a GC oven at 120 °C for 40 min. The determination of the potential of acrylamide formation is a fast and well reproducible method and it correlates with acrylamide contents in French fries, chips, and hash brown prepared from the same batch of potatoes [137]. Studies with this method demonstrated that the content of reducing sugars in the raw potatoes correlated with the potential of acrylamide formation, whereas sucrose, free asparagine and any other free amino acid did not correlate [127, 150, 151]. Two of these studies are part of this thesis and will be discussed later on [150, 151]. Experiments with chips and French fries confirmed...
these results [158, 160-163]. Glucose and fructose were clearly shown to be the key precursors for acrylamide formation in potato products. A low content of glucose and fructose in the raw material is a prerequisite to prepare fried potato products with low acrylamide content. However, there is also a lower limit for reducing sugars to obtain products with desired color and flavor [146]. This demonstrates that the acrylamide formation is linked to the Maillard reaction which is a very important source for flavor and color [145]. The interrelation of acrylamide and browning via the Maillard reaction enabled the Swiss frying test, originally established to evaluate the suitability of potatoes for industrial purposes, to be an approximate, but fast and inexpensive method to estimate the content of reducing sugars and the acrylamide formation in potatoes [164]. Apart from the composition of the raw material, the shape has an influence on acrylamide formation. Increasing the surface to volume ratio led to higher acrylamide content in the fried products, i.e. thin potato slices formed more acrylamide than thick potato slices at comparable process conditions [165].

2.6.2. Influence of process
In the context of process conditions, pretreatments before frying (e.g. blanching, addition of additives) as well as the process itself (time and temperature; type of oil) have to be considered. From a chemical point of view, it is obvious that a higher temperature and/or prolonged heating lead to enhanced formation and elimination of acrylamide. This was observed in French fries [160, 166, 167], chips [153, 162, 168-171], and potato model systems [138, 139, 165]. Grob et al. showed that the frying temperature is a key factor for acrylamide formation and that most of the acrylamide is formed during the last two minutes of frying. Lowering the initial oil temperature from 180 °C to 170 °C allowed the preparation of French fries with lower acrylamide content and rendered the determination of the end point of frying less critical as shown in Figure 10 [166]. Similar results were also obtained by Matthàus et al. [160].

![Figure 10: Formation of acrylamide in French fries prepared in oil with different initial temperatures [166].](image-url)
Since production of chips implies the removal of most of the water, evaporation is very important. Water evaporation at temperatures below 100 °C occurs only if the pressure is reduced. Granda et al. developed a vacuum frying system where potato slices were fried for 8 min at 118 °C and a reduced pressure of 10 torr (= 13.3 mbar) and compared it to a traditional frying system (4 min at 165 °C, atmospheric pressure). The acrylamide contents in the chips were reduced by a factor 6 to 16 if the vacuum frying system was used instead of the traditional system [169]. These findings emphasize that frying temperature is the pivotal process parameter for acrylamide formation in fried potatoes. The influence of frying time becomes more prominent only at elevated temperatures [166, 169]. Over-frying is particularly critical at high temperatures and for potatoes with elevated content of reducing sugars (> 1 g/kg) [167]. Frying temperature and time are directly interrelated: A reduction of temperature implies a longer process to prepare a product of comparable culinary quality. Thus, frying to optimal culinary quality is a reasonable means to optimize the frying process in terms of time and temperature [166, 167]. A recent study showed that the development of the oil temperature was more important than the initial temperature because virtually all acrylamide was formed in the second half of the frying process [172]. The temperature at the end of frying depended on the initial temperature, the mass of potato sticks per liter oil, and on the frying equipment itself (heating power, regulation). Fiselier et al. found that French fries of optimum culinary quality and low acrylamide content (< 50 µg/kg) were prepared if the initial temperature (170 °C) dropped by about 20 °C (approximately 50 g potato per liter oil) and frying for 5 min [172].

Blanching or soaking of potato sticks/slices in water aim at the extraction of the precursors from the outermost layers. Sugars and free amino acids can be extracted before the preparation of French fries [166, 173] and chips [153, 163, 170, 171, 174]. Blanching in warm water was more effective in extracting glucose and asparagine than immersion in cold water [166, 168, 170, 175]. Grob et al. assumed that hot water made cell membranes more permeable which facilitated extraction. However, frying of blanched potato sticks (80 °C for 2 min) gave unsatisfactory crispiness and no lower acrylamide contents. The authors explained this effect with the higher permeability of cell membranes in the blanched tissue which caused a higher migration of water and precursors to the surface. This impaired the formation of a crispy crust, but nurtured the acrylamide formation. They concluded that pretreatment with cold or warm (not hot) water and pre-frying followed by main frying gave the best results in terms of acrylamide and culinary quality [166]. If always the same frying conditions were used, i.e. the end point was not determined by culinary aspects, blanching reduced the acrylamide content in most studies [153, 170, 175] except one [174].

Blanching is usually applied to inactivate enzymes, e.g. polyphenoloxidases in the raw material. However, a recent publication demonstrated that commercial blanching and pre-frying of potato sticks did not prevent the release of reducing sugars during storage of “fresh” potato sticks at 4 °C. This led to increased acrylamide formation during frying [173].

Another approach is the addition of acids to soaking solutions. At a lower pH the α-NH₂ group of asparagine gets protonated whereby the first step in the formation of acrylamide is hindered. Immersion of potato sticks in 1% or 2% citric acid solution (w/w) before frying reduced the acrylamide content by 73% and 80%, respectively. Compared to the treatment with pure water the effect of 1% citric acid (w/w) was still
a decrease of 67% (see Figure 11) demonstrating that citric acid caused the main effect [176].

![Figure 11: Effect of the pretreatment with citric acid on the acrylamide formation in French fries [176].](image)

However, the use of citric acid has some inherent limitations: Due to the acidic taste in the finished product, the concentration is restricted to ≤1% (w/w). And it is not suitable for roasted potatoes and hash browns. However, immersion of potato sticks into a warm aqueous solution of 0.75% citric acid (w/w) for 15 min decreased the acrylamide concentration of French fries by a factor of 2 while the product had no acidic taste yet [148]. Kita et al. reported that acetic acid was more effective and more favorable for sensory as compared to citric acid. Both acids extracted also more precursors compared to water and thus the lower acrylamide contents were a consequence of lower pH and better extraction [168].

Some groups investigated also the influence of amino acids on acrylamide formation in potato. Rydberg et al. showed that glycine, glutamine, and lysine (all added at 35 mmol/kg) reduced the acrylamide content in homogenized potatoes. They attributed this effect to a lower pH, competition for the reactive carbonyls between asparagine and added amino acids, and/or enhanced elimination of acrylamide [139]. Bråthen and coworkers reported that Blanching of slices in aqueous solutions of glycine and glutamine (0.02 M) reduced the acrylamide content in chips by about 50% (effect of pure water: 30%) and observed a trend for stronger effects from glycine. However, they found no consistent influence of Blanching in the preparation of French fries for both amino acids [177]. Kim et al. found that soaking of potato slices in glycine solutions (0.1 - 3%) for some minutes prior to frying reduced the acrylamide content of the fried chips by 50 to 90% [178].

The type of oil and its changes during heating cycles (e.g. oxidation and hydrolysis) have been suggested to influence acrylamide formation as well. Becalski and coworkers presumed a reduction in acrylamide formation if rosemary extract was
added to frying the oil and they suspected olive oil to enhance acrylamide formation compared to corn oil or paraffin. However, data for both aspects were limited in this study [81]. Vattem and Shetty reported that acrylamide formation was likely not of oxidative nature because antioxidants (e.g. herbal phenolics) did not decrease acrylamide contents [179]. Gertz and Klostermann stated that the type of oil and the use of “silicone” (dimethylpolysiloxane, E 900) as antifoaming agent influenced acrylamide formation in French fries due to an altered heat transfer [180, 181]. However, these data have not been confirmed yet. Grob et al. found that additives to oil did not significantly reduce the acrylamide content in an optimized frying process [166]. Matthäus et al. reported that neither the type of oil, nor the use of antifoaming agents, nor the progressive aging of the oil (oxidation) significantly changed the acrylamide content of French fries [160]. Mestdagh et al. also reported that the extent of oil oxidation and the amount of di- and monoglycerides in the oil (i.e. the extent of oil hydrolysis) had no significant influence on acrylamide formation [182]. In another study Mestdagh and coworkers found that the type of oil affected the acrylamide concentration neither in a model system nor in French fries [183]. Altogether, the oil seems to be of minor or even negligible importance for acrylamide formation in fried potatoes.

2.7. Acrylamide in bakery

Model studies are excellent tools to identify critical factors for acrylamide formation in bakery and have often been applied [184-187]. However, they have also some inherent limitations: No real product is prepared and thus no information on culinary quality is obtained. Furthermore, the concentration of relevant reactants for acrylamide formation, elimination, and suppression can be different in real food. Therefore, experiments with real products using original recipes and processes most similar to industrial production offer advantages and they can also easily identify critical factors and find options for mitigation [136, 188-194].

2.7.1. Influence of raw material and formulation

Three publications concerning this aspect are part of this thesis and will be presented and discussed later in the chapters 5 to 7 [136, 189, 190]. In contrast to potatoes, the amount of free asparagine is a critical factor in bakery and often correlates with the acrylamide content of the final product: The more asparagine is present, the more acrylamide is formed during baking. This was shown in model systems [184, 185, 187] as well as in real products such as gingerbread, bread, and crisp bread [136, 185, 191, 193, 195]. Flour is usually the main source for free asparagine, but almonds, spices, and honey can also contribute some part [136, 186, 190, 193]. The amount of free asparagine in flour/dough depends on several factors:
• **Variety:** Rye flours usually contain more free asparagine than wheat flours [125, 138, 192, 196, 197]. In European wheat, contents of free asparagine from 74 to 664 mg/kg are found [198].

• **Cultivation:** Fertilization, location, farming system, and maturity may also influence the concentration of free amino acids and sugars in cereals [193, 199].

• **Flour type:** A higher extraction during milling, i.e. inclusion of outer kernel layers (aleurone, bran) leads to higher contents of free amino acids and sugars [192-194].

• **Fermentation:** Yeasts and lactic acid bacteria consume free amino acids during dough fermentation [192].

However, the presence of free asparagine alone does not imply the formation of acrylamide in bakery. If no reducing sugars are present, e.g. if inverted sugar syrup is replaced by a sucrose solution, much less acrylamide is formed [136, 184, 189, 190]. This underlines the need of a reducing sugar for the formation of acrylamide from asparagine [130].

Apart from these acrylamide precursors, the baking agent has a very strong influence on the acrylamide formation in leavened bakery. Figure 12 shows results from experiments with wheat flour (270 mg/kg free asparagine), fructose (39% (w/w) in the flour) and baking agents (1% (w/w) in dough). The mixtures were heated at 150 °C for 30 min. The baking agent ammonium hydrogen carbonate (NH₄HCO₃, E 503) strongly promoted the acrylamide formation while Na₂CO₃ did not [184]. Similar results were obtained by Weisshaar [186]. In absence of reducing sugars, the promoting effect of NH₄HCO₃ was not observed [136, 187, 189, 190].

![Figure 12: Acrylamide formation in a model containing wheat flour and fructose (Fru), resembling sweet bakery. Data taken from reference [184].](image-url)

The anion partner of the ammonium salt played an important role, too. NH₄Cl and NH₄Br formed 10 to 20 times less acrylamide in a model system as compared to NH₄HCO₃ [184]. The ammonium ion obviously needs a strong proton acceptor to
form ammonia. In addition, it was also reported that large amounts of NH₄HCO₃ in the dough enhanced the elimination of added deuterated acrylamide [184, 187]. However, the enhanced elimination probably does not compensate for the promotion in sweet bakery. The promoting effect of NH₄HCO₃ on acrylamide formation was part of one of the studies belonging to this thesis and will be discussed later [136]. Two other studies also investigated this aspect and found that NH₄HCO₃ was not incorporated to acrylamide but probably reacted with sugars [184, 186].

Additives such as organic acids affect the acrylamide formation as well. Citric acid reduced the acrylamide content of gingerbread to about one third, but weakened also browning and leavening, and affected taste negatively [136]. Addition of glycine to the dough of flat breads reduced the acrylamide content by about 60 - 80% [177]. Kim et al. reported that lysine and glycine were more effective in reducing the acrylamide content of a fried wheat snack compared to cysteine. However, the acrylamide contents of these samples were far above the acrylamide contents usually found in bakery [178]. The effect of added amino acids is not explained only by a lower pH, as competition between glycine and asparagine for the carbonyls and/or elimination of formed acrylamide are also likely to play a role.

2.7.2. Influence of process conditions

Two studies of this thesis focused on this aspect as well and the results will be presented and discussed separately [136, 190]. Baking temperature and time obviously influence the acrylamide formation. In general, prolonged baking and high temperatures increased acrylamide contents in bakery products [185, 187, 191]. A lower baking temperature can reduce acrylamide contents of the product [191, 193, 194], but sensory quality can also be negatively affected [194]. After very long baking and/or at very high temperatures decreasing acrylamide concentrations were observed [185, 187]. Elimination of acrylamide became more prominent at drastic conditions [185] and was also affected by ingredients such as sugars, baking agents, amino acids, and proteins [184, 187]. However, it is questionable that a reduction of the acrylamide content of bakery can be achieved by enhancing elimination via drastic process conditions because the product is likely to become over-baked. The same applies to the temperature (T) dependency of the acrylamide content reported by Bråthen and Knutsen [185]: They found that acrylamide concentration was proportional to -T² which implies that baking at high temperatures lowers the acrylamide contents. However, the acrylamide contents in this study were very high (often exceeding 1000 µg/kg) and the process conditions were drastic. Thus, the results are hardly transferable to conditions used in industry.

It has to be taken into account that the temperature within the product stays remarkably below the oven temperature, especially as long as the evaporation of water is significant. The highest temperatures are reached in the crust, while the temperature in the inner part usually stays around 100 °C or slightly above [187, 191, 198]. The crust therefore usually contains 90% or more of total acrylamide [191, 193].

The development of browning during baking presents an important parameter. Browning often correlated with the acrylamide content, i.e. the darker the product was the more acrylamide it contained [136, 144, 191]. Acrylamide also correlated
with the amount of total pyrazines in a model system [144]. This demonstrates that acrylamide formation is directly linked to the Maillard reaction and that measures aiming at acrylamide mitigation may also affect color and flavor.

2.7.3. Mitigation concepts

Three sub-projects of the present thesis investigated this important aspect [136, 189, 190]. These results are not to be discussed extensively here, but will be presented in separate chapters.

The main starting points for decreasing the acrylamide content of bakery are:

- Baking agent
- Reducing sugars
- Free asparagine
- Organic acids
- Baking process

If the baking agent NH₄HCO₃ was completely replaced by NaHCO₃ a significant reduction of the acrylamide content was observed [136, 186-190]. The effect was particularly large if the dough contained appreciable amounts of reducing sugars [136, 189, 190].

A virtual depletion of reducing sugars by using only sucrose as sugar led to a strong decrease of acrylamide contents [136, 189, 190]. However, reducing sugars are also needed for Maillard browning and thus, this approach is successful only in products for which browning is not important.

The amount of free asparagine can be lowered by using flours with a low degree of extraction and by omitting almonds and potato flakes which are rich in free asparagine. Another approach is the application of an asparaginase which hydrolyses the amide group of asparagine. This enzyme can be applied as a pretreatment of the flour or straightforward during mixing and kneading. It was successful in a flour model [186], gingerbread [136], and crackers [190].

Addition of citric acid or tartaric acid reduces the acrylamide content, but the application is limited due to inhibited browning and acidic taste [136, 189]. Amino acids like glycine can reduce the acrylamide content and enhance the Maillard browning at the same time which is an advantage [136, 177, 187]. However, rather high amounts are needed and the impact on sensorial and toxicological aspects is yet unclear.

Lower baking temperatures can limit the acrylamide formation to some extent, but the impact on sensory properties is usually not negligible and different products may give contradicting results. A lower temperature at the end of the baking process may be a feasible approach to reduce the acrylamide content of some products [190, 193].

2.8. Acrylamide in roasted products

Coffee is the most important roasted product because it is consumed frequently and in appreciable amounts all over the world. Therefore, it contributes a significant part to the human exposure [88, 90, 92]. Apart from coffee, cocoa and roasted nuts may
also be of concern. For roasted products, literature is rather scarce as compared to fried potatoes or bakery. The following section will give a short overview on acrylamide in coffee and roasted almonds.

2.8.1. Coffee
Roasted coffee beans contain acrylamide in the range of 40 to 400 µg/kg with a mean value of about 200 µg/kg [85, 200-202] while dry instant coffee can contain more than 500 µg/kg [202]. Coffee substitutes like roasted chicory contain the highest amounts: Up to 4000 µg/kg were found [109, 201]. Acrylamide is almost quantitatively extracted during brewing and a ready-to-drink coffee contains about 0.6 to 1.6 µg/100 mL and acrylamide was rather stable in brewed coffee [202, 203]. Granby and Fagt found no significant differences between preparation techniques but determined higher acrylamide contents in coffee from medium roasted beans (about 10 µg/L) compared to dark roasted beans (about 5 µg/L) [203].
Acrylamide is not stable in coffee beans and in ground coffee during storage. Acrylamide contents decreased for 30% to 65% depending on the sample and storage time [201, 202]. Losses through evaporation or UV-induced polymerization can likely be excluded whereas reaction with reactive (flavor) compounds such as thiols might explain this instability [200, 201]. Interestingly, acrylamide is also not stable in other roasted products such as cacao and almonds [87, 201].
In contrast to other food categories, the commercial products are processed in the elimination phase of the roasting process (Figure 13). This means that coffee beans contain the highest amounts of acrylamide before they reach any commercial roasting degree and that a less intense roasting would lead to higher acrylamide concentrations. Elimination starts from the first minutes of roasting as shown with deuterated acrylamide in heated coffee powder [204]. Thus, elimination of acrylamide is a key process during coffee roasting. Model experiments showed that color changes (a-value) follow a similar pattern as acrylamide formation during dry heating of coffee, and a logarithmic correlation was found between acrylamide content and the a-value of roasted coffee [204].
The importance of the roasting process for flavor and color and the relatively narrow range for commercial products as well as the impact of elimination make mitigation for coffee particularly complicated.
2.8.2. Roasted almonds
Two studies of this thesis focused on roasted almonds and they are presently the only publications that give detailed information on the influence of raw material and the roasting process on acrylamide formation [87, 205]. These results will be presented and discussed in detail in the chapters 8 and 9. Raw almonds contain up to 2 g of reducing sugars and free asparagine per kg [206, 207]. As a consequence, appreciable amounts of acrylamide were found in roasted almonds [81, 87, 186]. It was shown that cut or grated almonds formed more acrylamide than whole almonds [186] and that the roasting temperature again has a very strong influence on the acrylamide formation during roasting [87].

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3. Potential of acrylamide formation, sugars, and free asparagine in potatoes: A comparison of cultivars and farming systems


3.1. Abstract

Glucose, fructose, sucrose, free asparagine, and free glutamine were analysed in 74 potato samples from 17 potato cultivars grown in 2002 at various locations in Switzerland and different farming systems. The potential of these potatoes for acrylamide formation was measured with a standardised heat treatment. These potentials correlated well with the product of the concentrations of reducing sugars and asparagine. Glucose and fructose were found to determine acrylamide formation. The cultivars showed large differences in their potential of acrylamide formation which was primarily related to their sugar contents. Agricultural practice neither influenced sugars and free asparagine nor the potential of acrylamide formation. It is concluded that acrylamide contents in potato products can be substantially reduced primarily by selecting cultivars with low concentrations of reducing sugars.

3.2. Introduction

Hemoglobin adducts of acrylamide and its metabolite glycidamide were observed in humans who had been occupationally exposed to acrylamide [1]. A high background level of adducts and metabolites found in persons neither occupationally exposed to acrylamide nor being smokers [2] led to further research to identify the source of the exposure. Increased levels of hemoglobin adducts found in rats fed with a fried diet revealed that acrylamide may be formed during heating of foodstuffs [3].

In early 2002, acrylamide was detected in a range of foods heated during production or preparation [4, 5]. Concentrations often exceeded 1000 µg/kg which caused a world-wide concern since acrylamide is classified as probably carcinogenic to humans (Group 2A) by the IARC [6]. Particularly high concentrations were found in products of plant origin heated to high temperatures, such as potato chips (US terminology), French fries, pan-fried potato products or crisp bread, whereas the contents in foods rich in protein were low [4]. Particular attention was paid to potato products because of the high acrylamide concentrations and the consumption rate as a staple food.

Acrylamide is formed in the Maillard reaction [7-12]. Heating of glucose with asparagine yielded acrylamide at a rate strongly increasing with temperature increasing from 120 °C to 170 °C. A pathway for the formation of acrylamide via Strecker degradation of asparagine with dicarbonyls was proposed [7]. It was also shown that acrylamide is directly generated from N-glycosides formed from sugars and amino acids during an early stage of the Maillard reaction [8]. Becalski et al. [9] found that 15N-acrylamide is developed when 15N-(amido)-labelled asparagine was
heated with glucose. A proposed pathway starting from asparagine via a decarboxylated Amadori product pointed up the importance of reducing sugars in acrylamide formation [12]. Consequently asparagine, glucose and fructose are considered to be the main precursors, asparagine with its amide group delivering the backbone of the acrylamide molecule. Potato tubers contain substantial amounts of the acrylamide precursors free asparagine, glucose and fructose [13] which may explain the high concentrations of acrylamide in certain potato products. Reducing sugars and free amino acids are also the precursors of flavor components and of browning formed in the Maillard reaction [14, 15] which means that acrylamide is generated parallel with flavors and browning.

Storage of potatoes at temperatures below 8-10 °C induces a strong increase in sugar contents: The phenomenon is commonly known as “low temperature sweetening” [16]. Potatoes of the cultivar Erntestolz stored at 4 °C for 15 days showed an increase in reducing sugars from 80 to 2250 mg/kg (referring to fresh weight). As a consequence, the potential of acrylamide formation at 120 °C rose by a factor of 28 [11]. Long term storage at higher temperatures may, however, be a problem concerning sprouting in late spring/early summer, which usually requires the application of sprouting inhibitors [17].

Different potato cultivars grown in 2002 at various locations in Switzerland and different farming systems were analysed for glucose, fructose, sucrose, free asparagine, and free glutamine. To confirm the interrelation of sugars and free asparagine with the formation of acrylamide, potatoes were subjected to a standardized heat treatment inducing formation of acrylamide in a controlled manner [18]. The procedure for determining this “potential of acrylamide formation” turned out to be more reproducible than the analysis of, e.g., potato chips or French fries produced under standardised conditions. The results of this test correlated with the acrylamide contents in products prepared from the same potatoes, such as French fries, pan-fried potato (hash browns), chips, and roast potatoes.

3.3. Material and methods

3.3.1. Collection of potato samples

Samples of the cultivars Agria, Appell, Bintje, Charlotte, Desirée, Eba, Naturella, Nicola, Panda, and Santana were collected in Switzerland by the Swiss College of Agriculture (Zollikofen, Switzerland) from end of August to end of September 2002. The potatoes were part of a three year on farm experiment on 93 plots (20 organic, 31 integrated, 42 conventional farming system) focusing on quality aspects of Swiss potato production. Within this project all relevant data concerning crop rotation, cultivation technique, and site parameters were collected and potatoes assessed for in terms of quality. According to a defined sampling plan, 55 tubers from 55 plants were taken from each field, making up a total of about 5 kg per sample. Storage conditions were 9 °C at 95-98% relative humidity for all cultivars except Charlotte which was stored at 6 °C and 90% relative humidity to reduce sprouting.

Samples of the cultivars Erntestolz, Hermes, Lady Claire, Lady Rosetta, Markies, Marlene and Panda were obtained from Zweifel Pomy-Chips AG (Spreitenbach, Switzerland), a producer of potato chips. These potatoes were harvested in
September 2002, stored at 10-12 °C and 90% relative humidity without application of sprout inhibitors and analysed in November 2002.

3.3.2. Sample preparation
12 to 15 tubers of a given sample were washed and, after the water was dripped off, cut lengthwise. From each tuber one half was grated (holes of 2.5 mm x 7 mm, as typically used to prepare hash browns). The grated material was thoroughly mixed and used for all analyses.

3.3.3. Analysis of acrylamide
For the determination of the potential of acrylamide formation [18], 20 g of grated potato was spread on a grid and placed in a pre-heated oven at 120 °C for 40 min. After measuring residual weight, water (to a total weight of 20 g) and methacrylamide (Fluka AG, Buchs, Switzerland) as internal standard (500 µg/kg, referring to fresh weight) were added. Acrylamide was then analysed as described by Biedermann et al. [19]: 10 g of sample were heated at 70 °C for 30 min, and extracted with 40 mL of 1-propanol (Scharlau, Barcelona, Spain). The 1-propanol/water was removed by azeotropic evaporation. Acrylamide was extracted from the residue with 3 mL of acetonitrile (Merck, Darmstadt, Germany) and twice defatted with hexane (Merck). To determine the overall yield of sample preparation, 10 µL of butyramide solution (25 µg/mL, corresponding to 500 µg/kg fresh weight; Fluka) was added as a second internal standard to 1.5 mL of defatted acetonitrile extract. The solution was injected on-column onto a short GC capillary column coated in the laboratory with Carbowax 20 M (Fluka). Mass spectrometry with positive chemical ionisation monitored the ions \( m/z \) 72 (acrylamide), \( m/z \) 86 (methacrylamide) and \( m/z \) 88 (butyramide). Results were calculated as acrylamide concentrations referring to fresh weight. If the overall yield was less than 40%, the analysis was repeated starting from the evaporation step.

3.3.4. Measurement of amino acids
Free asparagine and glutamine were determined by the method of Arnold et al. [20]: 10 g of grated potato was diluted with 60 g of bi-distilled water and 1 mL of glycine solution (10 mg/mL) was added as internal standard. After blending with a Polytron (Kinematica, Lucerne, Switzerland) 29 g of bi-distilled water was added and the mixture thoroughly shaken. After addition of 50 µL 1-octanol (Fluka) to break down foam, samples were left to settle for 1 h. If needed, samples were filtered (Schleicher&Schuell, Dassel, Germany). Prior to injection, amino acids were converted to their carbamates by reaction with 9-fluorenylethylchloroformiate (FMOC-Cl; Fluka). Samples were separated on a 250 x 4.6 mm i.d. column with a C8 packing (MOS Hypersil 5 µm; Bischoff, Leonberg, Germany) with a gradient of acetate buffer/acetonitrile. Fluorescence detection was at 265/340 nm.
3.3.5. Determination of sugars
Glucose, fructose, and sucrose were determined enzymatically using the test kit from Scil Diagnostics (Martinsried, Germany). A mixture of 20 g of grated potato with 60 g bi-distilled water and homogenised (Polytron). 5 mL of solutions Carrez I (150 g of potassium hexacyanoferrate(II) trihydrate per liter; Merck) and Carrez II (300 g of zinc sulfate heptahydrate per liter; Fluka) were added. The mixture was thoroughly shaken, the pH adjusted to 7.0 – 7.5 with a few drops of KOH solution (4 mol/L; Fluka), foam broken by addition of 50 µL of 1-octanol (Fluka) and the volume adjusted to 250 mL with bi-distilled water. Filtered samples (Schleicher&Schuell) were subjected to enzymatic analysis as described by the producer.

3.3.6. Statistical analysis
Univariate analysis of variance was performed using the software SPSS (SPSS Inc., Chicago, Ill., USA), version 11.0 for Windows. The level of significance $\alpha$ was set to 5%. Tukey-HSD and LSD were accomplished as Post Hoc tests.

3.4. Results and discussion
3.4.1. Precision of results
The reproducibility of determining the potential of acrylamide formation was checked on a sample of grated and homogenated Sirtema potato which was exposed to the heat treatment and analysed for acrylamide four times. At a mean result of 990 µg/kg the relative standard deviation was 2.0%.
Seven tubers from a lot of Agria potatoes were analysed individually. Sugar content and potential of acrylamide formation varied strongly (Figure 14), but correlated well with each other. The size of the seven tubers varied from small to oversize. However, no correlation between size and sugars or acrylamide potential was found. Because of the observed strong variation between the tubers of the same potato lot, at least 12 to 15 tubers were analyzed.
3.4.2. Concentrations of sugars and amino acids

The concentrations of the assumed precursors of acrylamide, i.e. glucose, fructose and free asparagine, are listed in Table 3 together with those of sucrose and glutamine. Glucose concentrations ranged from 40 to 2700 mg/kg, with the lowest values found in the samples of the cultivars Lady Claire and Marlene and the highest in Naturella and Nicola. Concentrations of fructose varied similarly, but were generally lower than those of glucose, again with the highest values in Naturella and Nicola. Concentrations of fructose were positively correlated with those of glucose ($R^2 = 0.9495$). Sucrose concentrations ranged from 160 to 1800 mg/kg and did not correlate with either glucose or fructose. The high sugar contents in the samples of the cultivar Charlotte were probably due to the lower storage temperature.

Free asparagine was found at concentrations between 1400 and 5170 mg/kg and therefore was generally more abundant than sugars. On a molar basis, the mean content of asparagine was 3.7 or 5.6 times higher than that of glucose or fructose, respectively. Concentrations of free glutamine were lower and ranged from 570 to 2520 mg/kg. Correlation between asparagine and glutamine was clearly weaker than that between glucose and fructose ($R^2 = 0.5930$). The two amino acids showed no correlation with the sugars.

Concentrations of glucose and fructose varied more strongly than those of asparagine: For the cultivar Agria (22 samples), the relative standard deviation was 28% for asparagine, but 65% and 69% for glucose and fructose. In the cultivar Bintje, the variation was similar with a relative standard deviation of the concentrations of 17% for asparagine, but 45% and 54% for glucose and fructose. Variations were broad also for the other two components analysed, as well as the other cultivars (see standard deviations in Table 3).

Several authors reported widely varying contents of fructose and glucose within a given cultivar as well as between potato cultivars. Mean contents (ranges) of reducing sugars in the cultivars Trent and Onaway were reported as 340 mg/kg.
(180 - 460 mg/kg) and 1640 mg/kg (820 - 2470 mg/kg), respectively [21]. For the cultivar Saturna, 1000 mg/kg, 800 mg/kg, and 1070 mg/kg were measured for glucose, fructose and sucrose, respectively [22]. Considerable variation was reported also for asparagine and glutamine: for the cultivar Pentland Dell, asparagine ranged from 2060 to 9310 mg/kg, glutamine concentrations from 1760 to 7660 mg/kg [23]. In Bintje potatoes, free asparagine varied from 1370 to 7600 mg/kg and strongly depended on fertilization [24]. Total reducing sugars and total free amino acids can vary considerably between different seasons, and storage temperature has a strong impact on the sugar content [23].

Table 3: Concentrations of sugars and free amino acids in different potato cultivars in mg/kg (referring to fresh weight). SD: standard deviation; n.d.: not determined; -: no standard deviation calculable; a: n = 2).

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Glucose</th>
<th>Fructose</th>
<th>Sucrose</th>
<th>Asparagine</th>
<th>Glutamine</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean</td>
<td>SD</td>
<td>mean</td>
<td>SD</td>
<td>mean</td>
<td>SD</td>
</tr>
<tr>
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<td>218</td>
<td>143</td>
<td>99</td>
<td>616</td>
<td>205</td>
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<tr>
<td>Appell</td>
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<td>-</td>
<td>780</td>
<td>-</td>
<td>510</td>
<td>-</td>
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<tr>
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<td>197</td>
<td>239</td>
<td>128</td>
<td>759</td>
<td>129</td>
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<tr>
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<td>336</td>
<td>737</td>
<td>293</td>
<td>629</td>
<td>316</td>
</tr>
<tr>
<td>Desirée</td>
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<td>-</td>
<td>990</td>
<td>-</td>
<td>760</td>
<td>-</td>
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<tr>
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<td>346</td>
<td>275</td>
<td>899</td>
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<td>76</td>
<td>67</td>
<td>89</td>
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<tr>
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<td>186</td>
<td>163</td>
<td>163</td>
<td>1597</td>
<td>318</td>
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<tr>
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<td>86</td>
<td>44</td>
<td>61</td>
<td>882</td>
<td>331</td>
</tr>
<tr>
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<td>29</td>
<td>53</td>
<td>1</td>
<td>1143</td>
<td>397</td>
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<tr>
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<td>-</td>
<td>30</td>
<td>-</td>
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<tr>
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<td>15</td>
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<tr>
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<td>-</td>
<td>1500</td>
<td>-</td>
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<tr>
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<td>1537</td>
<td>456</td>
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<tr>
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<td>88</td>
<td>1471</td>
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<tr>
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<td>540</td>
<td>-</td>
<td>1120</td>
<td>-</td>
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<tr>
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<td>95</td>
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<td>1579</td>
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<td>435</td>
<td>996</td>
<td>3086</td>
<td>3870</td>
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</tbody>
</table>

3.4.3. Potentials of acrylamide formation and correlations

The potentials of acrylamide formation were related to contents of reducing sugars and free amino acids. Thereby the sugar and asparagine concentrations were combined into the product of reducing sugars and asparagine. Figure 15 shows a strong correlation between this measure and the potential of acrylamide formation (data set including all 74 samples from 17 different cultivars). The formula \((0.5\cdot\text{glucose}+\text{fructose})\cdot\text{asparagine}\) assumes a bi-molecular reaction between sugar and asparagine as the rate-determining step for acrylamide formation. Glucose was
weighted half, because fructose was about twice as effective as glucose in supporting acrylamide formation [11].

![Graph showing correlation between acrylamide formation and product of reducing sugars and asparagine.](image)

**Figure 15: Correlation between the potential of acrylamide formation and the product of the concentrations of reducing sugars and asparagine.**

The validity of the assumed bi-molecular reaction was checked by considering some alternative ways of calculation:

- The analogous correlation based on moles instead of weight resulted in virtually the same coefficient ($R^2 = 0.9026$).
- Concentrations (weight or moles) referring to dry matter turned correlations to be slightly weaker ($R^2 = 0.8813$).
- The correlation of the potentials of acrylamide formation merely with the reducing sugars, i.e. $0.5 \times$ glucose + fructose was slightly weaker ($R^2 = 0.8768$).
- Asparagine alone showed no correlation with the potential of acrylamide formation ($R^2 = 0.0062$).

The high correlation between the potentials and the reducing sugars is primarily explained by the stronger variation and the lower values of the concentrations of the reducing sugars, while the asparagine contents were more stable and substantially higher. The small improvement of the correlation when considering the asparagine concentrations confirms this interpretation. It means that in practice fructose and glucose determine acrylamide formation, even though they just act as a mediator. This corresponds to the experience that the reducing sugars determine the browning and the flavor formation by the Maillard reaction [14].

The asparagine content did not correlate with acrylamide formation in French fries from the cultivar Eba [25]. Virtually the same strong correlation between the formula $(0.5 \times$ glucose + fructose) $\times$ asparagine and the potential of acrylamide formation was also observed in potato chips prepared from different cultivars [26]. Although sucrose may form acrylamide with asparagine [8] no correlation with the potential of acrylamide formation was observed ($R^2 = 0.0402$). Sucrose can contribute to non-enzymatic browning in model systems as well as in potato chips.
However, the far more efficient fructose and glucose seem to completely surpass the activity of sucrose. No correlation between the potentials of acrylamide formation and glutamine ($R^2 = 0.0375$) or dry matter ($R^2 = 0.0470$) was observed.

### 3.4.4. Influence of cultivars

For cultivars of which at least 3 samples have been analyzed, potentials of acrylamide formation are shown in Figure 16.

![Figure 16: Potential of acrylamide formation in different potato cultivars](image)

The cultivar Nicola had the highest potential of acrylamide formation (maximum, 2020 µg/kg), followed by Charlotte (maximum, 1700 µg/kg), while the cultivar Panda exhibited the lowest mean potential (80 µg/kg). The difference between the extremes corresponds to a factor of 28, reflecting the large differences in contents of reducing sugars.

The differences between cultivars Panda, Agria, Bintje and Eba did not turn out to be significant in univariate analysis of variance ($\alpha = 5\%$) and Tukey-HSD test. In contrast, Charlotte and Nicola were each significantly different from all other five cultivars. The glucose and fructose contents in Charlotte and Nicola were also significantly higher from the other four cultivars and significantly different from each other. Tukey-HSD test revealed no significant differences between Agria, Bintje and Panda in terms of glucose and fructose contents. Asparagine concentrations did not differ significantly in any cultivars tested.

The average age of the potatoes, defined as the time from harvest to analysis, was 97 days (72 to 113 days). The age neither correlated with sugars, nor with asparagine, glutamine or the potential of acrylamide formation.
3.4.5. Influence of the farming system

No influence of the farming system on glucose, fructose and asparagine concentrations was observed. Figure 17 shows asparagine concentrations of the cultivar Agria (data set with 21 samples) grown according to three different farming systems: organic (n = 6), conventional (n = 10) and integrated (n = 5). Statistical analysis revealed no significant differences.

![Figure 17: Asparagine concentrations in potatoes of the cultivar Agria from different farming systems (mean values; error bars are ± standard deviation).](image)

Nitrogen fertilization or the farming system did not significantly influence the potential of acrylamide formation, as shown in Figure 18 for the three farming systems and 57 samples of potato from 10 different cultivars. This is in agreement with the observation that the farming system did not significantly influence the contents of reducing sugars and asparagine. Thus the potential of acrylamide formation strongly depends on the cultivar, while cultivation technique only seems to have a marginal influence.
3.4.6. Conclusions

Data obtained from 74 different samples enable a first classification of 17 potato cultivars which are important in Switzerland with respect to their potential for forming acrylamide. Acrylamide formation in potatoes, determined as potentials at 120 °C, is proportional to the product of the concentrations of reducing sugars and asparagine. Sugar contents vary by a factor of 118 (single values) or 32 (average values for cultivars). The contents of asparagine are higher and vary far less, which explains why glucose and fructose were found to present the determining factor for acrylamide formation in potatoes.

Since there seems to be little possibility for varying the asparagine content, the reducing sugars are considered to be the components through which acrylamide formation can be reduced most efficiently. Neither the farming system nor the extent of nitrogen fertilization influenced the measured components and the potential of acrylamide formation which means that future efforts should focus on cultivar selection.

However, selection of cultivars only achieves this goal if at the same time storage temperatures below 8-10 °C are avoided in order to prevent substantial release of reducing sugars. In practice, optimization of cultivars and storage conditions are interdependent and many further criteria have to be met, which will need further research.

ACKNOWLEDGEMENT

We thank Cooperative Migros, COOP Switzerland and the Federation of Swiss Food Industries (FIAL) for financial support for this study.
3.5. Literature


4. Potential for acrylamide formation in potatoes: Data from the 2003 harvest

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4.1. Abstract

Reducing sugars, free amino acids, and the potential for acrylamide formation were determined in more than 50 potato samples from the 2003 harvest in Switzerland. The reducing sugar content strongly correlated with acrylamide, whereas no correlation was found between acrylamide and free asparagine or the pool of free amino acids. The content of reducing sugars and the acrylamide potentials were higher in most of the cultivars tested as compared to the samples from 2002. This result was probably due to the hot and dry summer in 2003. Monitoring sugars and amino acids during heating at 120 °C and 180 °C showed that glucose and fructose reacted much faster than sucrose and the amino acids. Glutamine was consumed to a larger extent than any other of the amino acids. During prolonged storage reducing sugars decreased considerably while changes in free amino acids were only moderate. Altogether, glucose and fructose remain the critical factors for acrylamide formation in potatoes and represent the most feasible way to reduce the acrylamide formation in potato products.

4.2. Introduction

Acrylamide is neurotoxic and classified as probably carcinogenic to humans (group 2A) by the IARC [1] and its detection in a broad range of heated foods with concentrations exceeding 1000 µg/kg [2] caused a world-wide concern. Acrylamide is formed concurrently to the Maillard reaction [3, 4] from a reducing sugar and the free amino acid asparagine which delivers the backbone for the acrylamide molecule [5, 6]. Since potatoes naturally contain large amounts of free asparagine and reducing sugars [7, 8] fried and baked potato products such as French fries, chips (American terminology), baked potatoes, and hash browns showed the highest acrylamide contents in foods, sometimes exceeding 3000 µg/kg [2, 9-11]. In order to decrease the acrylamide content of these products different approaches were made: Beside the optimization and strict control of the frying process [12], the addition of citric acid [13, 14], and the application of an asparaginase [6] were successful. However, in practice any technological effort to limit the acrylamide formation in heated potato products is in vain, if the raw material is not suitable. Glucose and fructose largely determine acrylamide formation in potatoes [7, 15] and an appropriate selection of the raw material in terms of reducing sugar content is crucial to reduce the acrylamide content [12, 16]. Thus, a detailed knowledge of the composition of the raw material and the influence of harvest and storage are of fundamental importance. The aim of the present study was to investigate potato samples of the 2003 harvest in Switzerland in terms of contents of reducing sugars, free amino acids, and the potential of acrylamide formation and to check the interrelations between these
parameters. Results from over 50 samples from 15 different cultivars were obtained and compared to the analogous study made in 2002 [7]. Furthermore, the changes of potato components during standardized heat treatments as well as during prolonged storage were monitored.

4.3. Material and methods

4.3.1. Collection of potato samples
Samples of cultivars Agria, Appell, Bintje, Charlotte, Desirée, Eba, Naturella, Nicola, Panda, and Santana were collected in Switzerland by the Swiss College of Agriculture (Zollikofen, Switzerland) from August to September 2003 according to a well defined sampling plan: 55 tubers from 55 plants were taken from each field, making up a total of about 5 kg per sample. The potatoes were part of a three year on-farm experiment with different farming systems (organic, integrated, conventional) focusing on quality aspects and monitoring all relevant data concerning crop rotation, cultivation technique, site parameters, and tuber quality. Samples of the cultivars Erntestolz, Hermes, Lady Claire, Markies, and Panda were obtained from Zweifel Pomy-Chips AG (Spreitenbach, Switzerland). In addition, some samples were purchased at local supermarkets (cultivars Agata, Appell, Bintje, Stella, Nicola, and Urgenta). All samples were stored at 9 °C and 95 - 98% relative humidity from harvest on until analysis (November and December 2003).

4.3.2. Sample preparation
At least 15 tubers of a given sample were washed, and after the water was dripped off, cut lengthwise. From each tuber, one half was grated. The grated material was thoroughly mixed and used for all analyses. For the determination of sugars and free amino acids 50.00 g of grated potato were homogenized (Polytron, Kinematica, Lucerne, Switzerland) with 100.00 g of deionized water.

4.3.3. Analysis of acrylamide
The potential of acrylamide formation was determined according to Biedermann et al. [9]: 20 g of grated potato was spread on a grid and heated in a GC oven at 120 °C for 40 min. After measuring residual weight (giving the dry matter), water (to a total weight of 20 g), and 500 µg/kg of internal standards were added. The internal standards were $^{13}$C$_3$-acrylamide (CIL, Andover, Massachusetts, USA) and methacrylamide (Fluka, Buchs, Switzerland), both dissolved in methanol (Fluka). Acrylamide was determined with a GC-MS method as described in [17]. GC-MS involved an 8000 series gas chromatograph with on-column injector (Fisons Instruments, Milan, Italy) coupled with a SSQ 710 quadrupole mass spectrometer (Finnigan Mat, San Jose, USA). The precolumn (TSP deactivated, i.d. 0.53 mm) and the separation column (BGB Wax, 12 m, i.d. 0.25 mm) were both from BGB Analytik (Böckten, Switzerland). GC and MS conditions were as described in [17]. Results were calculated as the potential of acrylamide formation referring to fresh weight.
4.3.4. Measurement of free amino acids

7.5 g of the potato slurry was weighed (under stirring) into a 100 mL flask, 1 mL of internal standard solution (DL-norleucine, 500 mg in 100 mL bi-distilled water, Fluka) and about 50 mL of 0.1 M HCl (Fluka) were added. 5 mL of Carrez I (150 g of \( K_4[Fe(CN)_6] \cdot 3H_2O \) per liter, Fluka) and Carrez II (300 g of \( ZnSO_4 \cdot 7H_2O \) per liter, Fluka) solutions were added and the mixture was intensively shaken. Foam was broken with 50 µL of 1-octanol (Fluka) and the volume adjusted to 100 mL with 0.1 M HCl (Fluka). After filtration (Schleicher & Schuell, Dassel, Germany), samples were diluted 1+4 with 0.16 M lithium citrate buffer (pH = 2.2, PVP physiological; Laborservice Onken, Gründau, Germany) and thoroughly mixed. Diluted samples were filtered through a 0.45 µm HPLC membrane filter (Titan; Infochroma, Zug, Switzerland) and subjected to analysis by cation-exchange chromatography followed by post-column derivatization with ninhydrin (Biochrom 30, Biochrom, Cambridge, UK) by using the physiological system (Biochrom) as described by the producer. Injection volume was 50 µL and quantification was done both by comparison with an external standard and the internal standard.

4.3.5. Determination of sugars

54 g of the potato slurry was weighed (under stirring) into a 250 mL flask and about 150 mL of deionized water was added. After Carrez clarification (5 mL of each Carrez I and II solution), foam was broken with a few drops of 1-octanol (Fluka), the pH adjusted to 7 with a few drops of KOH solution (4 mole/L, Fluka) and the volume adjusted to 250 mL with deionized water. After filtration (Schleicher & Schuell) fructose, glucose, and sucrose were determined enzymatically using the kit from Scil Diagnostics (Martinsried, Germany).

4.3.6. Statistical analysis

Statistical analysis was carried out using the software Microsoft Excel 2002 and NCSS 2001, Number cruncher statistical systems (Kaysville, Utah, USA).

4.4. Results and discussion

4.4.1. Tuber composition and acrylamide formation

Beside the known acrylamide precursors, i.e. glucose, fructose, and free asparagine, the whole pool of free amino acids was determined in all potato samples (Table 4). Repeated analysis of the same potato sample (cultivar Charlotte, n = 4) gave a mean acrylamide potential of 327 µg/kg with a relative standard deviation of 3.5% pointing out the good repeatability of this procedure and confirming that the variation of the procedure is much smaller than the variation between different potato samples.
Table 4: Potential of acrylamide formation, reducing sugars, and free amino acids in the analysed potato samples (mean values; relative standard deviation in brackets; samples obtained from supermarkets are not included. AA: acrylamide; Glc: glucose; Fru: fructose; Asn: asparagine; Gln: glutamine; Glu: glutamic acid; Asp: aspartic acid; TFAA: total free amino acids).
addition, the following free amino acids were detected: Alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, γ-amino butyric acid, histidine, and arginine.

Statistical analysis revealed only a significant difference between the acrylamide potentials of cultivar Eba and Panda. However, Nicola had a clearly higher potential than Panda, but due to the small number of samples (n = 3 for Nicola) it was not detected as significantly different. The situation was identical for glucose and fructose. The only statistically significant difference of the free asparagine content was found between cultivars Charlotte and Eba. More significant differences were found for aspartic acid: Eba contained least and was different from Charlotte, Bintje, and Panda (different from Eba and Agria). The pool of free amino acids was highest in cultivar Charlotte and significantly different from Eba and Agria. In 2003 many samples showed a “germination/shooting” which was probably due to the hot and dry summer. This phenomenon was strongest for Eba (significantly different from Panda, Nicola, Charlotte, and Agria), followed by Bintje (significantly different from Panda, Charlotte, and Agria) and Agria (significantly different from Bintje and Eba). This is probably a major cause for the elevated sugar contents in these three cultivars compared to the 2002 harvest [7, 22]. Cultivars used for the production of potato chips (particularly Lady Claire and Panda) were generally low in reducing sugars, but the low number of samples for these cultivars allowed no statistical distinction from other cultivars. However, it is well known that such cultivars are bred and selected for low sugar content.

Samples obtained from supermarkets contained generally more reducing sugars: The mean content was 6097 mg/kg (n = 6) with a maximum of 8542 mg/kg (cultivar Nicola). A sample of cultivar Bintje contained 6585 mg/kg reducing sugars which is far above the mean in Table 4. Free asparagine ranged from 2103 to 4470 mg/kg (mean 3174 mg/kg) which is within the range of the samples in Table 4. These results indicate that probably all the samples from the supermarkets had been stored at low temperatures which led to the well known phenomenon of “cold temperature sweetening” [21]. The effect of the elevated sugar contents on the potential of acrylamide formation was drastic: The potentials ranged from 1494 to 5585 µg/kg with a mean of 3737 µg/kg which is high with respect to the values shown in Table 4. Thus, commercial storage for the potatoes of the 2004 harvest must be adapted in order to limit the release of reducing sugars during storage.

All measured components were checked for correlations to the acrylamide potential (data from Table 4 only). Figure 19 shows the strong correlation between reducing sugars and the acrylamide potential. Glucose and fructose obviously determine the acrylamide formation in potatoes which was also shown in other studies [7, 15, 16, 23]. The following components correlated also strongly with the potential of acrylamide formation: Fructose (R² = 0.9404), glucose (R² = 0.9245), and the formula (0.5•glucose + fructose)•asparagine (R² = 0.9211). The content of free asparagine (R² = 0.0085), free glutamine (R² = 0.0043), free glutamic acid (R² = 0.0800), free aspartic acid (R² = 0.1792), total free amino acids (R² = 0.0057), the contribution of each of these free amino acids to the pool of free amino acids (R² < 0.083), the concentration of any minor free amino acid (R² < 0.14), and dry matter (R² = 0.1195) did not correlate with acrylamide formation. Glucose strongly correlated with fructose (R² = 0.9523) as seen in other studies [7, 8, 15].
Overall, these results show that the reducing sugars determine acrylamide formation. There is no general correlation between acrylamide and any of the free amino acids which fully confirms the results from the study of the 2002 harvest [7]. Combination of the data from 2002 and 2003 showed no correlation between N-fertilization and acrylamide potential ($R^2 = 0.038$), free asparagine, glucose, or fructose, which was also found for the 2003 data alone. In fact, cultivar and climate have been shown to be much more important for reducing sugars than any kind of fertilization [24]. Asparagine is much more abundant than glucose and fructose (Table 4), reacts much slower than these sugars (see results below) and is therefore not limiting - in fact the correlation between reducing sugars and the acrylamide potential turned out to be stronger if free asparagine was not taken into account. Data from French fries [15] showed no correlation between the acrylamide content and the concentration of free asparagine of the raw potatoes ($R^2 = 0.0001$), whereas acrylamide correlated with reducing sugars ($R^2 = 0.8182$) which corroborates our findings.

### 4.4.2. Comparison of the harvests in 2002 and 2003

The data obtained from the harvest 2003 were compared with those from 2002 [7], and the reducing sugar contents of the most important cultivars from both years are shown in Figure 20. In Switzerland as on the European continent in general [25], the summer of 2003 was extraordinary hot: The mean temperature of the summer months was over 5 standard deviations higher than the average of the period 1864-2000 and thus was the hottest summer in the past 500 years [26]. Mean day temperature in August 2003 was 5 °C higher compared to August 2002 and similar differences were recorded for other summer months in Switzerland [27, 28]. Potatoes from 2003 showed strongly elevated sugar contents, and the differences between the cultivars were less distinct than in 2002.
Figure 20: Concentrations of reducing sugars in different potato cultivars from the 2002 and 2003 harvest (error bars are ± standard deviation; n for 2003 see Table 4; n for 2002: Agria: n = 22, Bintje: n = 12, Charlotte: n = 8, Eba: n = 8, Nicola: n = 3, Panda: n = 4).

Charlotte showed a lower sugar content in 2003 due to a cooler storage temperature of the Charlotte samples in 2002 [7]. The differences for cultivar Nicola probably reflect just the common variation. The strongest increase in reducing sugars was found in cultivars Eba (+ 165%), Bintje (+ 146%), and Agria (+ 113%) and were all significant. These cultivars showed the most pronounced sprouting and the formation of a second tuber generation before harvest. Bintje and Eba are known to be particularly sensitive to stress like heat and drought [22]. The pronounced increase of reducing sugars in these three cultivars can be explained that way. Panda showed still low sugar contents, while they were again very high in cultivar Nicola as observed in 2002 [7]. The potential for acrylamide formation in 2003 showed exactly the same pattern because it strongly correlated with the average content of reducing sugars ($R^2 = 0.9787$).

No significant difference in the content of free asparagine was found between the two harvests. This shows that reducing sugars are much more influenced by cultivars and season, whereas free asparagine is relatively stable. The extraordinary hot and dry climate in 2003 was probably a major cause for the elevated levels of reducing sugars and, as a consequence, for the increased acrylamide potentials in some potato cultivars of the 2003 harvest. Climate, in particular temperature, has a strong influence on sugar metabolism in potatoes: Reducing sugars can vary by more than a factor of 4 for a given cultivar between different harvests [24]. High temperature (29 °C) decreased tuber growth [29] and led to a stronger incorporation of $^{14}$C into sucrose and less incorporation into starch [30]. Callus tissue of cultivar Russet Burbank was shown to contain more reducing sugars after 8 weeks at 30 °C as compared to 20 °C [31]. Thus, the climatic situation, harvest conditions, and storage must be taken into account if a reduction of acrylamide in potato products is being demanded. Since the year-to-year variability of the climate is expected to increase in the future [26] this could make the provision of potatoes with low sugar content more difficult.
4.4.3. Changes of potato components during prolonged storage

The changes in tuber composition during prolonged storage were monitored in three samples (cultivars Agria, Charlotte, and Bintje). Figure 21 shows that reducing sugars in the sample of cultivar Agria decreased steadily in the first 180 days of storage, while asparagine did not change a lot which was also reported in other studies [8, 20]. The changes in reducing sugar strongly correlated with the acrylamide potential \( R^2 = 0.9557 \). In the other two cultivars sugars decreased in a similar way, but asparagine and total free amino acids both increased after about 180 days which could be due to a proteinase activity [20]. The decrease of reducing sugars during storage at 8 °C is a clear advantage in terms of acrylamide. However, after approximately 150 days all samples started to sprout which is a disadvantage. To clarify the effect of storage at elevated temperatures (≥ 8 °C) in depth several projects are currently ongoing in Switzerland to check the suitability of the available cultivars for this storage technique [32]. Storage at 8 °C of suitable potato cultivars, eventually combined with the application of sprout inhibitors, might be a way to reduce the acrylamide content of fried or roasted potato products through the supply of raw material with low sugar content.

![Figure 21: Changes in tuber components in a sample of cultivar Agria during prolonged storage. (diamonds: glucose; squares: fructose; crosses: free asparagine; concentrations referring to fresh weight).](image)

4.4.4. Changes of potato components during the determination of the potential for acrylamide formation

Acrylamide, sugars and free amino acids in a potato sample of cultivar Bintje heated to 120 °C for 40 min were determined to monitor their reactivity and consumption. Glucose reacted slightly faster than fructose (Figure 22). Both reducing sugars had disappeared almost completely after 40 min, whereas sucrose did not react as expected which explains why there is no correlation between the sucrose content and the potential of acrylamide formation [7]. The apparent increase of the sucrose
content could be due to inhomogeneity in the sample or to a slight overestimation during the enzymatic determination because of the very low glucose content.

Figure 22: Changes in sugar contents and acrylamide formation during the determination of the potential for acrylamide formation (40 min at 120 °C. triangles: acrylamide; diamonds: glucose; squares: fructose; circles: sucrose; concentrations referring to fresh weight).

The changes of the free amino acids are shown in Figure 23. After 40 min 74% of the free asparagine was still present. Asparagine obviously reacts much slower than the reducing sugars (Figure 22), and only a small part of it is consumed during the heat treatment. The glutamine content decreased to a larger extent than any of the free amino acids: After 40 min only 40% of the initial content was found and its proportion within the amino acid pool was significantly smaller compared to the other free amino acids. Threonine, serine, isoleucine, tyrosine, phenylalanine, γ-amino butyric acid, and proline behaved similar to asparagine, and they all decreased by approximately 30%. In the first 10 min virtually no acrylamide was formed, but afterwards the acrylamide content increased in an exponential manner reaching 718 µg/kg after 40 min. A similar course of the acrylamide concentration in grated potato was described by Biedermann et al. [9].
In addition to the standard heat treatment, one experiment with heating at 180 °C for 20 min was carried out and samples were taken every 5 min. Figure 24 shows the course of the sugar and acrylamide concentrations. After 10 min more acrylamide was formed than after 20 min at 120 °C. Subsequently the increase was less pronounced which might be due to enhanced elimination of acrylamide or lack of reducing sugars. Glucose and fructose were depleted after 10 min and in contrast to heating at 120 °C; sucrose was consumed as well, but much slower than glucose and fructose: After 20 min still 61% of the initial sucrose content was found.
Thus, sucrose might be of some importance for acrylamide formation in strongly fried and roasted potato products. In fact, sucrose was suggested to contribute to nonenzymatic browning in potato chips [33], correlated with the acrylamide content of French fries [15], and it formed some acrylamide when pyrolyzed with asparagine [4]. However, the higher reactivity of glucose and fructose surpasses the influence of sucrose, and the reducing sugars remain the key factor for acrylamide formation in potato products.

At 180 °C the changes in free amino acids were more pronounced than at 120 °C (Figure 25). After 20 min, only 4% of the initial amount of asparagine was found, while glutamine was completely depleted already after 10 min confirming its higher reactivity. Asparagine and glutamic acid behaved similar: In the first 10 min about 85% was consumed, and after 20 min they were depleted. Aspartic acid, leucine, isoleucine, tyrosine, and proline were consumed to a considerably smaller amount: After 20 min at 180 °C 20 to 30% of their initial content was still present.

![Figure 25: Changes of free amino acids during heating at 180 °C for 20 min](crosses: asparagine; triangles: aspartic acid; plus: glutamic acid; squares: glutamine)

Aspartic acid and isoleucine are known to react much slower with carbonyls than other amino acids [34] which explains part of this observation. Glutamine was shown to be completely deamidated at 110 °C after 2 h, whereas glutamic acid, aspartic acid, and asparagine were stable which explains the large losses of glutamine during both heat treatments [35]. At 180 °C ammonia is also released from asparagine and aspartic acid [35]. The release of ammonia from free amino acids could be an important factor for the acrylamide formation in heated potato products because ammonia strongly enhances acrylamide formation [36]. However, no correlation was found between any of the free amino acids or the pool of free amino acids with the potential of acrylamide formation, and reducing sugars turned out to be the key factor for acrylamide formation in potato products as shown in various studies as well [7, 9, 15, 16].
ACKNOWLEDGEMENT
We thank Zweifel Pomy-Chips AG, Spreitenbach, Switzerland for supplying potato samples. Financial support was provided by the Swiss Federal Office for Public Health (BAG), the Federation of Swiss Food Industries (FIAL), COOP Switzerland, and Cooperative Migros.

4.5. Literature


5. Acrylamide in gingerbread: Critical factors for formation and possible ways for reduction


5.1. Abstract

The influence of ingredients, additives, and process conditions on acrylamide formation in gingerbread was investigated. The sources for reducing sugars and free asparagine were identified and the effect of different baking agents on acrylamide formation was evaluated. Ammonium hydrogencarbonate strongly enhanced acrylamide formation, but its N-atom was not incorporated into acrylamide, nor did acrylic acid form acrylamide in gingerbread. Acrylamide concentration and browning intensity increased both with baking time and correlated with each other. The use of sodium hydrogencarbonate as baking agent reduced the acrylamide concentration by more than 60%. Free asparagine was a limiting factor for acrylamide formation, but the acrylamide content could also be lowered by replacing reducing sugars with sucrose or by adding organic acids. It is concluded that a significant reduction of acrylamide in gingerbread can be achieved by using sodium hydrogencarbonate as baking agent, minimizing free asparagine, and avoiding prolonged baking.

5.2. Introduction

The detection of acrylamide in heated foodstuffs [1] led to a world-wide concern because acrylamide is a known neurotoxin and is classified as “probably carcinogenic to humans” (group 2A) by the IARC [2]. Particularly high concentrations exceeding 1000 µg/kg were found in heated potato products such as French fries and potato crisps [1, 3]. It was shown that acrylamide is formed in the Maillard reaction [4, 5]. A decrease of the acrylamide concentration at temperatures above 170 °C [4] pointed to the concurrent elimination of acrylamide which was first monitored in potatoes [6].

Asparagine is considered to be the main precursor for acrylamide formation in foods as shown with 15N-labeled asparagine [7]. Zyzak et al. provided evidence that asparagine delivers the backbone of the acrylamide molecule, whereas glucose is not incorporated into acrylamide [8]. However, a reducing sugar is needed for the formation of the Schiff’s base of asparagine which is transformed via an oxazolidin-5-one intermediate to a decarboxylated Amadori product that releases acrylamide [9]. In analogy to the formation of acrylamide from asparagine, the release of vinylogous compounds from other amino acids was assumed. Stadler et al. demonstrated that acrylic acid is formed in pyrolyssates of aspartic acid and sugars [10].

Gingerbread may contain up to 1000 µg of acrylamide per kg fresh weight. In Germany, acrylamide contents in gingerbread ranged from < 20 µg/kg to more than 8000 µg/kg with an average value of 481 µg/kg and a median of 231 µg/kg [11]. Konings et al. [12] measured acrylamide in Dutch gingerbread products ranging from 260 to 1410 µg/kg (average 890 µg/kg; median 1070 µg/kg). Since gingerbread is
consumed frequently and all over the year in the Netherlands, these products were estimated to contribute 16% of the total acrylamide exposure of the Dutch population [12]. In our laboratory preliminary analyses of typical Swiss gingerbread of the 2003 Christmas season showed acrylamide contents from 100 to 800 µg/kg. These relatively high concentrations are in contrast to the low content of free asparagine in cereal flours: In wheat flour free asparagine was determined in the range of 70 to 300 mg/kg [13, 14]. In comparison, potatoes contain up to 4200 mg of free asparagine per kg [15]. Therefore the question of an alternative mechanism of acrylamide formation in gingerbread was raised. It was shown that ammonium hydrogen carbonate (i.e. the typical baking agent for gingerbread in Switzerland) strongly enhanced acrylamide formation in a model system for bakery products [16] and that acrylic acid and ammonia form high levels of acrylamide in browning model systems [17]. Since heating of aspartic acid and glucose can produce acrylic acid [10], a mechanism other than the thermal degradation of asparagine might contribute to the high acrylamide levels in gingerbread.

The aim of the present investigation was to test the hypothesis if ammonia, originating from the baking agent, is incorporated into acrylamide, presumably via reaction with acrylic acid, and to find ways to reduce the acrylamide content in gingerbread. The influence of the type and the amount of baking agent and other additives on acrylamide formation was investigated. The paper reports results from more than 180 experiments with different gingerbreads produced in our pilot plant. The critical factors for the acrylamide formation in gingerbread are identified and discussed. Several possible ways to achieve a significant reduction of the acrylamide content in gingerbread are suggested.

5.3. Material and methods

5.3.1. Preparation of gingerbreads

All ingredients were obtained from JOWA AG (Volketswil, Switzerland), a Swiss producer of gingerbread. The gingerbread dough was prepared with flour (a 70/30 mixture of spelt and wheat, corresponding to a flour type 720), inverted sugar syrup, powdered sugar (sucrose), honey (American origin), water, spices, caramel coloring, whole egg, ammonium hydrogen carbonate (baking agent), and lezirol (100 g/L in water, containing milk protein, an acid regulator, lecithin, vegetable oil, and β-carotene). For the experiments with 15N-labeled baking agent a mixture of 15N-ammonium sulfate (CIL, Andover, Massachusetts, USA) and sodium hydrogen carbonate (Fluka, Buchs, Switzerland) was used. This mixture contained the same amounts of ammonium and hydrogen carbonate ions as the normal baking agent. Several other ingredients were used in addition to the original recipe: L-asparagine, L-lysine, glycine, L-cysteine, L-aspartic acid, citric acid, tartaric acid, and sodium hydrogen carbonate, all from Fluka. Asparaginase (from E. coli) was purchased from Fluka and Sigma-Aldrich (Steinheim, Germany), diluted with distilled water to a volume of 1.5 mL, and directly added in small portions to the dough during kneading (4 units per kg dough). Preparation of gingerbreads was performed as close to the industrial process as possible. The ingredients were mixed according to the prescription obtained from JOWA AG in a Z-kneader (Farinograph; Brabender, Duisburg, Germany, batch size 300 g dough). The dough was left at room temperature for 24 hours. After a short reworking, the dough was sheeted to 7 mm
thickness, cut into pieces of about 50 g (5 cm x 10 cm), glazed (mixture of cracked egg, sucrose and salt), and then baked in a programmed oven (Schaubackofen Thermody B 160c; Pitec, Oberriet, Switzerland) in a two step program at 180 °C for 3 min and at 190 °C for 7 min (standard conditions). These conditions gave a product very similar in terms of color, taste, volume, dry matter, and acrylamide to the product prepared by the industry. Immediately after baking, the gingerbreads were glazed with lezirol solution. Product temperature during baking was measured by inserting a temperature sensor (T51; Rotronic, Bassersdorf, Switzerland) in the center of the gingerbread and was recorded every five seconds with a computer.

5.3.2. Measurement of color, dry matter, and pH
Color was measured at three different positions on top of the gingerbread using an optical L*,a*,b*-analyzer (Chroma-Meter Cr-300; Konica Minolta Photo Imaging, Dietikon, Switzerland). For determination of the dry matter gingerbreads were cut and homogenized with a household-cutter (Moulinette; Moulinex, Paris, France), dried in an oven at 104 °C for 5 hours (in triplicate), and dry matter was determined gravimetrically. The pH was measured according to the method of the gingerbread producer: 5.00 g of homogenized gingerbread was mixed with 45.00 g of bidistilled water and the pH was determined in the slurry under stirring.

5.3.3. Determination of sugars
Glucose, fructose, and sucrose were determined enzymatically using the test kit from Scil Diagnostics (Martinsried, Germany). An appropriate amount of ingredient was weighed into a 100 mL flask and about 60 mL bidistilled water was added. After homogenization (Polytron; Kinematica, Lucerne, Switzerland) 5 mL of each Carrez 1 (150 g of potassium hexacyanoferrate(II) trihydrate per liter; Fluka) and Carrez 2 solution (300 g zinc sulfate heptahydrate per liter; Fluka) were added. The mixture was thoroughly shaken, the foam broken with a few drops of 1-octanol (Fluka) and the volume adjusted to 100 mL with bidistilled water. Filtered samples (Schleicher&Schuell, Dassel, Germany) were subjected to enzymatic analysis using the hexokinase/glucose-6-phosphate-dehydrogenase assay as described by the producer.

5.3.4. Measurement of free asparagine
An appropriate amount of the sample (generally 10 g) was weighed into a 100 mL flask and mixed (Polytron) with about 60 mL aqueous solution of trifluoroacetic acid (1 g/L; Fluka). 5 mL of both Carrez 1 and 2 solutions were added and the mixture intensively shaken. Foam was broken by a few drops of 1-octanol and the volume adjusted to 100 mL with trifluoroacetic acid solution. After filtration (Schleicher&Schuell) samples were diluted 1+1 with 0.16 M lithium citrate buffer (pH = 2.2, PVP physiological; Laborservice Onken, Gründau, Germany) and thoroughly mixed. Diluted samples were filtered through a 0.45 µm HPLC membrane filter (Titan; Infochroma, Zug, Switzerland) and subjected to analysis by cation-exchange chromatography followed by post-column derivatization with ninhydrin.
5.3.5. **Analysis of acrylamide**

Two whole gingerbreads, baked under the same conditions, were cut into small pieces and homogenized in a household cutter (Moulinex). Acrylamide was analyzed as described by Biedermann et al. [19]: To 10 g of sample and 30 g tap water, the internal standards (\(^{13}\text{C}_3\)-acrylamide in acetonitrile, CIL; methacrylamide, Fluka), were added in a concentration of 500 µg standard per kg sample. The mixture was intensively homogenized (Polytron). After swelling in a water bath at 70 °C for 30 min, 10 g of sample was extracted with 40 mL of 1-propanol (Fluka). After settling of the solids, 10 mL of the clear supernatant was subjected to azeotropic evaporation. Acrylamide was extracted from the residue with 3 mL acetonitrile (Fluka) and twice defatted with hexane (Fluka). To determine the overall yield of sample preparation, 5 µL of butyramide solution (25 µg/mL in acetonitrile; Fluka) was added to 1.5 mL of defatted acetonitrile extract which was then analyzed by GC-MS. GC-MS involved a 8000 series gas chromatograph with on-column injector (Fisons Instruments, Milan, Italy) coupled with a SSQ 710 quadrupole mass spectrometer (Finnigan Mat, San Jose, USA). The precolumn (TSP deactivated, i.d. 0.53 mm) and the separation column (BGB Wax, 12 m, i.d. 0.25 mm) were both from BGB Analytik (Böckten, Switzerland). GC conditions were as described in [19]. Mass spectrometry with positive chemical ionization monitored the ions \(m/z = 72\) (acrylamide), \(m/z = 75\) (\(^{13}\text{C}_3\)-acrylamide), \(m/z = 86\) (methacrylamide), and \(m/z = 88\) (butyramide). Results were calculated as acrylamide concentration in fresh gingerbread without corrections relative to dry matter. If the overall yield was less than 40%, the analysis was repeated from the evaporation step.

5.4. **Results and discussion**

5.4.1. **Influence of the baking agent**

To investigate the influence of ammonium hydrogencarbonate on acrylamide formation in gingerbread, this baking agent was added in different amounts (normal amount: 0.8 g/100 g dough) to the dough and samples were baked under standard conditions. Acrylamide concentrations are shown in Figure 26. Gingerbread prepared according to the recipe contained 501 µg acrylamide per kg with a relative standard deviation of 16% (n = 9). This is well within the range of reported acrylamide levels in gingerbread products [11, 12]. The amount of ammonium hydrogencarbonate had a very strong influence on the acrylamide formation: In its absence almost no acrylamide was formed (about 10 µg/kg), but the product was unsatisfactory because it lacked browning and leavening. When 0.4 g NH\(_4\)HCO\(_3\) /100 g were added, the acrylamide content decreased to one third (170 µg/kg) and the color was too bright, whereas 1.6 g/100 g led to a strong increase in the acrylamide content (880 µg/kg) and enhanced browning. Apparently, ammonium hydrogencarbonate strongly promotes the formation of acrylamide in gingerbread which was also shown in different model systems for bakery products [16, 20]. The amount of added
ammonium hydrogencarbonate correlated with pH and color (L-value): The more baking agent was added, the higher the pH and the darker the product was. Ammonia boosts the browning from the Maillard reaction which was also shown by Izzo and Ho [21].

Figure 26: Acrylamide content in gingerbreads produced with different amounts of ammonium hydrogencarbonate as baking agent (error bars are ± standard deviation, n = 2, except for 1.6 g/100 g).

To check if the promotion of acrylamide formation was due to the incorporation of ammonia into acrylamide and therefore due to an alternative mechanism, gingerbread with $^{15}$N-labeled baking agent ($^{15}$NH$_4$)$_2$SO$_4$ + NaHCO$_3$) was produced. In a control experiment with unlabeled ammonium sulfate and sodium hydrogencarbonate, a normal product with slightly increased acrylamide content was obtained. Gingerbreads with the labeled baking agent were baked under standard (3 min at 180 °C, and 7 min at 190 °C) and more drastic conditions (12 min at 200 °C) to enhance temperature dependent effects. No $^{15}$N-acrylamide was detected in any of the products. Addition of L-aspartic acid (1000 mg/kg), which is a precursor of acrylic acid [10], or acrylic acid (1000 mg/kg) resulted in no detectable $^{15}$N-acrylamide in gingerbread prepared with labeled baking agent. In standard samples and samples with added L-aspartic acid, only trace amounts of acrylic acid were detected. Addition of L-aspartic acid or acrylic acid to normal dough (each time 1000 mg/kg) did not lead to a significant increase in the acrylamide content: 551 and 573 µg/kg, respectively (the standard product of same batch contained 562 µg/kg). Thus, the N-atom from the baking agent is not incorporated into acrylamide and the amidation of acrylic acid by ammonia from the baking agent does not contribute to the high acrylamide content in gingerbread. A mechanism for acrylamide formation other than by thermal degradation of asparagine is unlikely in gingerbread. However, formation of acrylic acid from aspartic acid, and reaction of ammonia with acrylic acid leading to acrylamide was shown in model systems [10, 17]. Formation of acrylic acid from aspartic acid and glucose or fructose started at 150 °C [10], and reaction of ammonia with acrylic acid was performed at 180 °C for 30 min [17]. For the amidation of aliphatic acids by ammonia heating at 160 °C for 5 h is needed for a yield of about
Measurement of the temperature within gingerbreads during the baking process showed that the temperature stayed below 100 °C in the first 6 min due to water evaporation and did not exceed 110 °C until the end of the baking process (Figure 27). This demonstrates why the formation of acrylamide via amidation of acrylic acid is unlikely to occur in gingerbread. A possible explanation for the promoting effect of ammonium hydrogencarbonate on the acrylamide formation could be the reaction of asparagine with reactive carbonyls. Glyoxal and methylglyoxal are formed from glucose and fructose in Maillard reaction models [23] and have been shown to react faster with amino acids than glucose or fructose do [24]. Beside these compounds many other α-dicarbonyls and α-hydroxy-carbonyls are formed from reducing sugars in the Maillard reaction [25] and the sum of these reactive carbonyls might be responsible for the high yield of acrylamide. This hypothesis is supported by the finding that glyoxal and glyceraldehyde formed more acrylamide from asparagine than glucose did [8]. Thus, the promoting effect of ammonium hydrogencarbonate on the formation of acrylamide might be indirect by providing more reactive carbonyls originating from the reaction of ammonia with reducing sugars. The fact that almost no acrylamide is formed in gingerbread prepared without NH₄HCO₃ corroborates this hypothesis.

![Figure 27: Progression of the temperature within a gingerbread during the baking process (3 min 180 °C, 7 min 190 °C; three independent determinations).](image)

The influence of sodium hydrogencarbonate which is an alternative baking agent was also investigated (Figure 28). Its application reduced the acrylamide content to one third, while only 1.67 g NaHCO₃ led to a product with a color comparable to that of the standard product (L-value = 47.3): L-values were 45.1 (1.67 g NaHCO₃ /100 g) and 51.3 (0.83 g NaHCO₃ /100 g). However, the pH was significantly higher in the samples with sodium hydrogencarbonate: 8.2 and 8.8, respectively, when compared to the standard product (pH = 6.9), and the product had an alkaline taste. This sensory taint could be compensated by adding some citric or tartaric acid which might reduce the acrylamide formation even more as confirmed by preliminary experiments (results not shown). Sodium hydrogencarbonate allows the preparation of gingerbreads with a substantially lower acrylamide concentration, acceptable
browning and sensory properties (taste, volume) and is therefore a valuable alternative baking agent. These results also show that a more alkaline pH does not necessarily imply higher acrylamide content in gingerbread. Other factors such as the presence of ammonia have a stronger impact.

![Acrylamide contents in gingerbread with different baking agents](image)

**Figure 28: Acrylamide contents in gingerbread with different baking agents** (error bars are ± standard deviation; n = 9 for NH₄HCO₃; n = 2 for NaHCO₃).

### 5.4.2. Sources of sugars and free asparagine

The precursors for acrylamide formation in foods were determined in all of the ingredients, and their contribution to the total amount in the gingerbread dough was calculated (Table 5). The method for the determination of free asparagine was verified in a small inter-laboratory comparison: The flour lot was analysed by four laboratories with four methods (different extraction, separation and detection techniques; internal and external standard for quantification). The average concentration of free asparagine was determined to be 132 mg/kg with a relative standard deviation of 9.3% (n = 4). Flour was clearly the main source for free asparagine in the gingerbread dough. Honey contributed less than 10% of the free asparagine. The spice mixture contained almost twice as much free asparagine as the flour, but contributed only a marginal part. In contrast to free asparagine, the contribution to reducing sugars from the flour was negligible. The inverted sugar syrup, honey, and caramel coloring contributed together 99% of the total amount of glucose and fructose and outnumbered the part from the flour by far. Interestingly, honey was a source for all of the compounds that are involved in acrylamide formation. In literature very similar values are reported: Sporns et al. found about 37 g fructose, 33 g glucose, and 0 to 5.5 g sucrose per 100 g in various honeys [26]. Speer and Montag reported concentrations of free asparagine in the range of 3.6 to 81.8 mg/kg in different honeys [27]. In hard red spring wheat varieties approximately 0.02 g fructose, 0.04 to 0.07 g glucose, and 0.1 to 0.3 g sucrose per 100 g were found [28]. Concentrations of free asparagine in wheat are reported in the range of 69 to 297 mg/kg [13, 14]. The concentration of free amino acids and sugars in cereal
flours increases with a higher degree of extraction during milling [29]. Our values are all well in the range of those cited in literature.

Table 5: Concentrations of sugars and free asparagine in the ingredients of gingerbread (referring to fresh weight; n = 2; -: not detected).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Glucose [g/100g]</th>
<th>Fructose [g/100g]</th>
<th>Sucrose [g/100g]</th>
<th>Free asparagine [mg/kg]</th>
<th>Contribution to total glucose and fructose [%]</th>
<th>Contribution to total free asparagine [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flour</td>
<td>0.03</td>
<td>0.03</td>
<td>0.44</td>
<td>139</td>
<td>0.2</td>
<td>87.5</td>
</tr>
<tr>
<td>Inverted sugar syrup</td>
<td>34.52</td>
<td>31.74</td>
<td>4.54</td>
<td>-</td>
<td>52.3</td>
<td>-</td>
</tr>
<tr>
<td>Powdered sugar</td>
<td>-</td>
<td>-</td>
<td>96.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Honey</td>
<td>30.69</td>
<td>39.66</td>
<td>3.37</td>
<td>64</td>
<td>38.8</td>
<td>8.5</td>
</tr>
<tr>
<td>Caramel coloring</td>
<td>31.71</td>
<td>23.77</td>
<td>5.79</td>
<td>212</td>
<td>0.1</td>
<td>3.2</td>
</tr>
<tr>
<td>Spices</td>
<td>0.77</td>
<td>0.81</td>
<td>0.57</td>
<td>31</td>
<td>0.1</td>
<td>0.7</td>
</tr>
<tr>
<td>Whole egg</td>
<td>0.39</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

In one experiment, honey, inverted sugar syrup, and caramel coloring were replaced by sucrose solutions containing the same amount of sucrose instead of glucose and fructose. This virtual depletion of reducing sugars resulted in a reduction of the acrylamide content by a factor of 20 (acrylamide content 25 µg/kg), but also in insufficiently browned products. The lack of reactive carbonyls obviously led to a strong decrease of the acrylamide formation and the Maillard reaction in general which is in accordance with literature [8, 9, 16, 25, 30]. “White gingerbread” is a specialty in some regions of Switzerland, prepared with NH₄HCO₃ and sucrose instead of honey and inverted sugar syrup, and baked at 230 °C for 15 min. It contains only little acrylamide (< 10 µg/kg). Ammonium hydrogen carbonate is thus only a critical factor if reducing sugars are present, its promoting effect is not related to free asparagine, and sucrose does not contribute to the formation of acrylamide [15, 16].

One kg of fresh gingerbread dough contains about 80 mg of free asparagine, and after standard baking 500 µg acrylamide per kg is found. This corresponds to a yield of 0.6% acrylamide based on free asparagine which is higher than the usually reported yields of about 0.1% [4, 7, 17, 31]. Biedermann and Grob found even higher yields (3.5 and 4.8%) in model systems consisting of wheat flour, fructose, and ammonium carbonate [16]. These high yields are probably due to the high molar ratio of fructose/asparagine of 6, whereas in our study this ratio was almost 100 times larger. Thus, free asparagine is a limiting factor and its addition to the flour before preparing the dough led to drastic increases in the acrylamide contents of baked gingerbreads (Figure 29).
Addition of only 250 mg asparagine to 1 kg dough resulted in a four times higher acrylamide content. When 1000 mg/kg were added, the acrylamide concentrations rose to > 8000 µg/kg, whereas the color of the product was only somewhat darker as on standard gingerbread. This shows that free asparagine largely determines the acrylamide formation in gingerbread. Consequently, the decomposition of free asparagine prior to baking is supposed to result in a decrease in acrylamide formation. This hypothesis was tested by adding an asparaginase during dough preparation in order to hydrolyze the amide group of asparagine. Gingerbreads from this dough contained 228 µg acrylamide per kg (average of three independent experiments) which corresponds to a decrease of 55% of the normal acrylamide content. Taste and color were virtually identical to the standard product which is a clear advantage of this approach to reduce acrylamide. Analysis of the fresh dough treated with asparaginase revealed that it still contained 22 mg/kg free asparagine. Therefore about 75% of the total free asparagine had been degraded which explains why acrylamide formation was not fully inhibited. The incomplete hydrolysis was probably due to the limited mobility of the enzyme and the substrate within the dough. These results indicate that a significant reduction of the acrylamide content could be achieved by choosing ingredients with a lower content of free asparagine, or by applying an asparaginase. It was shown that incubation of a potato matrix with an asparaginase can effectively reduce the acrylamide content [8]. Thus, the application of this enzyme to different food matrices prior to heating should be further investigated.

5.4.3. Influence of process conditions

Gingerbreads were baked under various temperature-time combinations to investigate the influence of temperature and time on acrylamide formation. Figure 30 shows the acrylamide concentrations measured during baking at 180 °C and 200 °C. Although the temperature stayed below 100 °C in the first 6 min (see Figure 27),
some acrylamide was already formed in this period. Even in the raw dough traces of acrylamide were detected. It seems that the presence of ammonia allows the formation of acrylamide at temperatures <100 °C. Acrylamide formation at 60 °C and even at room temperature was reported in various model systems, provided that ammonium carbonate and fructose were present [16]. The acrylamide concentrations increased steadily in the first 20 min of the baking process. A linear rather than an exponential correlation between acrylamide concentration and time could be assumed. In contrast to French fries, where most of the acrylamide is formed in the last minute of the frying process [32], acrylamide was formed almost evenly and over a broader period of the baking process. At 200 °C the acrylamide contents were slightly higher than at 180 °C during the first 15 min. During baking at 180 °C the L-value and the acrylamide content were strongly correlated ($R^2 = 0.9474$): The darker the product was, the higher the acrylamide concentrations. The extension of the baking process beyond the necessary time (10 min) resulted in a further increase of the acrylamide content. Thus, prolonged baking or excessive browning should be avoided in order to minimize the acrylamide content.

Figure 30: Influence of temperature and time on the acrylamide formation during baking (squares: 180 °C, triangles: 200 °C).

A lower temperature combined with a prolonged baking time did not result in lower acrylamide contents if the same browning of the product was to be achieved. It was generally observed that a prolonged baking at lower temperatures resulted even in higher acrylamide content. For instance gingerbread baked at 160 °C for 20 min exhibited the same color as a sample prepared at 200 °C for 10 min, but the acrylamide contents were 910 and 440 µg/kg, respectively. Thus, a shorter baking at higher temperatures is more suitable to contain the formation of acrylamide in gingerbread. At all temperatures tested (160 °C, 180 °C, and 200 °C) the acrylamide content decreased after a baking time of 20 min pointing to some elimination of acrylamide. To determine the extent of elimination, $^{13}$C$_3$-acrylamide (500 µg/kg) was added to the dough during mixing, and gingerbreads were baked at 180 °C for different lengths of time. Analysis was performed by using only methacrylamide as internal standard. Figure 31 shows that the elimination of acrylamide took place from
the first minutes on, but its extent was limited: Only about one third of the added $^{13}$C$_3$-acrylamide was eliminated after 10 min, and after 28 min about 50% were still present. The elimination of acrylamide is of no practical importance because baking exceeding 10 min results in unacceptably dark and dry products with high acrylamide contents.

Dry matter content increased steadily during baking and reached about 85% after the standard baking process. Dry matter and acrylamide content both increased steadily during baking (e.g. at 180 °C) and were strongly correlated with each other ($R^2 = 0.9874$). However, addition of some extra water (5 g/100 g dough) during preparation of the dough led to a 25% increase in acrylamide. This shows that the observed correlation between dry matter and acrylamide content is rather coincidental than causal.

![Figure 31: Elimination of $^{13}$C$_3$-acrylamide in gingerbread during baking at 180 °C.](image)

5.4.4. Organic acids to reduce the acrylamide content

Various experiments with the addition of different organic acids were performed to check their ability to reduce the acrylamide content in gingerbread (Table 6). Addition of 0.5 and 1.0 g citric acid per 100 g dough resulted in a drop of pH to 5.6 and 5.0, respectively, and in a reduction of the acrylamide concentration by a factor of 4 and 40, respectively. At the same time browning was also substantially reduced. Gingerbread with 1 g citric acid per 100 g had a clearly acidic taste and its leavening was not sufficient, probably due to the forced protonation of NH$_3$ whereby the gas volume was reduced during baking. The reduction of the acrylamide formation by citric acid has already been reported in French fries and various model systems [33-35]. The protonation of the α-amino group of asparagine hinders the formation of the N-substituted glycosylamine which may explain the reduced acrylamide content and the lesser browning.
Table 6: Effect of the addition of organic acids on the acrylamide content, color, and pH
(n = 1; a: n = 9; b: n = 5; c: n = 2).

<table>
<thead>
<tr>
<th>Organic acid</th>
<th>Amount [mg/kg]</th>
<th>Acrylamide [µg/kg]</th>
<th>L-Value [-]</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>-</td>
<td>501&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Citric acid</td>
<td>5000</td>
<td>133&lt;sup&gt;c&lt;/sup&gt;</td>
<td>56.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10000</td>
<td>12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>55.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glycine</td>
<td>2000</td>
<td>430</td>
<td>41.2</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>10000</td>
<td>151</td>
<td>38.4</td>
<td>6.5</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>500</td>
<td>368</td>
<td>48.7</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>380</td>
<td>42.7</td>
<td>6.4</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>2000</td>
<td>587</td>
<td>41.9</td>
<td>6.8</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>2000</td>
<td>542</td>
<td>41.7</td>
<td>7.1</td>
</tr>
</tbody>
</table>

A moderate addition of L-glutamine, L-lysine, or glycine (2000 mg/kg dough) did not lower the acrylamide contents, but enhanced browning. This might be due to the higher number of available α-amino groups which undergo Maillard reaction resulting in more melanoidins. However, a large addition of glycine (10,000 mg/kg dough) reduced the acrylamide content to one third, while the browning was even stronger and the pH slightly lower. Glycine is known to strongly enhance browning [36] and to react readily with α-dicarbonyls [24]. Thus, the observed effect could be due to the competition of the amino acids for the reactive carbonyls and/or the elimination of formed acrylamide by a reaction with glycine. L-Cysteine showed a tendency to reduce the acrylamide content and the pH, but these samples had also an unpleasant taste and odor, assumedly caused by S-containing decomposition products of cysteine. In a potato model system the acrylamide content was reduced up to 92% if amino acids had been added [35]. However, the authors used far larger amounts of amino acids (5000 to 20,000 mg/kg) which probably explains the strong effect. Only a moderate addition of citric acid (< 5000 mg/kg) seems to be suitable to reduce the acrylamide content in gingerbread because it also affects browning, leavening and taste. Browning (e.g. in samples with an alternative baking agent) could be enhanced by addition of amino acids, in particular glycine. Further research is needed to find the optimal combination of process conditions, ingredients and additives to produce gingerbread of good quality and with low acrylamide content.

ACKNOWLEDGEMENT
We thank Horst Adelmann for pilot plant support, Maurus Biedermann and Koni Grob (Official Food Control Authority of the Canton of Zurich) for valuable support and cooperation in the acrylamide analysis, and JOWA AG for supplying ingredients for gingerbreads. Financial support was provided by the Swiss Federal Office for Public Health (BAG), COOP Switzerland, Cooperative Migros, and the Federation of Swiss Food Industries (FIAL).
5.5. Literature


6. Ways to reduce the acrylamide formation in cracker products


6.1. Abstract

The sources of reducing sugars and free asparagine of two different cracker products were identified, and acrylamide formation during baking was measured. The application of an asparaginas e decreased the acrylamide content by at least 70% in both products. Replacing ammonium hydrogencarbonate by sodium hydrogencarbonate as baking agent and replacing reducing sugars by sucrose resulted in almost 80% less acrylamide in the wheat cracker. Decreasing free asparagine and reducing sugars in the ingredients and a lower end-temperature during baking lowered the acrylamide content of the potato cracker by about 50%.

6.2. Introduction

The detection of acrylamide in a broad range of staple foods [1] led to world-wide activities to minimize the exposure because of the neurotoxic and carcinogenic properties of acrylamide [2]. Acrylamide is formed at elevated temperatures concurrently to nonenzymatic browning by the reaction of reducing sugars with the free amino acid asparagine, which delivers the backbone of the acrylamide molecule [3, 4]. The baking agent ammonium hydrogencarbonate strongly promotes the formation of acrylamide in bakery products [5, 6], but reducing sugars influence the acrylamide formation in these products as well [6, 7]. The aim of the present study was to identify the critical factors for acrylamide formation in a wheat cracker and a potato cracker, and to find ways to decrease their acrylamide content while maintaining the sensory properties.

6.3. Material and methods

Wheat crackers were prepared with wheat flour (type 400), water, sunflower oil, condensed milk, sucrose, inverted sugar syrup, NaCl, and baking agent (mixture of NaHCO₃, Na₄P₂O₇, and NH₄HCO₃). They were baked in an industry-scale oven at 220 - 230 °C for 6 min. Potato crackers contained potato flakes, water, wheat flour (type 550), sunflower oil, a starch preparation, NaCl, and were baked in an industry-scale oven with an increasing temperature profile of 190 - 230 °C for 6 min. All ingredients and prescriptions were obtained from Midor AG (Meilen, CH). Asparaginase from E. coli (Fluka, Buchs, CH) was diluted in water and added to the dough during kneading (about 300 units per kg). Acrylamide analysis was performed with the GC-MS method of Biedermann et al. [8], using 13C₃-acrylamide (CIL, Andover, Massachusetts, USA) and methacrylamide (Fluka) as internal standards.
Free amino acids were extracted with 0.1 M HCl (Fluka) and determined by cation-exchange chromatography followed by post-column derivatization with ninhydrin as described in literature [6, 9]. Glucose and fructose were determined enzymatically using the kit from Scil diagnostics (Martinsried, Germany).

6.4. Results and discussion

All ingredients of the two cracker products were analyzed for glucose, fructose, and free asparagine (Table 7). Flour was the only source for free asparagine in the wheat cracker, whereas glucose and fructose mainly originated from the inverted sugar syrup. The raw dough of the wheat cracker contained 190 times more reducing sugars than free asparagine, which therefore was a limiting factor for acrylamide formation and a starting point to decrease the acrylamide content in this product. The situation for the potato cracker was different: The potato flakes were the main source for free asparagine and for reducing sugars. The flour type 550 was a relevant source for reducing sugars only although it contained more of free asparagine and reducing sugar than type 400. The content of sugars and free amino acids in cereal flour depends on the degree of extraction during milling, and thus the flour type can influence acrylamide formation in bakery [10]. The dough of the potato cracker contained about twice as much free asparagine than reducing sugars. Compared to the wheat cracker, it contained 49 times more of free asparagine which shows the different nature of the two products. The potato cracker resembles more a potato than a bakery product.

Table 7: Sources of reducing sugars and free asparagine in the two cracker products (Glc: glucose; Fru: fructose; Asn: asparagine).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Glc + Fru [mg/kg]</th>
<th>Free Asn [mg/kg]</th>
<th>Contribution to total reducing sugars [%]</th>
<th>Contribution to total free Asn [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat cracker:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flour (type 400)</td>
<td>822</td>
<td>63</td>
<td>6.9</td>
<td>100</td>
</tr>
<tr>
<td>Sucrose</td>
<td>155</td>
<td>0</td>
<td>&lt; 0.1</td>
<td>0</td>
</tr>
<tr>
<td>Inverted sugar syrup</td>
<td>597275</td>
<td>0</td>
<td>92.8</td>
<td>0</td>
</tr>
<tr>
<td>Condensed milk</td>
<td>564</td>
<td>0</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>Raw dough</td>
<td>7228</td>
<td>38</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Potato cracker:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potato flakes</td>
<td>2088</td>
<td>6000</td>
<td>77</td>
<td>99.1</td>
</tr>
<tr>
<td>Flour (type 550)</td>
<td>1069</td>
<td>96</td>
<td>23</td>
<td>0.9</td>
</tr>
<tr>
<td>Starch preparation</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Raw dough</td>
<td>836</td>
<td>1869</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
This difference is also demonstrated by the acrylamide contents: On average, the wheat cracker contained 339 µg acrylamide per kg (relative standard deviation between batches: 9%) whereas the mean acrylamide content in the potato cracker was 833 µg/kg (relative standard deviation between batches: 55%). In overbaked potato crackers acrylamide contents exceeding 2000 µg/kg were determined. The higher acrylamide content and the stronger variation reflect the influence of the potato flakes and are typical for potato based products [1, 11, 12].

To reduce the acrylamide content of the wheat cracker, different approaches were made and the results are shown in Figure 32. If NH₄HCO₃ in the baking agent was replaced by NaHCO₃ the acrylamide content decreased by about 50% because the promoting effect of ammonia on acrylamide formation was eliminated [5, 6]. Replacing the inverted sugar syrup with sucrose decreased the acrylamide concentration by about 60% and a similar effect has also been observed in gingerbread [6]. The combination of these two approaches resulted in a 77% lower acrylamide content compared to the standard recipe and the product had good sensory properties and was well comparable to the standard product. The poorer leavening was corrected by adding some extra water to the dough which did not affect the acrylamide content.

![Figure 32: Effect of recipe changes on the acrylamide content of wheat cracker.](image)

In another experiment an asparaginase was added during the preparation of standard dough to decompose free asparagine and thus to eliminate the other crucial precursor for acrylamide formation. As a consequence, the product contained only little acrylamide (39 µg/kg), i.e. a reduction of 85% was achieved. Free asparagine was not fully hydrolyzed by this treatment which explains why still some acrylamide was formed. The efficacy and application of this enzyme was also shown in other matrices and deserves further investigation [4, 6]. Changes of baking conditions were
not suitable to limit the acrylamide formation if a product with acceptable sensory properties was to be attained.

Due to the strong variation of the acrylamide content in the potato cracker a reference sample (standard recipe) had to be taken at the same time for every experiment to allow a correct interpretation. By inverting the ratio of potato flakes to wheat flour in the recipe the content of free asparagine was lowered by 40% and reducing sugars were decreased by 16%. As a consequence the acrylamide content decreased by 50% (from 557 to 274 µg/kg). The application of an asparaginase decreased free asparagine even more: The enzyme treated dough contained only 21 mg free asparagine per kg, i.e. 80 times less than the standard dough and thus, these baked potato crackers contained 70% less acrylamide (143 µg/kg; reference product: 489 µg/kg). The advantages of the use of asparaginase are: Recipe and process conditions remain the same and the product has virtually identical sensory properties. Another strategy for this product was to optimize baking conditions. If the baking profile was altered to decreasing temperatures, i.e. starting at 230 °C and ending at 190 °C, the acrylamide content decreased by 59% (reference: 1186 µg/kg; altered baking profile: 484 µg/kg). The end conditions during the heating process are a critical factor for acrylamide formation in the potato cracker which was also observed for French fries [13].

6.5. Conclusions

The application of an asparaginase during dough preparation can reduce the acrylamide content of cracker products by at least 70% and thus deserves further investigations. The baking agent NH₄HCO₃ promotes acrylamide formation and its replacement by NaHCO₃ is another way to limit the acrylamide formation in crackers. Minimizing reducing sugars in the ingredients or the use of a sucrose solution instead of inverted sugar syrup can also help to produce crackers with lower acrylamide content. For potato crackers the end temperature of the baking process should be lowered to contain the acrylamide formation. These strategies allow for the production of wheat and potato cracker products with lower acrylamide content and good sensory properties.

ACKNOWLEDGEMENT

We thank Midor AG (Meilen, CH) for the use of plant facilities and for providing raw materials. Financial support was provided by the Swiss Federal Office of Public Health (BAG), Cooperative Migros, COOP Switzerland, and the Federation of Swiss Food Industries (FIAL).
6.6. Literature


7. Reducing the acrylamide content of a semi-finished biscuit on industrial scale

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7.1. Abstract

The baking agent, reducing sugars and organic acids are the ingredients that most influence the acrylamide formation in sweet bakery. Various experiments focusing on these components were performed with biscuits on industrial scale. The replacement of ammonium hydrogencarbonate by sodium hydrogencarbonate reduced the acrylamide content by about 70%. The use of a sucrose solution instead of inverted sugar syrup had a similar effect. Addition of some extra tartaric acid reduced the acrylamide content by about one third. The positive effects on the acrylamide content were still observed after a second baking process. These results show that mitigation in industry-scale based on the optimization of baking agent, reducing sugars, and organic acid is feasible and compliant to high quality standards.

7.2. Introduction

The detection of acrylamide, which has neurotoxic and carcinogenic properties [1], in a large variety of heated foods [2] led to numerous research projects dealing with mitigation [3]. Acrylamide is formed concurrently to the Maillard reaction by the reaction of the free amino acid asparagine with reducing sugars or other reactive carbonyls [4-6].

In certain bakery products acrylamide contents up to 1000 µg/kg were observed [7]. The highest contents were often found in products prepared with the baking agent ammonium hydrogencarbonate such as gingerbread products [8, 9]. Model experiments showed that ammonium hydrogencarbonate strongly promotes the acrylamide formation in sweet bakery [10, 11]. Replacing this baking agent by sodium hydrogencarbonate presents a very effective way to limit the acrylamide content of bakery [8, 12]. Beside the baking agent, the content of reducing sugars and free asparagine as well as the process conditions influence the acrylamide formation in bakery [8, 12-14]. However, most of the studies dealing with mitigation for bakery were performed with model experiments or in laboratory scale production. Thus information of implementation of such mitigation concepts in industrial processes is very limited. The purpose of this study was to check the feasibility of different approaches to reduce the acrylamide content of bakery on an industrial production scale. Results from experiments focusing on baking agent, addition of organic acid, and replacing inverted sugar syrup by sucrose solution are shown and interpreted.
7.3. Material and methods

7.3.1. Preparation of biscuits

The biscuits used in this study represent an existing semi-finished product which is not directly consumed but ground to crumbs and then added to other types of biscuits before baking as a component in the dough or as coating. One such product was based on sugar and egg white foam, the other one was an almond biscuit coated with chocolate. Batches of 410 kg of biscuit dough per experiment were prepared with the following ingredients which were all provided by Kambly SA (Trubschachen, Switzerland): Biscuit flour (wheat, corresponding to type 550, several batches used), water, powdered sugar (sucrose), vegetable fat, milk powder, inverted sugar syrup, sodium chloride, a starch preparation, and baking agent. The baking agent consisted of a mixture of 127 g of NH₄HCO₃ and 273 g of NaHCO₃ per 100 kg of dough. After mixing in a “Wendel kneader” (Diosna, Osnabrück, Germany) for two minutes, tartaric acid was added (195 g/100 kg) and the dough further kneaded. If NH₄HCO₃ was replaced by NaHCO₃, the amount of NaHCO₃ and tartaric acid was adjusted to obtain the same gas volume released from the standard baking agent.

The baking process (about 5 min) was carried out in the plant of Kambly SA in a continuous oven (Baker Perkins, Peterborough, UK) with 3 heating zones held at temperatures between 225 °C and 230 °C. The baking process for all experiments reported here was adjusted to a slightly darker baking degree to enhance effects on acrylamide formation. Before and after each experiment the standard dough was baked to obtain a direct reference. About 300 biscuits per experiment (taken over a period of 20 min) were sampled, whereby the first 5 min of production were not included for sampling. After baking the samples were subjected to a simple sensory test with pairwise comparison to reference samples by a small panel of trained people. Taste, leavening, texture, and color were tested. Criteria were acceptance of the product as such and similarity to the reference sample. Baked biscuits were stored at -24 °C and homogenized in a household cutter (Moulinette, Moulinex, Paris, France) before analysis.

7.3.2. Measurement of acrylamide

Acrylamide was determined with a GC-MS method [15] using 13C₃-acrylamide (CIL, Andover, USA) and methacrylamide (Fluka, Buchs, Switzerland) as internal standards, both added at 500 µg per kg of homogenized sample. The GC-MS equipment comprised an 8000 series gas chromatograph with on-column injector (Fisons Instruments, Milan, Italy) coupled to a SSQ 710 quadrupole mass spectrometer (Finnigan Mat, San Jose, USA) operated at conditions as described [15]. The precolumn (TSP deactivated, i.d. 0.53 mm) and the separation column (BGB Wax, 12 m, i.d. 0.25 mm) were both from BGB Analytik (Böckten, Switzerland).

7.4. Results and discussion

The baking process was characterized in relation to acrylamide content by multiple analysis of the standard model biscuit for intra-day (n = 4) and inter-day (n = 9) variation. The mean acrylamide content was 170 µg/kg and the relative standard
deviation was 9% between the different production days. The variation within the same production day was smaller (6%). The acrylamide content of a product was considered to be significantly lower compared to the standard product if the difference amounted to at least two times the standard deviation, i.e. 31 µg/kg. Standard biscuits baked to the normal baking degree contained about 100 µg/kg, i.e. less than the standard biscuits prepared for this study.

7.4.1. Influence of baking agent
The baking agent is known to strongly influence the acrylamide formation in bakery [8] and thus various combinations of baking agents were tested. Figure 33 shows the acrylamide content of the model biscuits prepared with different baking agents. If NH₄HCO₃ was not fully replaced by NaHCO₃ the acrylamide content did not decrease (variant A in Figure 33). In contrast, the complete replacement of NH₄HCO₃ by NaHCO₃ reduced the acrylamide content by over 70% (variant B in Figure 33). Similar results were also obtained in other bakery products [8, 11, 12]. Part of the effect on the acrylamide content might also be due to the presence of more tartaric acid when NH₄HCO₃ was fully replaced by NaHCO₃, a factor which is discussed below. All samples passed the sensory test (color, taste) set by the company. However, samples prepared without NH₄HCO₃ showed a somewhat lesser leavening as compared to the standard product. This difference was not problematic since it concerned a semi-finished product and its suitability for further use was not negatively affected. They were used as described above and the final products were sold on the regular market. This demonstrates that replacing NH₄HCO₃ by NaHCO₃ is a feasible way to significantly reduce the acrylamide content of biscuit products.

![Figure 33: Acrylamide content of model biscuits prepared with different mixtures of baking agents](image)

Figure 33: Acrylamide content of model biscuits prepared with different mixtures of baking agents (n = 9 for reference, relative standard deviation = ± 9%; n = 1 for other samples. R (reference): 127 g of NH₄HCO₃ + 273 g of NaHCO₃ + 195 g of tartaric acid per 100 kg of dough; A: 49 g of NH₄HCO₃ + 439 g of NaHCO₃ + 312 g of tartaric acid per 100 kg of dough; B: 537 g of NaHCO₃ + 439 g of tartaric acid per 100 kg of dough).
7.4.2. Influence of type of sugar
A reducing sugar is needed for the formation of acrylamide from asparagine [5, 16]. The use of a sucrose solution (non-reducing sugar) instead of inverted sugar syrup resulted in an acrylamide content of 46 µg/kg which is over 70% less compared to the standard product (170 µg/kg). This result confirms earlier findings that replacing reducing sugars by sucrose is an effective way to significantly reduce the acrylamide content of sweet bakery [8, 12]. The taste was not noticeably affected, but the products prepared with sucrose had a somewhat lighter color due to a lack of reducing sugars which are needed for Maillard browning [17]. These biscuits were also further used for final production, because the brighter color did not negatively affect the final product. Thus, replacing reducing sugars by sucrose is a feasible approach for bakery if the browning is not of primary importance.

7.4.3. Amount of tartaric acid
Tartaric acid (or citric acid) is often added to baking agents containing NaHCO₃ to enhance the leavening. The addition of acid lowers the pH and thus affects acrylamide formation [8, 18]. Two experiments with the addition of some extra tartaric acid were carried out (Figure 34). The standard product was prepared with 195 g of tartaric acid per 100 kg dough, and in the two experiments 244 g and 293 g were added per 100 kg of dough. All of the three types contained the standard amount of NH₄HCO₃ and NaHCO₃. The use of 244 g/100 kg tartaric acid decreased the acrylamide content by one third (114 µg/kg), while even more acid (293 g/100 kg) had a slightly higher effect (acrylamide content reduced by 44%) which was not considered to be significantly lower compared to the experiment with 244 g/100kg. These results show that the large effect observed in Figure 33 cannot be due to the presence of more tartaric acid only, but also due to the replacement of NH₄HCO₃ by NaHCO₃. It has also to be taken into account that most of the tartaric acid in the experiments shown in Figure 33 was neutralized by the larger amount of added NaHCO₃, whereas this was not the case in the experiments with extra tartaric acid shown in Figure 34. Even the product with the highest amount of acid had no acidic taste. Again, all products passed the sensory test and were used for final production. These results are well in line with effects of citric acid observed in gingerbread [8] and show that the addition of some organic acid is another way to significantly reduce the acrylamide content of bakery. However, the amount of acid to be added may be limited, mainly for sensory reasons [8], and no studies on shelf-life were performed because the product was directly used as an ingredient in two types of biscuits.
7.4.4. Acrylamide formation in the second baking process

The model biscuit investigated here is not directly sold but used as component of other biscuits. It was also tested if the final products had lower acrylamide content if one of the above mentioned approaches was applied in the production. The biscuit based on egg white foam and sugar contained 110 µg/kg acrylamide if the standard biscuit was used for coating. If the coating was prepared without NH₄HCO₃ (see Figure 33) the acrylamide content was some 30% lower (75 µg/kg). The situation was similar for the almond biscuit: Use of the model biscuit prepared without NH₄HCO₃ lowered the acrylamide content of the almond biscuit by about 30% (374 µg/kg versus 551 µg/kg). The mentioned acrylamide contents refer to the almond biscuit before coating with chocolate. The acrylamide content of the final almond product is thus substantially lower. Both final products passed the sensory test, i.e. their quality was considered equal to the normal product. These results show that the replacement of NH₄HCO₃ is still effective even in a second baking process.

The three major approaches for mitigation of acrylamide in sweet biscuits are: (1) Replacing NH₄HCO₃ by NaHCO₃, (2) use of a sucrose solution instead of inverted sugar syrup, and (3) moderate addition of organic acid (e.g. tartaric acid). In crackers a combination of the first two approaches was even more effective [12] which could also apply for other products. The measure focusing on the replacement of reducing sugars by sucrose is limited to products where the browning is not of primary importance. Experiments on industrial scale with a semi-finished biscuit gave evidence that these three approaches are feasible and that the sensory properties of the semi-finished product as well as of the finished products conform to a high-quality standard expected by the food industry and the consumer. The acrylamide content can be lowered by 30 to 70% depending on the product and measure applied. However, other groups of bakery products may behave differently: Especially the leavening and the texture might be critical points if the product is directly consumed.
Therefore, experiments have to be made to find the optimal way to reduce the acrylamide content.

ACKNOWLEDGEMENT
Financial support was provided by the Swiss Federal Office of Public Health (BAG), the Federation of Swiss Food Industries (FIAL), Cooperative Migros, and COOP Switzerland.

7.5. Literature


8. Acrylamide in almond products


8.1. Abstract

Acrylamide was determined in 86 different almond products such as roasted almonds, almond containing bakery, raw almonds, and marzipan. The highest acrylamide concentrations were found in dark roasted almonds, while only moderate acrylamide contents were determined in bakery. Roasting experiments at different process conditions showed that acrylamide increases with time and that temperature has a much stronger effect on acrylamide formation than time. During roasting reducing sugars are consumed faster and to a larger extent than free asparagine suggesting that the content of reducing sugars may be a critical factor for acrylamide formation in roasted almonds. Acrylamide was found to decrease in roasted almonds during storage at room temperature.

8.2. Introduction

The detection of acrylamide in a broad range of heated foods at concentrations sometimes exceeding 1000 µg/kg [1] led to world-wide activities to reduce the acrylamide content of foods because of its neurotoxic and carcinogenic properties [2, 3]. Acrylamide is formed at elevated temperatures and medium to low moisture contents concurrently to the Maillard reaction between reducing sugars and the free amino acid asparagine which delivers the backbone of the acrylamide molecule [4-6]. Exceedingly high amounts of acrylamide were detected in strongly heated potato products such as crisps, French fries and hash browns [1, 7, 8], which is at least partially explained by the high content of free asparagine and reducing sugars in raw potatoes [9]. It has been shown that in potatoes the reducing sugars largely control the extent of acrylamide formation whereas the more abundant free asparagine does not correlate with acrylamide content [9-11]. In contrast, free asparagine is often limiting in sweet bakery products and therefore plays a key role together with the baking agent ammonium hydrogencarbonate which strongly promotes the acrylamide formation [12-14].

Almonds contain both acrylamide precursors in appreciable amounts: The content of free asparagine is reported in the range of 2000 to 3000 mg/kg [15]. Glucose and fructose were determined from 500 to 1300 mg/kg, and sucrose contents ranged from 2500 to 5300 mg/kg [16]. As a consequence, the detection of acrylamide in roasted almonds in concentrations from 260 to 1530 µg/kg [7, 14] was not surprising. It was shown that the physical form of the almond (whole kernel vs. cut vs. ground) has an impact on the amount of acrylamide formed during heating [14].

The aim of the present study was to gain an overview of the acrylamide content of various almond products in Switzerland and to identify the products with the highest acrylamide concentration. Over 80 samples of raw, intermediate, and final products containing almonds were collected and their acrylamide content was determined.
Acrylamide formation during roasting was monitored and sugars and free amino acids in raw and roasted almonds were measured.

8.3. Material and methods

8.3.1. Collection of samples
Almonds (raw, salted, smoked, roasted, and caramelized), and almond containing products such as biscuits, cookies, cakes, bars, breads, pastry, and marzipan were obtained from supermarkets and Swiss food manufacturers. Almonds used for roasting experiments were all of Californian origin and supplied by ABC (Almond Board of California, Modesto, CA, USA). The following cultivars were investigated: Non Pareil, Price, Monterey, and Mission. Roasting experiments were performed as pile roasting (batch size: 200 g) using a fluidized-bed hot air laboratory roaster (G.W. Barth AG, Freiburg, Germany). All samples were homogenized in a household cutter (Moulinette; Moulinex, Paris, France) before subjected to further analysis.

8.3.2. Analysis of acrylamide
Acrylamide was determined with the GC-MS method described by Biedermann et al. [17]. The internal standards were $^{13}$C$_3$-acrylamide (CIL, Andover, Massachusetts, USA) and methacrylamide (Fluka, Buchs, Switzerland), both dissolved in methanol (Fluka) and 500 µg of each were added per kg sample. Extraction and clean-up was performed as described [17]. Measurement of acrylamide was done with an 8000 series gas chromatograph with on-column injector (Fisons Instruments, Milan, Italy) coupled to a SSQ 710 quadrupole mass spectrometer (Finnigan Mat, San Jose, USA). The precolumn (TSP deactivated, i.d. 0.53 mm) and the separation column (BGB Wax, 12 m, i.d. 0.25 mm) were both from BGB Analytik (Böckten, Switzerland). GC and MS conditions were as described in [17]. Spiking experiments were made by adding 10 µL of an aqueous solution of acrylamide (500 mg/L; Sigma-Aldrich, Steinheim, Germany) to 10 g of homogenized sample.

8.3.3. Determination of free amino acids in almonds
Homogenized almonds (ca. 10 g) were mixed with deionized water (1+2) and further homogenized to a slurry (Polytron; Kinematica, Lucerne, Switzerland). About 7.5 g of the slurry were transferred to a 100 mL flask and 1 mL of an aqueous solution of norleucine (5 mg/mL; Fluka) was added as internal standard. About 60 mL of 0.1 M HCl (Fluka) and 5 mL of each Carrez I (150 g of K$_4$[Fe(CN)$_6$]•3H$_2$O per liter, Fluka) and Carrez II solution (300 g of ZnSO$_4$•7H$_2$O per liter, Fluka) were added and the mixture was thoroughly shaken. Foam was broken with 50 µL of 1-octanol (Fluka) and the volume adjusted to 100 mL with 0.1 M HCl (Fluka). After filtration (602 H ½, Schleicher & Schuell, Dassel, Germany), samples were diluted 1+4 with 0.16 M lithium citrate buffer (pH = 2.2, PVP physiological; Laborservice Onken, Gründau, Germany) and thoroughly mixed. Diluted samples were filtered through a 0.45 µm HPLC membrane filter (Titan; Infochroma, Zug, Switzerland) and subjected to analysis by cation-exchange chromatography followed by post-column derivatization.
with ninhydrin (Biochrom 30, Biochrom, Cambridge, UK) by using the physiological system (Biochrom) as described by the producer. 50 μL were injected and quantification was done both by comparison with an external standard and the internal standard.

8.3.4. Measurement of sugars in almonds
10.00 g of homogenized almonds were mixed with deionized water (1+2), further homogenized (Polytron) and about 10 g of the slurry was weighed into a 100 mL flask. After Carrez clarification (5 mL of each Carrez I and II solution), foam was broken with a few drops of 1-octanol (Fluka), the pH adjusted to 7 with 4 M KOH (Fluka) and the volume adjusted to 100 mL with deionized water. After filtration (Schleicher & Schuell) fructose, glucose, and sucrose were determined enzymatically using the kit from Scil Diagnostics (Martinsried, Germany).

8.3.5. Determination of color and moisture content
Almonds were ground in a household coffee mill (Mio-Star, Migros, Zurich, Switzerland) and evenly spread on a Petri dish. The color was determined with a Minolta CR310 colorimeter (Konica Minolta Photo Imaging, Dietikon, Switzerland) measuring color according to the L*,a*,b* system. The lightness was used as a measure for roasting degree where L* = 100 means white and L* = 0 means black. Dry matter was determined gravimetrically: About 1 g of homogenized (Moulinette) almonds were weighed in a pre-dried metal dish and thoroughly mixed with 4 g of dried quartz sand (Fluka) and then dried in an oven at 103 °C for 4 h.

8.4. Results and discussion
8.4.1. Acrylamide in almonds and almond products
The acrylamide content of various almond containing products or prefabricates (totally n = 86) was determined. Spiking samples with acrylamide resulted in recoveries (r) ranging between 90% ≤ r ≤ 110%. The products were split into three groups: roasted almonds, bakery with almonds, and marzipan + raw almonds. The results are shown in Table 8.

Table 8: Acrylamide content of various almond products (RSD: relative standard deviation; n.d.: not detected).

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Roasted almonds</td>
<td>36</td>
<td>443</td>
<td>424</td>
<td>2147</td>
<td>n.d.</td>
<td>100</td>
</tr>
<tr>
<td>Bakery with almonds</td>
<td>34</td>
<td>196</td>
<td>103</td>
<td>1574</td>
<td>n.d.</td>
<td>168</td>
</tr>
<tr>
<td>Marzipan + raw almonds</td>
<td>16</td>
<td>4</td>
<td>0</td>
<td>42</td>
<td>n.d.</td>
<td>290</td>
</tr>
<tr>
<td>All products</td>
<td>86</td>
<td>250</td>
<td>91</td>
<td>2147</td>
<td>n.d.</td>
<td>149</td>
</tr>
</tbody>
</table>
The data in Table 8 demonstrate that the highest acrylamide concentrations were found in roasted almonds with most of the values being around 400 µg/kg. The highest concentration was found in dark roasted almonds used as an ingredient in the manufacture of biscuits. Most values for roasted almonds are within the range reported by other groups [7, 14], however acrylamide levels in roasted almonds exceeding 2000 µg/kg were not found so far. The median acrylamide content of roasted almonds was 4 times higher than the one from bakery with almonds which demonstrates that roasted almonds contain generally more acrylamide than almond containing bakery. In bakery, only 8 values exceeded 200 µg/kg. However, there was one biscuit product (from 2 batches) that contained 1307 and 1574 µg/kg which is extraordinarily high. The main reason for this high amount was the presence of ammonium hydrogen carbonate (E 503) and a large amount of reducing sugars. This baking agent was shown to strongly enhance acrylamide formation in bakery [12-14, 18]. This is pointed out by the fact that 5 out of 8 bakery products containing more than 200 µg/kg acrylamide were prepared with ammonium hydrogen carbonate. In marzipan, cooked, blanched and raw almonds usually no acrylamide (limit of detection: 20 µg/kg) or very low amounts were found. Acrylamide is formed at temperatures above 100 °C [19] and even some slightly roasted almonds contained no detectable acrylamide (Table 8) which explains these results. Relative standard deviation was high in all the three categories showing that there is considerable variation in the acrylamide content of almond products. Because roasted almonds contained most acrylamide, the focus of the present study was directed to this category and some further experiments were carried out.

8.4.2. Acrylamide formation during roasting of almonds

The data for roasted almonds in Table 8 show a considerable variation suggesting that the roasting process may have an important influence on the acrylamide formation. Therefore, samples from an industrial roasting experiment, performed at 150 °C, were taken between 15 to 22 min roasting time and analyzed for acrylamide. Figure 35 shows that the acrylamide content of roasted almonds increased in a linear manner with time. However, a non-linear or a decreasing course of the acrylamide content has to be expected at longer process times as observed in model systems [20] as well as in foods such as gingerbread [12], coffee [19] and potatoes [8, 21, 22].
Besides roasting time, the roasting temperature is assumed to influence the acrylamide formation during roasting, too. Therefore, roasting experiments using various temperature / time combinations were performed with a laboratory roaster and the results are shown in Table 9. The reproducibility of the roasting process was checked with four samples roasted at 180 °C for 4 min: The mean acrylamide content was 1834 µg/kg with a relative standard deviation of 8.5% which is well within the range of other process variations reported [12, 18]. The results in Table 9 show that only small amounts of acrylamide were formed at 130 °C. Acrylamide was not detected in the first 11 min of roasting and even after 40 min only 236 µg/kg were measured. In contrast, at 150 °C acrylamide formation was much larger and faster: After 15 min 35 times more acrylamide was found than after 16.5 min at 130 °C. At 150 °C 6.5 times more acrylamide was formed after 25 min compared to roasting at 130 °C for 40 min. This clearly shows that temperature has a much stronger influence on acrylamide formation than time has. Experiments performed at 150 °C and 180 °C indicated that prolonged roasting can result in lower acrylamide concentrations. Almonds roasted at 150 °C for 25 min contained substantially more acrylamide compared to the sample roasted for 30 min. Prolonged roasting at higher temperatures seems to favor the elimination of acrylamide relative to its new formation. A similar effect was also observed in roasted coffee beans [19] and gingerbread [12]. Altogether, these results suggest that the optimization of the roasting process in terms of temperature and time may be a way to reduce the acrylamide content of roasted almonds and detailed investigations are in progress to find optimal process conditions.
Table 9: Acrylamide content of almonds roasted under different temperature and time combinations (cultivar Price, values refer to fresh weight, n = 1, n.d. = not detected; a: n = 4).

<table>
<thead>
<tr>
<th>Temperature [°C]</th>
<th>Time [min]</th>
<th>Acrylamide [µg/kg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>130</td>
<td>2.5</td>
<td>n.d.</td>
</tr>
<tr>
<td>130</td>
<td>11.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>130</td>
<td>16.5</td>
<td>44</td>
</tr>
<tr>
<td>130</td>
<td>22.5</td>
<td>94</td>
</tr>
<tr>
<td>130</td>
<td>40.0</td>
<td>236</td>
</tr>
<tr>
<td>150</td>
<td>15</td>
<td>715</td>
</tr>
<tr>
<td>150</td>
<td>25</td>
<td>1547</td>
</tr>
<tr>
<td>150</td>
<td>30</td>
<td>1044</td>
</tr>
<tr>
<td>180</td>
<td>4.0</td>
<td>1834^a</td>
</tr>
<tr>
<td>180</td>
<td>7.0</td>
<td>1718</td>
</tr>
</tbody>
</table>

8.4.3. Changes of sugar and asparagine content in almonds during roasting

In parallel to the acrylamide analysis, the changes of the concentrations of sugars and free asparagine during roasting were determined. Table 10 shows the contents of sugars and free asparagine in raw almonds of different cultivars.

Table 10: Content of sugars and free asparagine in raw almonds (n = 2; values refer to fresh weight, RSD = relative standard deviation).

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Glucose [mg/kg]</th>
<th>Fructose [mg/kg]</th>
<th>Sucrose [mg/kg]</th>
<th>Free Asparagine [mg/kg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non Pareil</td>
<td>1566</td>
<td>867</td>
<td>32700</td>
<td>2175</td>
</tr>
<tr>
<td>Mission</td>
<td>2130</td>
<td>1151</td>
<td>38650</td>
<td>2041</td>
</tr>
<tr>
<td>Price</td>
<td>2339</td>
<td>1482</td>
<td>43430</td>
<td>2238</td>
</tr>
<tr>
<td>Monterey</td>
<td>2101</td>
<td>1103</td>
<td>40950</td>
<td>2474</td>
</tr>
<tr>
<td>RSD [%]</td>
<td>16</td>
<td>22</td>
<td>12</td>
<td>8</td>
</tr>
</tbody>
</table>

The main sugar in raw almonds was sucrose accounting for about 3 to 4% of the fresh weight. Glucose and fructose were determined in the range 1500 to 2300 mg/kg and 900 to 1500 mg/kg, respectively, which is similar to values reported from Italian almond varieties [16]. On a molar basis free asparagine is roughly as abundant as the reducing sugars: The ratio of the molar content of free asparagine to reducing sugars ranged from 0.8 to 1.2. Thus, the situation of the acrylamide precursors in almonds is different from the one in potatoes, where reducing sugars are limiting [9, 23, 24], or the one in sweet bakery, where asparagine is limiting [12, 18]. As can be seen from the relative standard deviation (RSD) the reducing sugars...
varied somewhat more between the cultivars than free asparagine. Therefore more extensive investigations will be undertaken in another study to check if there are significant differences between the different almond cultivars in terms of sugars, free amino acids, and acrylamide formed during roasting. Since reducing sugars and free asparagine are considered to be the main precursors for acrylamide formation in foods, their changes during two different roasting experiments were monitored (Table 11). Almonds from the same batch were roasted at 130 °C for 22.5 min (“light roast”) and at 180 °C for 7.0 min (“dark roast”).

Table 11: Reducing sugars, free asparagine, and acrylamide in raw and roasted almonds (cultivar Price, n ≥ 2; values referring to dry matter; n.d.: not detected)

<table>
<thead>
<tr>
<th>Roasting conditions</th>
<th>Glucose [mg/kg]</th>
<th>Fructose [mg/kg]</th>
<th>Free Asparagine [mg/kg]</th>
<th>Acrylamide [µg/kg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw</td>
<td>2860</td>
<td>2010</td>
<td>2200</td>
<td>n.d.</td>
</tr>
<tr>
<td>130 °C, 22.5 min</td>
<td>700</td>
<td>900</td>
<td>2100</td>
<td>79</td>
</tr>
<tr>
<td>180 °C, 7.0 min</td>
<td>110</td>
<td>140</td>
<td>1600</td>
<td>1718</td>
</tr>
</tbody>
</table>

Glucose and fructose were consumed faster than free asparagine: After roasting at 180 °C for 7 min only 4% of the initial glucose content and 7% of the initial fructose content was found, whereas the free asparagine content had decreased only by 27%. The effects were likewise but less pronounced for the roasting experiment carried out at 130 °C: Reducing sugar decreased by more than 50%, whereas still 95% of the initial asparagine content was found. A similar behaviour of these compounds was also observed in heated potatoes [24]. Glucose and fructose react much faster than free asparagine upon heating and therefore their content could directly influence the acrylamide formation. This was tested with three samples of raw almonds with a different content of reducing sugars. They were roasted at 160 °C for 10 min and the results are shown in Figure 36. A strong correlation was found between the glucose content before roasting and the acrylamide content determined after roasting. The correlation was similar for fructose (R² = 0.9474) and glucose + fructose (R² = 0.9802), whereas sucrose correlated only very weekly with acrylamide (R² = 0.3336). This indicates that besides the roasting process the content of reducing sugars in the raw almonds may be a critical factor for acrylamide formation in almonds. However, data are limited and further experiments are needed to fully corroborate and understand these interrelations.
Figure 36: Interrelation between glucose content in raw almonds and acrylamide determined in these almonds after roasting at 160 °C for 10 min.

As both acrylamide and color (melanoidins) are formed in the Maillard reaction [4, 20, 25], the color of roasted almonds was compared with their acrylamide content. The L*-value (brightness) of roasted almonds (cultivar Price) ranged from 46 to 71. The L*-value and the acrylamide content showed a fairly close correlation with $R^2 = 0.8384$: The darker the almonds were the higher the acrylamide content was. The brown skins of the almonds were a major cause for uncertainty in the color determination because they differ between varieties and their change in color during roasting is different compared to the kernel. A similar correlation between browning (L-value) and acrylamide content was observed in gingerbread [12] and fried potato slices [21].

8.4.4. Stability of acrylamide in roasted almonds during storage

The stability of acrylamide in foods during storage is another point of interest. It was shown that acrylamide is stable in starchy foods such as breakfast cereals, biscuits, and potato crisps during storage, whereas the content of acrylamide significantly decreased in coffee products and cocoa powder [26-28]. Data for roasted almonds were not available yet and therefore, some samples of roasted almonds stored in a sealed container were re-analysed after 100 days of storage at room temperature (Figure 37). In all samples a decrease in the acrylamide content was observed and it ranged from 20 to 57%. So far, no correlation between the decrease and initial acrylamide content or roasting degree was found. Interestingly, decreasing acrylamide contents were mainly reported for roasted products such as coffee and cocoa [26-28]. It can be assumed that reactive compounds formed during the roasting process may be responsible for the decrease of acrylamide in these products during storage.
Figure 37: Acrylamide content in samples of roasted almonds before (white bars: 1st analysis) and after storage of 100 days (grey bars: 2nd analysis).

ACKNOWLEDGEMENT

We thank the Almond Board of California for supplying raw almonds samples as well as Horst Adelmann, Marcel Leemann, Hanna Schneider, Ernő Staub, Véronique Rouvinez, and Rainer Perren for technical support in almond roasting and analysis. Financial support was provided by the Swiss Federal Office of Public Health (BAG), the Federation of Swiss Food Industries (FIAL), Cooperative Migros, and COOP Switzerland.

8.5. Literature

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[9] Amrein, T. M., Bachmann, S., Noti, A., Biedermann, M., Barbosa, M. F.,
[15] Seron, L. H., Poveda, E. G., Moya, M. S. P., Carratalà, M. L. M., Berenguer-
Gonde, P., Van Eijck, P., Lalljie, S., Lingnert, H., Lindblom, M., Matissesk, R.,


9. **Acrylamide in roasted almonds and hazelnuts**


9.1. **Abstract**

The influences of composition and roasting conditions on acrylamide formation in almonds and hazelnuts were investigated. 18 samples of almonds originating from the US and Europe were analyzed for sugars and free amino acids, and acrylamide formed during roasting was determined. Asparagine was the main free amino acid in raw almonds and correlated with the acrylamide content of dark roasted almonds. Roasting temperature was another key factor and had a very strong influence on acrylamide formation. Almonds of European origin contained significantly less free asparagine and formed significantly less acrylamide during roasting as compared to the American almonds. Roasted hazelnuts contained very little acrylamide because of the low content of free asparagine in the raw nut. Reducing sugars, although being consumed much faster than free amino acids in both types of nuts, were not decisive for the extent of acrylamide formation during roasting.

9.2. **Introduction**

Acrylamide has neurotoxic and carcinogenic properties [1] and is classified as probably carcinogenic to humans (group 2A) [2]. Therefore, its detection in a broad range of heated food products [3] led to an impressive number of research activities to investigate its formation, to monitor its sources in food, and to find ways for mitigation [4, 5]. Although the relevance of human exposure to acrylamide through the diet is not fully clarified yet, the world health organization (WHO) stated that acrylamide levels in food should be reduced because of public health concern [6]. Acrylamide is formed in the Maillard reaction by the reaction of the free asparagine and reactive carbonyls (e.g. reducing sugars) at temperatures above 120 °C [7-9]. Studies with stable isotope compounds have shown that the backbone of the acrylamide molecule purely originates from asparagine [8, 10]. However, the type of carbonyl has a strong influence on the amount of acrylamide formed from asparagine during heating [10, 11].

Because potato products like chips, hash browns, and French fries showed the highest acrylamide contents [3, 12] and thus may contribute a major part to human exposure [13] many research activities were conducted in this field. The optimization of the raw potatoes in terms of amounts of reducing sugars [14, 15] as well as the control of the frying temperature [15] are the main approaches to reduce the acrylamide content of these products. In contrast, for sweet bakery the baking agent ammonium hydrogen carbonate, the content of free asparagine, and the baking process were identified as critical factors for acrylamide formation and may provide options for mitigation strategies [16-18].

Comparatively little information is available on acrylamide in roasted nuts although the acrylamide content of roasted almonds can exceed 1000 µg/kg [12, 19, 20]. Almonds contain free asparagine and reducing sugars in appreciable amounts [19,
and the roasting temperature as well as the physical form (whole, sliced, cut) of the almond kernel strongly influence the acrylamide formation [19, 20]. The purpose of this study was to continue the investigations of our first study on almonds [19], particularly to obtain an overview of the composition of almonds and hazelnuts, to identify the critical components for acrylamide formation, and to monitor the formation of acrylamide as well as the consumption of its precursors during roasting. Almonds of 14 different cultivars originating from the US and Europe were analyzed for free amino acids and sugars, roasted under different conditions and analyzed for their acrylamide content. Interrelations between acrylamide, its precursors and the process conditions are shown and discussed.

9.3. Material and methods

9.3.1. Raw material and roasting

Samples of raw almonds of the 2003 and 2004 harvest were obtained from the Almond Board of California (ABC; Modesto, CA, USA) and from various Swiss food companies. The investigated cultivars were: (1) American: Butte, Carmel, Fritz, Mission, Monterey, Neplus, Nonpareil, Padre, Price, and Sonora; (2) European (Spain and Italy): Avola, Longuettes, Larguetta, Valencia, and bitter almonds. Raw hazelnuts from Turkey and Italy and roasted hazelnuts (14 - 18 min at 150 °C) were obtained from Wernli AG (Trimbach, Switzerland). Raw nuts were stored at 5 °C in the dark until analysis and roasting, while roasted samples were stored air tight at -24 °C until analysis. All samples were homogenized in a household cutter (Moulinette; Moulinex, Paris, France) before analysis.

Roasting experiments were carried out in batches of 200 g as pile roasting using a fluidized-bed hot air laboratory roaster (G.W. Barth AG, Freiburg, Germany). Hot air was directed through the pile from bottom to top with a flux of 3.5 m/s. Temperatures of hot air below, within, and above the pile of nuts were monitored with temperature sensors (T51.1, Rotronic, Bassersdorf, Switzerland). After roasting, samples were cooled with air until the temperature within the pile stayed below 30 °C. Standard roasting experiments were performed at 145 °C for 14 min (light roast) and at 165 °C for 12.5 min (dark roast), and roasting curves were obtained at 145 °C (0 - 32 min), 165 °C (0 - 30 min), and during roasting for 10 min (120 °C - 220 °C).

9.3.2. Analysis of sugars and free amino acids

Homogenized samples were mixed with deionized water and (1 + 4, e.g. 15 g of almonds + 60 g of water) and further homogenized (Polytron, Kinematica, Lucerne, Switzerland). For the determination of glucose, fructose, and sucrose 12 to 40 g of the slurry was weighed into a graduated flask (100 mL). After addition of some water 5 mL of each Carrez I (150 g of K₄[Fe(CN)₆]•3H₂O per liter, Fluka, Buchs, Switzerland) and Carrez II (300 g of ZnSO₄•7H₂O per liter, Fluka) solution were added and the mixture intensively shaken. pH was adjusted to 7 with a few drops of 4 M KOH solution (Fluka), foam was broken with 1-octanol (Fluka), and the volume was adjusted to 100 mL with deionized water. Samples were shaken and filtered (Schleicher & Schuell, Dassel, Germany) and then analysed using an enzymatic kit as described by the producer (Scil, Martinsried, Germany). For the determination of
free amino acids about 12 g of slurry was weighed into a 100 mL flask (graduated) and 1000 µL of a norleucine solution (internal standard, 5 mg/mL in deionized water; Fluka) as well as about 60 mL of 0.1 M HCl (Fluka) and were added. After Carrez clarification (see above) foam was broken with 1-octanol (Fluka), the volume adjusted to 100 mL with 0.1 M HCl and the samples thoroughly shaken. Samples were filtered (Schleicher & Schuell), diluted 1+4 (almonds) or 1+1 (hazelnuts) with 0.16 M lithium citrate buffer (pH = 2.2, PVP physiological; Laborservice Onken, Grünau, Germany), and mixed (Vortex, Bender & Hobelin, Zurich, Switzerland). Samples were filtered through a 0.2 µm HPLC membrane filter (Titan; Infochroma, Zug, Switzerland) and subjected to analysis by cation-exchange chromatography followed by post-column derivatization with ninhydrin (Biochrom 30, Biochrom, Cambridge, UK) by using the physiological system (Biochrom) as described by the producer. Injection volume was 50 µL and quantification was done both by comparison with an external and the internal standard. The external standard was an amino acid standard solution (Sigma, Steinheim, Germany) to which asparagine, glutamine, and norleucine (all from Fluka) were added. T-Test was performed as a two-tailed test (homoscedastic) by using Microsoft Excel 2002.

9.3.3. Determination of acrylamide

Acrylamide was determined with a GC-MS method [22] using 13C3-acrylamide (CIL, Andover, MA, USA) and methacrylamide (Fluka), both dissolved in methanol (Fluka), as internal standards. GC-MS involved a 2000 series “TRACE GC” gas chromatograph with on-column injector (Thermo Quest CE Instruments, Milan, Italy) coupled to a TSQ quadrupole mass spectrometer (Finnigan Mat, San Jose, CA, USA). The precolumn (TSP deactivated, i.d. 0.53 mm) and the separation column (BGB Wax, 12 m, i.d. 0.25 mm) were both from BGB Analytik (Böckten, Switzerland). GC and MS conditions were as described in ref. [22] with the following modifications: The pressure of the reagent gas methane was set to 2000 mtorr only to allow for a much lower background giving better sensitivity and lower detection limits. To minimize undesired fragmentation of analytes, the ion source was held at 120 °C which demanded frequent cleaning of the ion volume. The signal to noise ratios for the determination of the limit of detection (LOD) and the limit of quantification (LOQ) were set to larger than 3:1 and larger than 10:1, respectively. Caution: Acrylamide (CAS 79-06-1) is classified as toxic and may cause cancer. Wear suitable protective clothing, gloves and eye/face protection when handling acrylamide.

9.4. Results and discussion

9.4.1. Reproducibility of analyses and laboratory roasting process

The method for the determination of free amino acids in almonds was tested with a homogenized sample of Nonpareil almonds in a small inter-laboratory test with four Swiss laboratories (different techniques for extraction, separation and detection). The mean content of free asparagine was 1837 mg/kg with a relative standard deviation (RSD) of 2.0%; the concentration determined by our method was 1854 mg/kg which demonstrates the good performance of the method. In-house validation showed
recoveries of added asparagine of 96% and relative standard deviations (n = 4) of 5% (one homogenized sample) and 9% (four individual samples), respectively. Results were calculated based both on internal and external standards and differed usually only little (maximum 10%); results shown below were calculated with the external standard.

The reproducibility of the roasting process was checked with four individual roasting experiments at equal conditions (180 °C, 5 min) of Nonpareil almonds. The mean acrylamide content was 582 µg/kg with a RSD of 9.7% which is within the range of other process variations reported [16, 18]. Analysis of a homogenized sample of roasted almonds (n = 4) gave a mean acrylamide content of 494 µg/kg with a RSD of 2.7% demonstrating that the analysis varied less than the roasting process. Based on these results, differences between two samples larger than 20% (i.e. two times the variation of the roasting process) were considered to be significant. The analysis of a sample of roasted almonds spiked with different amounts of acrylamide (n = 5, 100 - 1000 µg/kg) resulted in a linear correlation between measured acrylamide contents and added acrylamide with R² = 0.9996 showing the good linearity of the analysis. Based on signal-to-noise (s:n) ratios with standards and real samples the LOQ was estimated to be 10 µg/kg (s:n > 10) and the LOD 4 µg/kg (s:n > 3). E.g. a sample of roasted hazelnuts (injection volume 2 µL) gave a s:n of 37:1 for the acrylamide peak (m/z = 72) corresponding to an acrylamide content of 14 µg/kg.

9.4.2. Variation of acrylamide and its precursors between individual almond kernels

It has been shown that sugars and free amino acids vary considerably between individual potato tubers from the same lot which also affected the potential for acrylamide formation [14]. To test if this fact applies also to raw almonds, individual kernels of the cultivars Butte, Nonpareil, and Monterey (harvest 2003) were analyzed separately for sugars and free amino acids. Results for cultivar Butte are shown in Table 12. The content of free asparagine varied considerably and to a larger extent than that of the sugars. A similar pattern was observed for cultivar Monterey (RSD = 68% for the free asparagine content), whereas for Nonpareil the content of free asparagine varied less (RSD = 30%). The RSD for glucose and fructose contents in all three cultivars ranged from 12 to 33%. The content of total free amino acids varied less in Nonpareil and Monterey as compared to Butte. These differences could be due to different stages of physiological maturity of the kernels [23] and are likely a major cause for the different degrees of browning between individual nuts from the same roasting lot [24, 25]. Altogether, these data show that individual almond kernels of the same batch can vary considerably in their content of acrylamide precursors, especially in free asparagine.
Table 12: Sugars and free amino acids in individual almond kernels of cultivar Butte (data referring to fresh weight, n = 10, RSD: relative standard deviation; Asn: asparagine; TFAA: total free amino acids).

<table>
<thead>
<tr>
<th>Value</th>
<th>Glucose [mg/kg]</th>
<th>Fructose [mg/kg]</th>
<th>Sucrose [mg/kg]</th>
<th>Free Asn [mg/kg]</th>
<th>TFAA [mg/kg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>596</td>
<td>220</td>
<td>38274</td>
<td>1334</td>
<td>3655</td>
</tr>
<tr>
<td>Median</td>
<td>586</td>
<td>232</td>
<td>38429</td>
<td>1114</td>
<td>2836</td>
</tr>
<tr>
<td>Min</td>
<td>482</td>
<td>152</td>
<td>28067</td>
<td>445</td>
<td>1796</td>
</tr>
<tr>
<td>Max</td>
<td>733</td>
<td>286</td>
<td>51121</td>
<td>3463</td>
<td>8011</td>
</tr>
<tr>
<td>RSD [%]</td>
<td>12</td>
<td>23</td>
<td>21</td>
<td>75</td>
<td>58</td>
</tr>
</tbody>
</table>

Therefore, the acrylamide content of roasted almonds was determined to check its variability between individual kernels (Figure 38). A sample of roasted almonds (150 °C, 22 min) obtained from industry was used for this purpose. The acrylamide content of individual roasted kernels varied strongly: The mean and median acrylamide content were 885 µg/kg and 872 µg/kg, respectively, but the values ranged from 334 µg/kg (minimum) to 1811 µg/kg (maximum) and the RSD was 47%. These differences reflect the individual composition of the almond kernels. As a consequence, batches of 200 g almonds (170 to 200 kernels) were used for all roasting experiments, and for analysis of precursors in raw almonds two samples of about 50 g each were analyzed per lot.

![Figure 38: Acrylamide content of individual kernels from the same batch of roasted almonds (white bars: individual kernels; horizontal line: mean value. Data refer to fresh weight, n = 1).](image-url)
Sugars and free amino acids in raw almonds and acrylamide in roasted almonds

Sugars and free amino acids were determined in 18 samples of raw almonds (14 different cultivars) grown in California and Europe. These almonds were subjected to two standard roasting processes, 14 min at 145 °C and 12.5 min at 165 °C, corresponding to a medium and to a dark roasting degree, respectively, and the acrylamide content was determined (Table 13).

Table 13: Sugars and free amino acids in raw almonds and acrylamide formed in two standard roasting processes (Glc: glucose, Fru: fructose, Suc: sucrose, Asn: asparagine, TFAA: total free amino acids, AA: acrylamide. n ≥ 2, except for acrylamide (n = 1); *: Almonds from harvest 2003 (all other samples were from harvest 2004); data referring to fresh weight; variation of analyses within a certain cultivar was smaller than 12% on average).

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Origin</th>
<th>Glc [mg/kg]</th>
<th>Fru [mg/kg]</th>
<th>Suc [mg/kg]</th>
<th>Free Asn [mg/kg]</th>
<th>TFAA [µg/kg]</th>
<th>AA 145 °C 14 min [µg/kg]</th>
<th>AA 165 °C 12.5 min [µg/kg]</th>
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<tr>
<td>Butte</td>
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<td>1610</td>
<td>920</td>
<td>38100</td>
<td>1040</td>
<td>3000</td>
<td>44</td>
<td>712</td>
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<tr>
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<td>1180</td>
<td>700</td>
<td>32100</td>
<td>2760</td>
<td>5390</td>
<td>200</td>
<td>1681</td>
</tr>
<tr>
<td>Nonpareil A</td>
<td>USA</td>
<td>1500</td>
<td>1020</td>
<td>41650</td>
<td>1380</td>
<td>3690</td>
<td>226</td>
<td>1110</td>
</tr>
<tr>
<td>Nonpareil B *</td>
<td>USA</td>
<td>1260</td>
<td>660</td>
<td>23760</td>
<td>1460</td>
<td>4060</td>
<td>251</td>
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</tr>
<tr>
<td>Nonpareil C</td>
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<td>45810</td>
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<td>1265</td>
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<tr>
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<td>USA</td>
<td>1800</td>
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</tr>
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<td>1940</td>
<td>4690</td>
<td>166</td>
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<tr>
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<td>1243</td>
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<td>36930</td>
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<td>2770</td>
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<tr>
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<td>47100</td>
<td>1710</td>
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<tr>
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<td>35970</td>
<td>2640</td>
<td>5510</td>
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<tr>
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<td>39460</td>
<td>1330</td>
<td>3660</td>
<td>73</td>
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<td>26750</td>
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<td>2640</td>
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<td>605</td>
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<tr>
<td>Valencia A</td>
<td>Spain</td>
<td>1230</td>
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<td>38430</td>
<td>820</td>
<td>2880</td>
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<td>460</td>
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<tr>
<td>Valencia B</td>
<td>Spain</td>
<td>1660</td>
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<td>17390</td>
<td>560</td>
<td>2280</td>
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<td>250</td>
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<tr>
<td>Valencia C *</td>
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<td>2580</td>
<td>16110</td>
<td>490</td>
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<td>12380</td>
<td>520</td>
<td>2240</td>
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<td>202</td>
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<td>Longuettes</td>
<td>Spain</td>
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<td>30900</td>
<td>860</td>
<td>2640</td>
<td>66</td>
<td>664</td>
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<tr>
<td>Avola</td>
<td>Italy</td>
<td>4260</td>
<td>1560</td>
<td>15510</td>
<td>570</td>
<td>2350</td>
<td>95</td>
<td>353</td>
</tr>
</tbody>
</table>

Sucrose was the most abundant sugar in all samples with values ranging from 12,000 to 50,000 mg/kg. The mean glucose and fructose contents were about 2000 mg/kg (not including bitter almonds) and 1100 mg/kg, respectively which is in the range of other published values [19, 26]. The sample of bitter almonds contained a very large amount of glucose while the other components were in the range of the other European samples. There was a considerable variation of all parameters.
determined in the raw almonds indicating their different composition which is also noticeable within samples from the same cultivar (e.g. Valencia and Nonpareil).

Asparagine was the major free amino acid in all samples (500 - 2760 mg/kg) accounting for 20 to 50% of the pool of free amino acids. The other free amino acids were (ranges of all samples in parentheses): glutamic acid (270-700 mg/kg), aspartic acid (230 - 720 mg/kg), proline (120 - 630 mg/kg), alanine (110 - 210 mg/kg), valine (nd - 230 mg/kg), glutamine (40 - 250 mg/kg), \( \gamma \)-amino butyric acid (nd - 200 mg/kg), serine (40 - 120 mg/kg), threonine (30 - 430 mg/kg), glycine (40 - 100 mg/kg), phenylalanine (nd - 120 mg/kg), isoleucine (40 - 90 mg/kg), and leucine (40 - 70 mg/kg). In total, almonds contained a large pool of free amino acids: The mean value was 3560 mg/kg and values ranged from 2200 to 5500 mg/kg. Our present data on free amino acids are in the range of or slightly below published data [19, 21].

Light roasting at 145 °C resulted in low to moderate amounts of acrylamide (20 - 360 \( \mu \)g/kg) while during dark roasting at 165 °C up to 1500 \( \mu \)g/kg acrylamide was formed confirming previous findings that the roasting temperature has a very strong influence on acrylamide formation in almonds [19]. For both roasting processes a wide variation of the acrylamide contents was observed which probably reflects the different composition of the almonds. Part of these variations might also be due to inhomogeneities in samples and variation of analysis (see above). The RSD of the acrylamide contents of almonds roasted at 145 °C and 165 °C were 60% and 53%, respectively, which is close to the RSD of the contents of free asparagine (RSD = 56%) in the raw samples.

Interestingly, almonds of European origin contained on average about 2.7 times less free asparagine than American almonds which in turn contained somewhat less reducing sugars and 1.7 times more sucrose than the samples from Europe. A similar but less pronounced pattern of free asparagine in American and European almonds was reported by Seron et al. [21]. They found on average 1.6 times more free asparagine in American almonds (3660 mg/kg, \( n = 4 \)) than in European samples (2240 mg/kg, \( n = 11 \)) which turned out to be a significant difference in the T-test (\( p < 0.05 \)). In our study free asparagine accounted for 39% of the free amino acids in American almonds but only for 25% in European samples. As for acrylamide in dark roasted almonds, the comparison between American and European samples gave the same picture as for free asparagine. American samples contained on average 3.0 times more acrylamide than European almonds. The T-test revealed a highly significant difference between European and American samples for both criteria: \( p \) (free asparagine) < 0.001 and \( p \) (acrylamide, 165 °C 12.5 min) < 0.0001. The situation was similar but less pronounced in medium roasted almonds (145 °C, 14 min): American almonds again contained significantly more acrylamide than European almonds (\( p < 0.05 \)). Furthermore, American samples contained significantly more total free amino acids (\( p < 0.0005 \)), free glutamine (\( p < 0.00001 \)), isoleucine (\( p < 0.0001 \)), proline (\( p < 0.0001 \)), and sucrose (\( p < 0.001 \)) than European samples while the differences were less clear for the reducing sugars. However, to corroborate these differences between almonds from the US and Europe, more samples from further harvests have to be compared. Due to the limited number of samples it was not possible to identify statistical differences between almond varieties. To identify such differences much more samples per variety will have to be analyzed.

The results presented above suggest that the content of free asparagine in raw almonds determined the acrylamide formation. This hypothesis was tested by checking various combinations of acrylamide content and components determined in
raw almonds. A strong correlation was found between acrylamide and the content of free asparagine (Figure 39) confirming the key role of free asparagine for acrylamide formation in roasted almonds. Acrylamide correlated also with total free amino acids ($R^2 = 0.877$) which can be explained by the correlation between free asparagine and total free amino acids ($R^2 = 0.971$), respectively.

![Figure 39: Correlation between the acrylamide content of roasted almonds (165 °C 12.5 min) and the content of free asparagine in raw almonds (n = 1, data refer to fresh weight).](image)

In contrast, no correlation was found between acrylamide and glucose, fructose, or sucrose. This is different from potatoes where reducing sugars largely determine the acrylamide formation and free asparagine does not correlate with acrylamide [14, 27]. Furthermore, the earlier observed connection between the content of reducing sugars and acrylamide based on a very limited number of almond samples [19] has to be considered as rather coincidental than causal. Similar correlations between acrylamide in the heated product and free asparagine in the raw product were also observed in cereal model systems, breads, and gingerbread [16, 28].

Almonds roasted to a medium degree (145 °C 14 min) showed no correlation between the acrylamide content and any of the sugars and amino acids determined. This might be explained by the fact that after 15 min of roasting at 145 °C asparagine and amino acids in general had hardly been consumed and were thus not limiting as will be discussed in the next paragraph.

### 9.4.4. Formation of acrylamide and consumption of its precursors during roasting

Temperature and time are known to influence acrylamide formation [11]. To investigate this interrelation in detail roasting experiments at different conditions were carried out. Figure 40 shows the influence of roasting temperature on the acrylamide content of almonds roasted for 10 min. Acrylamide formation was observed from 140 °C onwards and increased strongly from 140 °C to 180 °C confirming the dominant impact of temperature on acrylamide formation in almonds [19]. At 190 °C
and 200 °C the acrylamide content decreased again showing that elimination of acrylamide exceeded the new formation which was also observed in a model system [7].

![Figure 40: Acrylamide content in almonds roasted for 10 min at different temperatures](image)

**Figure 40: Acrylamide content in almonds roasted for 10 min at different temperatures** (cultivar Butte, n = 1, data refer to fresh weight).

In our previous study [19] we reported a seemingly linear relationship between acrylamide content and roasting time at a constant temperature of 150 °C. However, data did not cover the full time range, and thus, extended roasting experiments were performed at 145 °C and 165 °C (Figure 41). During roasting at 165 °C acrylamide formation started after about 5 min only, and between 7.5 and 15 min an almost linear course was observed confirming previous results [19]. The delay of the onset of acrylamide formation was probably due to the time to heat the almonds to a temperature beyond 100 °C. After about 20 min of roasting the acrylamide content leveled off and decreased afterwards indicating that elimination exceeded net new formation.

Similar patterns of time dependency of acrylamide formation were also observed in model systems [29], roasted coffee beans [4], grated potato [30], and gingerbread [16]. In practice, roasting at 165 °C is an upper limit for almonds and samples roasted for longer than 10-15 min are considered “over-roasted” and bitter and therefore hardly suitable for direct consumption. Therefore, it seems unlikely that approaches based on elimination are feasible to reduce the acrylamide content of almonds. At 145 °C acrylamide formation started only after about 7 min and was much slower as compared to 165 °C. After 32 min the acrylamide contents were still below 900 µg/kg and new formation was yet larger than elimination as indicated by the still increasing acrylamide contents.
The consumption of sugars and free amino acids during roasting presents another point of interest (Figure 42). After 15 min of roasting at 145 °C still 95% of the initial content of free asparagine and 93% of the initial content of total free amino acids were found while less than 20% of the reducing sugars were left. Therefore, asparagine was not limiting at all at this roasting temperature which explains why no correlation between acrylamide and any amino acid was found for this roasting temperature (see above). Although the content of free asparagine determined the acrylamide formation in dark roasted almonds, its depletion was much slower compared to the reducing sugars (Figure 42).

At both temperatures the content of free asparagine slightly increased in the first minutes of roasting. A similar increase was also observed in rye and potato cakes [29], but can only partially be explained by the loss of water. After 5 min roasting at 165 °C still 96% of the initial content of free asparagine was found, while only 16% of the glucose and 38% of the fructose were left. After 15 min still half of the free asparagine was present while about 90% of the reducing sugars had already reacted. This might explain why no correlation was found between reducing sugars and acrylamide formation in standard roasting processes. The reducing sugars were depleted long before the acrylamide content reached its final level. Glucose was consumed somewhat faster than fructose, while the content of sucrose did not decrease to a significant extent in the first 15 min which is well in line with previous findings in roasted almonds [19] and with results from cereal and potato model systems [14, 29].

Figure 41: Development of acrylamide contents during roasting at 145 °C and 165 °C for different times (circles: 145 °C, squares 165 °C. cultivar Nonpareil, n = 1, data refer to fresh weight).
Among the free amino acids glutamine was depleted fastest (not detectable anymore after 5 min) while 60 to 80% of the other free amino acids were still present after 10 min. After 15 min 50% of the initial content of total free amino acids was found and the recovery of the main free amino acids were asparagine: 47%, glutamic acid: 19%, aspartic acid: 67%, proline: 54%, alanine 74%, valine: 40%, serine: 53%. The different extent of consumption of the individual amino acids at least partially reflects their different chemical reactivity which was also observed in potatoes and cereals [14, 29]. The fast depletion of glutamine in roasted almonds can have various reasons: If glutamine is heated with reducing sugars large amounts of 2-pyrolidinone and traces of 3-buteneamide are found [31]. Glutamine can also depleted through the loss of ammonia which takes place even under mild conditions [32] and/or by the formation of pyrrolidone carboxylic acid.

Interestingly, after 5 min 75% of the reducing sugars had disappeared although only 80 µg/kg acrylamide were formed corresponding to only 5% of its maximal content (see Figure 41). Reducing sugars were consumed very fast in the first 5 min of roasting, while asparagine decreased more uniformly. The loss of free asparagine was negatively correlated with acrylamide formation ($R^2 = -0.9836$) which was also observed in cereal and potato model systems [29]. The molar concentrations of reducing sugars and free amino acids in raw almonds at least partly explain these
observations: The median content of reducing sugars was 14 mmol/kg, while it was 42 mmol/kg for total free amino acids and 7 mmol/kg for free asparagine. Thus, the reducing sugars were confronted with about 2.5 times more free amino acids which would explain their fast degradation. A similar effect was also observed in heated potato cakes and the authors suggested that the greater loss of reducing sugars was due to the excess of free amino acids compared to the reducing sugars [29]. However, these different behaviors give also raise to the following hypotheses: (i) Formation of intermediates, e.g. Schiff base or decarboxylated Amadori product [9], did not require a large amount of energy since sugars were rapidly consumed in the first part of the roasting process when the temperature was still increasing. (ii) The release of acrylamide from such intermediates required more energy whereby the acrylamide formation was delayed compared to the consumption of reducing sugars. (iii) Sugars were degraded to fragments (e.g. desoxysones, glyoxal, etc.) under moderate conditions with amino acids acting as catalysts. Once these fragments were formed they readily reacted with free asparagine whereby acrylamide was formed. Hypothesis (iii) is supported by the findings that sugar fragments were formed under mild conditions from glucose and amino acids [33] and that sugar fragments such as glyoxal and hydroxyacetone formed more acrylamide than glucose when heated with asparagine [10, 11]. The determination of intermediates and sugar fragments was out of the scope of the project and thus these hypotheses can not be verified or rejected. However, monitoring intermediates and/or fragments of sugars and asparagine could be useful tools to understand the formation of acrylamide in food matrices.

9.4.5. Stability of acrylamide in roasted almonds

The stability of acrylamide in foods was unclear for some time and supposed to contribute to the wide range of reported acrylamide levels for a certain group of foods. Five samples of roasted almonds (medium roasting degree) were thus stored in sealed containers at room temperature for up to 300 days and reanalyzed. Figure 43 shows that in all five samples the acrylamide content decreased over the storage period. Between the first and last analysis the acrylamide contents had decreased significantly by 44% to 62% which confirms that acrylamide is not stable in roasted almonds [19]. Therefore, roasted almonds with a similar production date must be compared and roasted samples should be analysed quickly. A similar effect was recently reported for coffee and cacao where the acrylamide contents decreased for some 30% during storage [34]. As losses through evaporation or UV-induced polymerization can most likely be excluded, a reaction of acrylamide with Maillard reaction products from the roasting process, e.g. -SH or -NH₂ compounds [34], seems to be a reasonable explanation since acrylamide is known to easily react with thiol-compounds through a Michael addition [1].
9.4.6. Acrylamide and its precursors in hazelnuts

Roasted hazelnuts are widely used as ingredients in the manufacture of bakery and chocolate products but information on the acrylamide content of roasted hazelnuts is scarce. Therefore, samples of raw and roasted hazelnuts were obtained from industry and analyzed. Roasted hazelnuts as typically used for biscuits contained very little acrylamide (14 to 22 µg/kg). This can be explained by the very low content of free asparagine in the raw hazelnuts (Table 14).

Table 14: Sugars and free amino acids in raw hazelnuts and acrylamide in roasted hazelnuts

<table>
<thead>
<tr>
<th>Compound</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose [mg/kg]</td>
<td>970</td>
</tr>
<tr>
<td>Fructose [mg/kg]</td>
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<td>Sucrose [mg/kg]</td>
<td>29260</td>
</tr>
<tr>
<td>Free asparagine [mg/kg]</td>
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<tr>
<td>Free glutamic acid [mg/kg]</td>
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<td>Free alanine [mg/kg]</td>
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<tr>
<td>Free aspartic acid [mg/kg]</td>
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</tr>
<tr>
<td>TFAA [mg/kg]</td>
<td>1610</td>
</tr>
<tr>
<td>Acrylamide (145 °C 14 min) [µg/kg]</td>
<td>16</td>
</tr>
<tr>
<td>Acrylamide (165 °C 12.5 min) [µg/kg]</td>
<td>56</td>
</tr>
</tbody>
</table>

Figure 43: Changes of the acrylamide content in five samples of roasted almonds during prolonged storage at room temperature (n = 1, data refer to fresh weight).
Hazelnuts contained about 40 times less free asparagine as compared to almonds, whereas the sugar contents were similar. In hazelnuts free asparagine contributed only 2% to the pool of free amino acids in which glutamic acid (24% of total free amino acids), alanine (15%), and aspartic acid (10%) were the major free amino acids. Similar sugar contents were found by other groups [26, 35] while Alasalvar et al. reported somewhat higher contents of free amino acids but asparagine contributed again only little (about 7.5%) to the pool of free amino acids [35]. Hazelnuts subjected to the same roasting processes as the almonds (see Table 13) contained only little acrylamide: Roasting at 165 °C for 12.5 min produced dark roasted hazelnuts but even in this sample the acrylamide content stayed below 60 µg/kg. This is about 15 times less compared to the average acrylamide content in almonds roasted under the same conditions which underlines the difference between these two nuts.

Formation of acrylamide in hazelnuts roasted at 150 °C was very slow (Figure 44). After 10 min less than 10 µg/kg were determined and even after 18 min only ~25 µg/kg was found. Sugars were consumed in a similar manner as compared to almonds: After 10 min over 80% of the reducing sugars were degraded while 70% of the free asparagine and almost 80% of the total free amino acids were still present. Sucrose was not significantly depleted during the whole roasting process.

After 18 min 50% of the free amino acid pool was still found, while only about 10% of the reducing sugars were left. Aspartic acid and citrulline were the most stable amino acids, while glutamine was again very unstable (not detectable after 8 min), and still 40% of the initial content of free asparagine was found after 18 min. Amino acids obviously reacted much more slowly than reducing sugars as was also observed in potatoes, almonds and cereals [14, 19, 29].

![Figure 44: Consumption of reducing sugars and formation of acrylamide during roasting of hazelnuts at 150 °C](origin: Italy; n = 1, data refer to fresh weight; squares: acrylamide, circles: fructose, diamonds: glucose).
Overall, the following conclusions can be drawn: (i) Almonds form much more acrylamide during roasting than hazelnuts which is explained by the far larger content of free asparagine in almonds. (ii) Beside the concentration of free asparagine the roasting temperature is the main controlling factor for acrylamide formation in almonds. (iii) By selecting almonds low in free asparagine and by reducing the roasting temperature a significant reduction of the acrylamide content can be achieved. However, as development of flavor and color is also dependent on chemical composition and roasting conditions further research is needed to find the optimal combination of raw material and process conditions in order to obtain a product both with low acrylamide content and favorable sensory properties.

Acknowledgement

We thank the Almond Board of California, Olo Marzipan AG, Wernli AG, and Midor AG for supplying raw almonds and hazelnuts as well as Horst Adelmann and Hanna Schneider for technical support in roasting experiments and amino acid analysis. Financial support was provided by the Swiss Federal Office of Public Health (BAG), the Federation of Swiss Food Industries (FIAL), Cooperative Migros, and COOP Switzerland.

9.5. Literature


10. General discussion

In this chapter, the seven publications that were published during this thesis will be commented. The discussion is divided into three groups: potatoes [1, 2], bakery [3-5], and roasted almonds [6, 7]. After a comparison of these three categories, an outlook for the next steps in the respective fields of research is given.

10.1. Potatoes

Swiss potatoes of the 2002 and 2003 harvests were analyzed for sugars, free amino acids, and their potential for acrylamide formation [1, 2]. The study of the 2002 harvest was the first large study on potato composition with respect to acrylamide formation, cultivars, and farming practice. The project of the following harvest gave new results on the consumption of precursors during heating, demonstrated the importance of year-to-year differences, and confirmed the results of the previous study. In both studies the reducing sugars determined the acrylamide formation while the content of free asparagine in the raw potatoes did not correlate with the acrylamide contents. Studies with French fries and chips confirmed these findings [8-10]. However, it is somewhat surprising that asparagine was consistently not found to determine the acrylamide formation in potato products although it is the key precursor. The following aspects may explain this phenomenon.

- Potatoes contained about 1000 mg/kg of reducing sugars, 3000 mg/kg of free asparagine, and 7000 mg/kg of total free amino acids. This corresponds to 5.5 mmol of reducing sugars and 44 mmol of free amino acids per kg of fresh weight [1, 2]. Thus, about 8 times more free amino acids and 4 times more free asparagine were present compared to glucose and fructose. From a stoichiometric point of view it is evident that reducing sugars are limiting which may explain the correlation of reducing sugars and acrylamide in potatoes.
- Reducing sugars were consumed much faster during heating as compared to free asparagine [2, 11]. Thus, glucose and fructose became limiting before free asparagine. On one hand, this pattern might be due to different chemical reactivities, and on the other hand the differences mentioned above in molar concentrations also explain the fast depletion of reducing sugars.
- The content of reducing sugars varied stronger between different cultivars as compared to the concentration of free asparagine [1, 2, 8]. From a mathematic point of view, a parameter that varies only little (as free asparagine) is unlikely to correlate with a parameter with strong variation (as acrylamide) which may explain the absence of a correlation between free asparagine and acrylamide.
- Additional precursors for acrylamide exist. 3-aminopropanamide was detected in raw potatoes and it formed acrylamide at high yields upon heating [12]. Furthermore, aspartic acid is also present in raw potatoes (about 400 mg/kg) [2] and it formed acrylic acid and acrylamide upon pyrolysis with glucose [13, 14]. Although these compounds cannot explain the whole acrylamide formation, they are not negligible and may partly explain the absence of a correlation between free asparagine and acrylamide.
Fructose and glucose are very soluble in water, while asparagine is only slightly soluble. At room temperature about 990 g of glucose, but only 22 g of asparagine are soluble in one liter water [15, 16]. During the first 10 min of heating most of the water is evaporated and asparagine is likely to become insoluble earlier than the reducing sugars. Thus they retain their molecular mobility longer which may contribute to their faster consumption.

As glucose and fructose are the key factors, their concentration is important primarily for mitigation of acrylamide in potato products. If no raw material with a low content of reducing sugars is provided, all measures focusing on the process (e.g. frying temperature) are in vain or not fully effective. The content of reducing sugars is influenced by storage temperature, cultivar, climate, and eventually farming practice which therefore must be taken into account as well:

- Storage below 8 °C induces the release of reducing sugars [17] and must be avoided for potatoes used for frying [18]. Reconditioning at 12 - 15 °C can eliminate most but not all of the negative effect of cold storage [10]. Storage at temperatures <10 °C prevents the increase in sugars, but demands for the application of sprouting inhibitors.
- Significant differences were detected between cultivars [1]. These differences can be utilized through an appropriate selection of cultivars for fried potato products.
- The climate, particularly temperature and drought, can cause drastic increases of reducing sugars as observed in 2003 [2]. As climate cannot be influenced, the selection of cultivars which are unsusceptible to heat and drought is important.
- In both studies no relationship between acrylamide and farming system or fertilization was found [1, 2]. However, N- and K-fertilization might have some influence [19], but other factors such as storage and cultivar are more important.

On the process side, the frying temperature is clearly the most important factor. Acrylamide formation is much faster at higher temperatures and the effect is particularly strong towards the end of frying [2, 20, 21]. Measures like extraction of precursors (blanching, soaking) or addition of organic acids might be beneficial as well but their use is often limited for sensory reasons.

Altogether, the provision of potatoes with low content of reducing sugars and the control of the frying temperature are the most feasible and promising ways to reduce the acrylamide content of fried potato products. However, there is also a lower limit for the frying temperature and the content of reducing sugars: To prepare a product of desired sensory quality about 200 - 400 mg/kg of reducing sugars are needed for the generation of flavor and color [22, 23]. The temperature should exceed 140 °C at the end of frying to obtain good crispiness and flavor and to avoid oiliness [20, 21].
10.2. Bakery

The three studies with bakery products were all performed using original recipes and ingredients. Gingerbreads were prepared with pilot plant equipment [3], while crackers and biscuits were produced with industrial equipment and at large scale [4, 5]. These projects were thus close to industry which facilitated the implementation of results into industrial processes considerably. The gingerbread study was the first report on the successful application of asparaginase, alternative baking agents, and organic acids in a real bakery product [3]. The preparation of real products was a major advantage over studies with model systems because the influences on color, texture, and flavor were taken into account as well.

In contrast to potatoes, the content of free asparagine in the dough had a strong influence on the acrylamide formation in gingerbread and crackers [3, 5]. The more free asparagine was available the more acrylamide was formed. This finding was confirmed in other bakery products [24-26]. This can be explained from a stoichiometric point of view for both gingerbread and wheat crackers. The amount of reducing sugars in the raw dough outnumbered the one of free asparagine by far, e.g. in gingerbread by a factor of 1,600. Thus, free asparagine was limiting. Flour is usually the main source for free asparagine and contains more reducing sugars than free asparagine. The flour used for the wheat cracker contained 4.6 mmol/kg glucose + fructose but only 0.47 mmol/kg free asparagine. Furthermore, cereal flours contain about 12,000 mg/kg maltose [11] which is an additional reactant for acrylamide formation. If inverted sugar syrup or honey is part of the recipe, the amount of reducing sugars increases drastically and free asparagine becomes even more limiting. This explains why the content of free asparagine has a strong impact on acrylamide content in bakery and often correlates with it.

Some approaches to limit acrylamide formation aim at free asparagine. Its content in dough depends on several factors:

- Type of cereal: Rye contains more free asparagine than wheat [11].
- Type of flour: The content of free asparagine increases with a higher degree of extraction during milling [27].
- Fermentation: Yeasts and lactic acid bacteria consume free amino acids during fermentation [28]. In addition, yeast use sugars as carbon sources.
- Additional ingredients: Potato flakes and almonds are rich in free asparagine.

Selection of ingredients with low content of free asparagine decreases its amount in the dough and thereby the formation of acrylamide during baking. The application of an asparaginase is an attractive alternative because it only degrades the key precursor, while the recipe, the process, and consequently the sensory properties of the product remain the same. The addition of an asparaginase during the mixing of the dough decreased the acrylamide content in gingerbread by 55% and in crackers by over 70% [3, 5]. Thus, asparaginase is an option for mitigation and deserves further investigation. The disadvantages are its yet high costs and some legislative as well as toxicological aspects need to be clarified.

Asparaginase did not completely prevent acrylamide formation because asparagine was not fully degraded. For example, about 25% of the free asparagine was still present in the treated gingerbread dough before baking. Thus only a large decrease in free asparagine will significantly reduce the acrylamide content. From this point of view, it is questionable if a substantial progress is made in sweet bakery with the
optimization of flour type and source. But for unsweetened products like bread and crisp bread, this approach may be successful because the amounts of free asparagine and sugars are decreased [27].

However, the amount of free asparagine is not the only critical factor for acrylamide formation. The baking agent and the reducing sugars play a very important role, too. In all three studies, the baking agent NH₄HCO₃ strongly enhanced the acrylamide formation [3-5]. Interestingly, this effect diminished [4, 5] or completely disappeared [3] when the main sources for reducing sugars were replaced by sucrose solutions. The small amounts of acrylamide in products prepared without addition of reducing sugars can be explained by the presence of glucose, fructose, and maltose in the flour or by the eventual hydrolysis of sucrose or starch. The hypothesis that the promoting effect of NH₄HCO₃ was due to amidation of acrylic acid by NH₃ released from the baking agent turned out to be wrong although this pathway was reported in model studies [13, 14]. As shown in our gingerbread study the temperature within the product stayed below 110 °C during the whole process, which was not high enough to enable this pathway. In contrast Yaylayan and coworkers obtained their results in pyrolysis experiments at 350 °C [13] which demonstrates that model systems may behave in a different way and are sometimes not directly comparable to real food.

The fact that the effect of NH₄HCO₃ on acrylamide formation depended on the presence of reducing sugars and that the yield of acrylamide was higher if NH₄HCO₃ is present, gave raise to another hypothesis. The promoting effect of NH₄HCO₃ might be indirect by the reaction of ammonia with reducing sugars which provides reactive sugar fragments. These fragments may react more efficiently, under milder conditions, and at higher yields with free asparagine as compared to hexoses. Figure 45 gives a schematic overview on this hypothesis.

![Hypothesis on the promoting effect of NH₄HCO₃ on the formation of acrylamide from asparagine and reducing sugars.](image)

Figure 45: Hypothesis on the promoting effect of NH₄HCO₃ on the formation of acrylamide from asparagine and reducing sugars.
The following facts support this hypothesis:

- If no NH₄HCO₃ was added to gingerbread dough, almost no acrylamide was formed, although reducing sugars and asparagine were present [3]. The thermal conditions within the product were not drastic enough for a substantial acrylamide formation.
- NH₄HCO₃ enhanced the acrylamide formation only in presence of reducing sugars but not with sucrose [3-5]. This indicates an interaction of NH₄HCO₃ with the carbonyl group of glucose and fructose which is not possible with sucrose.
- Sugar fragments like glyoxal and methylglyoxal were formed already at 100 °C in Maillard model systems [29]. Therefore, these fragments can be formed already during the early phase of baking and also within the product where the temperature never reaches values above 100 °C.
- Glyoxal, acetol, or glyceraldehyde formed more acrylamide in reaction with asparagine compared to glucose [30, 31]. This might explain the higher yields of acrylamide per mole asparagine in presence of NH₄HCO₃. Furthermore, sugar fragments may form acrylamide at a lower temperature compared to glucose because of their higher reactivity.
- Fragmentation of sugars increases the number and the reactivity of carbonyls reacting with asparagine which enables the higher yields of acrylamide.
- Acrylamide was formed in model studies with NH₄HCO₃ at 60 °C and even at room temperature [32]. The available data on acrylamide formation from hexoses cannot explain these findings.
- Preliminary experiments with model systems showed that sugar fragments such as glyoxal and methylglyoxal formed more acrylamide in reaction with asparagine at 120 °C compared to glucose. Furthermore, the formation of such α-dicarbonyls was observed in model systems of glucose and NH₄HCO₃ under mild conditions. Further investigations in this field are planned.

If NH₄HCO₃ is replaced by NaHCO₃ as baking agent, the acrylamide content is substantially lower [3-5]. However, NH₄HCO₃ and NaHCO₃ have different properties and therefore some adjustments have to be made.

- NH₄HCO₃ releases gas (CO₂ and NH₃) mainly during the baking process, while NaHCO₃ (particularly in combination with acids) reacts already at room temperature. This can affect the volume, porosity, and texture of the product.
- NH₄HCO₃ releases the double gas volume per mole compared to NaHCO₃. For the replacement of NH₄HCO₃, best results are obtained if the amount of NaHCO₃ is adapted to obtain the same total gas volume [4].
- NaHCO₃ needs a good proton-donor to be neutralized and to release CO₂ efficiently. Thus, leavening can be improved by addition of organic acids, e.g. tartaric or citric acid which also eliminates the alkaline taste [4].
- Leavening by NaHCO₃ is also facilitated if some extra water is added whereby the viscosity of the dough is reduced [5].
- If ammonia contributes to the flavor of the product, e.g. some gingerbread products, the replacement of NH₄HCO₃ by NaHCO₃ may be critical for sensory reasons.
Interestingly, some 170 µg/kg acrylamide was still formed in gingerbread if NH₄HCO₃ was fully replaced by NaHCO₃ whereas almost no acrylamide (about 10 µg/kg) was found if no baking agent at all was used [3]. This effect is difficult to explain and only speculative statements can be given: The pH of the product with NaHCO₃ (pH 8.2) was higher compared to the one without baking agent (pH 5.4). A higher pH might facilitate the acrylamide formation in several ways:

- The α-NH₂ group of asparagine is less protonated (pKₐ = 8.8 [15]) which enables the formation of the imine. Thus the first step in the formation of acrylamide is facilitated.
- A more alkaline environment can enhance the formation of reactive dicarbonyls via sugar fragmentation catalyzed by α- and ε-NH₂ groups of amino acids [33].
- More alkaline conditions may favor the formation of a 2-azaallyl anion from the Schiff base which can also generate acrylamide whereas more acidic conditions favor the Amadori rearrangement or even the hydrolysis of the Schiff base [34].

Experiments with citric and tartaric acid clearly showed that an acidic pH in the dough lowered acrylamide concentrations in bakery [3, 4] which indirectly supports the hypothesis illustrated above. But acidification is limited because of unpleasant taste and poor development of browning and flavor. Acrylamide, flavor and color are interrelated through the Maillard reaction and thereby measures aiming at Maillard reaction might not be applicable in practice. This is also true for to the replacement of reducing sugars by sucrose which was very effective in gingerbread, wheat crackers, and biscuits [3-5]. Asparagine needs a reducing sugar to form acrylamide [35] and sucrose was probably not hydrolyzed during baking. However, if browning is important for the product character, e.g. for gingerbread, this approach is not suitable, because the lack of reducing sugars hindered the formation of melanoidins in the Maillard reaction [3, 23]. But for the other products investigated, this approach was feasible and the sensory properties of the products were good [4, 5].

The addition of amino acids can decrease the acrylamide content of bakery, as shown with glycine in gingerbread [3] and other products [36]. Competition for the carbonyls between asparagine and glycine and/or elimination of acrylamide by reaction with glycine (e.g. Michael addition type) may have caused this effect. Interestingly, added amino acids on the one hand slightly lowered the pH and decreased the acrylamide content, but on the other hand they intensified browning [3]. This can be explained by the higher number of α-NH₂ groups available for Maillard reaction. Although the sensorial and toxicological aspects need to be clarified yet, this approach has one important advantage: Intensified browning combined with lower acrylamide contents. In all other experiments acrylamide was coupled with browning. Thus the addition of glycine or other amino acids may be an option, eventually in combination with other measures, to reduce the acrylamide content in bakery.

On the process side, time and temperature obviously influence acrylamide formation which in turn is often correlated with browning [3-5]. The influence of temperature is rather product specific: Shorter baking at higher temperature led to lower acrylamide contents in gingerbread with the same browning, but a lower temperature towards
the end of baking decreased the acrylamide concentration in potato crackers. Thus, no general rule can be given and baking has to be optimized for each product. However, avoiding excessive browning limits the acrylamide formation in all products. Altogether, acrylamide formation is more complex in bakery compared to potatoes because the dough consists of different components. However, this complexity also offers more options to decrease the acrylamide content, particularly through additives mixed to the dough. The promoting effect of NH₄HCO₃ is still to be elucidated. Further investigations on the formation of dicarbonyls as sugar fragmentation products may provide deeper insight in this field.

10.3. Roasted almonds

Raw almonds contain considerable amounts of free asparagine and reducing sugars but only little water (about 5%). Therefore, they present an ideal matrix for acrylamide formation during heating which firstly was observed by Becalski et al. and Weisshaar [37, 38]. However, the different aspects of the roasting process and the stability of acrylamide in roasted almonds were first investigated in our studies [6, 7]. Analysis of various products showed that roasted almonds contained more acrylamide as bakery with almonds, while marzipan, raw or blanched almonds contained no acrylamide [6]. A temperature below 100 °C does not allow for acrylamide formation in almonds. In bakery other ingredients (see above) are more relevant than almonds themselves [6]. But due to their large content of free asparagine, they may contribute to acrylamide formation in bakery, especially when added ground or as coating/decoration [4, 38].

The content of free asparagine and the roasting temperature are the two critical factors for acrylamide formation in almonds. The more free asparagine raw almonds contain the more acrylamide is formed during roasting [7]. This rule applies to hazelnuts as well which contain only little free asparagine and form only small amounts of acrylamide. Interestingly, significant differences were detected between European and American almonds: The samples from the US contained more free asparagine and formed more acrylamide in standard roastings than the European samples [7]. To date it is unclear if this is due to different farming practices (e.g. fertilization) or if it indicates differences between the cultivars. The roasting temperature was the most important process parameter. Roasting at 165 °C instead at 145 °C for 10 min boosted the acrylamide content by a factor of 9, while roasting for 15 min instead of 10 min at 165 °C increased it only by a factor of 1.6 [7]. During prolonged roasting the acrylamide concentration declined because elimination outnumbered new formation. Furthermore, both asparagine and sugars were almost depleted at that time which rules out substantial formation. This was also observed in roasted coffee beans [39]. In contrast to almonds, commercial roasting of coffee is performed at conditions where elimination of acrylamide exceeds new formation by far. At such conditions almonds become over-roasted and are no more suitable for consumption. Thus enhanced roasting is not a way to reduce the acrylamide content of roasted almonds. On the contrary, a lighter roasting degree will limit the acrylamide content in almonds because acrylamide correlated with the darkness (L-value) and redness (a-value) of the roasted almond [7].
Coffee, cocoa and roasted almonds have in common that acrylamide is not stable during storage [6, 7, 40]. It is assumed that during roasting reactive (flavor?) compounds (e.g. thiols) are formed that react with acrylamide. But to date, no evidence for that has been reported yet.

10.4. Comparison of potatoes, bakery, and almonds

Potatoes, dough, and almonds are rather different as raw materials, but all form acrylamide during heating. In Table 15 some factors for acrylamide formation are listed. In all categories the key precursors are present, but large differences within and between categories exist.

Table 15: Comparison of potatoes, dough, and almonds as raw materials (values are rounded mean values and refer to dry matter, except for water).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Potato</th>
<th>Dough</th>
<th>Almond</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water content [%], fresh weight</td>
<td>80</td>
<td>40</td>
<td>5</td>
</tr>
<tr>
<td>Free asparagine [mmol/kg]</td>
<td>100</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Glucose + fructose [mmol/kg]</td>
<td>15 - 60</td>
<td>1 - 100</td>
<td>20</td>
</tr>
<tr>
<td>Surface : volume ratio</td>
<td>Medium to high</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Promoting additives</td>
<td>No</td>
<td>No / Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

During frying of potatoes a large amount of water evaporates which explains the delay of acrylamide formation. But due to their very high contents of free asparagine and reducing sugars, they can form large amounts of acrylamide to which 3-aminopropanamide might also contribute. The range of acrylamide contents in potato products is very broad (100 - 3000 µg/kg) because the amount of reducing sugars varies strongly. Although almonds contain 10 times less asparagine compared to potatoes, they can form up to 2000 µg/kg acrylamide because of their high surface to volume ratio and their low water content which allows temperature to quickly exceed 100 °C. The acrylamide contents in bakery are often low, because the raw dough contains only little free asparagine and because the surface to volume ratio is low. The amount of glucose and fructose can be very low (e.g. in breads) or very high (e.g. in sweet bakery like gingerbread) which makes comparisons within this category difficult. Furthermore, maltose might also contribute to acrylamide formation. Because the strong promoter NH₄HCO₃ is used in some bakery products, the acrylamide content can exceed 1000 µg/kg in spite of the low concentration of free asparagine.

The composition of individual potato tubers and almond kernels varied strongly within the same lot which was reflected in the acrylamide formation [1, 7]. These differences are likely due to different maturity of the individual tubers and kernels. This is important for sampling otherwise non-representative results may be obtained.

The amount of free asparagine in the raw material was crucial for the acrylamide formation in bakery and almonds, whereas the reducing sugars determined the acrylamide formation in potatoes. During roasting of almonds the reducing sugars were consumed much faster than free asparagine [7], as was also observed in grated
potatoes [2]. Nevertheless the amount of free asparagine in raw almonds correlated with acrylamide but the reducing sugars did not [7]. This seems contradictory to the results obtained with potatoes [1, 2].

The following hypothesis might explain this phenomenon: One of the precursors (free asparagine or reducing sugars) may correlate with acrylamide if the following conditions are fulfilled:

1. It is consumed to a significant part (> 40 %) and still decreasing at the time when acrylamide is determined.
2. If it is depleted long before the acrylamide content is determined, it cannot correlate with acrylamide.
3. If it is only consumed to a small extent (< 20 %) it cannot correlate with acrylamide either.
4. The acrylamide content is determined before elimination outnumbers new formation (i.e. it is still increasing).

In summary, the acrylamide content correlates with that precursor which becomes limiting at the time acrylamide is determined. The following aspects may support this hypothesis and explain the difference between potatoes and almonds:

- A correlation between asparagine and acrylamide was observed in almonds roasted at 165 °C for 12.5 min. At this time the reducing sugars had already been depleted for several minutes. If this applied to all almond samples, the content of reducing sugars could not correlate with acrylamide because acrylamide was determined at a time when there were no more differences between reducing sugars in the different samples.
- In contrast, free asparagine was depleted only by some 50% after roasting at 165 °C for 12.5 min. Therefore differences between the contents of free asparagine of the almond samples were still existent at that time which may explain the correlation between asparagine and acrylamide.
- After roasting at 145 °C for 14 min no correlation was found between asparagine and acrylamide (R^2 = 0.21). This is explained by the minimal losses of free asparagine (< 5%) at this time. Thus asparagine was far from becoming limiting which corroborates the statements above.
- In grated potatoes, free asparagine was consumed to some 30% only, while the reducing sugars were just about to become depleted after heating at 120 °C for 40 min [2]. This fact and the molar excess of amino acids compared to reducing sugars may explain the correlation of reducing sugars with the acrylamide content in potatoes.
- In grated potatoes reducing sugars correlated with the acrylamide after heating at 120 °C for 40 min (R^2 = 0.84, p < 0.05) but this correlation disappeared (R^2 = 0.33, p = 0.32) after an additional heating at 160 °C for 20 min when reducing sugars probably had been completely exhausted [41].

As a conclusion, the content of acrylamide precursors and their molar ratios in the raw material are of fundamental importance for acrylamide formation. But a correlation between a precursor and acrylamide depends also on the moment when acrylamide is determined, on the concentration of the precursor at that time, and on the presence of promoters (e.g. NH₄HCO₃) and inhibitors (e.g. organic acids).
The different consumption patterns of reducing sugars and free amino acids during heating [2, 7, 11] give rise to a new hypothesis: Acrylamide is only formed in substantial amounts after glucose and fructose have been degraded to reactive fragments. Sugar fragmentation takes place at relatively mild conditions (around 100 °C) and is “catalyzed” by amino acids [23, 29]. This would explain why sugars are degraded right from the beginning of heating when temperatures are still moderate (< 120 °C). It would also explain why reducing sugars are virtually depleted before substantial amounts of acrylamide are formed and before free amino acids are consumed. Once these fragments are formed and the temperature increases further then asparagine likely reacts rather with the more reactive fragments than with native glucose or fructose. The fact that sugar fragments like glyoxal and hydroxyacetone formed more acrylamide in reaction with asparagine than glucose under the same conditions [30, 31] supports this hypothesis. It is also compatible with the hypothesis made for the promoting effect of ammonia (see Figure 45). From this point of view the concentration of free amino acids receives another meaning: The more free amino acids are present the faster the reducing sugars might be degraded and the better the acrylamide formation is enabled. The absence of positive correlations between acrylamide and the content of free amino acids is not necessarily contradicting. The more free amino acids are available, the more reactants other than asparagine are available and they compete against asparagine for the carbonyls. This is consistent with the observation that addition of amino acids decreased the acrylamide content in bakery [3, 36].

10.5. Outlook

The content of reducing sugars and the frying temperature are identified as the critical factors for acrylamide formation in potato products. Thus the next steps should focus on the following:

- Optimization of storage to keep the content of reducing sugars low. Cold storage (< 8 °C) combined with reconditioning or warm storage (> 10 °C) combined with the application of sprout inhibitors are known techniques which should be optimized with respect to acrylamide formation.
- Selection of potato cultivars that have a low content of glucose and fructose and that are not susceptible to climatic influences such as drought and heat.
- Improvement of frying technique: The equipment should allow for an exact regulation and eventually programming of the temperature during frying, e.g. a decreasing temperature profile.

Breeding potato cultivars with a much lower content of free asparagine might be another approach. But concepts aiming at sugars and temperature seem to be more promising and can be implemented in a shorter time.

For bakery products, the most feasible ways to reduce the acrylamide content are avoiding NH₄HCO₃ and controlling reducing sugars and free asparagine. If asparaginase will become commercially available at competitive prices and no toxicological limitations hinder its application, this approach will gain much importance and relevance. Thus investigations on application and activity of this
enzyme in food matrices are advisable. Classical fermentation is another approach to eliminate free asparagine in the dough and may be an option for some chemically leavened products. Addition of amino acids, e.g. glycine, to decrease acrylamide and enhance browning at the same time, also deserves further research. The mechanism and the sensorial and toxicological aspects should be checked as well. This can be done with labeled acrylamide or labeled amino acids which allows for the specific detection of the formed derivatives. As the acrylamide formation is particularly complex in bakery, it is imperative to perform experiments with real products under most realistic conditions. Otherwise results may be obtained that are not transferable to the product of interest.

To elucidate the mechanism how NH₄HCO₃ enhances the acrylamide formation, sugar fragments such as dicarbonyls should be monitored and experiments with ¹⁵N-labelled NH₄HCO₃ might provide further insight into its effect and fate during baking.

Investigations on free asparagine are the starting point for almonds. As a first step, differences between the different cultivars must be evaluated. This can be done in a way like the studies with Swiss potato cultivars [1, 2]. Then the impact of origin (America versus Europe) and farming practices (in particular fertilization) needs to be clarified. For the time being, the selection of almonds with low content of free asparagine but high content of other free amino acids might be a way to produce roasted almonds with less acrylamide. Additives such as organic acids are probably not practicable for whole almonds, but the initial water content might be an option which should be kept in mind.

To better understand the acrylamide formation in food from a chemical point of view, the following points are interesting:

- Monitoring of acrylamide, its precursors, and their intermediates in food. This may clarify why some precursors are critical factors and correlate with acrylamide formation while other are not. The identification of the rate limiting step and compound will provide a basis to control the formation.
- Determination of kinetic parameters, e.g. activation energy, for the formation of acrylamide, color, and flavor compounds. Thereby, process conditions might be found where acrylamide is not coupled to flavor or color.
- Formation of flavor and color in parallel to acrylamide formation. Mitigation of acrylamide in food will only succeed if the sensory quality of the product is not negatively affected. Analysis of key flavor compounds and sensorial tests are helpful to find the optimal solution.
- Identification of the products when acrylamide is eliminated. Experiments with ¹³C-labeled acrylamide are advisable and the compounds formed in reactions with amino acids, proteins, sugars, and flavor compounds as well as acrylamide polymers as such are to be looked for. Knowledge about the formation and nature of these compounds may open new ways for mitigation.
- Relevance and occurrence of other precursors for acrylamide formation. 3-aminopropanamide (3-APA) and eventually aspartic acid in potatoes, carnosine, creatine, and aspartic acid in meat, maltose in flours, and β-alanine are interesting compounds in this respect.
- In addition to the biochemical formation of 3-APA from asparagine, the thermal generation of 3-APA should be further investigated. The formation of 3-APA
during heating of food and the occurrence of 3-APA in raw, semi-finished, and finished products should be checked.

- Formation of other vinylogous compounds such as acrylic acid, styrene, and N-alkyl derivatives of acrylamide.

Apart of these chemical aspects the toxicological relevance of acrylamide in food needs yet to be clarified. Acrylamide is likely to be of greater importance than other heat-induced toxicants such as polycyclic hydrocarbons or heterocyclic amines. However, more evidence for the toxicity of dietary acrylamide is needed to support the endeavors of industry to reduce the acrylamide content in all food products.

### 10.6. Literature


List of publications

The following seven sub-projects were published in peer-reviewed journals during the present thesis:


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