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Agronomic Strategies to Reduce Potential Precursors of Acrylamide Formation in Cereals

Dissertation

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12 Jahre,

1 Monat,

3 Wochen

und 2 Tage…

„…Ich habe fertig“

Giovanni Trapattoni

Gewidmet meiner Frau Sabine und meinen Kindern Muriel, Aurelie und Emilian

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Acronyms

AA	acrylamide
Asn	asparagine
$CaCO_2$	calcium carbonate
CIAA	confederation of the food and drink industry
CP	crude protein
EU	European Union
ha	hectare
M	molar mass
N	nitrogen
Na HSO_3	sodium bisulfite
NM	Nahrungsmittel
QTL	quantitative trait locus
IARC	international agency for research on cancer
S	sulfur
SDS	sodium dodecyl sulphate
SV	sedimentation value
*, **, ***	level of confidence (*<0.05, **<0.01, ***<0.001)
%	percent
R^2	coefficient of determination

1 Introduction

1.1 Acrylamide in food products

Next to food security the safety of food is the most desirable goal in food science. As food products are expected to contribute to human health and constitution, harmful substances such as residues of pesticides, mycotoxins, heavy metals, and environmental pollutants should be avoided. Besides naturally occurring contaminants in raw material, harmful substances can also be formed during food processing, e.g. nitrosamines, furan and acrylamide (AA). Normally most of these substances are easly to remove before food processing occurs, e.g. by choice of raw material. However, if the contaminant is formed during the processing of food products, this is much more difficult. Since 2002, the food contaminant Acrylamide (AA) has drawn attention worldwide.

AA is widely used as poly-AA in industry applications like plastics, colours, for the treatment of waste water in paper manufactures and as a flocking agent to build tunnels. Mono-AA is regarded as potentially neurotoxic, manipulating male reproduction, possibly causing birth aberrations and eventually even causing cancer (Friedman, 2003). The International Agency for Research on Cancer announced that AA is classified as probably carcinogenic to humans (IARC, 1994).

Until 2002, there had been no information on the fact that AA can be relevant within foods. However, a study of Tareke et al. (2000) revealed a link between heated food products and AA. Two years later, it was again the research group of Tareke et al. (2002), who finally proved, that AA is present in cooked food. Hence, AA is closely bound to the human diet.

Further studies then showed that AA is mainly formed during heat processing of carbohydrate rich foods based on potatoes and cereals (Mottram et al., 2002, Stadler et al., 2002). In this context the two main precursors of AA formation, reducing sugars and the amino acid free asparagine (Asn), react within the Maillard reaction (a non-enzymatic browning reaction) to form AA (Delatour et al., 2004).

Up to now, considerable progress has been made in methods of analysis, occurrence, formation, chemistry, toxicology, and potential health risk of AA in the human diet (Mottram et al., 2002, Stadler et al., 2002, Wenzl et al., 2003, Friedmann and Levine, 2008, Taeymans et al., 2004, Zhivagui et al., 2019).

However, in their published review on AA, Raffan and Halford (2019) described the monitoring history of AA in several food groups. They reported that after starting the implementation of strategies to reduce AA in 2002, AA was successfully decreased in food products. Yet, over the following years this effect diminished, and fluctuating or even higher levels of AA emerged. As a consequence, in 2017 the European Commission announced regulation that aims at limiting the level of AA in food products consistently (Commission Regulation, EU 2017/2158). A section in the regulation detailed that AA in foodstuffs potentially increase the risk of generating cancer for consumers across all ages. Thus, the commission regulation fixed benchmark levels for different food groups that must not be exceeded. Hence, food industry and gastronomy face the challenge of establishing mitigation strategies to meet benchmark levels for AA. Finally, at present, AA is seriously considered to potentially raise the risk of cancer for humans (Konings et al., 2010).

To date, many studies have been published investigating different food products regarding their contribution to daily AA exposure, including potatoes, cereals, coffee, olives, almonds, chestnuts, dried fruits, cooked rice and tomato sauce (Roach et al., 2003, Grob et al., 2003, Amrein et al., 2004a & 2005, Andrzejewski et al., 2004, Tateo et al., 2007, Karasek et al., 2009). During the last years barely any food group has not been analyzed for AA. Nevertheless, main research has focused on potatoes and cereals as both food groups, next to coffee, contribute the most to daily AA intake (Dybing & Sanner, 2003, Raffan & Halford, 2019).

In this context, studies focused on potato products revealing the impact of cultivars, storing temperatures, amount of fertilizers and climatic conditions (Weisshaar & Gutsche, 2002, Grob et al., 2003, Levine et al., 2005, Sohn & Ho, 1995). Additionally, changing process conditions e.g. frying temperature, time of heating or adding additives were investigated for their potential to minimize AA formation (Foot et al., 2004, Jung et al., 2003).

However, although strongly heat-treated potato products can contain much more AA than products based on cereals, foods such as biscuits, bread, rolls and crisp bread account for about 25 to 45 % of the dietary AA intake in Germany (European Food Safety Authority, 2011). The main reason is the high daily per capita consumption of bread of almost 240 g. By contrast potato chips and French fries contribute only 24 g (Claus et al., 2008). Thus, cereal products play an important role for the daily AA intake.

As reducing sugars are the limiting factor of AA formation within potatoes, for cereal based products the amino acid free Asn is the main precursor that limits the level of AA during food processing (Becalski et al., 2004, Weisshaar, 2004, Surdyk et al., 2004). The first efforts to reduce AA in cereal food products were similar to potatoes and focused on food processing. Changing processing steps during food production like heating temperature or heating time showed to be very promising applications to reduce AA in the final product (Surdyk et al., 2004). Finally, many studies were accomplished investigating the effect of post-harvest processing measurements to affect mostly AA formation pathways. This also included storage conditions and milling to reduce or dilute the AA precursor free Asn. The next section presents a short overview of these strategies.

Technological strategies to minimize AA

Amrein et al. (2004b) discovered the effect of ammonium hydrogencarbonate as a baking agent for the processing of gingerbread. By replacing ammonium hydrogencarbonate with sodium hydrogencarbonate the AA level dropped by 60 %. Additionally, they explored that AA level was raised by prolonging the browning intensity. The same research group showed, that replacing reducing sugars like fructose or glucose with sucrose decreased AA formation.

Prolonging dough fermentation time could be a further feasible way as Fredriksson et al. (2004) and Claus et al. (2007) reported a reduction of AA in yeast-leavened bread as a consequence of fermentation extension. By milling extraction rate (flour type) AA can be influenced by decreasing free Asn in flours containing less husk (Claus et al., 2006, Capuano et al., 2009). Haase et al. (2003) stated a minimization of AA by lowering the heating temperature while extending the heating time in rye bread. The same was found for wheat

bread of Claus et al. (2007). They also revealed that utilization of desk ovens is favourable to convection ovens in order to reduce AA.
A very high impact capacity on AA degradation is supposed by using different types of additives. There are some publications which tested various kinds of additives. Wakaizumi et al. (2009) showed the impact of filamentous fungi to decrease the content of AA in roasted green tea. They discovered the impact of 24 different strains of filamentous fungi on decreasing ability to AA. The addition of antioxidants was studied by Hedegaard et al. (2008). Rosmarinic rosemary extract, rosemary oil or dried rosemary leaves added to wheat dough reduced the amount of AA in wheat rolls by 57 to 67%. Similar effects were reported by Levine et al. (2009) as they analyzed the effect of adding $CaCO_2$. AA reduction was 36% if a 0.04 M solution of $CaCO_2$ was used. For the same inorganic salt Kukurova et al. (2009) carried out AA reductions of 60 to 90%. Different authors reported the effect of other inorganic salts like sodium chloride, potassium ferrocyanide and potassium iodate on AA formation (Moreau et al. 2009, Kolek et al. 2007). For each salt the effect of polymerization decreased AA formation. The impact of citric acid and glycine on AA was announced by Brathen et al. (2005) and Low et al. (2006). The application of the enzyme asparaginase reduced free Asn and is a promising way as it has been reported by different authors (Ciesarova et al., 2009, Capuano et al., 2009, Hendriksen et al., 2009). In wheat breads, the enzyme is able to reduce about 30 to 60% of free Asn (Tuncel et al., 2010). However, there are some difficulties with enzymes. As foodstuff is very complex (e.g. different components, pH-values, water contents), changes of the enzyme efficiency might occur. Tuncel et al. (2010) used a pea originated asparaginase and reached a high reduction of AA but at the same time unwanted rheological and sensory properties of the final product. Furthermore, production of asparaginase on a large scale is linked to high costs (Capuano & Fogliano, 2010).
Kotsiou et al. (2011) studied the impact of standard phenolic compounds and olive oil phenolic extracts. They discovered both a decrease and a strong increase of AA. If phenolic compounds like ferulic or gallic acid were applied, AA fell up to 70% but if olive oil phenolic compounds were used, AA increased. Zeng et al. (2009) published the impact of vitamins on AA reduction in a chemical as well as in a food model system. Only water-soluble vitamins like biotin, pyridoxine, pyridoxamine, L-ascorbic acid and nicotinic acid had a reduction potential of 34 to 51%. Claeys et al. (2005) and Claus et al. (2008) reported the impact of cysteine and lysine on AA for a model system and in dough. Both agreed that the addition of cysteine lowered the levels of AA. The beneficial impact of selected additives like ascorbic acid, $NaHSO_3$ and allicin was reported by Yuan et al. (2010) in an aqueous model system leading to AA inhibition of up to 50%.
In summary, it has been shown within cereal food production, that AA formation can be reduced by adjusting processing conditions such as the heating temperature and the heating time, pH, by changing the baking agents, by adding additives or by using enzymes. A further minimization can be achieved by elucidating the mechanistic pathways of AA formation and by removing precursors or intermediates. The approaches described above, often led to a significant AA reduction of 20 to 90%, depending on product group and type of modification.

However, the applicability of technological methods is restricted, because their use is often escorted by negative effects on rheological and sensory properties or on the quality and taste of the final products or they are simply too expensive to use (Tuncel et al., 2010, Capuano & Fogliano, 2010). Claus et al. (2008) reported the risk of decreasing consumers' acceptance by changes in recipe on an industrial scale.
In addition, Raffan & Halford (2019) reported in their review that food industry has not shown sufficient progress in reducing AA in their products, especially since 2011. They stated that raw material might be responsible for this. Thus, as precursors of AA mainly reducing sugars and free Asn can fluctuate in their content, for instance in cereal grain flour or potato tubers, technological treatments to reduce AA can fail. This makes reducing AA for food industry a huge effort.

	Different impact departments			
	pre-harvest	post-harvest (food processing chain)		
Category	**Agricultural strategies**	**Recipe or additives**	**Processing conditions**	**Final product preparation**
Potato products	+++	+	++	+++
Bread/Biscuits/ Bakery wares	+++	++	++	++
Breakfast cereals	+++	+	+	--
Coffee	--	--	+	++
Coffee substitutes	+	-+	-+	--

Figure 1: Impact power of different departments on AA formation of different food categories, +++ high impact, -- low or no impact (data based on Food Drink Europe CIAA Toolbox 2006)

Further, as it is visualized in Figure 1 (based on data of the Food Drink Europe CIAA Acrylamide Toolbox 2006) a very high impact on AA formation is presented only for the final products preparation during food processing. In contrast, for cereal and potato products agricultural strategies are very effective by reducing AA content in a previous step. This is highlighted by the red frame.

Hence, it seems highly promising to implement agronomic strategies in order to drop AA precursors. Thus, the following section depicts possible agronomic strategies in detail.

Agronomic strategies to minimize AA precursor free Asn in cereals

As the used raw material, notably the free Asn content in cereal grain, plays a central role for the AA formation potential, gaining information on how agronomic strategies can influence free Asn synthesis efficiently is essential. Managing free Asn synthesis during plant growth alongside the whole crop management chain – starting at crop rotation – will be the most promising approach (see Figure 2).

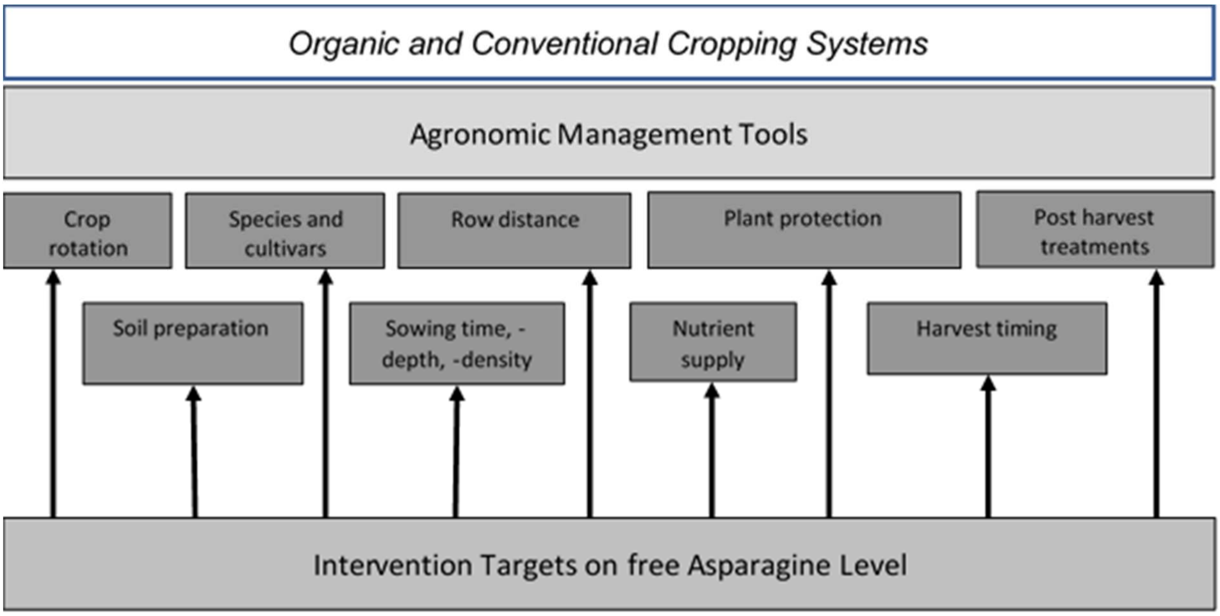

Figure 2: Agronomic strategies to influence formation of free Asn as precursor of AA alongside the plant production chain for cereals

Species and cultivars

Several studies revealed that cereal species differ in their Asn content and subsequently in their potential to form AA. Compared to wheat and spelt, rye usually delivers higher Asn levels (Claus et al., 2006, Curtis et al., 2009 & 2010). Durum, barley, oat and maize were investigated by Žilic et al. (2017). The range of free Asn followed the order rye, oat, maize and durum, while rye included the highest amount. Shewry et al. (2009) stated a higher level of free Asn in bran compared to white flour. As cereals differ in their grain size, form and distribution of components, variable levels of free Asn can occur due to a different husk fraction.

Moreover, cultivars within cereal species can differ in their precursor content as reported by several studies (Taeymans et al., 2004, Claus et al., 2006, Curtis et al., 2009 & 2010, Postles et al., 2013). Taeymans et al. (2004) reported a fivefold range for a set of European wheat cultivars while Claus et al. (2006) observed a high variability of Asn amounts in nine German winter wheat cultivars of up to a factor of three. Corol et al. (2016) analyzed 150 wheat genotypes as wholemeal samples and found differences of almost fivefold.

Thus, the choice of suitable cultivars with low Asn concentrations is considered as a promissing way to minimize AA formation potential. However, it has to be taken into account that climate and site-specific soil conditions may alter Asn amounts considerably (Curtis et al., 2009 & 2010). As the selection of species and cereals is a breeding issue, Rapp et al. (2018) investigated the possibility of breeding wheat with a low level of free Asn. They analyzed 149 wheat varieties and found large differences concerning the level of free Asn. But most important, they stated that free Asn and quality parameters relevant for the baking quality (e.g. crude protein and sedimentation value) did not correlate. Thus, breeding wheat cultivars low in free Asn will not affect baking behaviour. Finally, they pointed out, that combining an identified QTL (Quantitative Trait Locus) for Asn with a genome-wide prediction approach lead to a mean cross-validated prediction ability of 0.62.

Still missing in the field of cereals are studies analysing the impact of organic farming conditions on cereals' and cultivars' free Asn level.

Nutrient supply

A central measure in crop production is fertilization as it is closely related to increase yield and quality but can affect Asn levels as well. In this context nitrogen (N) level, application timing and N form can have a considerably impact on Asn contents in wheat (Weber et al., 2008, Martinek et al., 2009). High N availability during grain maturity leads to high crude protein contents, which are considered to increase free Asn levels significantly (Weber et al., 2008). Lea et al. (2007) stated that soluble Asn accumulates in plant parts when protein synthesis is low and there are large amounts of reduced N. This corresponds well with the findings of Pate et al. (1980), who reported Asn as highly related to N, since the amino acid is used in plants to transport and store N.
Postles et al. (2013) analyzed, that in rye free Asn level was influenced by variety and N supply. Moreover, some studies showed that sulfur deficiency sometimes dramatically increases Asn contents and thus the potential for AA formation (Muttucumaru et al., 2006, Granvogl et al., 2007, Elmore et al., 2008, Shewry et al., 2009). The immense increase of free Asn in wheat mainly appears within soils where sulfur has been deficient. Zhao et al. (1999) stated that this occurs because wheat uses free Asn to stock N in cases where sulfur is not available to synthesise proteins rich in sulfur. Thus, sufficient sulfur supply is expected to help reduce Asn levels in grains.
However, most of these results has been obtained in greenhouse pot experiments under sulfur deficient conditions. Curtis et al., (2018) presented a study where this subject was explored in a field trial. They found that in sulfur deficient soils free Asn was higher compared to the samples treated with sulfur.
As the fertilisation by N and sulfur is of great importance for Asn formation, both nutrients are included in the European Commission regulation (2017), which describes mitigation options to reduce AA.
However, studies investigating the effect of N on free Asn and simultaneously yield and baking quality comparing conventional and organic farming systems are still missing. Additionally, studies investigating the combined effect of sulfur types and different sulfur levels have not published to date.

Plant protection

Growing healthy plants is the main requirement to produce good yields and baking qualities. Only green leaves can run photosynthesis and produce assimilates. Cropping systems differ in their strategy to obtain healthy plants. In conventional farming systems the use of artificial pesticides is common. As artificial plant protection means are forbidden in organic farming, it is much more customary to select cultivars with a high natural resistance against diseases. Further strategies that have a positive side effect on disease pressure are crop rotation and soil preparation.
However, concerning free Asn, Martinek et al. (2009) reported that fungicide treatments prolonging green leaf area duration and delaying senescence can reduce free Asn concentration in grains. Similar results were presented by Lea et al. (2007), who reported that

free Asn levels increased if plants were infested with pathogens. They also revealed that stress conditions in general like mineral deficiencies, drought, salt and toxic metals, lead to an accumulation of Asn in plants. Studies of Navrotskyi et al. (2017) showed that the highest amount of free Asn was analyzed in wheat grains grown at a site with high disease pressure.

Further, Curtis et al. (2016) established a field trial including the effect of fungicide treatment on free Asn accumulation in wheat. They found significantly higher Asn levels if fungicides had not been applied for each cropped cultivar. However, there was also a big difference between cultivars meaning that cultivars did not react to fungicide treatment in the same way. Thus, plant attack by pathogens increasing free Asn depends on the cultivar, too. Similar findings were reported by Barbottin et al. (2005), concerning the effect of N remobilization during grain filling in wheat. They found out that disease resistant wheat genotypes seemed to be able to maintain a stable remobilization efficiency if disease pressure was high. In contrast, N remobilization efficiency decreased strongly in less resistant genotypes. This is an important outcome as cereal crop management under organic farming conditions does not use pesticides. Hence, in organic cropping systems, growing cultivars with a high resistance against diseases is of great importance.

Row distance, seed density

Row spacing and seed density will affect plant growth especially during the first growth stages (e.g. tillering, stem elongation). More space between plant rows serves two aims, weed handling and better nutrient supply particularly N. Seed density can also have an effect on nutrient availability as fewer plants per area lead to an increased nutrient supply for the single plant. Studies investigating the impact of row distance and seed density on grain yield, thousand kernel weight and quality parameters are available (Lafond, 1994, Hiltbrunner et al., 2005, Becker and Leithold, 2008), but concerning the level of free Asn in grain this issue has never been investigated before. However, as crude protein can be increased by both agronomic managements, an effect on free Asn as N storage pool (Lea et al. 2007) is suggested.

Next to row distance and seed density, sowing date and sowing depth should be mentioned as both have an effect on initial plant growth before and after winter season. Nevertheless, Motzo et al. (2007) reported that delaying the sowing date led to a 3 % increase (10.7 to 14.7%) in protein content in grains of durum wheat due to a decrease in grain weight. Thus, increasing protein can partially lead to an increase in free Asn as this amino acid is known to store N. To date, this subject has not been presented in scientific studies.

Harvest timing

The right harvest timing is an important criterion for safe storage including the stability of quality parameters. Pre-harvest sprouting can raise free Asn level dramatically. Pre-harvest sprouting mostly occurs if the harvest is delayed due to rainy and warm weather conditions. Lea et al. (2007) found profound evidence that seed germination increased the level of free Asn in different kinds of plants. Simsek et al. (2014) analyzed the effect of pre-harvest sprouting on physiochemical chances of proteins in wheat. They showed a clear significant correlation of free Asn with sprouting score, endoprotease activity and protein

degradation. Navrotskyi et al. (2017) reported that a delayed harvest can cause elevated free Asn content by excess solar radiation.
Hence, harvest conditions within cereals is of interest concerning the level of free Asn. Going further, there is a need for information of how grain development can influence free Asn content. Protein level rises during several growing stages and will finally be diluted by a higher starch storage in the grain. Thus, it can be assumed that different maturation stages of cereals also influence free Asn formation. However, no study investigating such an issue has been carried out.

Crop rotation, soil preparation

Both agronomic measurements, crop rotation and soil preparation, mainly affect soil fertility, water infiltration of the soil, weed pressure and disease pressure. Hence these tools only influence free Asn as a side effect. However, reduced tillage or no-tillage as well as a tight crop rotation could raise disease pressure, thus plants are subject to more stress conditions. Martinek et al. (2009) and Lea et al. (2007) reported higher Asn levels when cereal plants were attacked by pathogens or other stress conditions. Thus, choosing appropriate crop rotations and soil tillage that lower stress conditions and strengthen the fitness of plants might also have an impact on free Asn accumulation in cereals.
Additionally, soil tillage and crop rotation are assigned to optimize grain yield and quality. As free Asn is linked to N physiology in plants and this again refers to grain yield and quality traits like protein, an effect on free Asn is supposed.
However, studies investigating crop rotation and soil preparation on free Asn are not available.

Location and Year

Climate conditions mostly differ from year to year and locations can be very heterogenous. Therefore, it is barely imaginable that locations and years do not impact free Asn synthesis. Both these environmental conditions can affect plant growth including plant physiology. As for cereals weather conditions can highly influence plant growth especially during critical growing stages like stem elongation and grain maturation, it seems quite logical that these have an impact on free Asn synthesis.
Taking a look at the literature, Weber et al. (2007) revealed a high impact of the specific year on free Asn amount within different cereals. The harvest year had a significant effect mainly because of differences in growing temperature and sunshine duration as both have an impact on elevating protein and amino acid contents in grain and flour. Curtis et al. (2009 & 2010) reported that environmental conditions given by different growing locations can considerably influence free Asn levels of wheat and rye genotypes.
Investigations of Corol et al. (2016) showed that genotype only had a minor effect on the variation of free Asn within 26 wheat lines tested at different locations in Europe (France, United Kingdom, Hungary and Poland) and grown between 2005 and 2007. Only 13% of the variation in free Asn could be explained by the genotype, while 36% was the result of the environment. This shows that growing cereals at locations that differ highly regarding climate conditions and soils lead to a high range of free Asn.

Barbottin et al. (2005) stated in their study that, next to the genotype, N remobilization during grain filling in wheat depends on environmental and site conditions. As free Asn is mainly involved in N mobilisation in plants, an effect of environmental conditions on Asn can be expected. Rapp et al. (2018) analyzed 149 wheat varieties for Asn across three locations. They observed a high interaction of genotype × environment for Asn content. Shewry et al. (2010) reported the impact of temperature and water availability during grain growth on grain ingredients. They investigated 26 genotypes grown at different sites, and reported that mean temperature and precipitation was either positively or negatively linked to phytochemical contents during grain maturation or to water-soluble arabinoxylan fibre in bran and white flour.

Locations were found significantly to influence SDS unextractable polymeric protein parameters (Ohm et al., 2017). This study also showed a negative correlation between SDS unextractable polymeric protein parameters and free Asn. Thus, it can be assumed that locations will have an impact on free Asn as well. Lea et al. (2007) postulated that stress conditions can significantly raise free Asn in plants. Since environmental conditions can induce stress like drought and high disease pressure, climate factors may have an impact on fluctuating free Asn levels.

Ultimately, weather conditions can hardly be influenced and thus a proper selection of cultivars, which fit to the given environmental condition of the location and which are stable in a low production of free Asn, is essential.

Post-harvest treatments

To date, no study is available which describes the effect that e.g. grain storage might change free Asn level in cereals. There is evidence for potatoes that low temperatures during storage of around 4°C increase the limiting AA precursor the reducing sugars in tubers (De Wilde et al., 2005). But there is no indication that the similar effect applies to cereal grains. However, wet and warm storage conditions affect grain quality parameters and favour seed germination. In this context free Asn is highly present during seed germination (Lea et al., 2007) and the degradation of grain ingredients like proteins (Simsek et al., 2014). Thus, post-harvest conditions regarding grain storage, which force sprouting raise free Asn and need to be avoided.

Organic vs. conventional cropping systems

Most of the studies concerning agronomic measurements to influence free Asn were conducted solely for conventional crop production. However, organic farming applies different agronomic practices such as fertilization strategies, crops are grown without synthetic fertilizers, pesticides and processing aids and specifically bred varieties are used (Kunz et al., 2006). Thus, the question arose if cereal production systems (organic vs. conventional) differ considerably in their impact on AA formation potential. If this is verified, there could be a natural potential for AA reduction.

Springer et al. (2003), investigated the Asn level in two organically and two conventionally cropped rye cultivars and found higher Asn contents in the organically produced rye. Nevertheless, no additional information about the location, growing conditions and agronomic

management were given. Amrein et al. (2004a) did not find a significant impact of production systems when they analyzed the effect of conventional and organic farming systems on level of reducing sugars, free Asn and AA formation within potatoes.
At the start of this thesis, studies investigating the effect of organic versus conventional cropping practice on grain yield, quality traits, free Asn and finally the AA content of cereal products had not been published.
Thus, in view of the continuously rising demand for organically produced food products above all in Germany and the assumption that organically produced foodstuffs are considered to be healthier than conventionally produced products, closing this knowledge gap in an essential task.

1.2 Aim of the thesis

Based on the current state of the art, research gaps were identified to establish the main aims of this thesis. These aims focused on the impact of cropping systems, fertilization strategies including conventional and organic cropping procedures, the impact of row distance and seed density and the impact of cereal species and cultivars grown under both cropping systems on free Asn, AA formation, grain yield and flour quality parameters.

This framework led to the following hypotheses:

1. The cropping system has an impact on yield, quality parameters, free Asn, and eventually AA formation potential due to different agronomic crop management strategies e.g. plant protection and crop rotation.
2. Organic cropping strategies lead to a lower level of free Asn in cereal grain because of a lower nutrient supply e. g nitrogen and the use of farm fertilizer, which is considered being more difficult to steer in terms of mineralisation.
3. High nitrogen levels in order to obtain a high protein content in conventional farming systems cause an accumulation of amino acid free Asn due to downsized protein syntheses shortly before the harvest, what leads to an accumulation of Asn.
4. Up to a certain level of fertilized nitrogen, no matter which kind of nitrogen fertilizer is used or how the nitrogen application is split, free Asn content is only affected to a minor extent if a sufficient protein synthesis is maintained.
5. An additional sulfur fertilisation will not have an impact on free Asn formation if soils are well supplied with sulfur, due to a maintained formation of sulfur-rich amino acids, which prevent the accumulation of free Asn in grain.
6. Larger row distances and lower seed densities are agronomic strategies that will change yield and quality parameters e.g. crude protein, but without a significant impact on free Asn level due to a better utilization of nitrogen concerning protein synthesis.
7. Cereals grown under low nutrient supply in organic farming systems highly differ in their content of free Asn due to differences in grain shape, size and composition.
8. Selecting single cultivars of wheat, spelt and rye, seems a feasible strategy to lower free Asn in grain, as single cultivars can be identified with an inert reaction towards climate conditions concerning free Asn accumulation.
9. Breeding cultivars low in free Asn is expected to be a promissing approach because of a high heritability of the trait free Asn.

1.3 Thesis composition and structure

In order to investigate these hypotheses six field trials were carried out. The obtained results are presented in the following five chapters including five peer reviewed publications.

The **first paper** describes the first insight into the dynamics of AA and investigated whether cropping systems have an impact on free Asn, the main precursor of AA in cereals. As no level of free Asn was known for organically grown wheat cultivars, a focus was drawn on the evaluation of free Asn content in organically grown wheat cultivars.

Paper 2 details the comparison of organic vs. conventional cropping systems. For this purpose, the same cereal species and cultivars were grown in an orthogonal field trial under both conventional and organic farming conditions.

Since nutrient supply is a key factor in agriculture, in **paper 3** the effect of nitrogen and sulfur on free Asn is investigated. For conventional farming some publications are available which studied the impact of both nutrients. However, there is no study that investigated the impact of nitrogen and sulfur in both conventional and organic cropping systems for the same species and cultivars. Especially because organic farming systems applies lower levels of nutrients, mineral fertilizers are forbidden and the entire system follows a circular flow including large crop rotations, gaining insight into the impact of organic cropping systems on free Asn was essential.

Further, **paper 4** investigates whether special farming applications used in organic farming can influence free Asn as well as grain quality parameters. In organic cropping systems larger space between plant rows is a common practice. More space serves two aims, weed handling and a higher nutrient supply, especially with nitrogen. Seed density can also have an effect on nutrient availability, so different seed densities were tested, too.

Paper 5 deals with the evaluation of free Asn contents within organically grown species and cultivars of species. As the ancient cereal einkorn (*Triticum monococcum* L.) and emmer (*Triticum dicoccum* L.) have not at all been investigated for their AA formation before, this paper presents completely new findings. To implement free Asn in breeding programs for cereals with a low level of free Asn, the heritability of this trait was also tested. The heritability should reveal how strong the trait free Asn is bound to the cultivar or whether environmental conditions are the more decisive factor.

Within the five papers above, the particular results for the respective hypotheses have been already discussed. Thus, the **general discussion** depicts further aspects and strategies which might influence free Asn and AA formation. Agronomic strategies are particularly emphasised. Also included in this chapter is the presentation of results gained by additional field trials and laboratory work, carried out within the framework of this thesis. The last chapter within the general discussion highlights research approaches for future projects which are crucial for the advancement of the field. Finally, the overall **summary** presents the major outcomes of the thesis stating, which agronomic strategies can be transferred into practice and which are the most promising ones to lower free Asn in the long-term.

2 Publications

The main part of the thesis consists of 5 publications, that have been published in peer reviewed international journals. Several further publications from conferences or non-peer-reviewed journals are listed in the Appendix.

Below are the references for citation.

Paper 1

Stockmann, F.; Weber, E.A.; Graeff, S.; Claupein, W. **Influence of Cropping Systems on the Potential Formation of Acrylamide in Different Cultivars of Wheat**. In "Proceedings of the 16th IFOAM Organic World Congress", Modena, Italy, 16–20 June 2008. The paper was peer reviewed and published within an international conference proceeding.

Paper 2

Stockmann, F.; Weber, E.A.; Mast, B.; Schreiter, P.; Merkt, N.; Claupein, W.; Graeff-Hönninger, S. **Acrylamide-Formation Potential of Cereals: What Role Does the Agronomic Management System Play?** *Agronomy* **2019**, 9, 584. doi.org/10.3390/agronomy9100584

Paper 3

Stockmann, F.; Weber, E.A.; Schreiter, P.; Merkt, N.; Claupein, W.; Graeff-Hönninger, S. **Impact of Nitrogen and Sulfur Supply on the Potential of Acrylamide Formation in Organically and Conventionally Grown Winter Wheat**. *Agronomy* **2018**, *8*, 284. doi.org/10.3390/agronomy8120284

Paper 4

Stockmann, F.; Weber, E.A.; Merkt, N.; Schreiter, P.; Claupein, W.; Graeff-Hönninger, S. **Impact of Row Distance and Seed Density on Grain Yield, Quality Traits, and Free Asparagine of Organically Grown Wheat**. *Agronomy* **2019**, 9, 713. doi.org/10.3390/agronomy9110713

Paper 5

Stockmann, F.; Weber, E.A.; Mast, B.; Schreiter, P.; Merkt, N.; Claupein, W.; Graeff-Hönninger, S. **Evaluation of Asparagine Concentration as an Indicator of the Acrylamide Formation in Cereals Grown under Organic Farming Conditions**. *Agronomy* **2018**, *8*, 294. doi.org/10.3390/agronomy8120294

2.1 Paper 1: "Influence of cropping systems on the potential formation of acrylamide in different cultivars of wheat"

Stockmann, F.; Weber, E.A.; Graeff, S.; Claupein, W. **Influence of Cropping Systems on the Potential Formation of Acrylamide in Different Cultivars of Wheat**. In Proceedings of the 16th IFOAM Organic World Congress, Modena, Italy, 16–20 June 2008.

For conventional farming some references are available describing the impact of agronomic practices to influence the level of Asn and by this the AA formation potential in food products. Yet, no information on the impact of cropping systems were available especially for organic farming practices. But as cropping systems differ in the used agronomic practices like the use of fertilizers and cultivars, filling this gap was essential.

Thus, the paper describes the first results whether cropping systems could have an impact on free Asn, the main precursor of AA in cereals. Special emphasize has been given to the evaluation of free Asn content in organically grown wheat cultivars.

The results clearly showed that cropping systems affected AA precursor free Asn. Organically grown wheat cereals reached a lower Asn concentration in the grains leading to a lower AA formation potential. Nevertheless, within the organcícally grown wheat samples significant differencs were also found.

The paper was peer-reviewed and published within an international conference proceeding.

16th IFOAM Organic World Congress, Modena, Italy, June 16-20, 2008
Archived at http://orgprints.org/view/projects/conference.html

Influence of cropping systems on the potential formation of acrylamide in different cultivars of wheat

Stockmann, F.[1], Graeff, S.[2], Weber, A.[3] & Claupein, W.[4]

Key words: acrylamide, asparagine, production systems, cultivars, food products

Abstract

Acrylamide (AA) – probably carcinogen – is thermally created in carbohydrate-rich food (e. g. cereals) within the Maillard-Reaction by the reaction of asparagine and reducing sugars. First steps to decrease AA focused on changes in the technological food production process. However, these possibilities are limited due to occurring taste anomalies and consumer tolerance. Therefore, it might be an alternative to influence the precursors of AA. Up to now, multiple studies considering the influence of fertilisation, species, and cultivars on the content of asparagine (Asn) and reducing sugars have been carried out. But there is still a lack of information about the influence of the production system on the AA level. It can be expected that the amount of AA is different and might be lower in organic production systems, because of the difference in nitrogen management (amount and type). The aim of this study was to check organically and conventionally grown wheat samples of different cultivars for the level of the precursor Asn and the AA-formation-potential. The samples were obtained from locations in Switzerland and Germany. Partial significant differences in the amount of Asn and in the AA-formation-potential suggested an influence of the production system and thus a further chance to intervene.

Introduction

The health and safety of foodstuff nowadays is a very important aim even in industrialised countries (Nau et al. 2003). Normally the avoidance of food toxicants is quite well manageable. But for substances, created through food processing, like acrylamide (AA), it is problematical. Acrylamid, a so called “foodborne toxicant”, was first reported in 2002 by the Swedish National Food Administration in connection with food products. It is formed in carbohydrate-rich food stuffs like potatoes and cereals within the Maillard-Reaction (Mottram et al. 2002), where the amino acid asparagine (Asn) and reducing sugars (e. g. glucose) react by thermal processes to AA (Stadler et al. 2002). In this context, Asn is the limiting factor of the formation of AA in cereals (Weisshaar 2004). Furthermore, according to the IARC (International Agency for Research on Cancer 1994) it is a probably carcinogen substance. Consequently many efforts have been undertaken to understand the syntheses, the metabolism, the toxicology, the formation and, in the end, what can be done to minimize the amount of AA in foodstuffs. Up to now, a focal point was to find minimization strategies by changing technological food production steps. It has been successfully shown, that the

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amount of AA can be minimized by changing temperature, pH, time of heating, backing agents and adding additives. However, the possibility to change technological process steps is limited, because of negative impacts on the quality of produced products. Modifications in technical food processing are linked to changes in taste, smell and texture and thus consumers' acceptance can be endangered. Therefore, additional strategies are needed to minimize AA. An alternative to reduce AA might be to limit the contents of precursors (e.g. free Asn) by using crop species and cultivars with lower levels. Furthermore, different amounts of N-fertilization can also increase the Asn level. Weber (2007) investigated different conventionally grown cereal species and cultivars of wheat, spelt and rye and found different levels of Asn. Weber et al. (2008) also found that conventional N-fertilization levels can increase the level of Asn. As organic farming uses different N-fertilization strategies e.g. crop rotation, enhancement of mineralization and organic N-fertilizer that lead to crude protein levels below 13 %, it can be expected that the amount of Asn in organic grain samples is lower. Thus it seems that organic products can contain a lower amount of AA and by this might have an advantage for consumers' health. Up to now only few studies have investigated the impact of organic production systems on levels of AA in foodstuffs, wherefore further research is necessary.

Hence the aim of these study was i) to evaluate differences in Asn content of organically and conventionally grown wheat cultivars, ii) to compare the content of Asn and AA-formation-potential between organically and conventionally grown wheat cultivars and iii) to evaluate the correlation between Asn and AA-formation-potential of organically and conventionally produced wheat cultivars.

Materials and methods

The organically produced 16 wheat samples (Wiwa, MAA 48, TEPP 117, Wenge, Pollux, Ataro, Aszita, AIRA 28) were obtained from field trials of two different locations (Vielbringen, Montezillon) in Switzerland from the seed breeder Peter Kunz in 2004/2005. The selected two locations were in the Regions Chaumont (Montezillon: 770 m above sea level) and Bern (Vielbringen: 560 m above sea level). The annual precipitation ranged about 800 – 1030 mm. Annual average temperature ranged from 6.2 to 9.9 °C. The field trials were arranged in a block design (randomised), with two replications on a sandy loamy soil. Liquid manure was applied as N-source, previous crops at both locations were grass-clover ley.
The conventionally produced 16 wheat samples (Enorm, Altos, Monopol, Batis, Elvis, Tiger, Cubus, Transit, Tommi, Natutastar, Magnus, Terrier, Dekan, Punch, Manhatten, Wasmo) were collected from the experimental station Ihinger Hof of the University of Hohenheim (south-west of Germany, 48° 44' N; 8° 56' E, mean annual precipitation 693 mm, mean temperature 8.1 °C). The samples were arranged in a block design (randomised) with three replications. The soil texture was classified as loamy clay. Sugar beet was cultivated as previous crop. N-fertilization was applied as KAS in accordance to the quality levels of wheat (quality level E, A, B, K) from 170 kg N ha^{-1} to 200 kg N ha^{-1}. The selected cultivars were used because of their relevance in the applied production system.
The lab analyses of Asn and the AA-formation-potential in the grain samples were done according to Weber et al. (2008). Asn- and AA-data were analyzed according to the experimental design with a linear mixed model. Analyses of variance (ANOVA) were performed using PROC GLM of the SAS 9.1 statistical software package (SAS Institute Inc., Cary, NC, USA). Tukey-Tests were carried out for comparison of means using the procedure PROC MIXED. All effects were set as fixed.

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Results and discussion

Figure 1 shows the results of Asn levels [mg 100 g^{-1} in DM] in organically produced wheat. The content of Asn differed significantly between the chosen cultivars. Asn levels ranged from 7.8 mg 100 g^{-1} DM as minimum to 13.8 mg 100 g^{-1} as maximum with a mean of 10.7 mg 100 g^{-1} DM. Against this the conventionally produced wheat cultivars (results not shown) had significant differences by a mean content of 15.2 mg 100 g^{-1} DM, with a minimum of 7 mg 100 g^{-1} DM and a maximum of 21.2 mg 100 g^{-1} DM. The higher Asn contents in grain samples of conventional production systems might be the result of higher N-fertilization levels which can significantly enhance the amount of Asn (Weber et al. 2008). Means over two organic locations did not indicate significant differences in Asn level, but comparing the same cultivars a significant difference was given wherefore it is concluded that the location could have an effect.

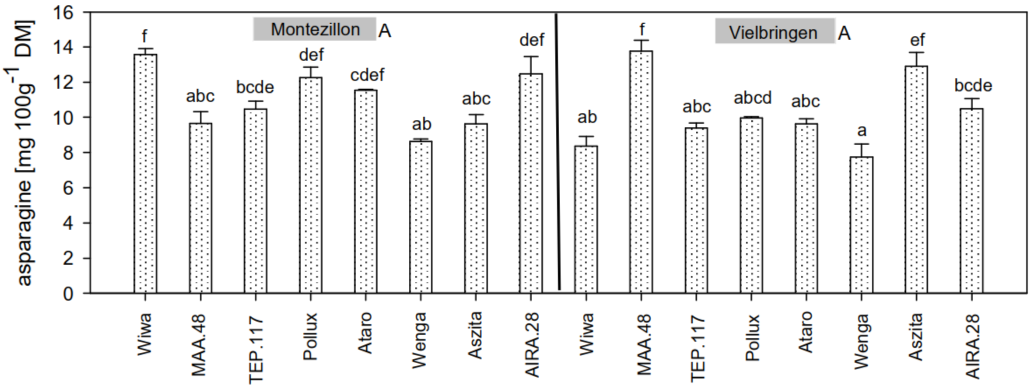

Figure 1: Asparagine levels [mg 100 g^{-1}] of analysed organic wheat cultivars. Cultivars with the same letters did not differ significantly (α<0.05).

In a further step, the content of Asn and AA-formation-potential were analysed and compared between the two production systems (figure 2 I & 2 II). The results showed a statistically significant difference in the amount of Asn (figure 2 I) as well as in AA-formation-potential (figure 2 II) between the systems. Asn potential of organically grown cultivars was significantly lower than in conventionally grown cultivars. Further, the same effect was found for the amount of AA-formation-potential (Fig. 2 II). Wheat cultivars of organic production systems had a significant lower level (almost four times) than conventionally produced samples.

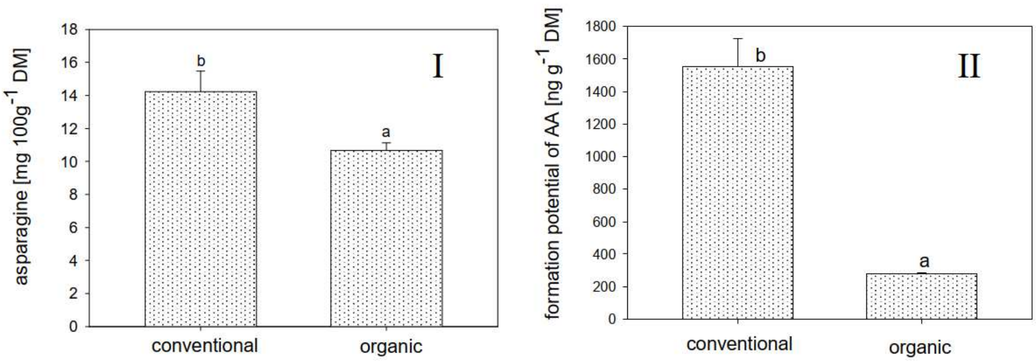

Figure 2: Comparison of means of (I) asparagine [mg 100 g^{-1}] and (II) acryl-amide (AA) formation potential levels [ng 100 g^{-1}] of analysed wheat cultivars. Production system with the same letters did not differ significantly (α<0.05).

The correlation between Asn and AA (figure 3) was only significant for the conventional samples. Due to a narrow range of Asn in organic samples, no correlation was obtained.

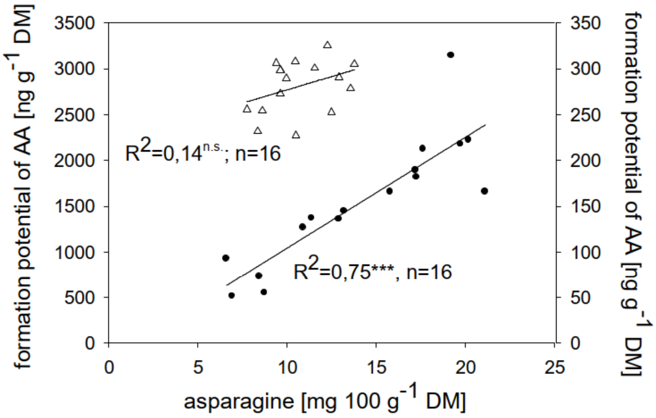

Figure 3: Correlation between asparagine (Asn) and acrylamide (AA) formation potential of conventionally (R^2=0.75*, black dots, left y-axis) and organically (R^2=0.14$^{n.s.}$, white triangles, right y-axis) grown wheat.**

Conclusions

The results of the study indicated significant differences in the content of Asn and the final AA-formation-potential within organically produced wheat cultivars. A possible strategy to minimize AA in foodstuffs seems to be the selection of wheat cultivars low in free Asn. Further, the location might to have an impact on the content of free Asn when comparing single cultivars. The production system seems also to have an influence. However, no clear statement can be given because of different cultivars and locations. Further research on the overall influence of the production system is needed.

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2.2 Paper 2: “Acrylamide-formation potential of cereals: what role does the agronomic management system play?”

Stockmann, F.; Weber, E.A.; Mast, B.; Schreiter, P.; Merkt, N.; Claupein, W.; Graeff-Hönninger, S. **Acrylamide-Formation Potential of Cereals: What Role Does the Agronomic Management System Play?** *Agronomy* 2019, 9, 584.

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The previous paper presented a first insight into the differences of organically grown wheat cultivars concerning free Asn and the impact of cropping systems on the level of free Asn. The results showed a significant difference between wheat cultivars, which were grown under organic management. The production system seems to have an influence. However, no clear statement could be derived, because different cultivars and locations were investigated. Thus, the need was born to investigate this subject in more detail using the same cultivars for both systems and expanding the research to the cereals spelt and rye. The overall aim was to investigate the impact of the single cropping system on AA. For this reason, an orthogonal two-year field trial was established under practical farming conditions typical for conventional as well as for organic cropping systems. In both systems the same species (wheat, spelt and rye) and cultivars were grown, but the agronomic management like fertilization and weed control as well as pesticide treatments were different.

The results reported in paper 2 showed that the cropping system had a significant impact on grain yield, level of free Asn and quality parameters. Across all species, free Asn contents in the flour were 26% lower under organic conditions compared to conventional farming. Wheat cultivars, in particular, showed that a maximum reduction of 50% in free Asn content was possible, if produced organically.

The paper was published within the special issue “Organic vs. Conventional Cropping Systems” of peer-reviewed open source journal MDPI Agronomy, covered in Scopus (2018 Cite Score: 2.59).

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Article

Acrylamide-Formation Potential of Cereals: What Role Does the Agronomic Management System Play?

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Abstract: As bakery products contribute considerably to the daily intake of the carcinogen acting substance acrylamide (AA), the aim of this study was to evaluate the impact of the management system (conventional vs. organic farming) on AA precursor levels of free asparagine (Asn) across different cultivars of the cereal species, namely winter wheat (*Triticum aestivum*), winter spelt (*Triticum aestivum* ssp. *spelta*) and winter rye (*Secale cereale*) with simultaneous consideration of gained grain yields and flour qualities. For this purpose, orthogonal field trials were established at two sites in Southwest Germany over two growing seasons (2006–2007 and 2007–2008). The results indicated a significant impact of the management system on free Asn contents in white flour. Across all species, free Asn contents in the white flour was 26% lower under organic compared to conventional farming. The impact of the management system on individual cultivars was obvious with a maximum reduction in free Asn contents of 50% in wheat cultivars if organically produced (e.g., for cultivars Ludwig, Privileg, Capo). For spelt, a significant impact of the management system was only found in 2008 with a reduction in free Asn of up to 25% if organically produced. Across both cropping systems, cultivar Franckenkorn reached the lowest levels of free Asn. For rye, a significant impact of the management system was observed only in 2007 with 33% higher Asn amounts in the conventional management system. Independent of the cropping system, rye reached the highest levels of free Asn followed by wheat and spelt. Depending on species, there was also an impact of the two systems on crude protein. The organically cropped wheat had a significantly lower level, but this was not observed for spelt and for rye only in 2007. The possible reason for the low free Asn content in the organically produced wheat flour could partially be the lower crude protein amount. Furthermore, the results indicated that lower AA contents in bakery products can be achieved by proper selection of species (e.g., 66% lower if rye is replaced by wheat) and cultivars. With an appropriate choice of the cultivar, a reduction of up to 65% was possible within wheat, along with a reduction of 44% within spelt and 12.5% within rye. In summary, the results indicated that organically produced wheat especially offers the opportunity to significantly lower the AA potential of bread and bread rolls by the choice of raw materials low in free Asn.

Keywords: acrylamide; free asparagine; management systems; organic; conventional; agriculture; cereals; species; cultivars; product quality

1. Introduction

Due to a current announced regulation of the European Commission [1] food industry and gastronomy face the challenge of establishing immediate mitigation strategies and benchmark levels for acrylamide (AA).

More than 15 years after the first findings of the food-borne toxicant AA in starch-rich heated foods, the dietary intake of AA is now seriously regarded to potentially increase cancer risk for humans [2]. Therefore, AA in foodstuffs should be minimized to a level as low as reasonably achievable.

Since 2002, it was successfully shown that AA is formed during a thermal treatment of carbohydrate rich food like cereals and potatoes [3,4], where 'reducing sugars' (mostly glucose and fructose) react with the amino acid free asparagine (Asn) within the Maillard reaction [5]. While reducing sugars are the limiting precursor in heated potato products [6], free Asn is the limiting precursor for processed cereal based products [7–10]. Although strongly heated potato products can contain much more AA than cereal based products, foods like bread, rolls, biscuits and crisp bread contribute to about 25% to 45% of the dietary AA intake in Germany [11]. This is mainly due to the high daily per capita consumption of bread of almost 240 g [12].

It has been shown that the amount of AA can be minimized by adjusting processing parameters such as pH, heating temperature and time of heating, changing baking agents, as well as adding additives, or by elucidating the mechanistic pathways of AA formation and eliminating precursors or intermediates [6,12–17].

Modification of recipes or processing conditions often led to significant reductions in AA levels of the final products. However, their applicability is restricted because their use is often accompanied by negative effects on taste or quality of the final products, or their use is simply too expensive. The used raw material, notably the Asn precursor content in cereal grains, plays an important role for the AA formation potential. Consequently, one alternative to reduce AA in the final product might be minimizing Asn contents in cereal grains by agronomic management.

Several studies showed that cereal species differ in their Asn levels and in consequence in their acrylamide formation potential. Rye usually has higher Asn levels compared to wheat and spelt [7,8,18]. Moreover, cultivars can differ considerably in their precursor content as shown by several studies [7,8,18–20]. Taeymans et al. [20] reported a fivefold range for a variety of European wheat cultivars, and Claus et al. [7] found a variability of Asn contents in nine German winter wheat cultivars of up to a factor of 3. Corol et al. [19] reported differences of 150 wheat genotypes analysed as wholemeal samples of almost 5-fold. Thus, selection of suitable cultivars with low Asn contents is considered as a feasible way to minimize AA formation potential, although it has to be taken into account that environmental conditions (site-specific soil properties and climate) may alter Asn contents considerably [8,18].

Fertilization is a key measure in crop production that increases yield and quality and affects Asn levels as well. Nitrogen amount and timing of application, as well as nitrogen form can affect Asn contents in wheat considerably [21,22]. Especially high nitrogen availability during grain filling leading to high crude protein contents is considered to increase free Asn levels significantly [22]. Postles et al. [23] found for rye that free Asn was influenced by variety and nitrogen supply. Moreover, sulphur deficiency can dramatically increase Asn contents and thus the AA formation potential [24,25]. Furthermore, fungicide application promoting leaf area duration and delaying senescence can reduce free Asn content in grains [21]. Further, environmental conditions given by different growing locations can highly change the level of free Asn within wheat genotypes [19].

However, most of the studies were accomplished for conventional crop production only. Thus, the question arose if there might be a considerable difference between organic and conventional cereal production systems in their impact on AA precursor content and AA formation potential as organic farming uses different agronomic practices, like nitrogen fertilization strategies, and to some extent different cultivars. In a study by Springer et al. [26], the comparison of Asn contents of two organically and two conventionally produced rye cultivars revealed higher Asn contents in the organically produced rye but no additional information about site, growing conditions and management were given. Preliminary studies of Stockmann et al. [27] reported an effect of the cropping system, as wheat cultivars grown under organic conditions showed a significantly lower amount of free Asn when compared to conventionally grown wheat cultivars. Further studies investigating the effect of organic versus conventional growing practice on yield, quality, and the latter AA content of cereal products have, to date, not been published.

With regard to the steadily rising demand for organically produced products in Germany and the association that organically produced foodstuffs are considered per se healthier than conventionally produced ones, filling this knowledge gap is essential.

Therefore, the main focus of this study was to compare different wheat, rye and spelt cultivars grown under conventional and organic farming conditions over two consecutive growing seasons regarding their free Asn content and AA formation potential under the consideration of yield and quality. Special emphasis was given towards analysing the impact of the management system on free Asn content and AA formation.

2. Materials and Methods

2.1. Experimental Sites

To transfer results into practice and obtain realistic results under typical management conditions of each production system, field trials were installed at two different sites located ~20 km away from each other, both in South-West Germany. The sites are long-term managed by either organic or conventional farming procedures e.g., fertilization, soil preparation and crop rotation. This must be taken into account in relation to the obtained results. The field trial under conventional management was carried out over two consecutive growing seasons (2006–2007; 2007–2008) at the experimental station Ihinger Hof, Renningen (48°44′ N 8°55′ E; average annual temperature 8.3 °C; average annual rainfall 693 mm) of the University of Hohenheim. The same trial was accomplished in parallel under organic management at the experimental station for organic farming of the University Hohenheim, Kleinhohenheim, Stuttgart (48°44′ N 9°12′ E; average annual temperature 8.8 °C; average annual rainfall 700 mm). Detailed data on temperature and rainfall during the seasons 2006–2007 and 2007–2008 for both sites are depicted in Table 1.

Table 1. Temperature and rainfall during the experimental seasons 2006–2007 and 2007–2008 at the locations Ihinger Hof, Renningen and Kleinhohenheim, Stuttgart, both in South-West Germany).

Location	Ihinger Hof (Conventional)		Kleinhohenheim (Organic)	
2006/2007	Temp. (°C)	Rainfall (mm)	Temp. (°C)	Rainfall (mm)
September	16.3	50	17.4	59.2
October	11.9	74	12.9	80.0
November	7.1	22	7.8	25.2
December	3.4	22	4.0	31.2
January	4.4	49	5.0	22.2
February	4.4	69	5.2	102.0
March	5.3	50	6.5	80.2
April	12.2	1	13.7	0.2
May	14.1	104	15.0	142.0
June	17.7	107	17.7	140.8
July	16.9	69	17.8	91.0
August	16.4	64	17.3	69.0
Mean/Sum	11.4	681	11.7	843.0

Table 1. *Cont.*

Location	Ihinger Hof (Conventional)		Kleinhohenheim (Organic)	
2007/2008	Temp. (°C)	Rainfall (mm)	Temp. (°C)	Rainfall (mm)
September	11.8	48	12.9	56.2
October	8.2	7	9.3	15.4
November	3.2	63	3.6	100.2
December	0.8	53	1.3	61.6
January	3.1	39	4.0	47.2
February	3.6	21	4.8	27.4
March	4.3	64	5.1	86.4
April	7.5	103	8.4	105.2
May	14.8	101	15.7	76.8
June	17.2	93	17.6	109.6
July	17.7	57	18.5	61.2
August	16.8	99	17.7	112.6
Mean/Sum	9.1	748	9.9	860.0

Soil Conditions at the Experimental Sites

The field trials at Ihinger Hof (conventionally managed) were carried out on loess derived soils with soil textures of silt (2006–2007) and silty clay (2007–2008) with sugar beet as the previous crop for both years. N_{min} values at start of the vegetation period (end of March) in 2006 and 2007 in a soil depth of 0–90 cm were 2.4 and 31 kg NO_3-N ha^{-1}, respectively. The soils at Kleinhohenheim (organically managed) were loess derived with a loamy soil texture with faba beans as the previous crop for both years. N_{min} values at start of vegetation period in 2006 and 2007 in a soil depth of 0–90 cm were 41.8 and 58.7 kg NO_3-N ha^{-1}, respectively.

In Table 2 the main characteristics of the soil chemical analysis for both sites are presented.

Table 2. Main characteristics of the soil chemical analysis for both sites over both growing seasons.

Site	Management System	PH Value	P_2O_5 [mg 100 g^{-1}]	K_2O [mg 100 g^{-1}]	Mg [mg 100 g^{-1}]
Ihinger Hof	conventional	7.0	34.0	31.0	27.0
Kleinhohenheim	organic	5.8	8.6	27.0	13.0

2.2. *Experimental Design*

Ten winter wheat, five winter spelt and five winter rye cultivars (Table 3), suitable for conventional as well as for organic farming in Germany were tested in a randomized complete block design (plot size 4 × 6 m) with three replicates. To avoid neighbouring effects between the different crop species due to differences in plant height, species were separated by a border plot with a width of 2 m. Species groups were randomly placed in each block and within each species group cultivars were arranged randomly.

Table 3. Cereal species and cultivars tested in the field trials.

Species	Cultivar	Quality Grade *
Winter wheat	Akteur	E
	Bussard	E
	Achat	E
	Privileg	E
	Capo	E
	Enorm (only at Ihinger Hof)	E
	Batis	A
	Naturastar	A
	Ludwig	A
	Astron	A
	Magnus	A
Winter spelt	Schwabenspelz, Ceralio,	-
	Oberkulmer Rotkorn,	-
	Franckenkorn, Schwabenkorn	-
Winter rye	Amilo, Nikita, Recrut	-
	Danko, Pollino	-

* refers to the German quality classes. E-wheat: highest baking quality, A-wheat: high baking quality.

2.3. Agronomic Practices

Primary tillage was done with a cultivator (15 cm depth) at Ihinger Hof and with a mouldbord plough (25 cm depth) at Kleinhohenheim. Seed bed preparation was accomplished by a power harrow at both experimental sites.

Sowing was done for all species on 12 October 2006 and 09 October 2007 at Ihinger Hof and on 19 October 2006 and 17 October 2007 at Kleinhohenheim. Winter wheat and winter spelt were sown on both locations with a sowing density of 350 seeds m^{-2}. Sowing density of winter rye was 300 seeds m^{-2}. Row distance was 0.10 m at Ihinger Hof and 0.12 m at Kleinhohenheim.

Nitrogen was applied as CAN (calcium ammonium nitrate: 13.5% Nitrate-N, 13.5% Ammonium-N) in the conventional trial. For winter wheat, total nitrogen amounts of 190 to 195 kg N ha^{-1} were applied in four to five rates according to the expected yield and the expected crude protein content (for details see Table 4). Winter spelt and winter rye cultivars were fertilized with 140 and 150 kg N ha^{-1} in four rates (Table 4). No further nutrients were applied during this field trial. However, during a 3–4 yr crop rotation a general fertilization of sulphur, phosphorous and potassium was carried out to maintain soil fertility.

Table 4. Time and amount of the N-fertilization for the conventionally produced species. The two dates refer to the application date for the single growing season.

Species/Quality Class	Time and Amount of N-Fertilization [kg N ha^{-1}]					
	N_{Total}	$N_{Start\ of\ Veg.}$	$N_{EC31/32}$ *	$N_{EC37/39}$	$N_{EC49/51}$	$N_{EC59/61}$
		08/03/2007 06/03/2008	02/04/2007 28/04/2008	07/05/2007 28/05/2008	24/05/2007 02/06/2008	31/05/2007 10/06/2008
E-Wheat	**190**	40	40	30	50	30
A-Wheat	**195**	55	40	50	50	-
Spelt	**140**	40	40	30	30	-
Rye	**150**	70	40	-	40	-

* EC: Growing stage of the plant.

In the organic trial, all species were supplied with 100 kg N ha^{-1} by liquid cattle manure (100 m^3 ha^{-1}: 1 kg N m^{-3}, total nitrogen content, 4% dry matter) in two rates a 50 m^3 ha^{-1} at start of vegetation and at start of stem elongation.

Growth regulators (Chlorcholinchloride and Trinexapac-ethyle), herbicides (Atlantis®: Iodsulfuron-methyl-sodium, Mefenpyr-diethyl, Mesosulfuron-methyl, Concert®: Metsulfuron-methyl, Thifensulfuron-methyl, Primus: Florasulam), fungicides (Juwel top®: Fenpropimorph, Epoxiconazol, Kresoxim-methyl) and insecticides (Bulldock®: beta-Cyfluthrin) were broadcast as needed in the conventional trial. No pesticides and no growth regulators were applied in the organic trial.

In 2008, the organic field was harrowed twice (31 March 2008 and 26 May 2008) to control weeds. Due to low weed densities, harrowing was omitted in the year prior.

Harvest was accomplished by a Hege 180 plot combine harvester (Hege, Eging am See, Germany) after grains had reached a dry matter content of 85%.

2.4. *Yield*

Grain yield of the cereal species and cultivars was determined by weighing the plot yield. Grain samples were dried at 105 °C for 24 h to determine grain moisture. Grain yields given refer to 86% dry matter content.

2.5. *Flours*

For the determination of quality parameters, the determination of the AA precursor content free Asn and the AA formation potential, grain samples were milled on a laboratory mill (Quadrumat Junior, Brabender, Duisburg, Germany) to obtain white flours. Ash content of flours was about 0.5% of flour DM. Flour moisture was calculated from the weight loss before and after drying of about 5 g flour at 105 °C for 24 h.

2.6. *Quality Analysis*

2.6.1. Crude Protein Content

Total grain nitrogen content was determined by Near-Infrared-Spectroscopy (NIRS, NIRS 5000, FOSS GmbH Rellingen, Germany). Calibration samples were analysed according to the Dumas Method [28] using a Vario Max CNS analyzer (Elementar, Hanau, Germany). The analysed final nitrogen content was multiplied by a factor of 5.7 [29] for wheat samples and 6.25 for spelt and rye samples.

2.6.2. Zeleny's Sedimentation Test

Zeleny´s sedimentation test was determined in wheat and spelt flours using 3.2 g flour according to ICC standard No. 116. The sedimentation values of the flours were adjusted to a 14% moisture basis.

2.6.3. Free Asparagin

Free amino acids were extracted with either 2 g of wheat or spelt flour or 1 g of rye flour and were mixed with 8 mL of 45% ethanol for 30 min at room temperature. After centrifugation for 10 min at room temperature with 4000 rpm and 10 min at 10 °C and 14000 rpm, the supernatant was filtered through a 0.2 μm syringe filter and poured into vials. Asparagine analysis was performed using Merck-Hitachi HPLC components. The pre-column derivatization with FMOC [30] was completely automated by means of an injector program. Subsequently, the derivatized Asn was separated on a LiChroCART Superspher RP 8 column (250 mm × 4 mm, Fa. Merck, Darmstadt, Germany) at a constant temperature of 45 °C. The fluorescence intensity of the effluent was measured at the excitation and emission maxima of 263 and 313 nm.

2.6.4. Acrylamide Formation Potential

The AA formation potential of cereal flour was assessed according to the AA contents of 5 g flour in 250 mL Erlenmeyer flasks after heating in an oven for 10 min at 200 °C. Due to the complexity of the acrylamide analysis, sample size was reduced to an overall number of 28 samples (6 winter wheat, 4 winter spelt and 4 winter rye samples from two investigation years).

Sample preparation was accomplished according to the test procedure 200L05401 [31] of the Chemische und Veterinäruntersuchungsamt (CVUA) Stuttgart.

After cooling down to ambient temperature, 100 mL of bidestilled water and 100 µL of D_3-Acrylamide were added as an internal standard to the heated flour samples in the Erlenmeyer flasks. To completely extract acrylamide from the flour, samples were put in an ultrasonic bath for 10 minutes at 40 °C. After adding 1 mL of Carrez I and II to each of the samples and shaking the flasks thoroughly, the samples were filtered using folded filter paper to separate the colloids and flour particles from the aqueous solution. Subsequently, samples were cleaned up by a solid phase extraction in a vacuum chamber after preconditioning the cartridges by 10 mL of bidestilled water and 10 mL methanol. After sample clean-up, about 1 to 2 mL of the eluate from each sample was filled in an autosampler vial and was deep frozen (−18 °C) until AA was determined by LC-MS-MS by the CVUA according to the test procedure 201L01301 [32]. The eluates were separated by a graphite or RP18-phase and detected by tandem-mass-spectrometer. Quantification was undertaken by using the isotope-labelled internal standard (D_3-Acrylamide).

2.7. Statistical Analysis

Yield and quality data (crude protein, sedimentation value), as well as free Asn were subjected to analysis of variance (ANOVA) using the Procedure MIXED from the statistical software package SAS 9.1. (SAS Institute Inc., Cary, NC, USA). If necessary, data were ln- or square root-transformed, to stabilize normal distribution and homogeneity of variance. A comparison of means was accomplished using the *t*-Test.

ANOVA was done in two steps: In a first step, the main effects year, management system, crop species and interactions were investigated. In a second step, crop species were analysed separately for determining potential varietal differences depending on year and management system. Only for the parameter AA, no statistical analysis was undertaken. This was because only single samples from one field replicate were selected by the level of free Asn to represent the whole range from low to high for each species. The analyses of AA were chosen to reveal if free Asn can serve as indicator for AA formation potential as it was reported by several studies.

3. Results

Grain yield, quality parameters and free Asn content of the three cereal species were significantly influenced by management system and year (Table 5). Additionally, the threefold interaction CS × Y × S was significant for all parameters except for the sedimentation value.

Table 5. F-values and *p*-values for main effects: management system (MS), species, year and interactions between factors on grain yield, crude protein content, sedimentation value and the Asn content of the flours.

		Grain Yield		Crude Protein		Sedimentation Value		Free Asn	
	DF [1]	F	*p* Value *	F	*p* Value	F	*p* Value	F	*p* Value
MS	1	101.92	***	266.94	***	15.41	***	41.60	***
Species (S)	2	40.77	***	710.29	***	118.62	***	470.25	***
Year (Y)	1	1.78	0.21	60.33	***	0.65	0.42	52.01	***
MS × S	2	1.79	0.17	69.49	***	19.59	***	7.29	***
MS × Y	1	1.47	0.25	73.02	***	0.05	0.82	0.02	0.89
S × Y	2	11.44	***	1.83	0.16	4.46	*	10.45	***
MS × S × Y	2	8.53	***	32.66	***	0.13	0.71	14.89	***

* level of confidence ($p < 0.05$ *, 0.01 ** or 0.001 ***), [1] degree of freedom.

Winter wheat (WW) and winter spelt (WS) grain yields were about 1.1 to 1.9 t ha^{-1} higher in the conventional system when compared to the organic management systems in both experimental years (Table 6). In contrast, conventionally produced winter rye only achieved higher yields when compared to the organic management system (by 2 t ha^{-1}) in 2008.

For both years, crude protein content of winter wheat was significantly higher under conventional conditions (Table 6). The mean protein content of conventionally grown samples was 13.37% compared to 9.72% if organically produced and by this 27% higher. Additionally, a significant year-to-year effect was obvious. In contrast, crude protein content of spelt was not significantly affected by the production system. Crude protein contents of conventionally grown rye were only significantly higher in 2007. In general, the year had a higher impact on the conventionally managed cereals. In 2007, the protein level was around 10% higher.

Sedimentation values of winter wheat for both years were considerably higher under conventional growing conditions when compared to organic farming (Table 6). For spelt, no effect was observed for either years.

Table 6. Grain Yield (GY), quality parameters (crude protein [CP], sedimentation value [SV]) and free Asn) of the three tested species depending on management system and year. Treatments within the same species (WW = winter wheat, WS = winter spelt, WR = winter rye) assigned by the same letters are not significantly different ($\alpha = 0.05$, *t*-Test).

		GY		CP		SV		Asn	
		[t ha^{-1}]		[%]		[mL]		[mg 100 g^{-1} DM]	
		Conventional	**Organic**	**Conventional**	**Organic**	**Conventional**	**Organic**	**Conventional**	**Organic**
WW	**2007**	7.70 c	6.55 b	14.18 c	9.77 a	37.00 b	29.09 a	16.07 c	12.67 b
	2008	7.01 b	6.04 a	12.56 b	9.66 a	40.47 b	31.56 a	14.97 bc	7.89 a
WS	**2007**	6.44 c	4.56 a	14.78 b	14.46 b	25.40 a	25.33 a	10.47 ab	12.24 b
	2008	6.75 c	5.50 b	13.16 a	13.23 a	24.03 a	24.81 a	11.44 b	8.62 a
WR	**2007**	7.54 bc	7.22 b	10.74 c	6.47 a			54.31 c	35.92 b
	2008	7.87 c	5.83 a	7.54 b	7.35 b			31.50 ab	30.14 a

3.1. Free Asn as Main AA Precursor

Free Asn content of the analysed white flours was significantly affected by management system, species, year and the interaction between these effects (Table 5). Rye had the highest Asn contents in both management systems and for both years with on average about 38 mg 100 g^{-1} flour-DM, followed by winter wheat with 13 and winter spelt with 11 mg 100 g^{-1} flour-DM (Table 6). Averaged across the three species, organically produced grain contained about 26% lower Asn contents compared to conventional production. Differences between management systems were most distinctive in winter wheat, with 22% to 47% lower Asn contents under organic production depending on the year (Table 6). Summing up years level of free Asn for conventional wheat samples were 15.52 mg 100 g^{-1} flour-DM and for organic wheat samples were 10.28 mg 100 g^{-1} flour-DM. Thus, the organically cropped wheat samples had a 33% lower level of free Asn. Asn contents of spelt and rye differed only significantly between the two management systems in single years (Table 6, Figure 1). Whilst Asn levels of spelt were not affected by the management system in 2007, organically produced spelt had a significantly lower Asn content of 8.6 mg 100 mg^{-1} flour-DM in 2008, which was about 25% lower than under conventional farming for the same year. However, spelt cultivars revealed to have significant effects on Asn levels irrespective of year and management system, with cultivars Frankenkorn and Schwabenkorn having the lowest and highest Asn contents (Franckenkorn: 7.5 mg 100 mg^{-1} flour-DM, Schwabenkorn: 13 mg 100 mg^{-1} flour-DM), respectively (Figure 1). In 2007, organically produced rye had significantly lower Asn contents while no significant differences in the Asn content occurred between the two management systems in 2008 (Table 6). By comparing the Asn response of the different cultivars in both management systems, four out of ten organically produced wheat cultivars had significantly lower Asn levels than their conventional counterparts. In 2007 and in 2008, Asn contents of each of the ten tested wheat cultivars were significantly lower under organic compared to conventional farming (Figure 1). In this context an interesting observation was the different responses of each cultivar to the management system concerning free Asn. Whereas free Asn of cv. Bussard hardly changed between both management systems in both years and in 2008 was even slightly higher in the organic trial, cv Privileg changed a lot. In between, cv. Naturastar showed small difference of free Asn in 2007 but a large difference in 2008.

In 2007, cvs Privileg and Naturastar had the highest Asn contents of 26.6 and 24.5 mg 100 g^{-1} under conventional conditions. All other conventionally grown cultivars had significantly lower levels than Privileg and Naturastar in a range between 12 and 16 mg 100 g^{-1} but did not differ significantly from each other (Figure 1). Cultivars Privileg and Naturastar also showed the highest Asn contents under organic conditions (18.5 and 24.8 mg 100 g^{-1}), which were significantly higher than from organically produced cultivars Bussard, Achat, Batis, Astron and Magnus with Asn contents ranging between 11.5 and 14 mg 100 g^{-1} flour-DM. In 2007, the significantly lowest Asn value under organic farming was recorded for cultivar Ludwig with 6 mg 100g^{-1} followed by cultivar Capo with 9.1 and cultivar Akteur with 9.8 mg 100 g^{-1} flour-DM (Figure 1). These three were the only cultivars with Asn contents lower than 10 mg 100 g^{-1} flour-DM, whereas under conventional farming none of the tested cultivars had Asn contents below this level. Privileg and Naturastar were the cultivars with the highest Asn contents, as well as in 2008, under conventional farming. With levels of 31.6 and 32.1, they significantly outreached the levels of all other conventionally produced wheat cultivars, which were in a range between 10.9 and 14 mg 100 g^{-1} flour-DM, except cultivar Capo which had the significantly lowest Asn content of about 10.1 mg 100 g^{-1} flour-DM (Figure 1). Asn levels of all organically grown cultivars were lower than 10 mg 100 g^{-1} flour-DM, except for cultivar Privileg with 10.7 mg 100 g^{-1} flour-DM. Cultivars Capo, Astron and Achat had the significantly lowest Asn contents of all organically produced wheat cultivars in 2008 with values of 6.1 and 6.5 mg 100 g^{-1} flour-DM.

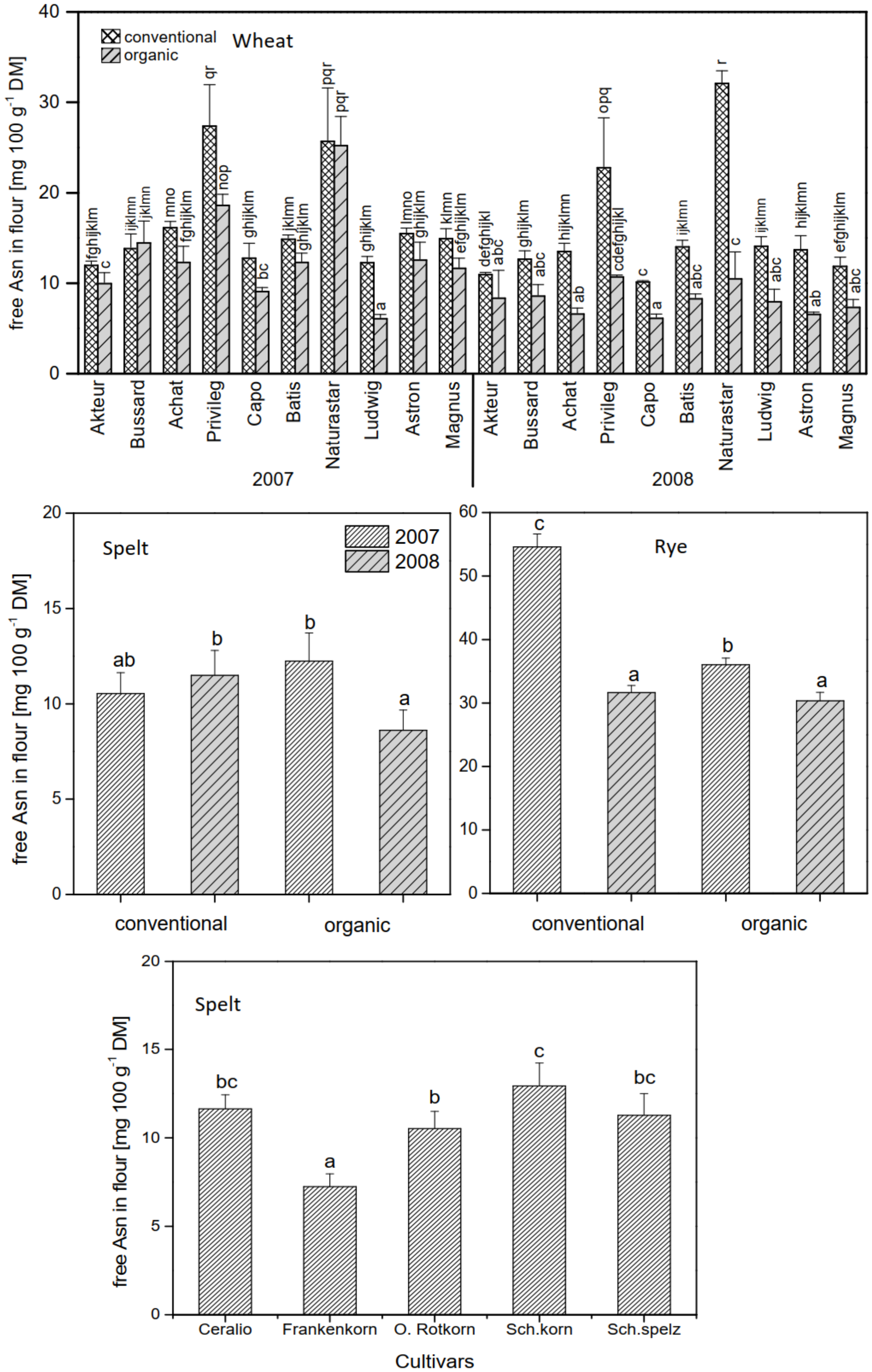

Figure 1. Free Asn content separated by species (wheat, spelt, rye), cropping system (organic, conventional), year (2007, 2008) and cultivars in dependence of their significant highest order interaction. Columns with different letters within cropping system and year indicate significant differences (α = 0.05, *t*-Test).

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3.2. Correlation of Crude Protein, Free Asn and AA

Between flours derived from the different wheat cultivars there was only a weak correlation of both parameters between different cultivars under both management practices in both years (R^2 = 0.18, Figure 2), meaning that cultivars with high crude protein contents did not show high Asn levels per se and vice versa. The correlation of crude protein and Asn of winter spelt was weak across the two management systems and years (R^2 = 0.1, data not shown) when averaged over cultivars, which was mainly due to a low variability of crude protein content between the management systems and years. Crude protein content and Asn content of rye correlated well with a R^2 = 0.8 (data not shown).

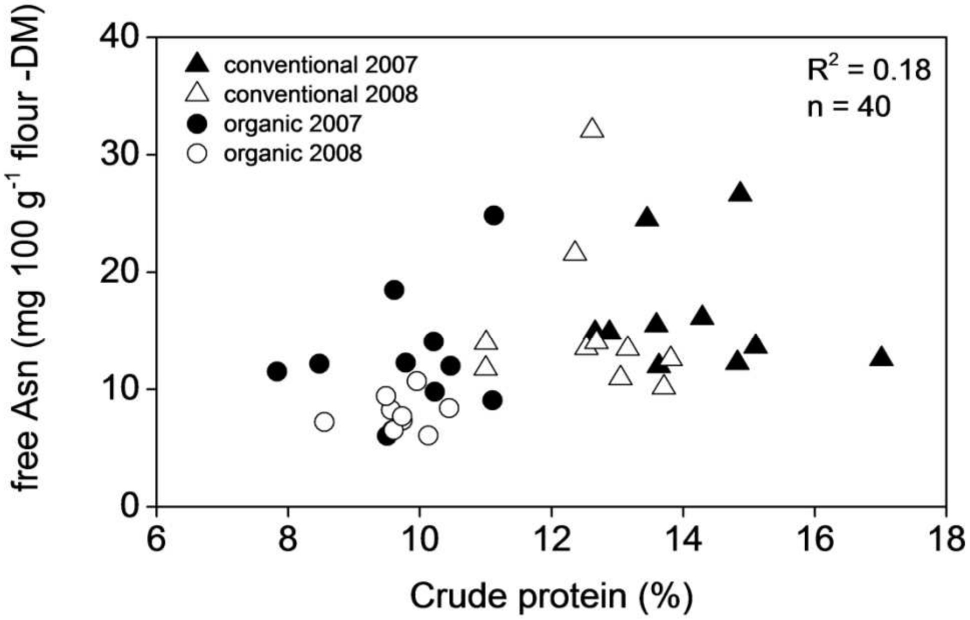

Figure 2. Relationship between crude protein content and free Asn levels in wheat flours produced under conventional and organic conditions in 2007 and 2008.

Free Asn and AA across management systems, years and species correlated well by R^2 = 0.64 (Figure 3A). Separating the management systems led within both systems to a strong relation of both traits (Figure 3B). The conventional crop management system showed an R^2 of 0.58, while for the organic management system the relation was even stronger with R^2 = 0.75. For wheat, both traits correlated with R^2 = 0.82 in 2007 and R^2 = 0.5 in 2008 for the conventionally produced samples and with R^2 = 0.76 in 2007 and R^2 = 0.86 in 2008 for the organically produced wheat flours.

Across management systems, free Asn and AA of spelt correlated by R^2 = 0.55. For organic samples, only the relationship was strong by R^2 = 0.83 but low for the conventional ones (R^2 = 0.4). The correlation within rye samples was generally lower by R^2 = 0.37 (across management systems), R^2 = 0.25 (organic samples) and R^2 = 0.5 (conventional samples).

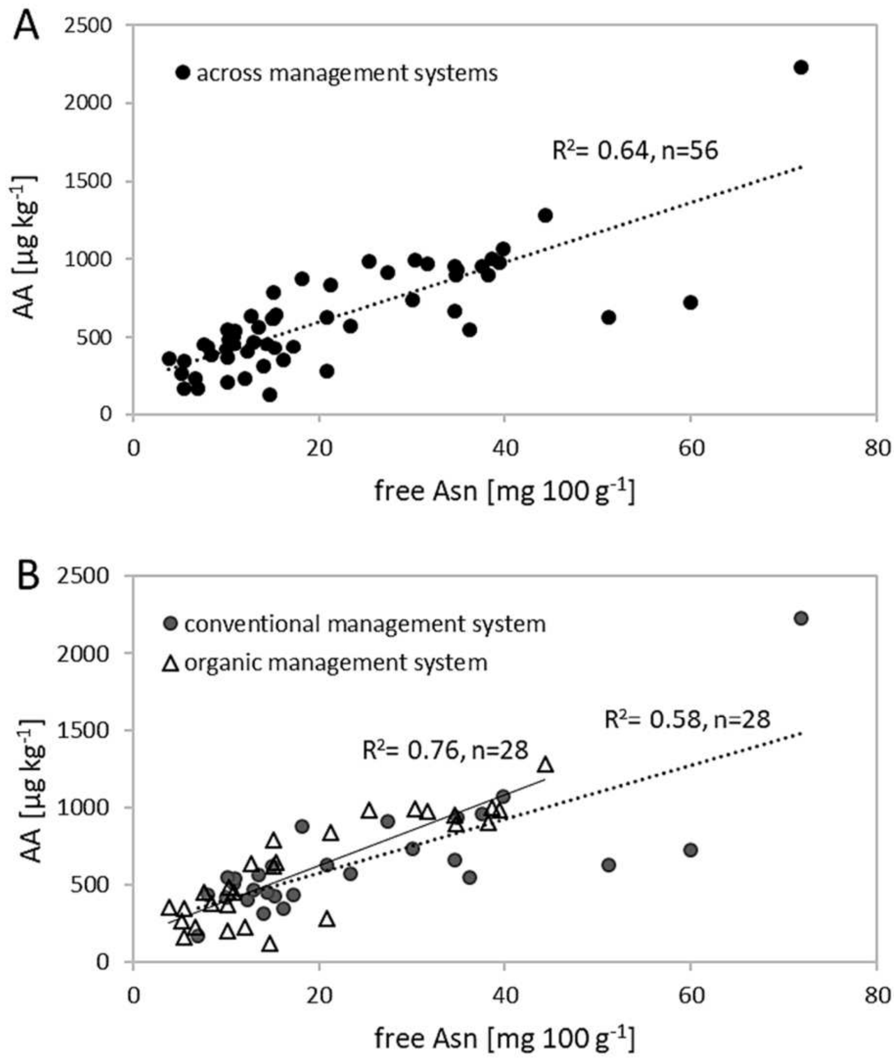

Figure 3. Relationship between free Asn in wheat flours and AA in heated wheat flours across management systems (**A**) and separated by conventional vs. organic management system (**B**).

4. Discussion

4.1. Grain Yield and Baking Quality

Grain yield of all three organically produced species was significantly lower compared to their conventionally grown counterparts. Organically grown wheat produced on average about 85% of the yield compared to conventionally gown. For spelt and rye the yield gap was even larger. Differences concerning grain yield of 20% to 30% between conventional and organic farming are described by other authors [33–35]. Determined lower protein contents of on average 3.5% across both years between conventionally and organically produced wheat corresponded well with results reported by Bilsborrow et al. [36] from a long-term field trial in the UK. Both difference in yield and crude protein content were most likely the result of differences in the availability of plant-available nitrogen between the two management systems as shown by other authors [36,37]. Especially, nitrogen availability influences grain yield [38] and crude protein content [22], which is an important quality parameter for baking quality of wheat due to its strong correlation with bread volume. Nitrogen fertilizer amounts in conventional farming are usually higher, mineral nitrogen is available faster than organic nitrogen. Thus, mineral nitrogen in conventional farming can be directed more precisely, leading to higher grain yields. Under organic conditions, there is often a mismatch between the plant-nitrogen demand and the mineralisation rate depending on environmental conditions e.g., temperature and soil moisture [39]. Moreover, higher weed, pest and disease pressure could also have been responsible for yield differences but were not assessed in our experiment as measures to control them under organic farming are very limited. Moreover, site-specific conditions usually strongly affect yield more than weeds, pests and diseases when crops are grown organically [40]. Thus, the manifold regulatory options in conventional management systems can compensate for location deficits better, leading to higher grain yields [41]. Consequently, if organically produced bakery goods are demanded, lower yields and lower baking qualities must be accepted [42], and bread bakery processing has to be adjusted to the lower protein contents of such flours [43] to achieve acceptable products.

However, the postulated levels of protein needed by industry are under discussion as there are some studies announcing that good baking quality of, for example, wheat flour, does not need high levels of crude protein [44]. Much more important are gluten content, gluten quality and sensory property. Thus, wheat cultivar cultivated under organic farming conditions can offer a good baking quality while having lower protein content. Furthermore, improving baking quality means also improving sensory properties. Torri et al. [45] reported that a treatment with mycorrhizal factors is able to raise the sensory properties of modern wheat varieties. No significant difference of spelt crude protein content between the two management systems could be observed. This was presumably due to only minor differences in nitrogen fertilizer amounts and nitrogen availability especially in the latter phase of grain filling.

4.2. Free Asn

Free Asn is the critical factor for acrylamide formation during processing of cereal based bakery products [12], and Asn contents in flour correlate almost linearly with the acrylamide content in heated flour [8] or in breads [7]. Free asparagine also proved to be strongly correlated with the AA formation in heated flour samples in our study. Rye Asn contents were considerably higher than that of wheat and spelt. This corresponds well with findings from Fredriksson et al. [46], Elmore et al. [47] and Claus et al. [7] who found up to 3 or 4 times higher Asn levels in rye compared to wheat or spelt. Therefore, the acrylamide formation potential of bakery goods made from rye flour is considered to be higher than from wheat or spelt [7,47]. In contrast studies by Curtis et al. [8] showed that AA formation in heated flour per unit Asn was much lower in rye than in wheat flour, suggesting that the higher Asn level compared to wheat does not inevitably mean that rye has a higher AA formation potential per se.

The amount of free Asn was strongly affected by the management system. Across all species, free Asn was 26% lower under organic conditions. Differences in the Asn level between the management systems were consistent across both experimental years for wheat, whereas spelt and rye showed inconsistencies. Organically produced wheat had a lower Asn content of about 33% compared to conventional production when averaged over years and cultivars, whereas Asn contents of organically produced spelt and rye were lower only in single years. Therefore, organically produced wheat particularly offers the opportunity to significantly lower the AA potential of bread and bread rolls via choosing a raw material low in free Asn.

However, in particular for wheat also, cultivars had a significant impact on free Asn formation. While cv Bussard showed only a slight difference between management systems in both years, cv Privileg changed to a large content in 2007 and 2008. Interestingly, cv Naturastar seemed to be highly influenced by year, as in 2007 the differences between management systems were hardly given while in 2008 the distinction was huge. The reason that the cvs reacted in three different ways could be the quality class of the cvs. Referring to the German quality classes Bussard and Privileg belong to E-wheats (highest baking quality) while Naturastar belongs to A-wheats (high baking quality). Thus, E- and A-wheats differ in the protein synthesis capacity. Furthermore, climate conditions (temperature and rainfall) during grain filling (June–August) may have influenced free Asn level in those cvs. However, only small changes in both, temperature and rainfall from June to August were observed in this study. Nevertheless, several studies showed that climate conditions (growing temperature, sunshine duration and water availability) during grain growth can highly influence Asn formation, protein synthesis, amino acid composition, nitrogen remobilization and phytochemicals in cereal grains [7,8,18,48,49]. In this context, further research especially revealing the reason why cultivars differ in their Asn formation potential is essential.

Weber et al. [22] investigated the impact of different nitrogen amounts and timings on the resulting Asn levels in flours from a conventionally produced winter wheat cultivar. They found a close correlation between crude protein and Asn content. Furthermore, it was predominantly the 'late applied nitrogen' that led to crude protein contents of more than 13% which significantly increased free Asn compared to the unfertilized control [22]. Winkler and Schön [50] found a close correlation between nitrogen concentration and free Asn in barley grain, as well as Curtis et al. [8] found in rye grain. As nitrogen fertilization in conventional farming is usually applied at higher amounts, and availability of mineral nitrogen is also higher than found in organically applied nitrogen, the higher level of free Asn under conventional farming presumably resulted from the differences in the nitrogen supply and availability between both systems during the phase of grain filling. In this context, it is quite likely that applying lower nitrogen in the conventional trial would match the organic trial, with the same lowering effect of protein levels as well [51,52]. However, it seems not to be that easy as a set of organically cropped cereal species and cultivars were investigated by Stockmann et al. [53] for their content of free Asn. The samples were only marginally supplied with nitrogen, but a high range of free Asn comparing species and cultivars within species was observed. Moreover, a broad range of crude protein was reported (8.7% to 13.7% across years and wheat cultivars). Thus, reducing nitrogen to reduce levels of free Asn appears to be a feasible way, but some other factors must be considered too. Finally, we suggest that the level of nitrogen available for plants must fit to the synthesised crude protein level to lower free Asn.

In general, winter spelt and winter rye have a lower N-demand compared to wheat. This led to a smaller difference in the crude protein contents within the two species between the conventional and organically grown grains. Thus, this may explain the less pronounced impact of the management system on the Asn contents within winter spelt and winter rye in a single year. Springer et al. [26] found higher amounts of Asn in organically produced cultivars compared to those conventionally produced. They assumed that the higher share of the seed coat on the grain under organic cultivation might have been responsible for the higher contents of Asn found in the organically cropped cultivars. This effect could not be observed in our study. Nevertheless, as reported by Corol et al [19], wholemeal flour, can reach higher levels of free Asn in a range of up to 32 to 156 mg 100 g^{-1} which is about 6 to 10-fold above our reported results within wheat flour.

Differences in the free Asn content between management systems especially in wheat could also be caused by diseases caused by fungus. Martinek [21] found that fungicide application promoting leaf area duration and delaying senescence can reduce free Asn content in grains. In our study, only in the conventional trial fungicides were applied. Thus, the level of free Asn could have been raised in the organic samples. However, as the level of free Asn in general was lower in organic samples, impact of nitrogen seems much higher than the effect of fungicides. However, the infestation of plant leaf by fungus should be considered as causing higher levels of free Asn. Cultivars owning a high baking quality do not inevitably have to be linked with high Asn contents and ultimately a high AA formation potential. This is suggested by the weak correlation found between crude protein and free Asn contents of different wheat cultivars. Furthermore, the results showed that cultivars combining high or acceptable baking quality and low Asn contents are already available and that the AA formation potential could further be lowered by selecting and cultivating appropriate cultivars, irrespective of management systems, if the information on genotypic disposition regarding Asn accumulation would be available.

Sulphur can have a high impact on free Asn in case of a deficiency in soil [25]. Within the conventional system, only mineral nitrogen fertilizer CAN was applied but no additional sulphur. In contrast, the organic management system was fertilized by manure that can contain sulphur. Thus, some differences concerning free Asn between both management systems could have been caused by the influence of sulphur. However, this was not analysed in detail.

Moreover, it has to be considered that environment (=location and year) can affect Asn levels considerably, as also shown by Curtis et al. [18] for wheat and by Curtis et al. [8] for rye. However, up to now information on how soil type, temperature and precipitation affect grain-Asn accumulation is missing [54]. Within wheat genotypes grown at six locations in Europe, Corol et al. [19] described that only 13% of the free Asn variability was explainable by heritability while about 36% was caused by environment. This must be also taken into account for the interpretation of our data, as the locations for the conventional and for the organic trials were located within a distance of 20 km. Therefore, cultivars must be tested at different locations for at least two years before recommendations or selection of cultivars can be announced for further specific breeding programs targeted towards lowering the AA precursor contents.

5. Conclusions

The study aimed to assess the effect of organic versus conventional farming practices on free Asn contents in flours of winter wheat, winter spelt and winter rye. The management system significantly affected Asn levels. The effect was most noticeable and consistent for winter wheat, where Asn levels were lowered on average by 33% if cultivars were organically grown. Organically grown spelt and rye showed lower Asn contents only in single years. Different intensities of plant-available nitrogen during critical phases of grain development between both systems are considered to be mainly responsible for differences in Asn accumulation. These differences were most evident for wheat due to its high N demand and the close correlation between nitrogen supply and baking quality. Therefore, organically produced wheat flour can be well regarded as having a lower AA formation potential than conventionally produced wheat, despite also having lower yields. Furthermore, the weak correlation between crude protein and Asn content between different cultivars suggested that choice and cultivation of cultivars combining low Asn contents with adequate baking quality can help in lowering the amount of dietary AA intake from cereal based bakery goods, irrespective of whether the raw material originates from organic or conventional farming. Especially low-Asn cultivars with high stability over different environments are of great interest for future research in this area.

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2.3 Paper 3: "Impact of nitrogen and sulfur supply on the potential of acrylamide formation in organically and conventionally grown winter wheat"

Stockmann, F.; Weber, E.A.; Schreiter, P.; Merkt, N.; Claupein, W.; Graeff-Hönninger, S. **Impact of Nitrogen and Sulfur Supply on the Potential of Acrylamide Formation in Organically and Conventionally Grown Winter Wheat**. *Agronomy* 2018, *8*, 284.

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As nutrient supply is a key factor in agriculture, this paper examined the effect of nitrogen and sulfur fertilization on free Asn level and simultaneously on yield and quality parameters in winter wheat. For conventional farming some publications investigated the implication of both nutrients on free Asn. But the issue of comparing cropping systems with a special emphasis on organic farming methods concerning nitrogen, has never been analyzed before. The fact that under organic farming conditions a much lower level of nutrients is applied, artificial fertilizers are forbidden and the entire systems follows a circular flow including large crop rotations, made it essential to gain insight into this topic. In addition, different sulfur fertilizing strategies in terms of S-amount and S-type were applied. The impact on free Asn formation was investigated.

The results in paper 3 showed that nitrogen fertilization, significantly influenced grain yield, and baking quality in both cropping systems. In contrast, up to a certain amount of nitrogen, free Asn was only affected to a small extend. In particular, within the organic farming samples there was no significantly higher free Asn analyzed if nitrogen was raised or the nitrogen form was changed. The late nitrogen fertilization step within the conventional nitrogen trial significantly increased crude protein content, while for free Asn no clear effect was found. Furthermore, neither type nor amount of sulfur fertilization influenced free Asn significantly. However, also cultivars influenced the free Asn level significantly as cultivar Capo exhibited the lowest AA formation potential at a nitrogen supply of 180 kg N ha^{-1} while at the same time reaching a crude protein content > 15% (conventional) and > 12% (organic). Thus, lowering free Asn by regulating nitrogen treatments should not necessarily affect baking quality. Finally, it was proven that free Asn amounts in wheat varied widely both within cultivars and between cropping systems.

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Impact of Nitrogen and Sulfur Supply on the Potential of Acrylamide Formation in Organically and Conventionally Grown Winter Wheat

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Abstract: In a two-year field trial, the effect of nitrogen (N) and sulfur (S) fertilization was investigated on grain yield, grain quality parameters, formation of acrylamide (AA), and the precursor free asparagine (Asn) in organically and conventionally produced winter wheat cultivars. In both production systems, different types, amounts, and temporal distributions of N were tested. The the effect of S fertilizer types and amounts on free Asn was only tested in the conventional farming system. Within both cropping systems, grain yield and baking quality were significantly influenced by N treatment while the effect on free Asn was only minor. Especially within the organic farming system, increasing N fertilization levels did not increase free Asn significantly. A slight trend of increasing free Asn levels with an intensified N supply was observed, especially in the presence of crude protein contents of 14% or higher. However, only N amounts of 180 kg N ha^{-1} or higher increased the probability of high free Asn contents considerably, while N supply below that amount led to free Asn values similar to the unfertilized controls. The results indicated that good baking quality can be achieved without significantly increasing free Asn levels. In addition, cultivars affected the levels of free Asn significantly. Compared to cv. Bussard and Naturastar, cv. Capo exhibited the lowest AA formation potential at an N supply of 180 kg N ha^{-1} while simultaneously reaching a crude protein content > 15% (conventional) and > 12% (organic). Thus, it seems that cultivars differ in their ability to store and incorporate free Asn into proteins. Over all trials, a relation of free Asn and AA was shown by R^2 = 0.77, while a relation of free Asn and protein was only R^2 = 0.36. Thus, lowering free Asn by adjusting N treatments should not necessarily affect baking quality. S fertilization within conventional farming did not change the free Asn amount or crude protein significantly, probably due to the fact that soil was not sulfate-deficient. In summary, it was evident that free Asn amounts in wheat varied widely both within cultivars and between cropping systems. In order to clearly unravel genotypic differences and their interaction with environmental factors and especially N fertilization, further research is needed.

Keywords: acrylamide; asparagine; agriculture; nitrogen; sulfur; fertilization; cereals; cropping system

1. Introduction

Food industry and gastronomy are facing a big challenge because current regulation of the European Commission [1] was announced that limits the level of acrylamide (AA) in cereal food products and requires that minimization strategies are applied.

AA—a probable carcinogen to humans—is formed in carbohydrate-rich food (e.g., cereals and potatoes) thermally by means of the Maillard-reaction, where free Asn and reducing sugars react [2–4]. Its discovery in 2002 by a Swedish research group [5] gained immediate attention by health authorities worldwide. Intense efforts were undertaken to gather information about the synthesis, toxicology, and formation routes of AA and led to several approaches to minimize the amount of AA in foodstuffs. Studies have successfully shown that the limiting factors for AA formation in potato products are the concentrations of reducing sugars, while for cereal products, the content of free Asn is the limiting factor [6–9]. Although strongly heated potato products can contain much more AA than cereal-based bakery products, bread and bread rolls contribute to about 25% to 45% of the dietary AA intake in Germany, due to the high daily per capita consumption of almost 240 g [10,11].

In the context of the newly released EU regulation, AA has gained a renewed interest. Currently, the food business is forced to reduce the presence of acrylamide in foodstuffs where raw materials contain its precursors by laying down appropriate mitigation measures.

Initial efforts focused on finding ways to lower AA by modified processing steps alongside the food production chain; including changing heating temperature, heating duration, as well as changing the recipe [6,9,12–14]. Moreover, some studies investigated the efficacy of the use of additives and the enzyme asparaginase during processing for lowering AA [13,15,16]. Although modification of processing conditions often led to significant reductions in AA levels, these treatments are also expensive, not feasible for the food industry, or affect taste, texture, color, and aroma compounds, which often impair consumer acceptance. A more practical solution for the food industry is to lower the AA formation potential in cereal-based bakery wares by using raw materials low in precursors of AA. Thus, flours with a low level of free Asn will gain the interest of the food industry. In this context, it is important to implement agronomic measures that will produce raw material low in free Asn, which will consequently minimize AA in the final product.

Up to now, several studies showed that cereal species differ in their Asn levels and consequently in their AA formation potential. Rye usually has higher Asn levels compared to wheat and spelt [7,8,17]. Moreover, cultivars can differ considerably in their precursor content as shown by several studies [7,8,17–19]. Taeymans et al. [19], which reported a 5-fold range for different European wheat cultivars and Claus et al. [7] found a variability of Asn contents in nine German winter wheat cultivars of up to a factor of three. Postles et al. [18] compared five rye cultivars and found significant differences concluding that there is a genotype control of free Asn. Thus, selecting suitable cultivars with low Asn contents is considered as a feasible way to minimize AA formation potential. However, it has to be taken into account that site-specific and climatic conditions may alter Asn contents considerably [8,17]. Furthermore, crop management practices, such as fungicide applications promoting leaf area duration and delaying senescence, can also reduce the free Asn content in grains [20].

Fertilization is a key measure in crop production to increase yield and quality affecting Asn levels as well. Studies of Weber et al. [21] and Martinek et al. [20] showed that N amount and the timing of application, as well as N form, can affect Asn contents in wheat considerably. Up to now, information about the impact of N supply under organic farming conditions on the level of free Asn has been scarce. Since organic farming systems can only use organic fertilizers whose N release is slow and availability for plants more uncertain than from mineral N fertilizers, the knowledge gained from mineral N fertilization experiments on Asn cannot be transferred directly to organic farming. Preliminary studies of Stockmann et al. [22] reported a cropping system effect, where wheat cultivars grown under organic conditions showed a significantly lower amount of free Asn when compared to conventionally grown wheat cultivars, presumably due to the lower N availability under organic conditions.

Moreover, several studies showed that S deficiency sometimes dramatically increases Asn contents and thus the AA formation potential [23–26]. Thus, a sufficient S supply is expected to help reduce Asn levels in grains. However, such results were obtained mostly from greenhouse pot experiments under S deficient conditions. Information from field experiments with no explicit induced S deficiency are rare, and no information on the effect of different S fertilizer forms on free Asn is currently available.

Thus, the present study aimed to comparatively investigate the impact of organic and mineral N fertilization (amount, type, and time of application) on the AA precursor Asn under conventional and organic farming conditions. Additionally, the effect of S supply (amount, type and time of application) under varying N fertilization intensities was investigated under field conditions for its impact on the content of free Asn under conventional farming conditions. The following hypotheses were tested:

- The amount and timing of N fertilization affect yield, grain quality, and the content of free Asn in winter wheat, irrespective of its form (organic or mineral).
- Due to a slower release rate and thus a lower availability of organic N, its effect on grain quality and free Asn is less pronounced compared to the application of mineral N.
- The type and amount of S fertilizer affect free Asn accumulation in wheat flour, especially under high N amounts.

2. Materials and Methods

2.1. Site Description

Grain and flour samples were obtained from three field trials during two consecutive growing seasons in 2006/2007 and 2007/2008. All trials were carried out by the Institute of Crop Science, University of Hohenheim. The conventional N and S trials were conducted at Ihinger Hof (conventional farming research station), while the organic N trial was conducted at Kleinhohenheim (organic farming research station).

The conventional research station Ihinger Hof is situated 25 km west of Stuttgart, Germany in the district of Boeblingen (48.74° N, 8.92° E) at an altitude of 450–508 m above sea level. Average air temperature during the growing season from October 1 to July 31 was 9.7 °C in 2006/2007 compared to 8.0 °C in 2007/2008. Total precipitation was 546 mm in 2006/2007 compared to 600 mm in 2007/2008 (Figure 1).

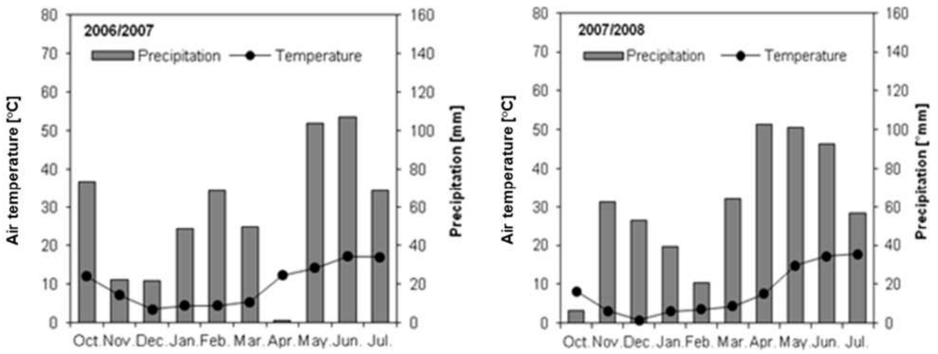


Figure 1. Air temperature (●) and precipitation (bars) at the trial site Ihinger Hof for the growing seasons 2006/2007 and 2007/2008.

The field trial was carried out on vertic Luvisol (2006–2007) and vertic Cambisol (2007–2008) soils according to the World Reference Base [27]. Those soils provide well drained, highly fertile conditions for wheat production. Soil analyses for mineral N content were taken in spring 2007 and 2008. In 2007, mineral N content was 2.1 kg ha^{-1} within a soil horizon of 0 to 60 cm compared to 22 kg ha^{-1} in 2008.

The research station for organic farming, Kleinhohenheim, has 64 ha of farmland, half of which is arable land and the remaining half meadows. It is located 435 m above sea level in the southern peripheral part of Stuttgart, Germany (48.74° N, 9.20° E). The average air temperature from October to July was 10.6 °C in 2006/2007 compared to 8.8 °C in 2007/2008. Precipitation for the growing season of 2006/2007 was 715 mm compared to 691 mm in 2007/2008 (Figure 2).

The soil at the trial site in Kleinhohenheim falls under the Luvisol type. It is characterized by a nearly 2 m thick horizon of loess to loamy clay. Therefore, it features a high water holding capacity and is well suited for agricultural purposes. In spring 2007, mineral N content was 35 kg ha^{-1} within a soil horizon of 0 to 60 cm compared to 62 kg ha^{-1} in 2008.

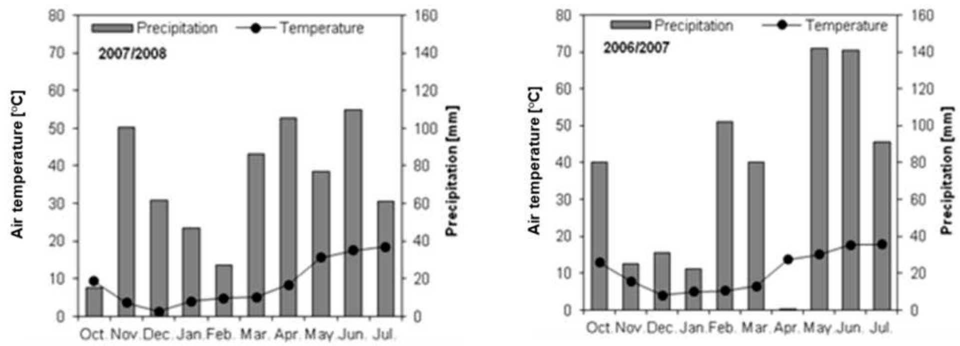


Figure 2. Air temperature (●) and precipitation (bars) at the organic trial site Kleinhohenheim for the growing seasons 2006/2007 and 2007/2008.

2.2. Experimental Design

Each field trial was set up as a randomized block design with three repetitions. While in the conventional N and S trial, common conventional farming methods, including chemical weed and pest management, were applied, the organic N trial was conducted according to standards of organic farming.

2.2.1. Conventional N Trial

The conventional N trial aimed at determining the impact of both the amount and the temporal distribution of N fertilization on potential AA formation. The total amount of N fertilization given as CAN (calcium ammonium nitrate: 13.5% Nitrate-N, 13.5% Ammonium-N) varied from 0 kg N ha^{-1} (control plots) to 180 kg N ha^{-1} and fertilizer was applied on up to five different dates as shown in Table 1. In each treatment, a late N fertilization (Zadoks 49/51, Zadoks 55) application was integrated and marked 'late'. The same three winter wheat cultivars, as used in the organic trial, were tested. For E-grade cultivar Bussard, eight N treatments plus one untreated control treatment were tested. Cultivar Naturastar (A-grade), as well as Capo (E-grade), were only tested with N 180-late plus 0 kg N ha^{-1} (control treatment. In this context E-grade and A-grade refers to the German baking quality classification system for wheat cultivars. E- and A-grade wheat cultivars reach the highest baking qualities including high levels of crude protein (12.7% to 13% and higher) and sedimentation values (31–37 units and higher).

Table 1. Treatments of the conventional N trial differing in winter wheat cultivar (Bussard: B, Naturastar: N, Capo: C), amount of N fertilization, and temporal distribution of N fertilization.

Treatment	Wheat Cultivar	Total N (kg ha^{-1})	Vegetation Start (kg N ha^{-1})	Zadoks * 31/32 (kg N ha^{-1})	Zadoks 39 (kg N ha^{-1})	Zadoks 49/51 (kg N ha^{-1})	Zadoks 55 (kg N ha^{-1})
Control	B	0	-	-	-	-	-
N60-late	B	60	-	30	-	30	-
N60	B	60	30	-	30	-	-
N100-late	B	100	30	-	40	30	-
N100	B	100	30	30	40	-	-
N140-late	B	140	30	30	30	50	-
N140	B	140	50	40	50	-	-
N180-late	B	180	30	40	30	40	40
N180	B	180	60	60	60	-	-
Control	N	0	-	-	-	-	-
N180-late	N	180	30	40	30	40	40
Control	C	0	-	-	-	-	-
N180-late	C	180	30	40	30	40	40

* [28].

In both years, sugar beet was the preceding crop. A few days prior to seeding, plots were tilled with a combination of cultivator and disk harrow (depth of tillage: 15 cm) and the seedbed was prepared with a tine harrow. Seeding was carried out on 12 October in 2006 and on 9 October in 2007 at a seeding density of 350 kernels m^{-2}. Harvest dates were 23 July in 2007 and 28 July in 2008. A plot combine harvester (Hege Maschinen GmbH, Hohebuch, Germany) was used.

2.2.2. Organic N Trial

The organic N trial aimed at determining both the impact of the amount and temporal distribution of N fertilization on potential AA formation. In addition, different types of organic N fertilizer were tested: cattle slurry (1 kg N m^{-3} total N content, 4% dry matter), horn meal (12% total N content), or a combination of both was used. The total amount of N fertilization varied from 0 kg N ha^{-1} (control plots) to 180 kg N ha^{-1}. Fertilizer was applied on up to three different dates (see Table 2). Winter wheat cultivars Bussard, Capo, and Naturastar were used. For E-grade cultivar Bussard, seven different N treatments plus one untreated control treatment were tested, whereas in A-grade cultivar Naturastar and E-grade cultivar Capo, one N treatment (180 kg N ha^{-1}) plus the control treatment (0 kg N ha^{-1}) were tested.

Table 2. Treatments in the organic N trial differing in winter wheat cultivar (Bussard: B, Naturastar: N, Capo: C), amount of N fertilization and temporal distribution of N fertilization.

Treatment	Wheat Cultivar	N Fertilizer	Total N (kg ha^{-1})	Vegetation Start (kg N ha^{-1})	Zadoks 31/32 (kg N ha^{-1})	Zadoks 39 (kg N ha^{-1})
Control	B	Control	0	-	-	-
S50	B	Slurry	50	50	-	-
S100	B	Slurry	100	50	50	-
S50-H50	B	Slurry & horn meal	100	50 slurry	-	50 horn
S100-H20	B	Slurry & horn meal	120	50 slurry	50 slurry	20 horn
H60	B	Horn meal	60	30	-	30
H120	B	Horn meal	120	40	40	40
H180	B	Horn meal	180	60	60	60
Control	N	Control	0	-	-	-
H180	N	Horn meal	180	60	60	60
Control	C	Control	0	-	-	-
H180	C	Horn meal	180	60	60	60

2.2.3. Conventional S Trial

Contrary to the aforementioned trials, in the S trial, only the winter wheat cultivar Enorm (E-grade) was tested. The trial aimed at examining the influence of variable amounts, types, and temporal distributions of S fertilization. Total S application varied from 0 kg N ha^{-1} (control plot) to 60 kg S ha^{-1}. As S fertilizer, kieserite, epsom salt, elemental S, or a combination of kieserite and epsom salt was used. S was applied on four different dates, as detailed in Table 3. N fertilization was also varied in the S trial. Treatments received a total amount of N given as CAN (calcium ammonium nitrate) of either 120 or 200 kg ha^{-1} while control treatments remained unfertilized.

Table 3. Treatments in the S trial differing in amount, type (Kieserit: K, Epson salt: Ep, Kieserite + Epson salt: KEp, Elemental sulfur: elS) and temporal distribution of S fertilization and in amount of N fertilization (N1: 120 kg N ha^{-1}, N2: 200 kg N ha^{-1}).

S Fertilization (kg ha^{-1})							
Treatment	**Total S**	**Vegetation Start**	**Zadoks 37/39**	**Zadoks 49/51**	**Zadoks 55**	**S Fertilizer**	**Total N (kg ha^{-1})**
Control	0	-	-	-	-	-	0
Control-S	20	20	-	-	-	K	0
K20-N1	20	20	-	-	-	K	120
K20-N2	20	20	-	-	-	K	200
K40-N1	40	20	20	-	-	K	120
K40-N2	40	20	20	-	-	K	200
K60-N2	60	60	-	-	-	K	200
Ep-N1	6	-	2	2	2	Ep	120
Ep-N2	6	-	2	2	2	Ep	200
KEp26-N1	26	20	2	2	2	KEp	120
KEp26-N2	26	20	2	2	2	KEp	200
elS N1	5,6	2.8 (Zadoks 25)	2.8 (Zadoks 32)	-	-	eS	120
elS N2	5,6	2.8 (Zadoks 25)	2.8 (Zadoks 32)	-	-	eS	200

Plant production was carried out according to common conventional practice. The trial included the same procedures according to plant protection, plant growth regulators, previous crop, soil and seedbed preparation, sowing date, and density as well as harvest procedure and harvest date as described for the conventional N trial.

2.3. Yield

Grain yield of the different trials was determined by weighing the plot yield. Grain samples were dried at 105 °C for 24 h to determine grain moisture. Grain yields given refer to 86% dry matter content.

2.4. Flour

For the determination of grain quality traits, free Asn, and the AA formation potential, grain samples were milled on a laboratory mill (Quadrumat Junior, Brabender, Duisburg, Germany). Ash content of flours was approximately 0.5% of flour DM. Flour moisture was calculated from the weight loss before and after drying approximately 5 g flour at 105 °C for 24 h.

2.5. Crude Protein

Total grain N content was determined by near-infrared spectroscopy (NIRS, NIRS 5000, FOSS GmbH, Rellingen, Germany). Calibration samples were analyzed according to the Dumas Method [29] using a Vario Max CNS analyzer (Elementar, Hanau, Germany). The analyzed final N content was multiplied by a factor of 5.7 to obtain crude protein content.

2.6. Sulfur

Flour samples of the S trial were determined by a CNS elemental analyzer (Vario max CNS, Elementar Analysensysteme GmbH, Hanau, Germany). The values refer to dry mass.

2.7. Zeleny's Sedimentation Test

Zeleny´s sedimentation test was performed using 3.2 g flour according to ICC standard No. 116. The sedimentation values of the flour were adjusted to 14% moisture basis.

2.8. Free Asparagine

Free amino acids were extracted from 2 g of wheat flour and were mixed with 8 mL of 45% ethanol for 30 min at room temperature. After centrifugation for 10 min at room temperature with

4000 rpm and 10 min at 10 °C and 14000 rpm, the supernatant was filtered through a 0.2 μm syringe filter and poured into vials. Asn analysis was performed using Merck-Hitachi HPLC components. The pre-column derivatization with FMOC [30] was completely automated by means of an injector program. Subsequently, the derivatized Asn was separated on a LiChroCART Superspher RP 8 column (250 × 4 mm, Fa. Merck, Darmstadt) at a constant temperature of 45 °C. The fluorescence intensity of the effluent was measured at the excitation and emission maxima of 263 and 313 nm.

2.9. Acrylamide Formation

The AA formation potential of wheat flour was assessed according to the AA contents of 5 g flour in 250 mL Erlenmeyer flasks after heating in an oven for 10 min at 200 °C. Sample preparation was accomplished according to the test procedure 200L05401 described by Weißhaar [31].

After cooling the samples down to ambient temperature, 100 mL of bidistilled water and 100 μL of D_3-Acrylamide were added as an internal standard to the heated flour samples in the Erlenmeyer flasks. In order to completely extract acrylamide from the flour, samples were put in an ultrasonic bath for 10 min at 40 °C. After adding 1 mL of Carrez I and II to each of the samples and shaking the flasks thoroughly, the samples were filtered using folded filter paper to separate the colloids and flour particles from the aqueous solution. Subsequently, samples were cleaned by a solid phase extraction in a vacuum chamber after preconditioning the cartridges with 10 mL of bidistilled water and 10 mL methanol. After sample clean-up, about 1 to 2 mL of the eluate from each sample were filled in autosampler vials and deep frozen (−18 °C) until AA was determined by LC-MS/MS by the CVUA according to the test procedure 201L01301 [32]. The eluates were separated by a graphite or RP18-phase and detected by a tandem-mass spectrometer. Quantification was undertaken by using the isotope-labelled internal standard (D_3-Acrylamide).

2.10. Statistical Analyses

For each trait listed in the previous section, analysis of variance (ANOVA) was performed using the procedure PROC MIXED of the statistical software package SAS 9.2 (SAS Institute Inc., Cary, NC, USA). ANOVA was done in two steps: in a first step, the main effects of year, treatment, and interaction were investigated. In a second step, years were analyzed separately for determining potential treatment differences. Thus, letters within the treatment refer to single years. If within the same year treatment was not significant no letters appear. If the treatment was significant, but the interaction and the year were not, years were combined (see Table 4).

Table 4. Grain yield (GY), sedimentation value (SV), crude protein (CP), and free Asn of the conventional and organic N trial in dependence on fertilization and year for winter wheat cultivar Bussard.

Conventional

Treatment	GY (t ha^{-1})			SV (mL)			CP (%)	Free Asn (mg 100 g^{-1})		
	2007	2008	mean	2007	2008	mean	07/08	2007 *	2008	mean
Control	4.4 a	3.5 a	3.9	23.5 a	33.5 a	28.5	10.2 a	11.7	10.3 ab	11.0
N60-late	5.7 b	4.4 b	5.1	29.7 ab	43.8 bc	36.7	12.0 b	9.8	8.2 a	9.0
N60	5.9 b	4.7 bc	5.3	28.5 ab	39.7 ab	34.1	11.2 b	8.8	8.9 a	8.9
N100-late	6.3 bc	5.2 cd	5.7	34.0 b	49.3 cd	41.7	13.0 c	11.2	9.1 a	10.2
N100	6.8 cd	5.3 cd	6.1	34.7 bc	45.2 bcd	39.9	12.5 c	8.6	9.9 ab	9.2
N140-late	6.9 cd	5.5 de	6.2	37.3 bcd	49.5 cd	43.4	14.1 e	11.5	15.4 b	13.5
N140	7.5 e	6.1 ef	6.8	37.2 bc	47.0 bcd	42.1	13.1 d	12.1	11.8 ab	12.0
N180-late	7.2 de	5.8 def	6.5	46.5 d	54.2 d	50.3	15.2 g	13.7	15.2 b	12.4
N180	7.7 e	6.2 f	6.9	43.7 cd	47.2 bcd	45.4	14.2 f	12.3	17.8 b	13.0
Year (mean)	6.5 b	5.2 a		35 a	45.5 b		n.s.	10.6	11.4	

Organic

Treatment	GY (t ha^{-1})	SV (mL)			CP (%)			Free Asn (mg 100 g^{-1})		
	07/08	2007	2008	mean	2007	2008	mean	2007	2008	mean
Control	4.2 ab	28.5	40.2	34.3 a	9.1	11.0	10.1 a	9.8	7.2	8.5
S50	4.5 bc	31.3	41.2	36.3 ab	10.2	11.4	10.8 ab	10.0	7.4	8.7
S100	5.1 d	36.0	43.0	39.5 bc	10.7	12.0	11.4 bc	10.8	8.7	9.8
S50-H50	4.6 bcd	32.3	43.5	37.9 abc	10.5	11.8	11.1 bc	12.4	7.3	9.8
S100-H20	4.9 cd	35.3	43.5	39.4 bc	11.1	12.3	11.7 c	10.4	7.5	9.0
H60	4.2 ab	31.2	44.8	38.0 abc	10.1	12.0	11.1 bc	11.2	6.4	8.8
H120	4.2 ab	34.8	47.3	41.1 c	10.7	12.2	11.5 bc	11.1	6.8	9.0
H180	3.9 a	42.5	51.7	47.1 d	12.6	13.4	13.0 d	11.8	8.2	10.0
Year (mean)	n.s.	34.0 a	44.4 b		10.6 a	12.0 b		10.9 b	7.5 a	

Years are shown separately if the year or the interaction of year-treatment was significant. However, for the treatment letters only refer to the single year ($\alpha = 0.05\%$, Tukey test). For the year different letters assign significant differences ($\alpha = 0.05\%$, Tukey test). Where no letter appears, no significant differences were found or the interaction was significant but within the single year the treatment was not significant. For crude protein (within the conventional trial) and grain yield (within the organic trial), only the treatment was significant; therefore, only means of both years separated by treatment are given.

In order to ensure normal distribution and equality of variances, the data was transformed where necessary. Means were analyzed for statistically significant differences employing the Tukey range test. As a level of significance, $\alpha = 0.05$ was chosen.

3. Results and Discussion

3.1. Conventional and Organic N Trials

Grain yield and sedimentation value of conventionally produced cultivar Bussard were significantly influenced by the main effects treatment and year, but the interaction was not significant. Only N treatment affected crude protein content significantly, while free Asn showed significant differences concerning treatment and treatment-year interaction.

Yields were higher in 2007 than in 2008 with an average yield across all N treatments of 6.5 t ha^{-1} in 2007 and 5.2 t ha^{-1} in 2008. Yields increased with the applied amount of N and were highest with 6.8 and 7.0 t ha^{-1} across both years in treatments fertilized with 140 and 180 kg N ha^{-1}, respectively. Nevertheless, the maximum grain yield was reached at an N supply of 140 kg N ha^{-1}: A further increase in N supply did not lead to a further significant increase in grain yield. The treatments with an emphasized late application rate of N showed slightly reduced grain yields compared to their respective counterparts (Table 4). Regarding the influence of total N fertilization independent of their distribution, increases in total N fertilization generally increase grain yield, unless fertilization exceeds a certain maximum [33]. In both trial years, the grain yield results confirmed this assumption. The baking quality increased with increasing N input. The treatment 180 kg N ha^{-1} with an emphasized late application rate led to the highest crude protein content of 15.2%, which was 5% higher than in the unfertilized control. Similar to the crude protein content, the protein quality assessed by the sedimentation test also increased with increasing N supply and was highest in treatment 180-late with a mean value of 50 mL over both years. The sedimentation values can partially be influenced by the amount and a late N fertilization [34,35]. This was also confirmed in this study, as sedimentation values increased with increasing N contents in the grain. The highest sedimentation values were reached by the highest N supply independent of the cropping system. Free Asn in the flour of cultivar Bussard was less influenced by N supply in 2007 under conventional farming, as the treatments with intensive N application did not show significantly higher Asn values compared to the unfertilized control (about 11.7 mg 100 g^{-1} flour-DM). However, in 2008, free Asn contents of cultivar Bussard were significantly different when comparing N60-late, N60, and N100-late with N140-late, N180, and N180-late (Table 4). The highest free Asn contents of 17.8 mg 100 g^{-1} flour-DM were found when N amounts of 180 kg ha^{-1} were applied in 2008 without a late application rate. Determined values were about 43% higher than the free Asn value of the unfertilized control. Furthermore, the temporal distribution of N fertilization had no significant effect on free Asn levels. Results from Woolfolk et al. [36] stated, that a late foliar application after flowering increased the total N content. They concluded that a late N supply, before or shortly after flowering may significantly enhance grain N content and finally the crude protein amount in winter wheat. Winkler and Schön [37] found an increase of free Asn with increasing grain N concentration in barley. According to those studies, late N fertilization treatments may have led to an increased level of both crude protein and free Asn. However, only a significant increase of crude protein by late fertilization was found; therefore, it is assumed that synthesis of free Asn is genetically determined, and differences between cultivars will occur.

Under organic conditions, grain yield was only significantly influenced by treatment but not by year or treatment-year interaction. Hence, grain yield of cultivar Bussard is displayed combining years 2007 and 2008. Sedimentation value and crude protein content were both significantly influenced by the treatment and year but not by treatment-year interaction. In contrast to the conventional trial, under organic farming conditions free Asn content was only affected by year, but not by N treatment.

The highest grain yields (5.1 t ha^{-1}) were achieved when slurry was applied with amounts of 100 kg N ha^{-1}. The achieved grain yields were about 20% higher than the unfertilized control.

The application of horn meal solely; however, did not increase grain yield significantly. This suggests that the mineralization of horn meal was slow, leading to late N availability. The high sedimentation value and crude protein content also indicated a late N availability.

Sedimentation value and crude protein content were lower in 2007 than in 2008 (34 compared to 44 units and 10.6% compared to 12.0%). Sedimentation values of the treatments S50, S50-H50 and H60 did not significantly differ from the control treatment while the treatments S100, S100-H20, H120, and H180 increased the sedimentation value significantly compared to the unfertilized control. The highest sedimentation value of 47 units was found when 180 kg horn meal ha^{-1} was applied. Compared to the unfertilized control, crude protein content increased significantly by about 1 to 2.9% if cv. Bussard was fertilized with N except for the application of 50 kg N ha^{-1} given as slurry. An amount of 180 kg N ha^{-1} horn meal led to the highest crude protein content of 13%, which was about 23% higher than the unfertilized control. For flour of organic origin, a lower baking quality is accepted. To reach a good baking quality, Brunner [38] recommends a sedimentation value of 34 units and a crude protein content of 11.6%. Regarding our results, all treatments exceeded the suggested sedimentation value, while only treatments S100-H20 and H180 achieved the values for crude protein. However, a clear year effect was observed in this study as previously found by other authors for durum wheat [39,40]. Specifically, in this study, significantly lower sedimentation values and crude protein values in 2007 compared to 2008 may be attributed to a higher N leaching-caused by the higher rainfall amount from May to July in 2007 (about 370 vs. 260 mm).

If organically produced bakery goods are demanded, lower yields and lower baking qualities must be accepted [41]. Bread bakery processing has to be adjusted to the lower protein contents of such flour [42] to achieve acceptable products.

Free Asn contents were higher in 2007 (11%) than in 2008 (7.5%) and tended to increase with increasing amounts of N from 8.5 to 10 mg 100 g^{-1}, however, no statistically significant difference could be found.

When comparing the free Asn contents of the three winter wheat cultivars dependent on N supply (unfertilized control vs. 180 kg N ha^{-1}), year, N treatment, cultivar, and the interaction year-nitrogen was significant under conventional farming, while N treatment, cultivar, and the interaction year-cultivar were significant under organic farming (Table 5).

Table 5. F-values and *p*-values of -free Asn separated by cropping system for the main effects year, nitrogen, and winter wheat cultivar as well as forinteractions between main effects, df = degree of freedom.

		Free Asn			
Effect		**Conventional**		**Organic**	
	df	***f*-Value**	***p*** [1]	***f*-Value**	***p***
Year (Y)	1	4.69	*	2.92	n.s.
Nitrogen (N)	1	11.46	**	17.11	***
Cultivar (C)	2	20.25	***	36.24	***
C × N	2	0.81	n.s.	2.29	n.s.
Y × N	1	5.49	*	0.00	n.s.
Y × C	2	0.03	n.s.	4.25	*
Y × C × N	2	0.74	n.s.	1.72	n.s.

[1] level of confidence ($p < 0.05$ *, 0.01 **, 0.001 ***, n.s., not significant).

The three conventionally cropped winter wheat cultivars differed in their capacity to store free Asn in the flour, with Capo showing the lowest value of 6.8 mg 100 g^{-1}, followed by Bussard with 10.3 mg 100 g^{-1}, across years and N treatments (Table 6). Cultivar Naturastar reached the highest level of free Asn (17.42 mg 100 g^{-1}). Also, the application of organic N increased free Asn contents in flour when averaged across years and cultivars. Cultivars differed in the same ascending order under organic conditions as under conventional conditions, with Capo having a free Asn content of

6.5 mg 100 g^{-1}, Bussard 7.2 mg 100 g^{-1}, and Naturastar 11.3 mg 100 g^{-1} flour-DM averaged across years and N treatments. Though Bussard and Naturastar had a slight trend to produce less free Asn in 2008 compared to 2007 if organically grown and under N supply, Capo had a slightly higher free Asn content of about 2.5 mg 100 g^{-1} in 2007 compared to 2008 if N was applied. In contrast, if cultivars grow under conventional farming conditions all three cultivars had in 2008 a higher level of free Asn. Thus, besides the year the cropping system seems to effect free Asn formation.

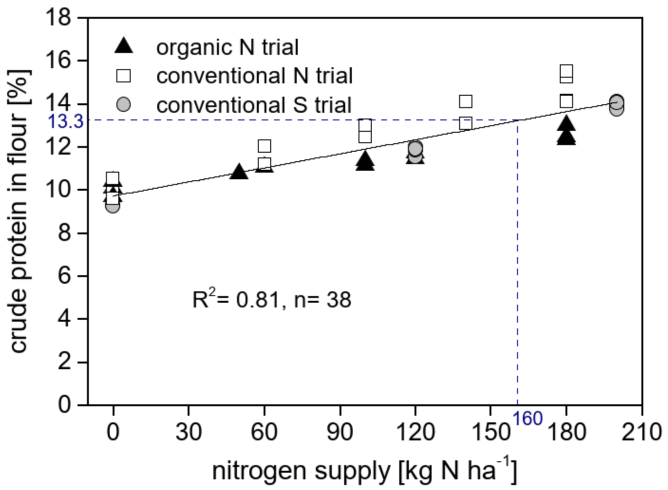
Table 6. Free Asn content of conventionally and organically grown winter wheat cultivars separated by N treatment and year.

		Conventional			Organic		
		Free Asn (mg 100 g^{-1})			Free Asn (mg 100 g^{-1})		
Wheat Cultivar	**Treatment**	**2007**	**2008**	**07/08**	**2007**	**2008**	**07/08**
Bussard	Control	11.7 ab	10.3 ab	11.0	9.8 a	7.2 a	8.5
	N180	13.7 ab	15.2 bc	14.4	11.8 ab	8.2 a	10.0
Naturastar	Control	13.2 ab	13.3 abc	13.3	11.3 ab	11.4 ab	11.3
	N180	15.9 b	17.4 c	16.6	16.6 b	14.5 b	15.6
Capo	Control	6.8 a	7.6 a	7.2	6.9 a	6.5 a	6.7
	N180	8.8 ab	11.8 abc	10.3	7.3 a	9.8 ab	8.6
Year		11.7 a	12.9 b		10.6	9.6	

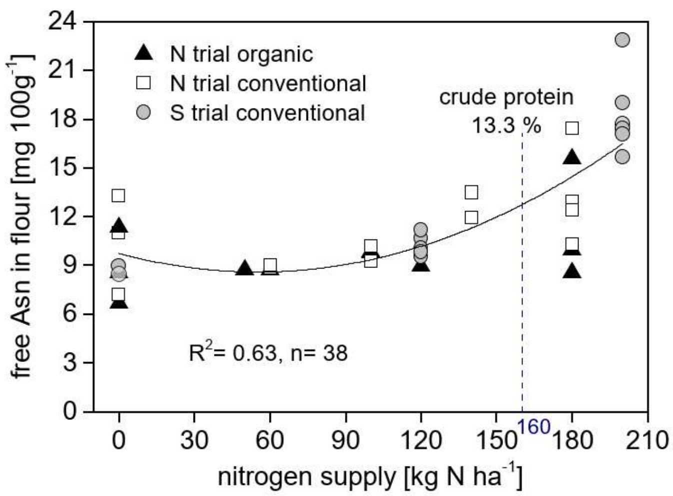
Different letters assign significant differences (α = 0.05%, Tukey test).

Finally, across years, N treatments and cropping systems cultivar Capo was found to exhibit the lowest free Asn level by up to 22% lower amounts when compared to Bussard and 42% when compared to Naturastar. When comparing the same N treatments, significant differences between cultivars were also found by Weber et al. [21]. Stockmann et al. [43] found a reduction potential of free Asn of around 60% for wheat cultivars grown under organic cropping terms. Postles et al. [18] analyzed a significant increase in free Asn by up to 29% if tested rye cultivars were supplied with 200 kg N ha^{-1} compared to 1 kg N ha^{-1}. Nevertheless, they reported, that independent of N supply differences between cultivars in free Asn was not affected by N nutrition. Thus, combining cropping practices like N fertilization and choosing cultivars including a low potential to form free Asn will more effectively reduce free Asn than applying single measurements.

When pooling the means of free Asn values from the three field trials across both experimental years and correlating them with the N supply, a clear trend of increasing free Asn levels with an intensified N supply was obvious (Figure 3). Contrary to a linear effect of increasing N amounts on crude protein content, the effect on free Asn followed a more quadratic function with moderate free Asn levels up to N amounts of 140 kg N ha^{-1}. Amounts of 180 kg N ha^{-1} or higher increased the probability of high free Asn contents considerably, while N supply below that amount led to free Asn values that did not differ considerably from the unfertilized controls. Similar findings were described by Weber et al. [21] investigating one E-wheat cultivar (Enorm). They achieved an increase in free Asn by raising the level of N at different steps. Depending on the year, they found a significantly higher amount of free Asn at a level of 140 kg N ha^{-1}. According to the German Bundessortenamt, high baking quality can be expected from wheat lots (conventionally cropped) with crude protein contents of 13.3% or higher. According to the regression line, this critical crude protein content was met already with N amounts of 160 kg N ha^{-1} in the experimental years. In order not to exceed N supply, farmers are encouraged to carefully choose the amount of N as baking quality will not be affected negatively.



(**a**)



(**b**)

Figure 3. Impact of N supply on crude protein (**a**) and free Asn (**b**) content, separated by trial. The scattered line shows which N supply needed crude protein when E-wheat was reached and how much free Asn was formed.

The overall correlation between crude protein and free Asn was relatively weak (Figure 4), due to the fact that mean values of different cultivars and different trials were pooled. However, it was clear that considerably increased free Asn contents were found primarily if crude protein contents were 14% or higher. Also, a high scattering of free Asn, especially within untreated control without N supply and 180 kg N ha^{-1}, was present. Thus, it has to be considered that environment (=location and year) can affect free Asn levels considerably, as also shown by Curtis et al. [17] for wheat and by Curtis et al. [8] for rye. There is now clear evidence that free Asn accumulates in most, if not all, plant organs during periods of low rates of protein synthesis and a plentiful supply of reduced nitrogen [44]. However, up to now information on how and why soil type, temperature, and precipitation affect grain Asn accumulation is missing. Corol et al. [45] stated that especially during grain development low rainfall and high temperatures increased free Asn amount in grain. This has to be taken into account when interpreting our data, as the climate conditions during 2007 and 2008 could have had an impact.

In addition, a poor relation of crude protein and free Asn was found for both N trials, whereas the conventional S trial showed a good correlation for both traits (R^2 0.71). This means that a higher amount of crude protein may lead to higher levels of free Asn. Corol et al. [45] correlated free Asn with different quality traits of wheat wholemeal, and the closest relation was found for free Asn and protein content ($r = 0.507$). Marschner [46] reported an increase of amides if N fertilization was increased. Similar results concerning soluble N were reported by Gianibelli and Sarandon [47]. Acknowledging that S fertilization had no effect on the level of free Asn, the increase in both crude protein and free Asn was mainly due to the high N treatment of 200 kg N ha^{-1}. Therefore, this high N supply could have led to an accumulation of soluble N, mainly as free Asn.

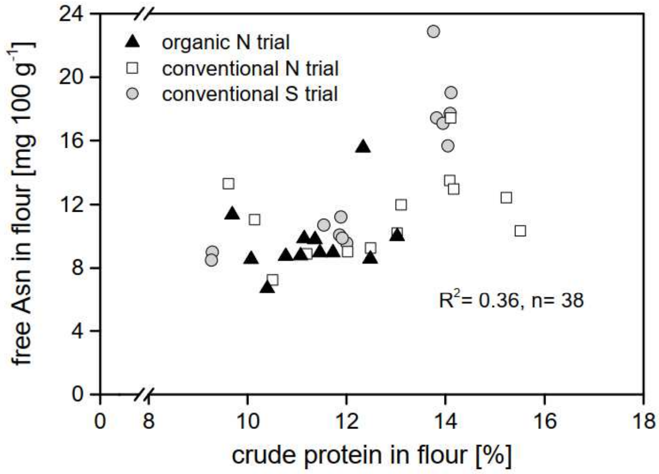


Figure 4. Correlation of crude protein and free Asn of winter wheat, separated by trial.

In addition to environmental conditions and N treatments, the cropping system also had an impact on free Asn (different symbols in Figure 3). Across N treatments and cultivars, organically treated samples (black triangles) showed up to 18% lower free Asn compared to conventional farming. While for single cultivars, a reduction of 23% was possible by choosing organically grown cultivars. This may favour the assumption that the level of free Asn is generally lower in organic farming systems due to a lower N supply. This is in agreement with studies of Stockmann et al. [22], who realized a significant reduction potential of free Asn (up to 30%) if wheat cultivars were grown under organic farming conditions.

3.2. *Conventional S trial*

Grain yield, crude protein, and sedimentation values were significantly influenced by treatment and year, but not by the interaction of both. Free Asn content was significantly influenced by the treatment and year-treatment interaction, but not by year. Since S level of flour samples was only influenced significantly by treatment, it is given as mean of both years.

Independent of S and N treatment, grain yield in 2007 ranged from 4.2 to 7.5 t ha^{-1} and from 4.3 to 7.5 t ha^{-1} in 2008 (Table 7). While N application led to a significant yield increase, S supply did not change grain yield significantly. Similar results were found by Pompa et al. [48] and Rossini et al. [40], where the effect of a foliar S supply was tested and no significant effect on grain yield was found.

Randall et al. [49] and Luo et al. [35] recommended that plants did not suffer from S deficiency if grain S concentration is higher than 0.12% and N/S ratios in grains are below 17:1. All grain S concentrations analyzed in this trial, including the control treatments, exceeded 0.12% (Table 7) and the N/S ratio was below 17:1. Thus, it can be assumed that no S deficiency occurred. In addition, Dai et al. [50] reported that the time of S availability is important as sufficient S supply, especially during grain filling, will invert high levels of free Asn. It can be assumed that in our trial soil S availability during grain filling was sufficient.

Crude protein contents ranged from 9.6% to 14.6% in 2007 while in 2008 it was significantly lower, ranging from 8.5% to 13.9% (Table 7). N fertilization levels of 200 kg ha^{-1} resulted in significantly higher crude protein contents of around 14%. Comparing type and amount of S fertilization within the same N amount applied, only a significant impact on crude protein was found for K20-N2 and elS-N2. The S content of flour samples varied from 0.13% to 0.19% across years (Table 7). All treatments except treatment KEp26-N1 produced significantly higher S contents than both control treatments. Consistent effects were found neither for the type nor for the amount of S supply.

The analysis of flour concerning free Asn showed means varying from 6.9 to 21.9 mg 100 g^{-1} in 2007 while in 2008 means ranged from 9.3 to 23.8 mg 100 g^{-1} (Table 7). The S supply had no influence on free Asn et al.

Weber et al. [51] investigated the effect of S fertilizer kieserite and an additional N supply of 180 kg ha^{-1} and found similar results. They concluded that an additional S application for lowering free Asn is not constructive if the S amount within the soil is sufficient. Nevertheless, if soils are poor in S, free Asn level can increase dramatically. This was revealed by Muttucumaru et al. [25], who showed that free Asn content in wheat grain increased up to 30 times under S deficiency. Similar results were reported by Granvogl et al. [24] from a greenhouse experiment with a summer wheat cultivar. They found a high increase of free Asn in flour of S poor wheat, which finally revealed the AA formation strongly. However, it might be a cereal species influenced output as Postles et al. [18] reported that there was no effect of S increasing free Asn in grain samples of five rye cultivars. Thus, it seems that S fertilization is more linked to protein-rich cereal species above all wheat, where S is needed to form storage proteins, not accumulating free Asn. Köhler et al. [52] and Shewry et al. [26] postulated that storage protein composition changes if S availability is limited, due to a limited formation of S rich protein fractions. They concluded that this leads to an increase of protein fractions low in S and boosts the amount of N structures, e.g., aspartic acid and free Asn. Besides those results, the working group of Postles et al. [18] also stated that S supply could minimize the effect of high N availability on free Asn formation. They found two cultivars which showed a reduced level of free Asn if high N was available and S was applied. Curties et al. [53] reported that in case of S supply the level of free Asn was less influenced by year and the cultivar was more stable in producing free Asn amounts. In our study, similar effects of S on free Asn formation were not found. There was no clear reduction effect of S comparing N1 with 100 kg N ha^{-1} and N2 with 200 kg N ha^{-1} within the single S treatments. However, one needs to keep in mind that, in our study, N was not applied without S. Maybe a treatment of N1 (100 kg N ha^{-1}) and N2 (200 kg N ha^{-1}) without an S application could have revealed other results. Nevertheless, most studies concerning the effect of S on free Asn formation were carried out as pot trials, including soils poor in S as well as field trials where S was deficient [24,25].

Table 7. Grain yield and grain quality traits of winter wheat cultivar Enorm of the S trial dependent on N and S fertilization and year.

	GY (dt ha^{-1})			CP (%)			SV (mL)			S (%)	Free Asn (mg 100 g^{-1})		
Treatment	**2007**	**2008**	**07/08**	**2007**	**2008**	**07/08**	**2007**	**2008**	**07/08**	**07/08**	**2007**	**2008**	**07/08**
Control	4.2	4.7	4.5 a	9.8	8.5	9.3 a	28.3	27.3	27.8 a	0.137 ab	8.6 ab	9.4 a	9.0
Control-S	4.3	4.3	4.3 a	9.6	8.8	9.2 a	28.2	28.7	28.5 a	0.133 a	6.9 a	10.1 a	8.5
K20-N1	6.7	7.1	6.9 b	12.0	11.4	11.7 b	37.3	41.0	39.2 b	0.155 cd	9.5 b	11.9 abc	10.7
K20-N2	7.1	7.2	7.2 cde	14.6	13.9	14.3 d	44.8	50.2	47.5 e	0.174 cde	21.2 c	16.8 d	19.0
K40-N1	6.7	7.0	6.8 b	12.0	11.6	11.8 b	38.2	39.3	38.8 bc	0.163 cd	10.6 b	9.5 a	10.1
K40-N2	7.2	7.1	7.2 cd	14.3	13.8	14.1 cd	45,2	49.3	47.3 e	0.175 de	16.6 c	18.9 de	17.8
K60-N2	7.2	7.4	7.3 def	14.5	13.6	14.1 cd	43.5	48.5	46.0 de	0.168 cde	16.8 c	14.5 cd	15.7
Ep-N1	6.7	7.0	6.9 b	12.4	11.5	12.0 b	38.7	40.2	39.5 bc	0.160 cd	9.8 b	9.3 a	9.6
Ep-N2	7.3	7.5	7.4 def	14.2	13.5	13.9 c	43.0	47.2	45.1 de	0.169 cde	20.3 c	14.6 cd	17.5
KEp26-N1	6.8	7.1	7.0 bc	12.1	11.7	11.9 b	39.5	40.2	39.9 c	0.153 bc	10.2 b	12.2 abc	11.2
KEp26-N2	7.5	7.5	7.5 f	14.5	13.6	14.1 cd	42.7	45.3	44 d	0.172 cde	20.2 c	13.9 bdc	17.1
elS N1	6.6	7.2	6.9 b	12.1	11.5	11.8 b	39.0	41.0	40 bc	0.190 e	9.4 b	10.4 ab	9.9
elS N2	7.3	7.5	7.3 ef	14.2	13.5	13.9 c	44.3	46.5	45.4 de	0.175 de	21.9 c	23.8 e	22.9
Year (mean)	6.6 a	6.8 b		12.8 b	12.1 a		39.4 a	41.9 b		n.s.	14.0	13.5	

Different letters within analyzed trait and year displays significant differences (Tukey test, α = 0.05). Letters only appear where the main effects or interactions were significant.

4. Conclusions

The scope of this paper was to examine the impact of N and S supply in organic and conventional wheat cropping systems with regard to their potential for AA minimization. Grain and flour samples from three different field trials, which had been carried out for two consecutive growing seasons, were analyzed. In addition to AA, free Asn, grain yield, and grain quality, with a focus on baking quality, were determined. The results of this study strongly suggest that crop- and agronomy-based studies could provide a significant contribution in reducing the levels of acrylamide in processed foods by lowering the relevant precursors in the raw material. N fertilization, significantly influenced grain yield, and baking quality in both cropping systems. Particularly within organic farming, an increased N treatment did not enhance free Asn, but baking quality could be influenced positively. The late N fertilization step within the conventional N trial significantly increased crude protein content, while for free Asn no clear effect was found. Furthermore, neither type nor amount of S fertilization influenced free Asn significantly. That suggests that on soils, which are not deficient in S, an additional S supply will not affect free Asn formation.

For free Asn, a clear impact of cultivars was shown. Capo was found to exhibit the lowest AA formation potential over the treatments 0 and 180 kg N ha^{-1}. Interestingly, this cultivar reached a high crude protein (15% if conventionally cropped and 12.5% if organically cropped) at an N supply of 180 kg N ha^{-1}, but at the same time the lowest level of free Asn. This leads to the assumption that cultivars differ in their genetic potential to form free Asn under increased N supply. Thus, concerning new wheat cultivars, the potential of forming low free Asn amounts accompanied by a good baking quality should be part of breeding programs. Overall, determination of the factors and mechanisms that influence free Asn accumulation may ultimately be manipulated to give safer food products to consumers. Therefore, acrylamide in food is an agronomic as well as a food science issue, and agronomists, breeders, and farmers must be engaged in addressing it.

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2.4 Paper 4: "Impact of row distance and seed density on grain yield, quality traits and free asparagine of organically grown wheat."

Stockmann, F.; Weber, E.A.; Merkt, N.; Schreiter, P.; Claupein, W.; Graeff-Hönninger, S. **Impact of Row Distance and Seed Density on Grain Yield, Quality Traits, and Free Asparagine of Organically Grown Wheat**. *Agronomy* **2019**, 9, 713.

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Based on the results of paper 3, in particular the results of the organically treated varieties, the question arose whether special management applications used in organic farming could influence free Asn and quality traits as well. This was investigated in paper 4. Using a larger space between plant rows is a known practice in organic cropping systems. More space serves two aims, first a better weed handling and second a better nutrient supply to the plant especially with nitrogen. Seed density can also have an effect on nutrient availability, as it delivers more space to single plants, therefore different seed densities were tested, too.

The results indicated that the expansion of row distances can increase quality traits in the case of protein and sedimentation value. Seed density was highly related to grain yield and test weight. Most important, free Asn, which is related to AA formation potential, was only affected to a minor extend by both treatments. Thus, larger row distances can be recommended in order to raise baking quality in organic farming systems without simultaneously affecting free Asn.
A high relation between the number of grains per spike and free Asn was found (R^2=0.72). This opens new insights into Asn synthesis during grain development and offers the opportunity to predict free Asn formation without expensive and time-consuming chemical analyzes.

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Article

Impact of Row Distance and Seed Density on Grain Yield, Quality Traits, and Free Asparagine of Organically Grown Wheat

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Abstract: Organic farming faces challenges providing sufficient nutrient supply as manure and crop rotations are often the major nutrient inputs. Larger row distances and fewer seed densities can support nitrogen availability by giving more space to the single plant. As free asparagine (Asn) the main precursor of acrylamide (AA) in plants is closely related to nitrogen uptake and storage, the question arose whether free Asn will be affected by row distance and seed densities in organic farming. This study investigated the effect of row distance and seed density on yield, yield components, baking quality, and free Asn in organic farming. A two-year field trial was carried out including two winter wheat cultivars, two row distances, and two seed densities. Year and cultivar highly influenced all traits. The impact of both treatments was mainly caused by interaction. Nevertheless, enlarged row distances raised baking quality, while free Asn was changed to a minor extent. Thus, we recommend larger row distances for raising baking quality without increasing free Asn. Seed density is of minor relevance. The close relation found between free Asn and grains per spike (R^2 = 0.72) indicates that smaller grains contain more Asn than bigger grains. This opens new insights into Asn synthesis during grain development and offers a potential prediction of Asn amounts.

Keywords: acrylamide; free asparagine; organic farming; yield; grain size; quality; food safety; row distance; seed density; cereals

1. Introduction

Securing of food quality is currently a major task for the scientific community. In this context, ensuring the absence of harmful substances in foods that can cause cancer is of high relevance. Until the year 2000, the food born toxicant Acrylamide (AA) was not known to be present in food products. That changed when Tareke et al. (2002), stated that carbohydrate rich food products contain AA [1]. Now nearly two decades after the first discoveries of AA in food, the European Commission [2] announced a regulation which restricts AA contents in cereal food products and forces the implementation of harm minimization strategies if benchmark levels are exceeded. Since that announcement, the food industry has faced the major challenge of reducing the risk of AA appearing in their food products.

AA is formed during the Maillard reaction in carbohydrate rich material like cereals and potatoes where free asparagine (free Asn) and reducing sugars react under heat treatment [3–5]. Up to now, the lowering of AA was mainly achieved by reducing the process temperature and heating time. Further studies investigated the effect of adjusting processing parameters such as pH, changing baking agents, adding additives, or by elucidating the mechanistic pathways of AA formation and eliminating precursors or intermediates [6–14].

Also, enzymes like asparaginase are able to reduce free Asn the AA formation that would occur as a result of its reaction with sugars, however the production of enzymes can be expensive and leads to undesired changes in taste and product quality [15]. Moreover, the efficiency of enzymes is affected by the enzyme dose, reaction time, temperature, and pH [16], meaning that efficiency of the enzyme asparaginase used to reduce free Asn depends on the food that is produced.

For all foods where free Asn is the factor that limits the formation of AA [7], all processing strategies aim to prevent the formation of AA by changing or reducing free Asn shortly before or during food processing.

While most of the above-mentioned strategies successfully lead to a lower AA formation, their ability to lower AA depends on the raw material used. Depending on crop management and environmental conditions, the amount of free Asn in foods like cereal flours can highly fluctuate from year to year, which means lowering AA using technological and food processing strategies can fail.

As the amount of free Asn in plants is affected by nitrogen, free Asn might be influenced by crop management strategies like nutrient supply, fungicide treatment, and the choice of cereal species and cultivars [17–20]. In crop production, fertilization is a central strategy to raise grain yield and quality traits, but it can affect Asn levels as well. In addition to the nitrogen application content, the timing of nitrogen fertilization and different kinds of nitrogen fertilizer can considerably affect Asn amounts in wheat [17,19,20]. Especially high nitrogen supply during grain maturation which heads to high crude protein concentrations seems to increase free Asn levels significantly [17]. Postles et al. [21] found for rye that free Asn was influenced by variety and nitrogen supply. Moreover, sulfur deficiency can dramatically increase Asn accumulation in grain, leading to high AA formation potential [18,22]. Furthermore, fungicide treatments increasing green leaf area periods and prolonging senescence can downsize free Asn content in grains [19]. Further, environmental conditions given by different growing locations can highly change the level of free Asn within wheat genotypes [23]. Lea et al. [24] reported in their review that stress conditions during growth like drought, salt, toxic metals, and disease pressure can also lead to an increase in free Asn. However, reference [25] suggested that some climate conditions that may induce stress conditions like elevated ozone do not seem increase free Asn, although interestingly crude protein content does increase as a result of these conditions.

Further, different investigations revealed that cereal species vary in their Asn levels and as a result in their AA formation potential. Rye normally provides higher Asn amounts in the grain than wheat and spelt [26–28]. Additionally, cultivars within cereal species can differ highly in their precursor content of free Asn, which was reported by numerous studies [21,26–29]. A 5-fold range of free Asn within a diversity of European wheat cultivars was reported by Taeymans et al. [29] while Claus et al. [26] found up to a factor of three diverse free Asn levels in nine German winter wheat cultivars. Corol et al. [23] reported almost 5-fold differences between 150 wheat genotypes analyzed as wholemeal samples. Thus, identifying cultivars providing naturally a low Asn content is considered to be a reasonable approach to minimize AA formation potential. Nevertheless, climate conditions (sunshine duration and rainfall) and soil properties can also significantly alter Asn contents [27,28], which should be taken into account during cultivar selection.

The studies mentioned above exclusively completed their research using conventional farming methods. Studies investigating free Asn in cereals cultivated under the guidelines of organic farming systems are rare. Kunz [30] announced that for breeding wheat cultivars under organic farming conditions, plants must have completely different characteristics than under conventional practices, e.g., a higher accumulation of gluten under a lower nitrogen supply, longer stems, a long terminal internode, loose ears or a faster transfer of nutrients into the grain, more weed competitiveness, and compatibility with harrowing and hoeing. Thus, the results of studies done for conventional farming cannot simply be transferred to organically cultivated crops.

In this context, Stockmann et al. [31] investigated organically and conventionally cropped cereals to determine their content of free Asn. The used species and cultivars were the same for both systems, while only the crop management differed. They found a high impact of the cropping system for wheat in particular, as the organically grown wheat cultivars had a significantly lower level of free Asn.

In addition, Stockmann et al. [20] examined the effect of nitrogen on free Asn formation by comparing conventional cropping methods with organic ones. They found that the wheat samples produced under organic farming conditions showed no significant increase in free Asn if nitrogen levels were raised. Significantly higher levels of free Asn were only found within the conventionally treated wheat samples when nitrogen amounts of 180 kg N ha^{-1} or higher were applied, which led to crude protein contents over 14%. They concluded that until a certain level of nitrogen was reached which included a sufficient protein synthesis, free Asn would not be significantly affected. This is in agreement with Lea et al. [24], who stated that large amounts of nitrogen during a phase of low protein synthesis will increase free Asn.

In contrast, a set of organically cropped cereal species and cultivars were investigated by Stockmann et al. [32] for their content of free Asn. The samples were only marginally supplied with nitrogen, however a high range of free Asn comparing species and cultivars within species were reported. Thus, only reducing nitrogen could lead to failure to reduce the levels of free Asn. Particularly if a sufficient baking quality is needed, nitrogen supply should be adequate to help obtain marketable flours. In this context, baking properties are highly related to crude protein (gluten content), the sedimentation value, and falling number since these traits affect the dough preparation and bread volume [33].

However, nitrogen supply in low input farming systems is generally lower. Hence, strategies are needed to ensure there is a certain amount of crude protein to obtain a good baking quality.

Regarding organic farming, growing wheat in a larger row distance is a known agronomic strategy. In addition to providing better weed control, the main reason for this agronomic management tool is better nitrogen availability for each single plant. Thus, larger row distances can lead to a better baking quality in terms of quality traits like crude protein and the sedimentation value [34].

In addition, lowering the seed density could also support the effect of a better nutrient supply of the single cereal plant, as different plant densities per unit may change plant architecture in terms of the number of spikes per m^2 and grains per spike.

As free Asn is closely related to nitrogen uptake, storage and transport within plants [24,35], the question arose whether the level of free Asn and finally AA formation would be affected by a larger row distance and a lower seed density.

As such, a two-year field trial was established to investigate (i) the impact of row distance and seed density on yield, quality aspects and free Asn of two winter wheat cultivars, and (ii) the relation between the grain number per spike, crude protein, free Asn, and AA formation.

2. Materials and Methods

2.1. Experimental Site

The field trial was carried out over two consecutive growing seasons (2006–2007; 2007–2008) at the experimental station for organic farming of the University of Hohenheim, Kleinhohenheim, Stuttgart (48°44′ N 9°12′ E; average annual temperature 8.8 °C; average annual rainfall 700 mm).

The research station is located 435 m above sea level in the southern peripheral part of Stuttgart, Germany.

Detailed data on temperature and rainfall during the seasons 2006–2007 and 2007–2008 are depicted in Figure 1.

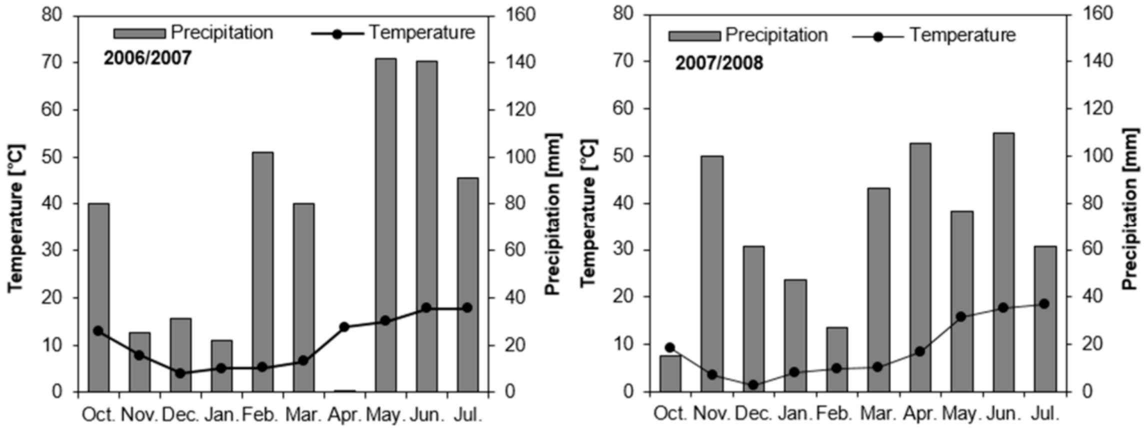

Figure 1. Air temperature (●) and precipitation (bars) at the organic trial site Kleinhohenheim for the growing seasons 2006/2007 and 2007/2008.

The soil at the trial site in Kleinhohenheim falls under the Luvisol type. It is characterized by a nearly 2 m thick horizon of loess to loamy clay. Therefore, it features a high-water holding capacity and is well suited for agricultural purposes. In spring 2007, mineral N content was 35 kg ha^{-1} within a soil horizon of 0 to 60 cm compared to 62 kg ha^{-1} in 2008. In Table 1, the main results of the soil chemical analysis are presented.

Table 1. Main characteristics of the soil chemical analysis across both growing seasons.

Humus Content (%)	Soil Acidity (pH)	P_2O_5 (mg 100 g^{-1})	K_2O (mg 100 g^{-1})	Mg (mg 100 g^{-1})
2.34	6.8	21.0	18.5	11.0

2.2. Experimental Design

The field trial was set up as a randomized block design with three repetitions (plot size 4 × 6 m). As the trial was established according to the standards of organic farming (e.g., no artificial fertilizer, no pesticides). The previous crop in both years was winter wheat, while a 2-yr wheat clover grass mixture was grown in the years before.

Two different winter wheat cultivars (cv. Bussard and cv. Naturastar), two row distances, and two seed densities were tested. The tested treatments are shown in Table 2.

Table 2. Applied cultivars, row distances, and seed densities in the field trials.

Cultivar	Row Distance (cm)	Seed Density (Grains m^{-2})
Bussard (E-wheat *)	R1 = 12.5	A1 = 350
	R1 = 12.5	A2 = 250
	R2 = 30.0	A1 = 350
	R2 = 30.0	A2 = 250
Naturastar (A-wheat *)	R1 = 12.5	A1 = 350
	R1 = 12.5	A2 = 250
	R2 = 30.0	A1 = 350
	R2 = 30.0	A2 = 250

* refers to the German quality classes. E-wheat: highest baking quality, A-wheat: high baking quality.

2.3. *Agronomic Practices*

Primary tillage was done in both years with a moldboard plough (25 cm depth). Seed bed preparation was accomplished using a power harrow.

Sowing was done on 19 October 2006 and 24 October 2007. In total 100 kg N ha^{-1} were applied as liquid cattle manure (100 m^3 ha^{-1}: 1 kg N m^{-3} total nitrogen content, 4% dry matter) which was split into two rates of 50 m^3 ha^{-1} at the start of vegetation and at the start of stem elongation.

No pesticides and no growth regulators were applied. If necessary, weeds were treated by a currycomb. Infestation of diseases was monitored, but the outcome showed no significant infestation.

Harvest was accomplished by a Hege 180 plot combine harvester (Hege, Eging am See, Germany) after grains had reached a dry matter content of 85%.

2.4. *Analyses of Yield and Yield Components*

2.4.1. Yield

Grain yields was determined by weighing the plot yield. Grain samples were dried at 105 °C for 24 h to determine grain moisture. Grain yields given refer to 86% dry matter content.

2.4.2. Thousand Kernel Weight

Thousand kernel weight (TKW) was determined by counting 1000 grains, which were dried to absolute dry matter content by a Contador® seed counter (Pfeuffer GmbH, Kitzingen, Germany).

2.4.3. Test Weight

The test weight was determined by a cereal sampler (Pfeuffer GmbH, Kitzingen, Germany) after drying grain samples to absolute dry matter content by using a grains volume of ¼ L.

2.5. *Grain Quality Analyses*

2.5.1. Flours

For the determination of quality parameters, the determination of the AA precursor content free Asn, and the AA formation potential, grain samples were milled on a laboratory mill (Quadrumat Junior, Brabender, Duisburg, Germany). Ash content of flours was about 0.5% of flour DM. Flour moisture was calculated from the weight loss before and after drying of about 5 g flour at 105 °C for 24 h.

2.5.2. Crude Protein Content

Total grain nitrogen content was determined by Near-Infrared-Spectroscopy (NIRS, NIRS 5000, FOSS GmbH, Rellingen, Germany). Calibration samples were analyzed according to the Dumas Method [36] using a Vario Max CNS analyzer (Elementar, Hanau, Germany). The analyzed final nitrogen content was multiplied by a factor of 5.7 [37] for the wheat samples.

2.5.3. Hagberg Falling Number

The Hagberg falling number was determined in line with ICC standard No. 107 using a PerCon 1600 Falling Number machine (PerCon, Hamburg, Germany) and 7 g of flour (weight adjusted for moisture concentration to 15%).

2.5.4. Zeleny's Sedimentation Test

Zeleny's sedimentation test was determined in wheat flours using 3.2 g flour according to ICC standard No. 116. The sedimentation values of the flours were adjusted to a 14% moisture level.

2.5.5. Free Asparagin

For free amino acids, extraction 2 g of wheat flour were mixed with 8 mL of 45% ethanol for 30 min at room temperature. After centrifugation for 10 min at room temperature with 4000 rpm and 10 min at 10 °C and 14,000 rpm, the supernatant was filtered through a 0.2 µm syringe filter and poured into vials. Analysis of free Asn was performed using Merck–Hitachi HPLC components. The pre column derivatization with FMOC [38] was completely automated by means of an injector program. Subsequently, the derivatized Asn was separated on a LiChroCART Superspher RP 8 column (250 mm × 4 mm, Fa. Merck, Darmstadt, Germany) at a constant temperature of 45 °C. The fluorescence intensity of the effluent was measured at the excitation and emission maxima of 263 and 313 nm.

2.5.6. Acrylamide Formation Potential

The AA formation potential of wheat flour was assessed according to the AA contents of 5 g white flour in 250 mL Erlenmeyer flasks after heating in an oven for 10 min at 200 °C. Due to the complexity of the AA analysis, sample size was reduced to an overall number of 16 samples.

Sample preparation was accomplished according to the test procedure 200L05401 [39] of the Chemische und Veterinäruntersuchungsamt (CVUA) Stuttgart.

After cooling the heated flour samples down to ambient temperature, 100 mL of bidestilled water and 100 µL of D_3-Acrylamide were added as an internal standard to the heated flour samples in the Erlenmeyer flasks. To completely extract acrylamide from the flour, samples were put in an ultrasonic bath for 10 min at 40 °C. After adding 1 mL of Carrez I and II to each of the samples, and shaking the flasks thoroughly, the samples were filtered using folded filter paper to separate the colloids and flour particles from the aqueous solution. Subsequently, samples were cleaned up by a solid phase extraction in a vacuum chamber after preconditioning the cartridges by 10 mL of bidestilled water and 10 mL methanol. After sample clean-up, around 1 to 2 mL of the eluate from each sample was filled in an autosampler vial and was deep frozen (−18 °C) until AA was determined by LC-MS-MS by the CVUA according to the test procedure 201L01301 [40]. The eluates were separated by a graphite or RP18-phase and detected by tandem-mass-spectrometer. Quantification was undertaken by using the isotope-labeled internal standard (D_3-Acrylamide).

2.6. Statistical Analyses

For each trait listed in the section above, analysis of variance (ANOVA) was performed using the procedure PROC MIXED of the statistical software package SAS 9.2 (SAS Institute Inc., Cary, NC, USA). ANOVA was done for the main effects of year, treatment (row distance, seed density), cultivar, and all interactions. A mixed-linear model approach was used. All effects were taken as fixed.

In order to ensure normal distribution and equality of variances, the data was transformed if necessary. Means were analyzed for statistically significant differences using the Tukey range test. As a level of significance, $\alpha = 0.05$ was chosen. For analyzing the coefficient of determination concerning the grains per spike, crude protein, free Asn, and AA, a linear regression was performed using the software package of Sigmastat 4.0 (Systat Software Inc., Cranes Software, San Jose, CA, USA).

3. Results and Discussion

3.1. Yield and Yield Components

Grain yield was significantly affected by year (Y), seed density (SD), and the interaction cultivar (Cv) × row distance (RD) (Table 3). As the interactions Y × Cv × SD, Cv × SD × RD and Y × Cv × RD × SD were not significant for any tested trait, it was not listed in Table 3.

Table 3. F-values and *p*-values for all main effects and interactions, where at least one tested trait had a significant impact on grain yield [kg ha^{-1}], thousand kernel weight (TKW) and quality parameters: Test weight (TW), falling number (FN), crude protein (CP), sedimentation value (SV), and free Asn of flours.

	Grain Yield		TKW		TW		FN		CP		SV		Free Asn	
	F	*p* *	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*
Year (Y)	61.4	***	28.4	***	239.7	***	14.3	***	4.4	*	138.1	***	30.9	***
Cultivar (Cv)	n.s.		56.7	***	n.s.		147.5	***	n.s.		61.6	***	26.1	**
Row Distance (RD)	n.s.		n.s.		n.s.		n.s.		n.s.		n.s.		n.s.	
Seed Density (SD)	5.9	*	n.s.		5.6	*	n.s.		n.s.		n.s.		n.s.	
Y × Cv	n.s.		4.5	*	8.4	**	n.s.		n.s.		61.6	***	27.1	***
Y × RD	n.s.		n.s.		n.s.		n.s.		12.9	**	12.3	**	n.s.	
Y × SD	n.s.		n.s.		n.s.		n.s.		n.s.		n.s.		n.s.	
Cv × RD	6.4	*	n.s.		n.s.		n.s.		n.s.		13.2	**	25.9	**
Cv × SD	n.s.		n.s.		n.s.		n.s.		n.s.		n.s.		n.s.	
RD × SD	n.s.		n.s.		n.s.		n.s.		n.s.		n.s.		20.4	*
Y × Cv × RD	n.s.		n.s.		n.s.		n.s.		n.s.		7.5	*	n.s.	
Y × SD × RD	n.s.		n.s.		n.s.		n.s.		n.s.		n.s.		18.1	*

* level of confidence ($p < 0.05$ *, 0.01 **, 0.001 ***, n.s. not significant).

Comparing years, in 2007 a grain yield of 3740 kg ha^{-1} was harvested while in 2008 the average was 4350 kg ha^{-1}, around 700 kg ha^{-1} higher. In addition to the year, the higher seed density of 350 grains m^{-2} led to a significantly higher grain yield. The higher seed density resulted in a grain yield of 4190 kg ha^{-1} compared with 4020 kg ha^{-1} when using the smaller seed density. This was likely the result of more spikes m^{-2} as the number of spikes m^{-2} increased by the higher seeding rate (Table 4). Indeed, less spikes m^{-2} could only partially be compensated by an increased number of grains per spike (Table 4). Similar results were observed by Landon 1994 [41] and Arduini et al. [42], who investigated the effect of seeding rate on the grain yield of wheat. Both reported a compensation by either a higher number of grains per spike or a higher kernel weight. Gooding et al. [43], stated in their study that a lower seed density was compensated by a larger level of tillers and grain numbers per ear. In our work the effect of an increased number of tillers or a higher thousand kernel weight was not determined, while the number of grains per spike increased. However, the number of grains per spike could not compensate for the effect of a lower seed density on yield.

Row distance only had a significant impact on grain yield for some of the cultivars (cv). In this context, grain yield was significantly lower for the larger row distance of 30 cm if cv Bussard was grown and differed by around 300 kg ha^{-1}. For cv Naturastar, the row distance had no significant effect. Whereat, a yield increasing tendency was observed by enlarging the row distance but without being significant. The different reactions of both cultivars regarding row distance might be related to the varying structure of spikes per m^2 and grains per spike. As shown in Table 4, cv Bussard responded to the larger row distance with a higher reduction of spikes m^{-2} compared to cv Naturastar (Table 4). Landon et al. [41] developed a different effect of row distance in their study where increasing the row distance led to a higher grain yield due to an increased number of kernels per spike. However, in this study the number of grains per spike only marginally changed in the case of cv Bussard and the spikes per m^2 decreased (Table 4). Thus, the larger row distance was not compensated by an increased number of spikes, nor by more grains per spike of cv Bussard (Table 4).

Table 4. Results of yield components (spikes m^{-2} & grains $spike^{-1}$) of the different treatments (*n* = 24).

Cultivar	Treatments		Results				
	Row Distance	Seed Density	Spikes		Grains		Grain Yield
	(cm)	(Grains m^{-2})	(m^{-2})		($Spike^{-1}$)		(kg ha^{-1})
Bussard (E-wheat)	12.5	350	440	(±2.3)	29	(±2.0)	4230
	12.5	250	373	(±26.8)	29	(±4.4)	4170
	30.0	350	308	(±100.1)	28	(±3.1)	4050
	30.0	250	292	(±30.5)	30	(±3.1)	3760
Naturastar (A-wheat)	12.5	350	390	(±17.5)	30	(±2.6)	4250
	12.5	250	300	(±12.3)	38	(±2.4)	4020
	30.0	350	350	(±57.3)	32	(±0.5)	4240
	30.0	250	244	(±10.3)	38	(±5.2)	4140

The Thousand kernel weight (TKW) was significantly affected by the year, the cultivar, and the interaction of both (Table 3). Neither row distance nor seed density had a significant effect. This is in contrast to a study carried out by Hiltbrunner et al. 2005 [44], who reported an increase in TKW if the row distance was expanded. Nevertheless, the year had a significant effect as TKW was lower in 2008 (38.4 g) than in 2007 (40.5 g). Across years, the TKW of cv Bussard was 40.9 g, which was significantly higher than Naturastar at 38.0 g. This fits well to the monitored level of grains per spike, which were lowest for cv Bussard (Table 4). This leads to the assumption that the grains of cv Bussard were bigger and thus a heavier TKW was reached. As cv Naturastar is known for reaching a high grain yield with a higher level of grains per spike, this leads to the suggestion that grains of this cv were generally smaller, leading to a lower TKW.

For the interaction Y × Cv in 2007, the highest TKW of 42.4 g was observed for cv Bussard, while TKW was lowest (37.4 g) for cv Naturastar in 2008. Finally, TKW was much more affected by the cultivar and year than by row distance or seed density.

Older studies reported that test weight can serve as a marker for flour yield [45]. Newer findings have not supported this statement [46]. However, test weight is still used in some countries as a quick test for grain quality. Higher amounts indicate rounder grains, leading to a better milling behavior and thus a higher flour yield. In contrast, smaller grains can include an uneven shape and thus provide lower test weights. In our study, test weight was significantly influenced by year, seed density, and the interaction Y × Cv (Table 3). In 2007, the test weight was 80.9 kg hL^{-1}, which was significantly higher than for 2008 (78.5 kg hL^{-1}). Cultivar only had a significant effect in interaction with the year. Compared to Bussard (80.6 kg hL^{-1}), cv Naturastar in 2007 reached a much higher amount (81.2 kg hL^{-1}), while in 2008 there was no statistically proofed difference between the two (78.4 and 78.7 kg hL^{-1}, respectively).

Next to the year, the most relevant factor for test weight was seed density. A lower seeding rate (250 grains m^{-2}) led to a significantly lower test weight of 79.5 kg hL^{-1}, while the seeding rate of 350 grains m^{-2} provided a test weight of 79.9 kg hL^{-1}. This can partly be explained by the differences in spikes per m^2 and the grains per spikes. Spikes per m^2 were higher if 350 grains m^{-2} were sown, leading to a lower number of grains per spike (Table 4). This leads to the assumption that grain size was bigger and thus the test weight also increased. This is well guided by the TKW, as cv Bussard with the smaller number of grains per spike reached the highest TKW, which was most likely caused by larger grains. Schuler et al. [47] investigated the impact of seed and spike characteristics on test weight. They reported that number of seeds in spikes and test weight had a negative correlation of r = 0.41. Hence, if the seeds per spike increased, the test weight decreased. This fits well to the results of this study as lowering seed density to 250 grains m^{-2} increased the number of grains per spike, especially for cv Naturastar (Table 4). We assumed that the higher number of grains per spike led to a smaller grain size, which may explain the lower test weight. Finally, the lower seed density led to less spikes per m^2 and this was likely compensated by a higher rate of grains per spike along with smaller grains.

3.2. Baking Quality Traits

Falling number (FN) is a baking quality trait, as it refers to water absorption during dough preparation. Thus, effective preparation of dough requires a sufficient FN. Delayed grain harvest can cause pre-harvest sprouting causing a higher activity of enzymes (amylase). This may lead to a lower FN as consequence of polysaccharides decomposition (amylose and amylopectin) and thus affecting baking quality [48]. FN was significantly influenced only by year and cultivar (Table 3) but was not affected by row distance or by seed density. The mean FN was 244 s (cv Bussard) and 332.5 s (cv Naturastar). Brunner [49], recommended that for organically produced wheat flours, FN should range between 160 and 280 s. They stated that such flours deliver a sufficient baking quality, including a normal, elastic well pored crumb and an adequate gas holding capacity. Thus, referring to reference [49] the FN results revealed in our study indicates no negative effect on baking quality.

Crude protein (CP) is the most widely used method for estimating the baking quality of wheat flour other than gluten content. High levels of CP indicate a good preparation of foods such as biscuits. This trait was significantly influenced by year and the interaction Y × RD (Table 3, Figure 2A). Neither cultivar nor seed density had a relevant impact.

In 2008, CP content was 11.7% which was around 8% higher than in 2007 (10.6%). In general CP ranged from 10.4% to 12.2%. The high impact of the year can be explained by different weather conditions, especially during grain filling periods. In 2008, the temperature during grain filling period (May–July) was 1.4 °C higher than in 2007 (Figure 1). This fits well to the corresponding rainfall, which was around 140 mm higher in the period May–July for 2007 compared to 2008. These weather conditions led to a better CP synthesis during 2008 and thus to higher CP values.

That climate conditions especially during grain development can influence grain composition was reported by Fuhrer et al. [25], Shewry et al. [50], and Ohm et al. [51]. Further et al. [25] reported the effects of ozone on the grain composition. They observed an increased CP level. Shewry et al. [50] analyzed the impact of temperature and water availability during grain growth on grain composition. After the observation of 26 genotypes grown at different locations, they stated that mean temperature and precipitation was either positively or negatively related to phytochemical contents during grain growth, or to water-soluble arabinoxylan fiber in bran and white flour. As locations are closely related to environmental conditions like rainfall and sunshine, Ohm et al. [51] observed a significant impact of locations on SDS unextractable polymeric protein parameters.

Nevertheless, as Brunner [49] and Casagrande [52] announced that a CP content of at least 10.5% is required to match baking industry needs for organic flours, the CP levels accomplished in this trial were sufficient.

Overall, only row distance had a significant effect on CP if the years were separated. While in 2007 no statistical implication was analyzed, in 2008 the larger row distance of 30.0 cm significantly raised the mean CP content by nearly 12% (Figure 2A). This was around 5% more than for the smaller row distance, which reached a CP content of 11.4%.

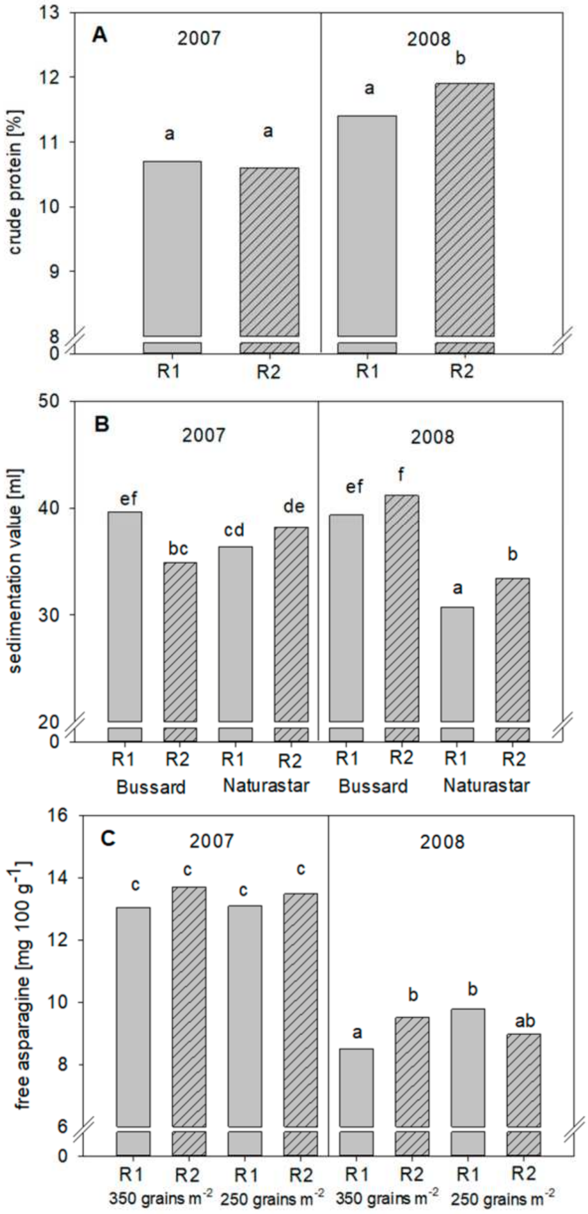

Figure 2. Level of crude protein (**A**) sedimentation value (**B**) and free Asn (**C**) in dependence of their highest significant interaction. Row distance R1: 12.5 cm (solid columns), R2: 30 cm (hatched columns). Seed density: 350 and 250 grains m^{-2}. If no cultivar or only one treatment appears, columns refer to averages. This was the consequence of there being no significant impacts of single treatments. Columns with different letters only within each single trait indicate significant differences ($\alpha = 0.05$, *t*-Test).

In fact, 12% CP is a well synthesized amount, as the required level of the baking industry [49,52] of at least 10.5% was outnumbered by 1.5%. The impact of row distance on CP was also investigated by Becker et al. [34] and Hiltbrunner et al. [44]. Both studies revealed a higher protein content if the row distance was enlarged. Thus, it can be assumed that increasing the row distance may provide an opportunity in organic farming to match needed protein concentrations.

Nevertheless, it has to be taken into account that lower grain yields and more weed management efforts must be accepted if raised CP levels are the main target. Selecting a fitting cultivar (cv) could diminish yield loss, as in our study cv Naturastar (A-wheat) did not respond by lower grain yields if row distance was increased. But this might be an effect of different wheat classes.

Sedimentation value (SV) is a key parameter for interpreting quality of CP and therefore is of high relevance for baking quality. Compared to CP, significant effects regarding SV were more distinct as significant differences were obtained for the effect of the year, cultivar, and the interactions Y × Cv, Y × RD, Cv × RD, and Y × Cv × RD (Table 3).

Concerning years, an SV of 37.3 mL was measured in 2007, while SV in 2008 was significantly lower, reaching 36.2 mL. As already explained in the CP section during grain filling, climate conditions, especially sun duration, could have affected this trait differently over different years.

Regarding the impact of row distance on CP and SV, both increased significantly in 2008 (Figure 2A,B). However, the effect was more consistent for SV, as a slight trend of increasing SV by a larger row distance of 30.0 cm was obvious in both years (Figure 2B). In general, SV ranged from 30.0 mL to 42.7 mL. The smallest level was obtained in 2008 for cv Naturastar if cropped in the narrow row distance, while the overall highest level was reached in 2008 by cv Bussard cropped in the larger row distance (Figure 2B). Expanding the row distance to 30.0 cm increased SV only slightly from 36.5 mL to 37.0 mL. The effect was most notable for cv Naturastar as in both years, SV increased under the larger row distance, although it was only significant in 2008. For cv Bussard, the effect was not consistent.

The impact of row distance on quality trait SV within organic farming systems was also investigated by Becker [34] and Germeier [53]. Both studies announced that SV significantly increased if row distances were expanded to either 50.0 cm [34] or 75 cm [53]. Indeed, the row distance in our study was only increased to 30.0 cm but partially reached the same result. We suppose that if row distance could be further increased, then the effect on SV could have been more pronounced.

Nevertheless, a mean SV of at least 34 mL seems to be sufficient for the baking industry [49]. That level was obtained in all treatments of the trial. Finally, larger row distances seem to support the requirements of the baking industry.

3.3. Free Asn and AA Formation Potential

Free Asn as main indicator for AA formation potential in cereals was significantly influenced by the year, the cultivar, and by the interactions Y × Cv, Cv × RD, SD × RD, Y × SD × RD (Table 3). Neither SD nor RD as single treatment significantly affected the free Asn amount.

In 2007, free Asn was significantly higher than in 2008 (13.3 mg 100 g^{-1} vs. 9.2 mg 100 g^{-1}). Separating years within 2007, the treatments, row distance, and seed density had no effect on free Asn levels at all. This trait ranged from 13.5 mg 100 g^{-1} to 13.7 mg 100 g^{-1} (Figure 2C). By contrast, significant changes were observed in 2008. If the higher seed density of 350 grains m^{-2} was chosen, increasing the row distance to 30 cm raised free Asn levels significantly from 8.5 mg 100 g^{-1} to 9.5 mg 100 g^{-1}. A lower planting density could have changed grains per spike as was shown in Table 4, especially for cv Naturastar. This cultivar showed a higher level of grains per spike if the seed density was decreased (30 to 38 and 32 to 38 grains $spike^{-1}$). By contrast, cv Bussard did not change the number of grains $spikes^{-1}$ if the seed density was lowered.

We suppose that smaller grains contain less starch and more soluble nitrogen (N) fractions, leading to higher CP levels or N fractions may be stored as free Asn. This fits well to the test weight, as it was stated above that bigger grains are expected to deliver higher test weights, including more starch. Furthermore, Figure 3A presents the relation between free Asn and grains $spike^{-1}$. In this context, more grains $spike^{-1}$ indicates an increase in free Asn. This supports the above-mentioned postulation of higher soluble N fractions in smaller grains.

However, as it is known that wholemeal flour contains more free Asn compared to white flour [23], the hull/grain ratio could have influenced the free Asn level since the proportion of hull can be higher if grains are smaller. In contrast, compared to the surface, bigger grains may have a lower proportion of hull. We measured the free Asn level of hull in our trial and analyzed a mean of around 53 mg 100 g^{-1}, and found almost 5-fold more Asn in hull compared to in white flour. This should also be taken into account.

Independent of the highest significant interaction for free Asn, a clear impact of the cultivar was obvious, as the level of free Asn almost was twice as high for cv Naturastar (14.2 mg 100 g^{-1}) compared to cv Bussard (8.8 mg 100 g^{-1}) (Table 5).

Table 5. Level of free Asn (mg 100 g^{-1}) across years influenced by cultivar and row distance. Different letters next to free Asn amount refer to significant differences.

Cultivar	Row Distance (cm)	Free Asn (mg 100 g^{-1})
Bussard	12.5	8.3 a
Bussard	30.0	9.4 b
Naturastar	12.5	14.4 c
Naturastar	30.0	14.1 c

Both cultivars differ in their quality class (Bussard: highest baking quality, Naturastar: high baking quality) and grain yield. Naturastar is related to higher grain yields while Bussard is a high protein wheat. We conclude that protein synthesis of Bussard leaves less soluble N in grain until harvest, while Naturastar used N for grain yield formation and lower protein synthesis, leading to the hypothesis of accumulating more soluble N fractions in grain. Those soluble N fractions may contain free Asn. This assumption is supported by the significant impact of cultivars on sedimentation value (SV), as this trait describes protein quality. Generally, SV was significantly higher for cv Bussard than for cv Naturastar (34.6 mL). Additionally, the higher Asn level of cv Naturastar fits well to the stated effect of smaller grains on Asn, since for cv Naturastar, grains per $spike^{-1}$ were much higher compared to cv Bussard, leading to smaller grains.

Other studies, conducted either under conventional farming or organic farming conditions, also reported that years and cultivars [19–21,23,26–29,31,32] have a major impact on free Asn levels in cereal grains. In this context free Asn in conventional trials normally indicate a higher level as well as a broader range. Stockmann et al. [31] reported an average of 15.5 mg free Asn 100 g^{-1} in white flour and a range of 12 to 32 mg free Asn 100 g^{-1} in conventionally cropped wheat cultivars. Nevertheless, our results of free Asn concentrations fit well to the references and are comparable.

The impacts of row distance and seed density has to date never been investigated before concerning free Asn. Stockmann et al. [20] investigated the effect of nitrogen (N) supply in organically grown wheat cultivars. They increased the N supply step by step to a maximum of 180 kg ha^{-1} and analyzed the impact on baking quality traits and free Asn. It was stated that a raised N supply increased protein significantly, but the free Asn level did not change significantly. Additionally, a high impact of cultivars under different N treatments was reported. The same was found in our study, as above all row distances were able to increase N availability and could have similar effects to those of N treatments.

Hence, those results support the assumption that raising nutrient supply by increasing row distance will increase the protein content and sedimentation value without elevating free Asn.

3.4. Relationship between Baking Quality, Yield Components, Free Asn, and AA

Free Asn as precursor of AA formation potential was not related to crude protein (Figure 3B, R^2 of 0.04). Thus, raising baking quality by using treatments like larger row distances does not increase AA formation potential in the case of free Asn. That is also indicated by the regression of crude protein and AA formation (R^2 = 0.53, Figure 3D). Studies are available reporting either a clear relation between free Asn and protein [23] or no such relation [51]. Thus, further studies investigating the relation between crude protein and free Asn, especially for wheat, are highly important.

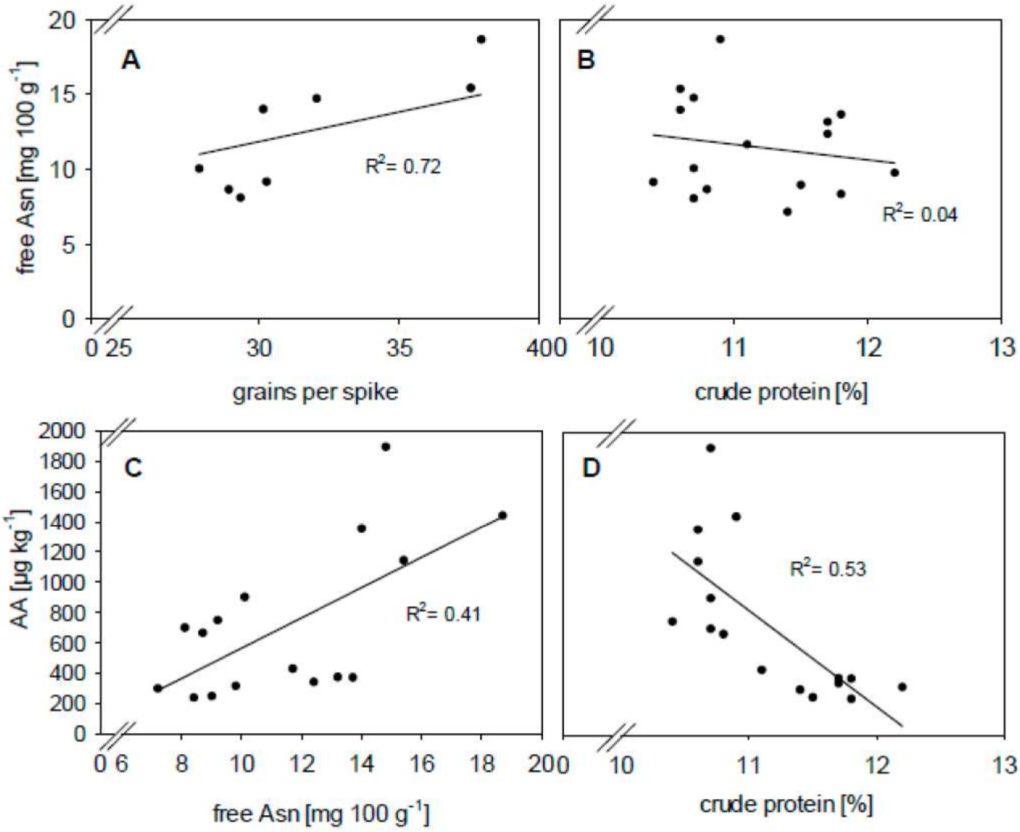

Figure 3. Relation of the traits free Asn * grains per spike (**A**), free Asn * crude protein (**B**), AA * free Asn (**C**) and AA * crude protein (**D**) in flours of wheat cultivars across years and treatments.

The relation between free Asn and AA formation in conventionally cropped cereals was reported in different studies [7,54]. However, for cereals grown under organic farming, this relation has not been investigated intensively. Across all treatments free Asn seems to be a main precursor of AA formation, as shown by an R^2 of 0.41 (Figure 3C). Thus, it seems that in organically grown wheat flours, similar mechanism pathways seem to be present during food processing to those for conventional flours. However, the relation was smaller, wherefore we suppose that other amino acids took part in AA formation. Such findings were also reported by Mottram et al. [4] and Stadler et al. [5].

To date, no study investigated the relation between grain number per spike and free Asn amount (Figure 3A). Interestingly, increasing grain numbers per spike increased the free Asn, as indicated by a close relation of $R^2 = 0.72$. In this context more grains per spike indicate a smaller grain size as the spike has only a defined size. Thus, it can be assumed that smaller grains might contain more free Asn. These findings correspond well with the results mentioned above, where the level of free Asn was highest for cv Naturastar, while also having a high number of grains. This outcome was additionally assisted by the analyzed sieve grading (data no shown), where grains were separated into four grain sizes (>2.8 mm, >2.5 mm, >2.2 mm, and <2.2 mm). In this context cv Bussard reached the biggest grain size fraction (>2.8 mm) for 60% to 80% of its kernels across various years, while for cv Naturastar the equivalent was only 40%. Most kernels of cv Naturastar were within the smaller grain size fractions. In addition, cv Naturastar also had a lower TKW. Overall, free Asn and TKW reached a negative regression of $R^2 = 0.71$ (2007) and 0.49 (2008), indicating that smaller grains led to lower TKW and thus an increase in Asn concentration.

Hence, all three traits (TKW, grains per spike, sieve grading) indicate smaller grains with higher free Asn concentrations. Such a relation (grain number per spike vs. free Asn) has not been observed by other studies before.

Nevertheless, it should also be taken into account that bigger grains might contain less free Asn as a consequence of a thinning effect. Transferring starch assimilates the grain short before the harvest could dilute the level of Asn in grain. However, our results do not support this assumption.

Moreover, Navrotskythe et al. [55] found that thousand kernel weight and kernel size correlated with free Asn by r = 0.3. This is also in contrast to the supposed dilution effect. Further, Navrotskythe et al. [55], reported that delayed harvest elevated free Asn concentration. Delaying harvest is linked to enhancing the possibility of pre-harvest sprouting, which leads to an increase in free Asn [56]. Taking these effects into account, the correlation between kernel weight/kernel size and Asn in the study of Navrotskythe et al. [55] could have been covered by the delayed harvest. However, a falling number was not mentioned in their study, which makes finding common relations between both studies difficult. Additionally, in our study no pre-harvest sprouting seemed to occur, as the falling number was not decreased.

In contrast to our results, no relation between free Asn and TKW respectively kernel weight was reported by Corol et al. [23]. They stated that free Asn is not determined by grain size and grain number per plant. Further, they found higher levels of free Asn in taller cereal plants. We suppose that taller plants differ in their grain number and structure compared to smaller ones. Moreover, larger stems may differ in their transferring ability of nitrogen during grain development leading to less nitrogen mobilization directed to the grains, which could have affected free Asn assimilation. However, it is worth noting that Corol et al. [23] investigated wholemeal flour. This was in contrast to our study as we used white flour, wherefore different outcomes can be expected. Nevertheless, it would be interesting to investigate if free Asn in smaller plants is increased by a higher number of grains per spike. Further, as we just cropped two cultivars, additional trials should be carried out, including an enlarged number of wheat cultivars focusing on grain structure and free Asn value.

The fact that smaller grains may contain more Asn can to some extent be explained by the higher proportion of hull in relation to the full grain size [57]. Studies of Corol et al. [23] did not support these results as they announced no relation between free Asn and yield of flour and bran. We also analyzed Asn concentration in hull and found up to fivefold higher levels compared to white flour. In addition, rye and einkorn seems to have much higher free Asn concentrations than wheat [32]. At the same time, both species have much smaller grains than wheat (TKW: rye: 28–36 g, einkorn: 21–35 g, wheat: 40–55 g). Moreover, protein fractions also differ a lot between these cereals, which could explain the different Asn levels.

In summary, the described relations above provide an absolutely new insight of Asn synthesis and interaction with other traits. This forces the need for future studies revealing the interactions of spike/grain structure and free Asn.

4. Conclusions

The study aimed to assess the impact of row distance and seed density on grain quality, yield components, and yield in organically grown wheats. Although all traits were influenced by year and mostly by cultivar, increasing the row distance also increased the baking quality traits of the crude protein level and the sedimentation value, while free Asn concentration was affected only to a minor extent. Thus, we recommend larger row distances as a feasible way of raising baking quality traits without increasing free Asn levels, which act as precursors for AA formation. Seed density seems to be of minor relevance, as it only affected grain yield and test weight. Nevertheless, as seed density may affect plant space, seed density should be taken into account in further studies, since it may lead to changes in baking quality traits. Zhang et al. [58] reported that increasing plant density had an effect on e.g., grain protein concentration, amount and composition of protein fractions as well as loaf volume in interaction with nitrogen supply. Baking quality traits increased upon increasing the plant density if the plants were highly fertilized with nitrogen, while it decreased if no nitrogen was fertilized. As organic farming is a so-called low input system, the effects of seed density on baking quality must be considered.

Moreover, gluten quality is also important, as described by Augspole et al. [59]. They reported significantly lower gluten content in wheat grains grown under organic farming conditions, while gluten was significantly stronger compared to conventionally cropped samples. Especially under organic farming conditions, strong gluten quality seems highly important for obtaining a good baking performance.

However, if higher yields are required, then seed density should not be diminished. In addition, the study revealed new relationships between yield components (grain structure, TKW, grains per spike) and free Asn. It seems that smaller grains contain more free Asn, which opens new insights into Asn synthesis during grain development.

Thus, future studies revealing the interaction of spike/grain structure and free Asn would be of great interest.

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2.5 Paper 5: "Evaluation of asparagine concentration as an indicator of the acrylamide formation in cereals grown under organic farming conditions"

Stockmann, F.; Weber, E.A.; Mast, B.; Schreiter, P.; Merkt, N.; Claupein, W.; Graeff-Hönninger, S. **Evaluation of Asparagine Concentration as an Indicator of the Acrylamide Formation in Cereals Grown under Organic Farming Conditions**. *Agronomy* 2018, *8*, 294.

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Cereal species and cultivars used under organic cropping conditions face different requirements. Kunz et al. (2006) stated that in order to breed cultivars for these requirements plants must have completely different characteristics than under conventional practices, e.g. a higher accumulation of gluten under a lower nitrogen supply, longer stems, a long terminal internode, loose ears or a faster transfer of nutrients into the grain, more weed competitiveness, and compatibility against harrowing and hoeing. Thus, results of studies done for conventional farming cannot simply be transferred to organically cultivated crops.

Paper 5 deals with the evaluation of free Asn contents within species and cultivars of species grown under organic cropping conditions. Most of the grown cultivars applied in this study, were especially bred for their use under organic farming conditions. As the ancient grains einkorn and emmer have not at all been investigated before in regard to their AA formation potential, this paper presents completely new findings. To implement free Asn in breeding programs for cereals, the heritability was tested, too. The heritability should reveal how strong the trait free Asn is bound to the cultivar or whether environmental conditions are the more decisive factors.

The results presented in this paper clearly show the impact of species and cultivars within organic farming on free Asn. A reduction potential of 85 % was reached if rye was replaced by spelt. Surprisingly, the species einkorn and emmer reached a very high free Asn content similar to rye. Heritability of the trait free Asn was high for wheat and spelt concerning locations. In contrast regarding years heritability was low for wheat but high for spelt and rye. Concerning organically cropped cereals, the relation of free Asn and AA formation has never been examined before. Across species and years, a high regression of $R^2 = 0.69$*, was found. Thus, the amino acid free Asn can serve as an indicator for AA formation. Finally, in organic farming systems, the level of AA in final food products can be highly influenced by a proper selection of species and cultivars.*

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Evaluation of Asparagine Concentration as an Indicator of the Acrylamide Formation in Cereals Grown under Organic Farming Conditions

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Abstract: This study investigated the impact of organically grown cereals on the level of free asparagine (Asn) with simultaneous consideration of grain yields and flour qualities over three growing seasons in Germany. Additionally, the relation of free Asn and acrylamide (AA) was investigated. By including free Asn results of a second trial site, heritability of the trait free Asn was calculated. Free Asn was significantly influenced by species and within species by cultivars. Rye showed the highest free Asn amount, followed by einkorn, emmer, wheat, and spelt. Replacing rye with spelt would reduce free Asn by 85%. Cultivars differed in free Asn by up to 67% (wheat), 55% (spelt), and 33% (rye). Year significantly influenced free Asn levels. Heritability was high for wheat and spelt concerning locations, but regarding years, heritability was low for wheat but high for spelt and rye. For organically grown cereals, the relation between free Asn and AA formation has never been investigated. Across species and years, a correlation of $R^2 = 0.69$ *** was found. Thus, free Asn can serve as an indicator for AA formation. In conclusion, the level of free Asn can be highly influenced by proper selection of species and cultivars.

Keywords: Acrylamide; free Asparagine; agriculture; organic farming; cultivars; cultivar selection; cereal production; cereals

1. Introduction

As a consequence of the carcinogenic potential of acrylamide [1] formed by heating carbohydrate-rich food materials such as cereals [2,3], the European Commission in 2017 has announced a regulation aiming to reduce the level of acrylamide (AA) in food products like cereals and potatoes [4]. Since April 2018, the food industry and gastronomy in Germany faces AA mitigation strategies and benchmark levels.

Besides reducing sugars, free Asn is known to be the main precursor for AA in food. Both sugar and free asparagine (Asn) react during the Maillard reaction to form AA [5–9].

However, after intensive research, several food processing measures have turned out to effectively lower the levels of AA [10–13]. Unfortunately, changes in processing measures often lead to impairment of taste, colour, and texture, or are cost expensive and thus could reduce consumer acceptance, which necessitates additional strategies.

As for cereals, free Asn is the key parameter for AA formation. Some studies investigated the effect of different additives or using the enzyme asparaginase to minimize free Asn [14–17]. Regardless, currently, most of these technological approaches have not been transferred into practice.

As free Asn is formed during crop growth, agronomic measures could reduce the amount of free Asn already in the raw material. This is also the favourable way of the food industry as no technological approach has to be applied or adjusted. Several studies proved that the level of free Asn can be considerably influenced by agronomic measures [5,6,18–24]. Multiple studies showed that cereal species differ in their Asn levels and consequentially in their AA formation potential. Typically, rye seems to have higher Asn levels when compared to wheat and spelt [5,6,18]. Moreover, cultivars can differ considerably in their precursor content, as shown by several studies [5,6,18,22,25,26]. Taeymans et al. [22] reported a 5-fold range for a selection of European wheat cultivars and Claus et al. [5] found a variability of Asn contents in nine German winter wheat cultivars of up to a factor of three. Similar results were found by Corol et al. [25]. They analyzed the Asn content in wholemeal of 150 wheat genotypes and found differences of almost 5-fold. Thus, selection of suitable cultivars with low Asn contents is considered as a feasible way to minimize AA formation potential, although it has to be taken into account that environmental conditions (site-specific and climatic conditions) may alter Asn contents considerably [6,18].

Fertilization is a key measure in crop production to increase yield and quality that can affect Asn levels as well. Nitrogen amount and timing of application, as well as nitrogen form can affect Asn contents in wheat considerably [20,23]. Especially high nitrogen availability during grain filling, which leads to high crude protein content, is considered to increase free Asn levels significantly [23]. Postles et al. [26] realized that high levels of nitrogen raised the amount of free Asn to about 29% when comparing N supply of 1 kg N ha^{-1} with 200 kg N ha^{-1}. Moreover, sulphur deficiency can dramatically increase Asn contents and thus the AA formation potential [19,21,27]. Furthermore, fungicide application promoting leaf area duration and delaying senescence can reduce free Asn content in grains [20,28].

However, most of the available studies on AA were carried out under conventional farming practices. This includes cultivars bred for conventional farming and adjusted for a high input of inorganic N fertilizer and crop protection measurements [29,30]. As organic farming methods differ considerably from those of conventional farming (e.g., fertilization treatment, plant protection, weed control, cultivars in use), results of studies done for conventional farming cannot simply be transferred to organically cultivated crops. Kunz [31] announced that for breeding wheat cultivars under organic farming conditions, plants must have completely different characteristics than under conventional practices, e.g., a higher accumulation of gluten under a lower nitrogen supply, longer stems, a long terminal internode, loose ears or a faster transfer of nutrients into the grain, more weed competitiveness, and compatibility against harrowing and hoeing. In addition, other cereal species like einkorn and emmer are used in organic cropping systems and have not been previously investigated regarding their level of free Asn in white flour. Also, for organically grown cereals, no information is available regarding the possible relation between free Asn and AA as it was found for conventionally cropped cereals. Moreover, in order to recommend cultivars low in free Asn to farmers, it would be important that the trait of free Asn is bound mainly to the cultivar and is only slightly influenced by the environmental conditions. Up to now, only few studies are available addressing the heritability of the trait of free Asn in cereal cultivars.

Hence, the aim of this study was to assess and compare the levels of free Asn in flours of different winter wheat, winter spelt, winter rye, einkorn, and emmer cultivars fully grown under organic farming conditions, over 3-years in Southwestern Germany. The major objectives were to investigate (i) if cereal species winter wheat, winter spelt, winter rye, einkorn, and emmer differ in their potential to accumulate free Asn in their flours, (ii) if Asn levels are affected by cultivar (cv) and year in the context of gained yields and qualities, (iii) to what extent the genotype contributes to the Asn accumulation in flours.

2. Materials and Methods

2.1. Site Description

The field trial was carried out over three consecutive growing seasons (2005–2006, 2006–2007; 2007–2008) at the Dörrmenz trial site (49°12′ N, 9°59′ E, average annual temperature 7.8 °C; average annual rainfall 790 mm). Dörrmenz is located in Southwestern Germany in the federal state of Baden-Württemberg, roughly 100 km north-east of Stuttgart. The site has been cropped under the guidelines of organic farming of the association of Demeter, referring to the highest and strongest organic farming guidelines in Germany.

In 2008, the same field-trial was accomplished on an additional trial site at the experimental station for organic farming of the University Hohenheim, Kleinhohenheim, Stuttgart (48° 44′ N 9° 12′ E; average annual temperature 8.8 °C; average annual rainfall 700 mm). Detailed data on temperature and rainfall for Dörrmenz during the seasons 2005–2006, 2006–2007, and 2007–2008 and Kleinhohenheim during the season 2007–2008 are depicted in Figure 1.

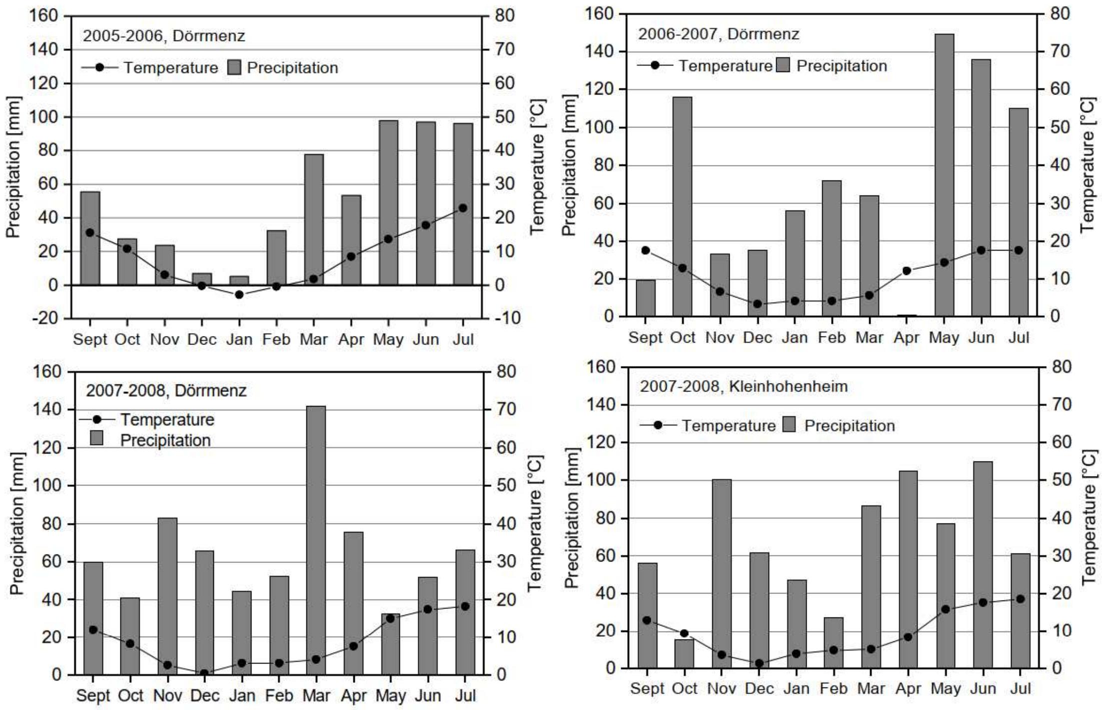

Figure 1. Temperature (●) and precipitation (bars) at the Dörrmenz organic trial site for the growing seasons 2005–2006, 2006–2007, and 2007–2008 and the Kleinhohenheim organic site for the growing season 2007–2008.

2.2. Site Conditions at the Experimental Sites

The field trial at Dörrmenz was carried out on shell lime derived soils with a soil texture of clayey loam with maize as the previous crop in 2006 and 2007, while oat was the previous crop in 2008. N_{min} values at the start of the vegetation period in a soil depth of 0–90 cm were 44 kg NO_3-N ha^{-1} in 2006 and 21 kg NO_3-N ha^{-1} in 2007 and 2008. The soils at Kleinhohenheim are loess derived with a loamy soil texture with faba beans as the previous crop. N_{min} content at autumn in 2007 in a soil depth of 0–90 cm was 42 kg N ha^{-1}.

2.3. Experimental Design

Nineteen winter wheat (*Triticum aestivum* L.), seven winter spelt (*Triticum aestivum ssp. Spelta* L.), and two winter rye cultivars (*Secale cereale* L.), mostly specified as organic cultivars, were grown under organic farming conditions at Dörrmenz over three consecutive growing seasons (2006–2008). In addition, ten rye, three einkorn (*Triticum monococcum* L.), and two emmer cultivars (*Triticum dicoccum* Schranck) were organically grown in single years (see Table 1). For analyzing the heritability of the parameter free Asn concerning the trait location the same species and cultivars were grown under organic farming conditions at a second location (Kleinhohenheim) in 2008.

Table 1. Tested cereal species and cultivars at the Dörrmenz and Kleinhohenheim trial sites over the years 2006–2008.

Species	Cultivar	Quality Grade *	Cultivation Year	Cultivated in 2008	
				Dörrmenz	Kleinhohenheim
Winter wheat	Akteur	E	2006, 2007, 2008	yes	yes
	Arctur	-	″	yes	yes
	Aszita	B	″	yes	yes
	Ataro	1	″	yes	yes
	Bussard	E	″	yes	yes
	Capo	E	″	yes	yes
	Cassia	TOP	″	yes	yes
	Clivio	1	″	yes	yes
	Karneol	-	″	yes	yes
	Laurin	TOP	″	yes	yes
	Magister	E	″	yes	yes
	Naturastar	A	″	yes	yes
	Pollux	1	″	yes	yes
	Scaro	TOP	″	yes	yes
	SpießHS154	-	″	yes	yes
	SpießHS226	-	″	yes	yes
	Tengi	TOP	″	yes	yes
	Wenga	TOP	″	yes	yes
	Wiwa	TOP	″	yes	yes
Winter spelt	Franckenkorn		2006, 2007, 2008	yes	yes
	O. Rotkorn		″	yes	yes
	Alkor		″	yes	yes
	Sirino		″	yes	yes
	Badengold		″	yes	yes
	Tauro		″	yes	yes
	Titan		″	yes	yes
	Samir		2008	yes	yes
	Zollernspelz		2008	yes	yes
Winter rye	Recrut		2006, 2007, 2008	yes	yes
	Lichtkornroggen		″	yes	yes
	Amilo		2008	yes	yes
	Carotop		2006, 2007	no	no
	Conduct		2008	yes	yes
	Crona		2006	no	no
	Hacada		2006	no	no
	Harca		2006, 2008	yes	yes
	HS Aman		2008	yes	no
	Firmament		2007, 2008	yes	no
	Lauropa		2008	yes	yes
	Rolipa		2008	yes	yes
Winter einkorn	Saffra		2006	no	no
	Albini		2007	no	no
	Terzino		2008	yes	no
Winter emmer	EM07		2007	no	no
	EM08		2008	yes	no

* Letters, numbers, and TOP refers to the German and Switzer quality classes (E and TOP: highest baking quality, A and 1: high baking quality), Minus means no information is available. ″ means see above.

At both locations, all cultivars were tested in a randomized complete block design (plot size 1.5×12 m) with three replicates. To avoid neighbouring effects between the different species, species were separated by a border plot with a width of 1.5 m. Species groups were randomly placed in each block, and within each species group, cultivars were arranged randomly.

2.4. Experimental Performance

At both locations, seed bed preparation in autumn took place with a power harrow followed by primary tillage with a moldboard plough (25 cm depth).

Sowing was done for all species on 12 October 2005, 12 October 2006 and 8 October 2007 at Dörrmenz and on 17 October 2007 at Kleinhohenheim. Sowing density was 400 seeds m^{-2} for winter wheat, 180 seeds m^{-2} for winter spelt, 300 seeds m^{-2} for winter rye, 350 seeds m^{-2} for einkorn, and 240 seeds m^{-2} for emmer. Row distance was 0.125 m at both sites.

Nitrogen was applied only in Dörrmenz as liquid cattle manure at start of vegetation in spring equally for all species. The applied amount of nitrogen was 40 kg N ha^{-1} in 2006, 25 kg N ha^{-1} in 2007 and 25 kg N ha^{-1} in 2008. At the Kleinhohenheim site (only tested in 2008), no nitrogen was applied due to faba bean being the previous crop. If necessary, a currycomb was used to reduce weeds.

Harvest was accomplished on 3 August 2006, 17 July 2007, and 1 August 2008 at Dörrmenz and on 2 August 2008 in Kleinhohenheim with a Hege 180 plot combine harvester (Hege, Hohebuch, Germany) after grains had reached a dry matter content of 85%.

2.5. Yield

Grain yield was determined by weighing plot yield. Grain samples were dried at 105 °C for 24 h to determine grain moisture. Given grain yields refer to 86% dry matter content.

2.6. Flours

For the determination of quality parameters, the determination of free Asn and the AA formation potential, grain samples were milled on a laboratory mill (Quadrumat Junior, Brabender, Duisburg, Germany). Ash content of flours was approximately 0.5% of flour-DM. Flour moisture was calculated from the weight loss before and after drying of approximately 5 g flour at 105 °C for 24 h.

2.7. Crude Protein Content

Total grain nitrogen content was determined by Near-Infrared-Spectroscopy (NIRS, NIRS 5000, FOSS GmbH Rellingen, Germany). Calibration samples were analyzed according to the Dumas Method [32] using a Vario Max CNS analyzer (Elementar, Hanau, Germany). The final nitrogen content was multiplied by a factor of 5.7 for wheat samples [33] and 6.25 for all other samples.

2.8. Zeleny's Sedimentation Test

Zeleny´s sedimentation test was determined by using 3.2 g flour according to ICC standard No. 116. The sedimentation values of the flours were adjusted to 14% moisture basis.

2.9. Hagberg Falling Number

The Hagberg falling number was determined according to ICC standard No. 107 using a PerCon 1600 Falling Number machine (PerCon, Hamburg, Germany), using 7 g of flour (weight adjusted for moisture concentration to 15%).

2.10. Free Asparagine

Free asparagine was extracted from either 2 g of wheat, spelt, einkorn or emmer flour or 1 g of rye flour. Samples were mixed with 8 mL of 45% ethanol for 30 min at room temperature. After centrifugation for 10 min at room temperature with 4000 rpm and 10 min at 10 °C and 14,000 rpm, the supernatant was filtered through a 0.2 μm syringe filter and transferred into vials. Asparagine analysis was performed using Merck–Hitachi HPLC components. The pre-column derivatization with FMOC [34] was completely automated by means of an injector program. Subsequently, the derivatized Asn was separated on a LiChroCART Superspher RP 8 column (250 mm × 4 mm, Fa. Merck, Darmstadt) at a constant temperature of 45 °C. The fluorescence intensity of the effluent was measured at the excitation and emission maxima of 263 and 313 nm.

2.11. Acrylamide Formation Potential

The AA formation potential of cereal flour was assessed according to the AA contents of 5 g flour in 250 mL Erlenmeyer flasks after heating in an oven for 10 min at 200 °C. Due to the complexity of the acrylamide analysis, sample size was reduced to an overall number of 21 samples (8 winter wheat, 5 winter spelt, 5 winter rye, 1 einkorn, and 1 emmer sample).

Sample preparation was accomplished according to test procedure 200L05401 [35] of the Chemische und Veterinäruntersuchungsamt (CVUA) Stuttgart.

After cooling down to ambient temperature, 100 mL of bidestilled water and 100 μL of D_3-Acrylamide were added as an internal standard to the heated flour samples in the Erlenmeyer flasks. To completely extract acrylamide from the flour, samples were put in an ultrasonic bath for 10 minutes at 40 °C. After adding 1 mL of Carrez I and II to each of the samples and shaking the flasks thoroughly, the samples were filtered using folded filter paper to separate the colloids and flour particles from the aqueous solution. Subsequently, samples were cleaned up by a solid phase extraction in a vacuum chamber after preconditioning the cartridges with 10 mL of bidestilled water and 10 mL methanol. After sample clean up, about 1 to 2 mL of the eluate from each sample was filled in an autosampler vial and was deep frozen (−18 °C) until AA was determined by LC-MS-MS by the CVUA according to test procedure 201L01301 [36]. The eluates were separated by a graphite or RP18-phase and detected by tandem-mass-spectrometer. Quantification was undertaken by using the isotope-labelled internal standard (D_3-Acrylamide).

2.12. Statistical Analysis

Yield and quality data (crude protein, falling number, sedimentation value), as well as free Asn of the location Dörrmenz were subjected to analysis of variance (ANOVA) using the Procedure MIXED from the statistical software package SAS 9.1. (SAS Institute Inc., Cary, NC, USA). When necessary, data was in- or square root-transformed, to stabilize normal distribution and homogeneity of variance. A comparison of means was accomplished using the t-test.

ANOVA was done in two steps: in a first step, the main effects year, species, cultivars, and interactions were investigated. In a second step, crop species were analyzed separately for determining potential cultivar differences depending on year. For the parameter AA, no statistical analysis was undertaken as, only single samples from one field replicate were selected for analysis. The impact of year, species, and cultivar ANOVA was only analyzed for Dörrmenz.

Heritability of the trait “free Asn content” was calculated from the variances according to Miedaner [37] for Dörrmenz in the years 2006, 2007, and 2008 and for Dörrmenz and Kleinhohenheim in 2008. In this context, the impact of location on free Asn was only used for the heritability.

3. Results and Discussion

3.1. Grain Yield, Quality and Free Asn

Note: Except for chapter “Impact of locations on free Asn, Heritability”, all results refer to the Dörrmenz location.

Grain yield, crude protein, and free Asn content at Dörrmenz were significantly influenced by species and year, as well as by their interaction (Table 2).

Table 2. F-values and p-values for main effects species, year, and interactions between factors on grain yield, crude protein content, and free Asn content of flours.

		Grain Yield		Crude Protein		Free Asn	
	DF	F	*p* [1]	F	*p*	F	*p*
Species (S)		7.69	***	62.08	***	134.83	***
Year (Y)		16.22	***	12.89	***	48.89	***
S × Y		25.49	***	9.36	***	16.78	***

[1] level of confidence ($p < 0.05$ *, 0.01 **, 0.001 ***, n.s. not significant).

Within the cereal species wheat and spelt, cultivar, year, and the interaction between cultivar and year affected grain yield, quality, and the Asn content considerably (Table 3). However, grain yield of wheat was not affected significantly by the cultivar × year interaction. Winter rye grain yield was significantly affected by cultivar and year but not by their interaction. Falling number of rye was neither effected by cultivar and year nor by their interactions. Free Asn of the tested rye cultivars was only affected significantly by year but neither by cultivar nor by the cultivar × year interaction (Table 3).

Especially year had a crucial impact on yield, quality, and Asn content. While yield and partially crude protein contents were significantly lower in 2007 compared to 2006 and 2008, the content of free Asn considerably increased in 2008 by about 35% in emmer and up to >100% in winter rye. While lower yields in 2007 were presumably the result of increased weed infestation due to a mild winter 2006–2007 and a reduced number of kernels per ear due to the absence of precipitation in April 2007, in 2008, free Asn contents were possibly affected by the comparably low precipitation from May until harvest in July (see Figure 1).

Grain yields of all species were generally low due to the low-input crop management (nutrient supply, and the omission of any weed control). While average grain yields across years varied less between winter wheat, winter spelt, and winter rye (from 3.5 t ha^{-1} of wheat to 3.8 t ha^{-1} of spelt), the yield potential of einkorn was about 1 t ha^{-1} and that of emmer about 0.5 t ha^{-1} lower (Table 4).

Crude protein contents were highest for spelt (mean 12.7%) and einkorn (mean 14.1%), while rye reached levels of around 7.7%. Winter wheat and emmer showed average protein contents of 10.6% and 11.2%. According to Brunner [38], crude protein contents of at least 10.5% for organically produced wheat and 11% for organically produced spelt are required to provide acceptable baking quality. Thus, mean values obtained within this work should offer a sufficient baking quality for organically produced wheat flour. Nevertheless, under organic farming, protein contents are known to be generally lower than under conventional farming [39]. As a consequence, bread bakery processing has to be adapted to the lower protein contents [40] to achieve acceptable organic bakery products.

According to Haglund et al. [40] dough from wheat flours with protein contents lower than 12% needs a longer dough mixing time. At crude protein levels below 8%, it was found that it was difficult to form an acceptable bread volume. Consequently, bakers are required to adjust their processing conditions for flours of organic origin. The sedimentation value, a further parameter to describe the baking quality, was determined from wheat samples and differed significantly between years, but in a very close range between 31 and 36 mL. Brunner [38] recommended that a range of 30 to 45 mL is needed for wheat, thus the achieved mean sedimentation values would fulfil processors requirements.

A low falling number gives evidence of pre-sprouting, and in consequence leads to an increased free Asn level due to an increased protease activity [5,41]. Falling number as a parameter to assess starch quality of bread rye lots was only marginally affected by year, ranging from 217 to 245 s. Values were within the required range [38] for which no impairment of baking quality is expected.

Table 3. F-values and p-values for main effects, cultivar, year, and interactions between factors on grain yield (GY), crude protein content (CP), sedimentation value (SV), falling number (FN), and free Asn content of flours in dependence of the species. Because only one cultivar of einkorn and emmer was cultivated during years both species were excluded for statistical analyses ($\alpha = 0.05$, *t*-test).

	Winter Wheat								Winter Spelt						Winter Rye					
	GY		CP		SV		Asn		GY		CP		Asn		GY		FN		Asn	
	F	*p* [1]	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*
Cultivar	6.1	***	42.0	***	27.5	***	16.7	***	19.1	***	446.0	***	57.0	***	80.5	***	1.2	n.s.	0.1	n.s.
Year	1616.6	***	273.2	***	41.9	***	264.1	***	540.7	***	469.7	***	204.4	***	28.2	***	1.3	n.s.	143.2	***
Cultivar × Year	1.5	n.s.	6.5	***	3.0	***	7.3	***	2.5	*	17.67	***	7.1	***	0.9	n.s.	0.5	n.s.	0.2	n.s.

[1] level of confidence ($p < 0.05$ *, 0.01 **, 0.001 ***, n.s. not significant).

Table 4. Grain Yield (GY), crude protein (CP), sedimentation value (SV), falling number (FN), and free Asn of the five tested crop species depending on year. Treatments in the same line assigned by the same letters are not significantly different ($\alpha = 0.05$, *t*-test).

	Winter Wheat			Winter Spelt			Winter Rye			Winter Einkorn			Winter Emmer *	
	2006	**2007**	**2008**	**2006**	**2007**	**2008**	**2006**	**2007**	**2008**	**2006**	**2007**	**2008**	**2007**	**2008**
GY [t ha^{-1}]	4.2 ef	2.1 a	4.2 ef	4.2 ef	2.4 ab	4.7 f	4.1 de	3.3 cd	3.4 cd	2.7 abc	1.8 a	3.1 bcd	1.9	4.2
CP [%]	11.6 e	9.9 c	10.4 d	13.4 fg	11.6 e	12.9 f	6.8 a	6.6 a	8.8 b	15.6 g	11.9 ef	14.8 fg	10.6	11.8
Asn [mg 100 g^{-1}]	8.1 b	8.2 b	13.2 c	6.6 ab	5.5 a	12 c	32.3 de	28.6 d	67.0 f	25.5 d	30.7 de	40.0 e	13.4	18.1
SV [ml]	31.8 b	30.6 a	34.7 c											
FN [s]							217 a	224 a	245 a					

* Emmer was not integrated into the statistical analysis, because it was not grown each year. Nevertheless, data are presented for comparing species. Different letters assign significant differences ($\alpha = 0.05\%$, Tukey test).

The potential to accumulate free Asn varied clearly between the species, with rye having the highest average Asn level across years of about 43 mg flour-DM, followed by einkorn with about 32 mg, emmer with about 16 mg and wheat and spelt with about 10 mg and 8 mg 100 g^{-1} flour-DM, respectively, across all years. Fredriksson et al. [42], Elmore et al. [43], and Claus et al. [5] reported up to 3 or 4 times higher free Asn levels in rye compared to wheat or spelt. This corresponds well with data obtained in this study for cereals produced under organic conditions. Therefore, the AA formation potential of bakery goods made from rye flour is considered to be higher than from wheat or spelt [5,43] independent of the cropping system. In contrast, studies of Curtis et al. [6] showed that AA formation in heated flour per unit Asn was much lower in rye than in wheat flour, suggesting that the higher Asn level compared to wheat does not inevitably mean that rye has a higher AA formation potential per se. In our study, however, AA formation potential was strongly correlated to the level of free Asn, as it was highest for rye and einkorn.

Within species grain yield of wheat and spelt varied only marginally between cultivars (cv). The mean standard deviation was only 0.38 t ha^{-1}, whereas year influenced grain yield much more, causing variations of up to 49% between years.

Wheat crude protein content ranged from 10.1% (cv Bussard) to 13.7% (cv Tengri) in 2006, while in 2007 it varied between 8.7% (cv Magister) and 10.7% (cv Arctur). In 2008, the lowest crude protein content was found for cv Akteur (8.7%) while cultivars Tengri and Wiwa showed the highest contents with 11.7%. Crude protein of spelt ranged across years between 9.5% (cv O.Rotkorn) and 16.1% (cv Sirino). Johansson and Svensson [44] investigated the effect of weather conditions on protein concentration of wheat cultivars for 21 years. They described that climatic conditions, especially temperature, during grain filling had a high impact on grain protein level of wheat. For spring wheat, they found differences of 34% concerning crude protein concentration. Thus, crude protein content obtained during this work was most likely influenced by weather conditions and cultivars.

Wheat sedimentation values varied depending on cultivar from 24 mL in 2007 to 43.3 mL in 2008. Especially cultivars Wiwa, Wenga, Spieß 2, Clivio, Pollux, and Scaro reached higher amounts in each year when compared to the other investigated cultivars.

Rye grain yield varied based on cultivar in 2006 from 3.5 t ha^{-1} (cv Corona) to 4.4 t ha^{-1} (cv Hacada), in 2007 at a lower level from 2.8 t ha^{-1} (cv Firmamant) to 3.7 t ha^{-1} (cv Recrut) and in 2008 from 2.1 t ha^{-1} (cv Firmament) to 4.2 t ha^{-1} (cv Lichtkornroggen). Crude protein content of rye was at an expected low level in 2006 and 2007 and ranged from 6.3 (cv Carotop) to 7.2 (cv Lichtkornroggen), while in 2008 the amount of crude protein increased by up to 2.1% and varied from 8.1% to 10.5% (cv Firmament). With regards to falling number, cultivars differed from 163 s (cv Hacada) to 224 s (cv Carotop) in 2006, while in 2007 cv Lichtkornroggen reached to lowest level of 208 s compared to cv Firmament, which reached the highest falling number with 272 s. The overall highest level of falling number was found in 2008 as cv Amilo obtained 303 s. However, there was no cultivar which fell below the required amount of at least 90 s, which is recommended by [38] as the lowest level needed by processors.

No comparison of cultivars from einkorn and emmer concerning quality parameters could be made as only one cultivar of each species was grown.

The level of free Asn for wheat was significantly influenced by cultivar and year (Table 3, Figure 2A). In general, the amount of free Asn varied between 5 and 22.4 mg 100 g^{-1}. Similar ranges are seen in other studies: Claus et al. [5] found a range of 8.7 to 24.9 mg Asn 100 g^{-1} in a variety of conventionally produced winter wheat cultivars. Stockmann et al. [45] investigated a variation of organically grown wheat cultivars and found significant differences between cultivars in their Asn level ranging from 8 to 14 mg 100 g^{-1}. Also, Taeymans et al. [22] investigated free Asn levels of a range of wheat cultivars mostly grown under European (UK) conditions during 2002. They found a broad range of 7.4 to 66.4 mg 100 g^{-1} including a five-fold difference between cultivars and a mean of 22 mg 100 g^{-1}. The level of free Asn within a set of 150 wheat cultivars was analyzed as wholemeal by Corol et al. [25]. The difference between cultivars were up to 5-fold. The high variation found by

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Taeymans et al. [22] and Corol et al. [25] could have been caused by including grain samples from various locations across Europe, as Curtis et al. [18] found that climatic conditions can influence the level of free Asn significantly. Also, stress conditions like plant disease and water limitations can have an impact on free Asn synthesis. Curtis et al. [28] realised that fungicide treatment can reduce the level of free Asn. As all cereal samples in this study were grown under organic farming conditions stress factors like diseases could have contributed to a higher variation in the level of free Asn.

Across years, cv Akteur was found to accumulate the lowest amounts of free Asn (6.9 mg 100 g^{-1}), while cv Arctur reached the highest average value of 13.8 mg 100 g^{-1}. Although the level of free Asn was generally influenced by year, Asn contents in some cultivars were more or less stable over all years (year-to-year variation < 2 mg 100 g^{-1} flour-DM: Akteur, Clivio, Spieß HS 154, Spieß HS 226, Tengri and Wenga). However, some cultivars were highly susceptible towards year-to-year effects (year-to-year variation > 6 mg 100 g^{-1} flour-DM: Ataro, Capo, Karneol, Laurin, Magister, Naturastar). A third group of cultivars showed Asn contents varying between years in the range of 2 and 6 mg 100 g^{-1} flour-DM (Cassia, Arctur, Aszita, Bussard, Pollux, Scaro, and Wiwa). Hence, selecting or breeding cultivars with low to medium Asn levels and a robust response towards varying climatic conditions seems a more suitable measure to lower AA formation potential in the long term, while selecting cultivars with the lowest Asn contents in single years may be a short-term solution.

The free Asn level of wheat differed between cultivars by up to 50% across years and up to 67% within single years. Assuming a linear increase of AA with increasing contents of Asn in the flour [5], choosing a proper cultivar could halve free Asn levels and thus minimize AA formation during processing considerably.

In regard to wheat, the amount of free Asn in spelt flour was significantly affected by cultivar and year (Table 3, Figure 2B). The free Asn content of spelt was higher in 2008 (mean 12.0 mg 100 g^{-1}) than in the other two years (2006: 6.5 mg 100 g^{-1}, 2007: 5.5 mg 100 g^{-1}). Comparing cultivars across years, free Asn amounts showed a range of 5.4 (O. Rotkorn) to 11.1 mg 100 g^{-1} (Sirino). Within single years, cv Tauro showed the highest content of 16.2 mg Asn 100 g^{-1}. Cultivars O. Rotkorn and Badengold had the lowest level of free Asn in each year. While free Asn levels of spelt varied to up to 54% by year-to-year effects, the variation between cultivars by up to 77% was much higher.

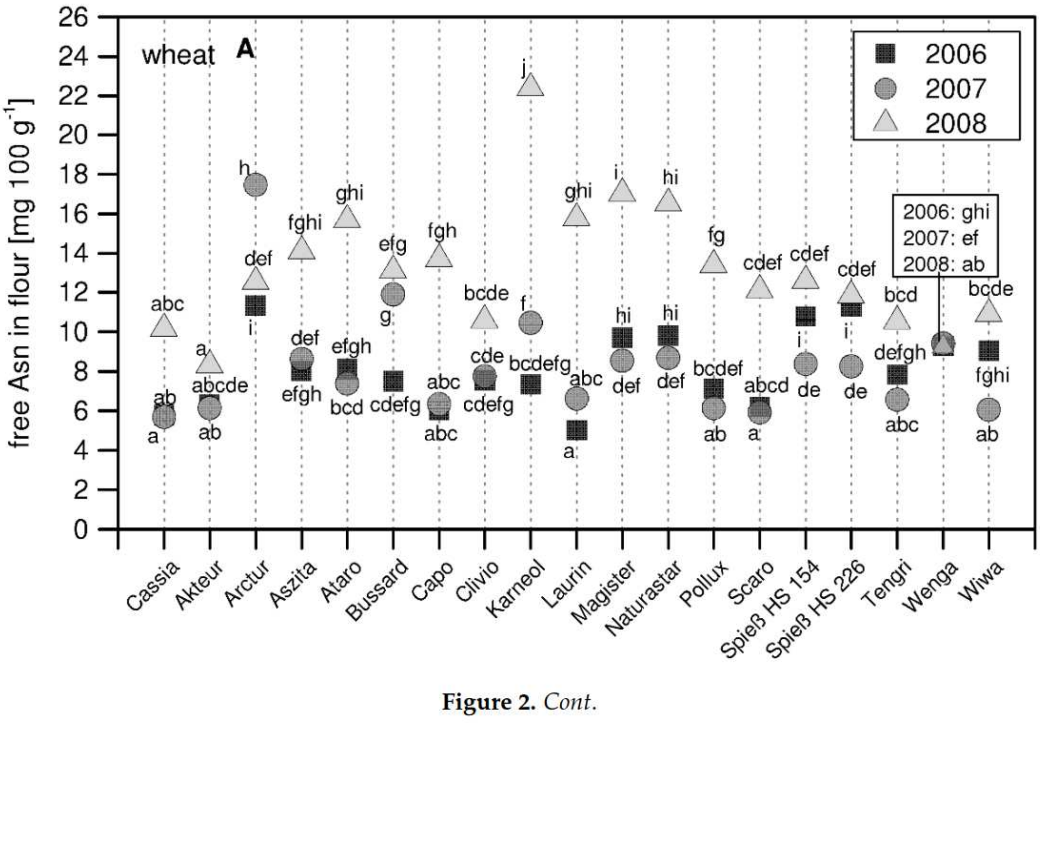

Figure 2. *Cont.*

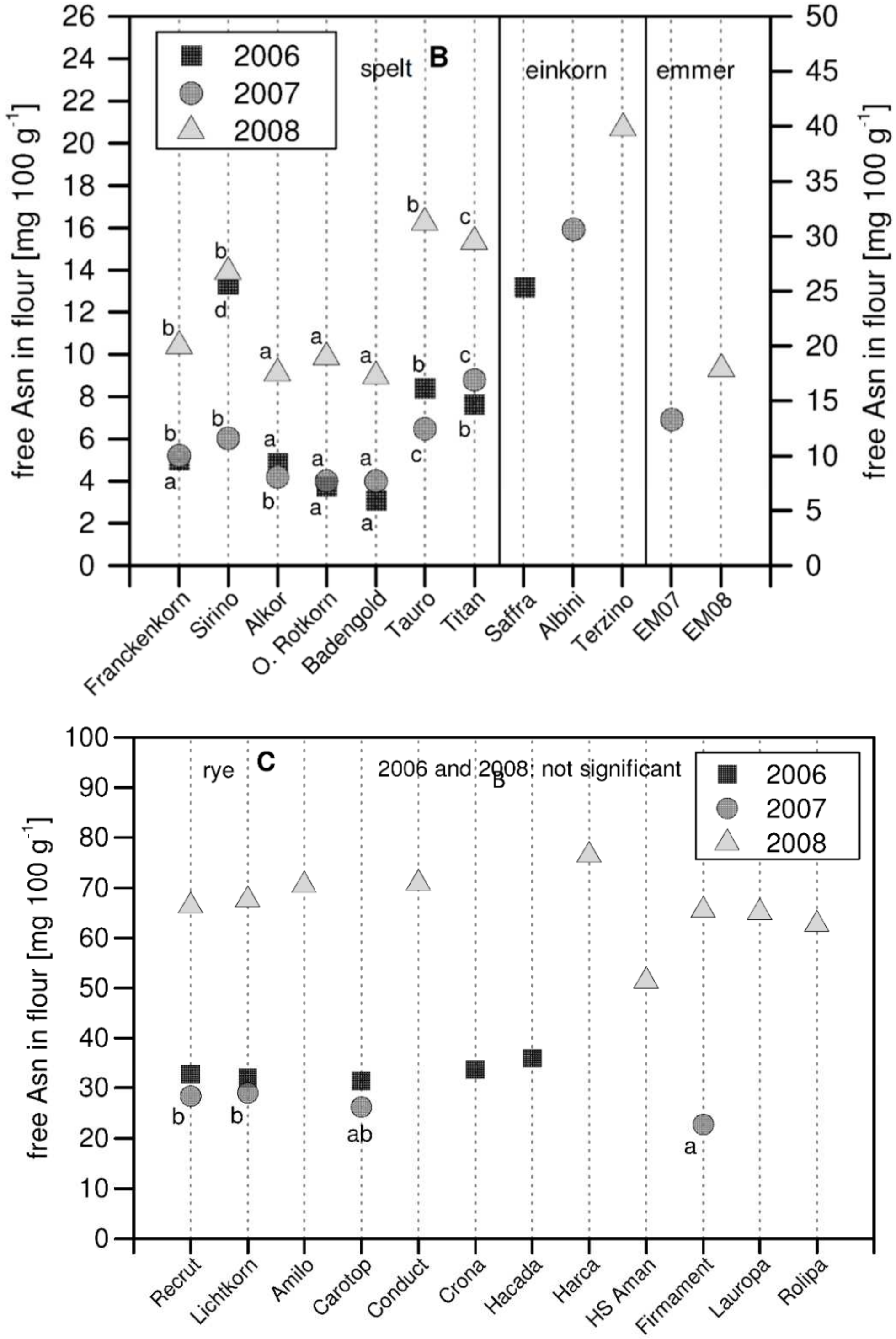

Figure 2. Free Asn content separated by species (**A**: wheat, **B**: spelt, einkorn, emmer, **C**: rye), year (2006, 2007, 2008), and cultivar. Symbols with different letters within the same year indicate significant differences (α = 0.05, *t*-Test) between cultivars. The right-hand y-scale of B refers to free Asn content of einkorn and emmer.

Information about free Asn of spelt in the literature is rare. Claus et al. [5] reported amounts of conventionally produced spelt cultivars of 6.5 to 12.2 mg 100 g^{-1} and by this, a somewhat smaller range than in our study. Similar results were found by Corol et al. [25] as the range of free Asn of spelt cultivars ranged from 0.60 to 0.79 mg g^{-1} DM in wholemeal flour.

To date, no previous studies have investigated einkorn and emmer for their amount of free Asn in white flour. Corol et al. [25] analyzed free Asn in einkorn and emmer but the samples were cropped under conventional cropping conditions and free Asn was analyzed in wholemeal. Thus, a comparison with our results is difficult. Nevertheless, the study showed that between six cereal species, einkorn reached the highest level of free Asn with 1.12 mg g^{-1} DM.

In our study, einkorn showed high Asn contents when compared to wheat of up to 40 mg Asn 100 g^{-1} (Figure 2B). It ranged from 25.5 mg Asn 100 g^{-1} in 2006 (cv Saffra) to 40 mg Asn 100 g^{-1} (cv Terzino) in 2008. This high level was otherwise only found in rye samples. With a mean of 15.7 mg Asn 100 g^{-1}, emmer cultivars showed comparable amounts when compared to wheat and spelt. Consequently, if einkorn is supposed to be used for preparing bread and rolls, high levels of AA in the final product are expected.

For rye there was no significant cultivar-dependent effect on free Asn, while year affected the Asn content significantly (Table 3, Figure 2C). While the average Asn levels across cultivars were comparable in 2006 and 2007 with about 30 mg 100 g^{-1} flour-DM, the levels more than doubled in 2008 with an average of 67 mg 100g^{-1} flour-DM.

These results are in agreement with studies of [5] who investigated the amount of free Asn in two rye cultivars and obtained a mean of 42.6 mg 100 g^{-1}. In studies by Springer et al. [46], a similar range was found with rye cultivars varying between 31.9 and 79.1 mg 100 g^{-1}. Studies by Stockmann et al. [45], who investigated the impact of cropping systems on free Asn, also found that rye cultivars had no significant impact on free Asn content no matter which cropping system was examined. A significant year impact was, however, present for conventionally treated cultivars. They found a free Asn content ranging from 31.5 to 52.3 mg Asn 100 g^{-1} for conventionally cropped samples and from 30.1 to 35.9 mg 100 g^{-1} for organically treated samples. Thus, it seems that lowering free Asn by choosing rye cultivars low in free Asn will be of minor relevance to reduce AA.

Overall, the amount of free Asn was highly influenced by species and cultivars. However, it has to be considered that abiotic factors can affect Asn levels as well, as shown for wheat by Curtis et al. [18] and for rye by Curtis et al. [6]. Until now, information on how soil type, temperature, and precipitation affect grain-Asn accumulation is lacking [47]. Investigations of Corol et al. [25] revealed that free Asn was increased if temperature was higher and precipitation was low during grain filling. In our study, temperature in June and July was similar across years and locations (see Figure 1), but during June and July rainfall was much lower in 2008 compared to 2006 and 2007 (see Figure 1). Therefore, we assume that the level of free Asn in our study was significantly influenced by year. However, as the main objective was to detect and breed cultivars with a low susceptibility to climatic conditions, cultivars with only a weak response year-to year variations (e.g., cv Wenga with a standard error across years of 0.1) are promising to effectively reduce the AA-formation potential by proper cultivar selection.

Therefore, cultivars should be tested over several years at different locations before recommendations can be made for breeding programs targeted towards lowering the AA precursor contents.

3.2. Correlation of Crude Protein, Free Asn, and AA

Free Asn is the critical factor for AA formation during processing of cereal-based bakery products [13]. Asn contents in flour of conventional origin correlated almost linearly with the AA content in heated flour or in breads [5,6].

The results of this work corresponded well with these findings as, averaged over species and cultivars, free Asn and AA correlated well with $R^2 = 0.69$ ***. The same was found if species were separated, as AA formation was correlated with free Asn (wheat R^2 0.66 *, spelt R^2 0.83 $^{n.s.}$, rye R^2 0.64 $^{n.s.}$).

However, cultivars with a high baking quality do not inevitably have to be linked with having high Asn contents and therefore a high AA formation potential. This is suggested by the weak correlation found between crude protein and free Asn contents across species (R^2: 0.07 $^{n.s.}$) and within wheat (R^2 0.00005 $^{n.s.}$). This is also proven by studies of Ohm et al. [48]. They found a negative correlation between baking quality parameters and free Asn within a set of hard red spring wheat cultivars. In contrast, crude protein and free Asn correlated within spelt by R^2 0.4 ** and rye by R^2 0.79 ***. The close correlation between crude protein and free Asn of rye could have been caused by higher nitrogen availability as crude protein was around 1 to 2% higher in 2008 than the other years, leading to levels of free Asn approximately twice as high. As crude protein has a low relevance for baking quality of rye, choosing cultivars low in free Asn is not thought to affect the baking quality of rye.

The results show that cultivars combining high or acceptable baking quality and low Asn contents are already available, and that the AA formation potential could further be lowered by selecting and cultivating appropriate cultivars.

3.3. Impact of Location on Free Asn, Heritability

Figure 3 shows a close correlation for wheat and spelt regarding locations, meaning that the ranking of the tested cultivars concerning their Asn contents was comparable at both locations, although the absolute contents varied at least partly. For the tested rye cultivars, the correlation of Asn levels was weaker than for wheat with R^2 = 0.38.

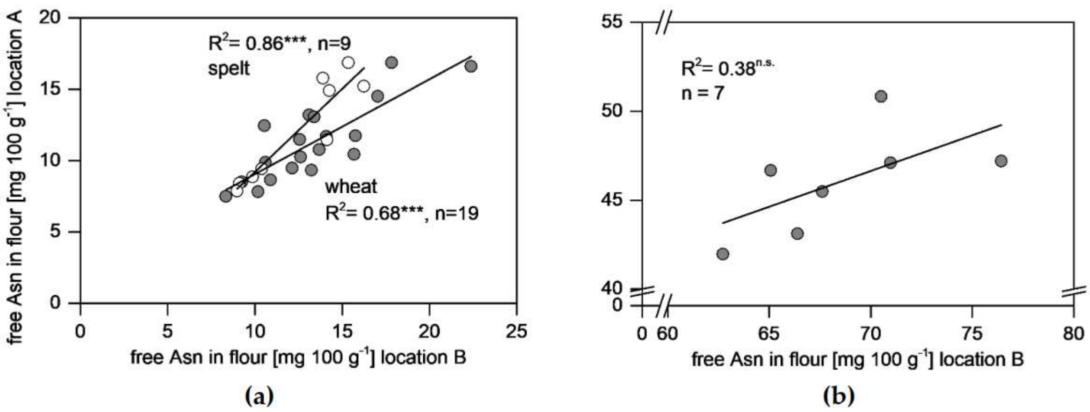

Figure 3. Relation of free Asn at two different locations of cultivars of wheat & spelt (**a**), and rye (**b**) during the growing season 2007/2008.

This was well-supported by the calculated heritability for the free Asn concentration of wheat cultivars grown at the Dörrmenz location compared to the Kleinhohenheim location in 2008. The calculated heritability value is, in general, an indicator to which extent a factor, e.g., level of free Asn, is genetically determined or influenced by environmental conditions [37]. Heritability close to 1 indicates a high impact of the genotype, whereas a low heritability (close to 0) shows a high impact of the environment. Wheat and spelt showed a very high heritability of 0.79 and h^2 0.91. Thus, the level of free Asn seemed to be bound closely to the cultivar. This was also expressed in the small variation of Asn contents between the two locations as free Asn levels changed only by 15% for wheat and 3% for spelt. In contrast, heritability for rye was 0.31 and the variation between locations regarding free Asn was about 32.8%.

A different outcome concerning heritability was obtained comparing the years at the Dörrmenz location. For wheat, a heritability of only 0.23 was found, whereas for spelt and rye, a higher impact of the cultivar was found with 0.67 for rye and 0.71 for spelt.

Investigations of Corol et al. [25] showed that genotype only had a minor effect on variation of free Asn within 26 wheat lines tested at different locations in Europe grown 2005 to 2007. Only 13% of variation in free Asn could be explained by the genotype, while 36% was the result of the environment. This is in contrast to our studies. The reason can be that the locations within the study of Corol et al. [25] were much more different. The tested wheat lines were cultivated in UK, France, Poland, and Hungary and thus growing conditions (climate conditions, soil type, cropping methods) were highly different.

In our studies, cultivars were grown at only two locations. This leads to the assumption that, if locations are similar, heritability will be much higher.

However, a high genotypic impact on the degree of free Asn accumulation is the prerequisite for the significance of cultivar selection or targeted breeding efforts to lower Asn storage in cereal grains and therefore to lower AA in cereal-based bakery ware.

Thus, further experiments aiming at elucidating significant climatic or soil-specific factors on Asn accumulation are necessary to better understand the role of site-specific conditions and genotype on Asn accumulation in grains.

4. Conclusions

This study aimed to evaluate the free Asn levels in flours of winter wheat, winter spelt, winter rye, winter einkorn, and winter emmer grown under organic farming practices. Results indicated that the level of free Asn can be highly influenced by proper selection of species and cultivars. The effect was most noticeable if rye was replaced by spelt, as the free Asn amount was lowered by up to 85%. Organically grown spelt reached the lowest level of free Asn, while einkorn obtained free Asn contents similar to rye. As crude protein content of einkorn and rye (only in 2008) were high but sedimentation values were very low, the higher Asn level of both species could be explained by a higher level of soluble nitrogen fractions within the grain mostly stored as Asn.

The level of free Asn followed the order rye > einkorn > emmer > wheat > spelt. Within species, cultivars had a high impact on free Asn amount, as differences of up to 77% were found.

A close relation between free Asn and AA was also found, while crude protein and free Asn did not show any correlation, especially for wheat, where crude protein is most important for baking quality. Thus, choosing cultivars low in free Asn could be a feasible strategy to reduce AA during processing without affecting baking quality.

As heritability was high for wheat and spelt in regard to location, the level of free Asn seems to be influenced by genotype, thus breeding programs specifically for low-Asn cultivars with stable response to different environments and years are of great interest. In this context, the most important question is why species and cultivars differ in their amount of free Asn. Therefore, a deep insight into Asn synthesis is needed to answer this question.

In general, the impact of year has to be considered as free Asn contents were significantly higher in 2008 than in 2006 and 2007. Rye was affected most by year, with levels of free Asn varying up to 32%, while variation of Asn was smaller in wheat and spelt.

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3 General discussion

Since 2002, the discovery of AA in food products has drawn world-wide attention. Potatoes and cereals were first studied extensively for their AA formation potential. To date, studies investigating AA levels of almost every food product have been carried out, including peaches, olives, dried fruits, tomato sauce, tea, different foods based on national specialities, oils and meat.

A clear relation between heat processing of such raw materials, the involved precursors of AA formation and the technological processing applications related to a decrease of AA, was revealed. First approaches focussed on adapting production measures, e.g. the heating procedure, additives and changes in recipe, while the use of enzymes was also investigated.
Evidence was also clear that agronomic strategies can highly minimize AA formation during processing by lowering the amino acid free Asn, the main precursor of AA in cereals. However, there were still some main agronomic aspects that had never been in focus to investigate.

Thus, the main aim of this thesis was to close existing knowledge gaps within the entire agronomic production chain, and to evaluate how free Asn can be influenced by crop management strategies. For this reason, the included papers highlighted the implication of cropping systems (paper 1 and 2), nutrient supply (paper 3), row distance and seed density (paper 4) as well as organically grown cereal species and cultivars (paper 5) on free Asn concentration in cereal grain. In each specific paper the results had been already discussed in detail. Nevertheless, taking a look at the agronomic management chain (Figure 2) there are some targets left, which may have an impact on AA formation potential. Thus, this chapter explores further agronomic strategies, which are supposed to influence the amino acid free Asn. This also includes results gained by additional field trials and laboratory work.

*Closely linked to the main research of the thesis, the question arose if the timing of harvest could affect free Asn (**chapter 3.1**). As a delayed harvest can cause pre-harvest sprouting leading to a high increase in free Asn, the investigation of this subject was considered as essential. Further, in paper 5 of this thesis the evaluation of organically grown species highlighted einkorn and emmer for their high free Asn level. Thus, both ancient cereals should be discussed in more detail for their contribution to AA exposure (**chapter 3.2**). In this context, special emphasis was given to the AA level of bread crust obtained from einkorn and emmer flours. As the pseudocereals amaranth and quinoa provide starch-rich grains and their use is partly similar to wheat, the idea was born to discuss them for their level of free Asn in flour and the AA formation in popped grains (**chapter 3.3**). At the end of this chapter strongly needed future research is discussed, as well as their prioritized implementation (**chapter 3.4**).*

3.1 Impact of harvest timing on free asparagine formation in cereals

The right cereal harvest timing mainly serves two aims. First, a low grain water content is important for a safe storage avoiding the infestation with mould. Second, a delayed harvest, accompanied by warm and wet weather conditions could lead to pre-harvest sprouting. Both negatively affect important baking quality traits like protein and starch.
Besides these aspects, free Asn is affected by harvest conditions, too. Simsek et al. (2014) reported a high increase in free Asn concentration following pre-harvest sprouting. In this context, pre-harvest sprouting mostly occurs during a postponed harvest accompanied by wet climate conditions. The research group further observed that, during activating the enzyme endoprotease, proteins were degraded, which is supposed to lead to an increase in free Asn.
These findings correspond with studies of Lea et al. (2007), which revealed the dramatic increase of free Asn by seed germination. As sprouting is nothing else than seed germination, raising Asn concentrations by sprouting is quite logical. This fits well to the plant development after sowing, as free Asn must be established due to its role within the nitrogen (N) metabolism concerning the ability of Asn to uptake, transport and store N in plants (Pate et al., 1980, Marschner et al., 1995).
Going further, Navrotskyi et al. (2017) reported a high elevation of Asn caused by solar radiation due to a delayed harvest. Next to a covered sprouting effect, it is quite imaginable that higher temperatures led to a fastened senescence, which could have forced the transfer of soluble N from different plant parts into the grain. Halford et al. (2015) reported that amino acids can highly accumulate during senescence. Thus, since the amino acid Asn is known as transport and storage form for N (Lea et al., 2007), elevated Asn amounts in grains can be explained by a higher transferring rate of soluble N. Furthermore, during phases of late N availability, proportion of amide-N, mostly free Asn could have been raised in grain (Schwab, 1936). Gianibelli & Sarandon (1991) observed an increase in soluble proteins above certain protein amounts. These soluble proteins could mostly contain free Asn and may appear during full grain maturity. In consequence, delaying harvest must be inhibited as it causes the increase of free Asn in several ways.
Motzo et al. (2007) reported a protein increasing effect of a later sowing date in durum wheat by up to 3 %. This was mainly due to a lower grain weight. They further found a high N translocation from vegetative plant parts towards grain. Around 78% of grain N was caused by shifting from plant tissue to grain. In this context different maturity stages of cereals might lead to varied N levels when comparing plant compartments.
Thus, the question arose if an earlier harvest could lead to a lower free Asn accumulation in grain. Studies investigating such an issue are not available. Hence, we established a small study investigating the effect of grain maturation on Asn amount.
As cropping systems differ in their applied crop management strategies, we analyzed the effect for both conventional and organic farming systems.

Material and methods

From the field trial in paper 2 during the growing season 2007/2008, grain samples of cereals wheat, spelt, rye and einkorn (Table 1) were collected weekly after milk ripeness of grain was reached (15/06/08 rye, 22/0/08 all other species) until full maturity six weeks later. Referring to Zadoks et al. (1974) this corresponds with growing stage 73 and 92. In this context both cropping systems (conventional and organic) were investigated separately (see paper 2). After spike sampling and drying, grains were threshed and milled to flour. The details of the experimental design, agronomical practices and applied methods concerning analyzes of grain yield, crude protein, sedimentation value and free Asn are presented in the chapter "Paper 2". Grain water content was analyzed by pulling grains out of spikes and weighting them before and after drying at 105 °C for 24 h.

Table 1: Used cultivars of cereal species wheat, spelt, rye and einkorn, separated by the cropping system (conventional and organic).

Species	Conventional trial cultivars	Organic trial cultivars
Wheat	Bussard(E*), Enorm(E), Ludwig(A*), Tommi(A)	Bussard(E), Akteur(E), Capo(E), Naturastar(A), Ludwig(A),
Spelt	Franckenkorn, Schwabenkorn	Schwabenspelz, Ceralio, Oberkulmer Rotkorn, Franckenkorn, Schwabenkorn
Rye	Nikita, Recrut	Amilo, Nikita, Recrut, Danko, Pollino
Einkorn	-	Terzino

*E: highest baking quality, *A: high baking quality

Results

Conventional trial:

Figure 3 shows that the Asn concentration in grain flour increased constantly starting two weeks before full maturity (grain harvest). Except for wheat cultivar Bussard, each cultivar regardless of cereal species increased significantly in grain Asn amount. The effect of harvest timing was also obvious for cultivar Ludwig, but without statistically significant proof. Comparing only the 5th and 6th harvest date, there were still significant differences in free Asn content of spelt and rye cultivars. For wheat only one in four cultivars showed a significant impact. Nevertheless, a grain harvest just one week prior to the "normal" harvest (6th harvest week) led to a significantly lower Asn level within each cereal species. Wheat cultivar Tommi and spelt cultivar Schwabenkorn doubled in Asn concentration within one week. For rye cultivars an increasing effect of around 20 to 30% was reached. It seems that protein synthesis decelerates towards grain harvest, which led to an increase of amino acid free Asn in grain.

Thus, dependent on cultivar choice, the strategy of an earlier harvest seems to be well applicable to reduce Asn in cereals.

However, also quality trait crude protein and water content of grains have to be considered as baking quality should not suffer and grain storage should not be endangered by this cultivating strategy.

Water content of grains at week 4 and 5 after milk ripeness ranged between 23 and 29% depending on species. For a safe storage water content should be around 15 to 16%. Thus, if lower levels of Asn are the main target drying costs must be taken into account before a safe storage is assured.

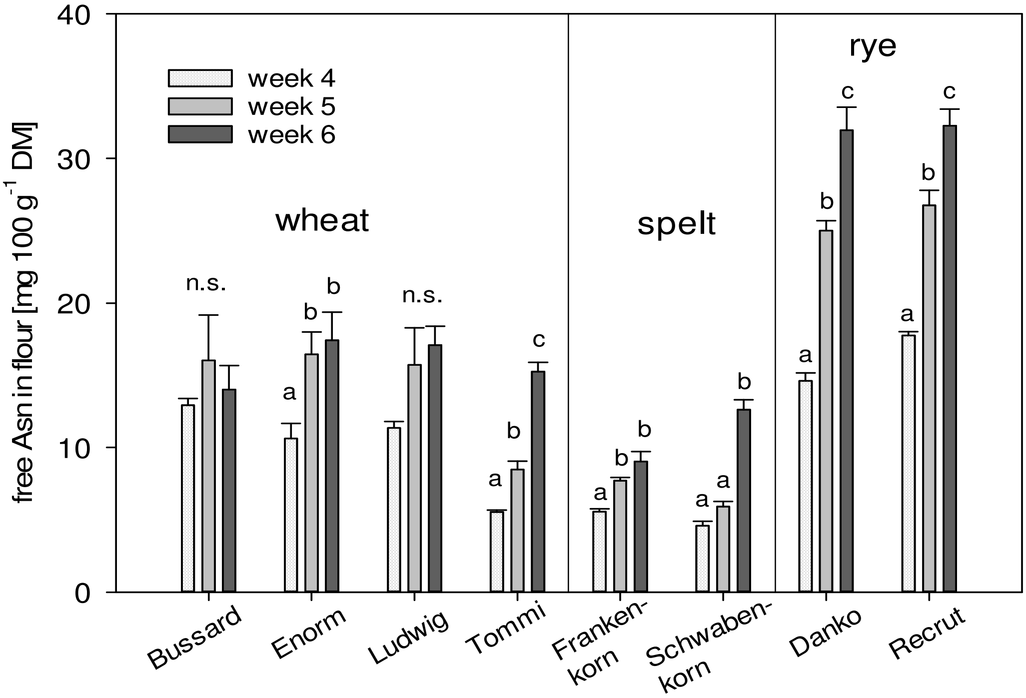


Figure 3: Free Asn level of 4th, 5th and 6th week after milk ripeness of conventionally cropped cereal species and cultivars. (Same letters indicate no significant difference, n.s. not significant, $\alpha \leq 0.05$, Tukey Test).

Nevertheless, most importantly, the crude protein as the main trait for the baking quality of wheat was not decreased by shifting grain harvest to 4th or 5th sampling date. Crude protein of 4th sampling date ranged between 12.5% to 15% for wheat and was around 16% for spelt. The level of protein increased towards the 5th sampling as it ranged between 13 and 16% (wheat) and 16.5 to 17% (spelt). Towards the 6th sampling level of crude protein dropped a little reaching 13 to 15.5%. This was most likely caused by a dilution effect as grain yield increased. A dilution effect of Asn concentration by starch during grain development due to higher yields was not observed.

The small decrease of crude protein content comparing 5th an 6th sampling can also refer to an increase in free Asn. Schwab (1936) reported a raised proportion of amide-N mostly free Asn in grain during phases of late N availability while an increase in soluble proteins above certain protein amounts was reported by Gianibelli & Sarandon (1991).

Organic trial:

Contrary to the conventional trial free Asn was only affected to a small extent by harvest timing (Figure 4) if cereals were treated organically. Wheat cultivars showed absolutely no effect on Asn level in case of an earlier harvest. However, depending on the cultivar for spelt and rye, a significant effect of harvest timing was obvious. For einkorn a similar trend was given but without statistically significant proof. Interestingly a slight decreasing effect within some wheat cultivars was analyzed. Lea et al. (2007) reported an elevated Asn amount if available N is high and crude protein synthesis is low. Keeping in mind that N supply in organic farming is much lower it can be proposed that the available N was fully integrated into protein synthesis compared to the conventional trial where N seemed to accumulate in grain. The increase of free Asn for both rye cultivars indicated an Asn accumulation in grain caused by a lower protein synthesis.

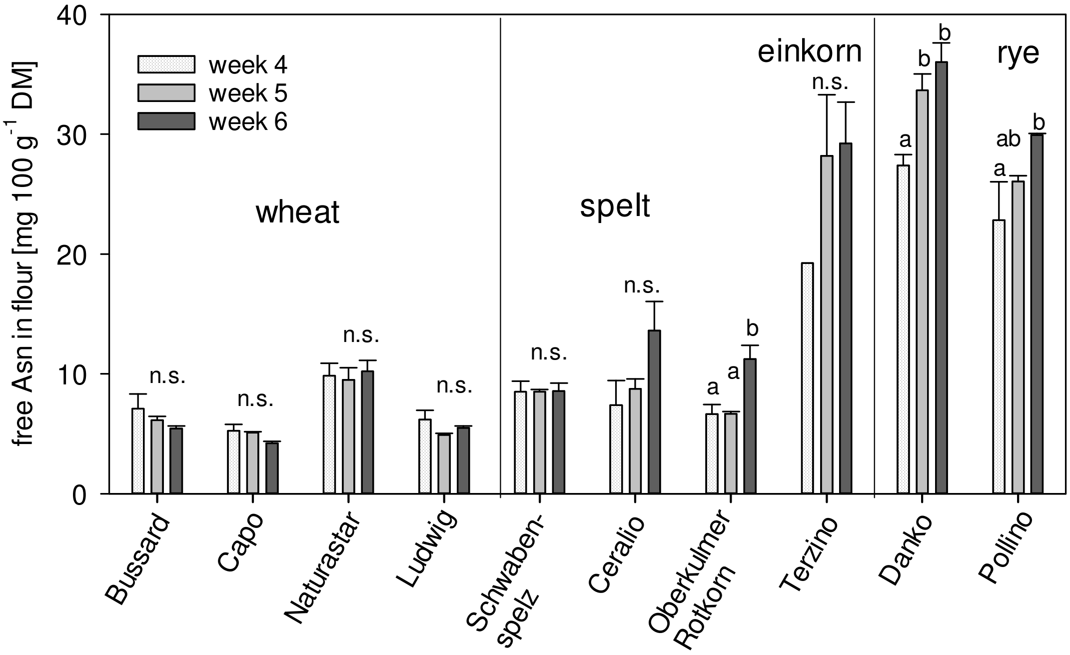


Figure 4: Free Asn level of 4th, 5th and 6th week after milk ripeness of organically cropped cereal species and cultivars. (Same letters indicate no significant difference, n.s. not significant, α≤0.05, Tukey Test).

The water content decreased within two weeks from 20 to 16.6% for wheat, 18 to 15.9% for spelt, 26 to 20.6% for rye and 30 to 17% for einkorn. At 5th sampling dry matter content for wheat and spelt was already sufficient for a safe storage. However, rye and einkorn samples have to be dried before storage.

Level of crude protein of wheat increased during 1th to 4th sampling and after that was stable reaching around 10.3% at 4th, 5th and 6th sampling. For spelt, rye and einkorn no clear effect of harvest timing could be seen as crude protein fluctuated reaching a final amount of 16% (spelt), 8.5% (rye) and 15% (einkorn).

Nevertheless, the values correspond well with the N content in straw samples which diminished from 1th to 4th sampling and then increased slightly until grain harvest within all species. This might be explained by several theories. Firstly, straw N level decreased as effect of transferring N into grain; secondly after grain development finished, N accumulated in straw. Additionally, it might be caused by an enrichment effect as other substances were transferred from straw to roots or grain or water loss.

As rye is known for lower protein levels, protein synthesis might be accomplished earlier leading to a higher accumulation of Asn in grain as a consequence of a higher transferring rate of soluble N fractions. In contrast einkorn also reached a high Asn concentration while crude protein was almost double as high compared to rye. However, N in straw of einkorn was higher than that found in rye and simultaneously N in straw of einkorn was diminished during senescence by a higher rate. This could have led to more soluble N being transferred into the grains of einkorn. This fits quite well to Geisslitz et al. (2019), who reported a high N efficiency of einkorn by calculating the ratio of kernel yield and level of N fertilizer.

Thus, it can be proposed that einkorn is able to transfer higher rates of N into the grain. Further, Lea et al (2007) stated that Asn is a major product during senescence of leaves. It can be supposed that dynamics of senescence differ between rye and einkorn leading to various levels of Asn. Nevertheless, also protein structure of both species differs, which enables similar Asn concentrations and at the same time different protein levels. As rye grain mostly includes the prolamin secalin which is very poor in sulfur rich amino acids, einkorn grains contain mostly gliadins (Miedaner & Longin, 2012, Richter, 1998). Thus, it can be supposed that both species accumulate Asn in grain for different reasons.

Nevertheless, above all the conventional cropping systems presented a high increase in free Asn during grain development. This fits well to findings of Raffan & Halford (2019), which showed that during grain maturity gene expression activity of Asn synthesis genes, e.g. Asn-synthetase, increased successively after the anthesis of wheat. Thus, the raising activity of genes responsible for Asn synthesis could have elevated the Asn amount during the last two weeks of grain maturity. The authors also reported in their review that nutrient supply has an impact on gene expression. A higher N availability within rye plants, 21 days after anthesis led to an increase of glutamine synthetase gene expression causing higher glutamine levels. As glutamine and Asn both are integrated into the N metabolism of plants, the expression of Asn-synthetase might be raised simultaneously by a higher N supply. This also means that if N supply after anthesis is low, gene expression is low. As the Asn levels shown in Figure 3 rose significantly above all within the conventionally cropped samples, it can be supposed that gene expression of Asn-synthetase was increased by a better N supply. In contrast, the organically cultivated cereals obtained a low N supply, which could have led to lower gene expression and consequently lower Asn synthesis. As N supply is within the tested cereal species especially for wheat most important, the higher N fertilization may explain the stronger reaction during the conventional trial and the different effect regarding both cropping systems seems to be quite logical.

Sticking to the N supply, some studies reported elevated Asn amounts depending on sulfur deficiency (Muttucumaru et al., 2006, Granvogl et al., 2007). Gao et al., (2016) reported an increasing effect for wheat on Asn-synthetase gene expression if sulfur deficiency occurs, while Postles et al. (2016) stated the same for rye. Such an effect could also explain the rising Asn levels in the conventional trial. However, no shortage of sulfur could be observed. Manure was fertilized in the organic trial. As manure can contain sulfur, an effect on gene expression behaviour by sulfur deficiency does not seem obvious. This fits well to the low response of the organically grown samples to harvest timing concerning Asn amount.

Nevertheless, site specific conditions like salt and osmotic stress were revealed by Wang et al. (2005) to increase gene expression of Asn-synthetase. As the trials were carried out at two different sites such effects must also be taken into account. Yet, weather conditions of both sites were very similar during the growing season 2007/2008.

Concluding remarks

Independent of the cropping system an early grain harvest could be an additional strategy to minimize levels of free Asn. This strategy seems most promising within conventional

farming systems. Baking quality traits were not affected negatively while grain yield lowered. Costs for extra drying of grain have to be considered.
Finally, it was obvious that species and cultivars differed in their reaction of harvest timing on free Asn accumulation. This shows that both, species and cultivars differ in their ability to transfer free Asn from straw into grain. Thus, when applying harvest timing as a strategy to lower free Asn, the right choice of cereal species and cultivars must also be taken into account.
However, there is a clear need for further research that reveals in much more detail how harvest timing can contribute to lowering free Asn levels in cereals.

3.2 Ancient cereals einkorn and emmer

Ancient cereal species like einkorn (*Triticum monococcum* L.) and emmer (*Triticum dicoccum* L.) have drawn consumers' interest during the last years as they promise a fine taste and healthy ingredients. From an agronomic point of view these grains provide a higher resistance towards pests and diseases and are so-called marginal crops (Kling et al., 2006, Wieser, 2006). Thus, they are mostly cropped under organic farming conditions.
Both species belong to the oldest cereal species which were already cultivated 10,000 years ago (Nesbitt & Samuel, 1995, Konvalina et al., 2008). Normally, each modern soft wheat cultivar originates from both ancient cereals.
Compared to modern wheats they have a smaller genome (Wieser et al., 2009), which may lead to lower baking properties. However, einkorn and emmer have a high content of minerals e.g. calcium, manganese and sulfur (Løje et al., 2003, Brandolini et al., 2008). Especially the high level of secondary compounds like yellow pigments, carotenoids and lutein particularly in einkorn makes them healthy cereals (Brandolini et al., 2008, Hidalgo & Brandolini et al., 2014). Although both cereals reach high crude protein contents, they have an inappropriate baking performance due to low protein quality (Longin et al., 2016). Contrary to the high nutritional assumption level of AA precursor free Asn was reported as very high for einkorn compared to different kinds of cereals (Stockmann et al. 2018, Corol et al., 2016). Thus, there might be a mismatch between healthy vs. harmful compounds.
To our knowledge only the two studies mentioned above investigated einkorn and emmer for their Asn levels. While Stockmann et al. (2018) just analyzed three einkorn and two emmer cultivars, Corol et al. (2016) analyzed free Asn in wholemeal flour. Thus, it seems necessary to investigate more samples of these ancient cereals concerning their Asn accumulation ability.
Additionally, AA level in bred crust of both kinds have never been reported. Hence, there is a need for such information to estimate AA exposure by the consumption of ancient cereals like einkorn and emmer. In summary, out of the highlighted results of paper 5, two central questions arose.

1. Why is the Asn concentration this high especially in einkorn?
2. Does the high Asn amount lead to high AA levels in baked bread?

For this reason, six einkorn and nine emmer flour samples and breads made from these flours were analyzed for their Asn content in flour and the AA formation in baked bread.

Material and methods

The flour and bread samples were obtained from the Department of Agronomy and Plant Breeding I of the University of Gießen. The samples originated from different millers. No information of cultivar, growing location, crop management and weather conditions were available. Nevertheless, all samples were grown under the conditions of organic farming. This allows for an estimation on cropping practices concerning N supply and pest treatments. The analyzes of free Asn were carried out as described in the material and methods section of chapter “Paper 2”. For investigating the formed AA content of bread, standardised baking trials were carried out at the Federal Centre for Cereal, Potato and Starch Technology, Detmold. Tin loaves (Kastenbrote) were baked using the following recipe:

580 g flour, 360 ml water, 2% baker's yeast, 1.5% salt, 1% sugar, 1% fat

After fermentation at 32°C for two hours, bread was baked at 210°C for 50 minutes.

As the study of Surdyk et al. (2004) revealed that around 99% of AA is located in crust the crust of each bread was removed, dried and finally crushed by a mortar for analyzing AA until a fine powder was obtained. Afterwards 5 g of the powder was put into a folded filter and doused by 50 ml iso hexane for fat removal. The sample was then dried overnight and put into an Erlenmeyer flask. The following preparation steps and the analyzes of AA content are described in the material and methods section of the chapter “Paper 2”.

Results

Presenting a mean value of 43.0 mg Asn 100 g^{-1}, einkorn flour samples significantly surpassed emmer who reached 27.0 mg Asn 100 g^{-1} (Table 2). While just a small range of Asn was found in einkorn samples, emmer samples more than doubled in free Asn concentration ranging from 16.4 to 39.5 mg 100 g^{-1}. Thus, after gaining these values, it seems that the ancient species einkorn and emmer have a high ability to accumulate free Asn in grains, similar to rye.

Table 2: Free Asn level of einkorn and emmer flour samples. Different small letter within species and capital letter between both species show significant differences (α≤0.05, Tukey Test).

Einkorn samples	free Asn [mg 100 g^{-1} DM]	Emmer samples	free Asn [mg 100 g^{-1} DM]
E1	43.0 abc	EM1	16.6 d
E2	45.9 a	EM2	28.6 c
E3	44.6 ab	EM3	16.4 d
E4	41.5 bc	EM4	39.5 a
E5	42.1 bc	EM5	34.8 b
E6	41.1 c	EM6	27.7 c
		EM7	32.7 b
		EM8	18.4 c
		EM9	28.0 d
Mean	43.0 A		27.0 B

Observating the AA formation in bread, this trait behaved in a similar way as Asn did. The highest AA amount was found for einkorn by 498 µg kg^{-1} including a small range of AA (350 to 498 µg kg^{-1}). Emmer samples reached the lowest AA content of 54 µg kg^{-1} and ranged high until 237 µg kg^{-1}. Formation of AA in bread crust and free Asn in flours showed a high relation by R^2 0.6*** (Figure 5).
In contrast, there was no relation found between the parameter crude protein*free Asn and crude protein*AA, shown by R^2 of 0.03 & 0.05.

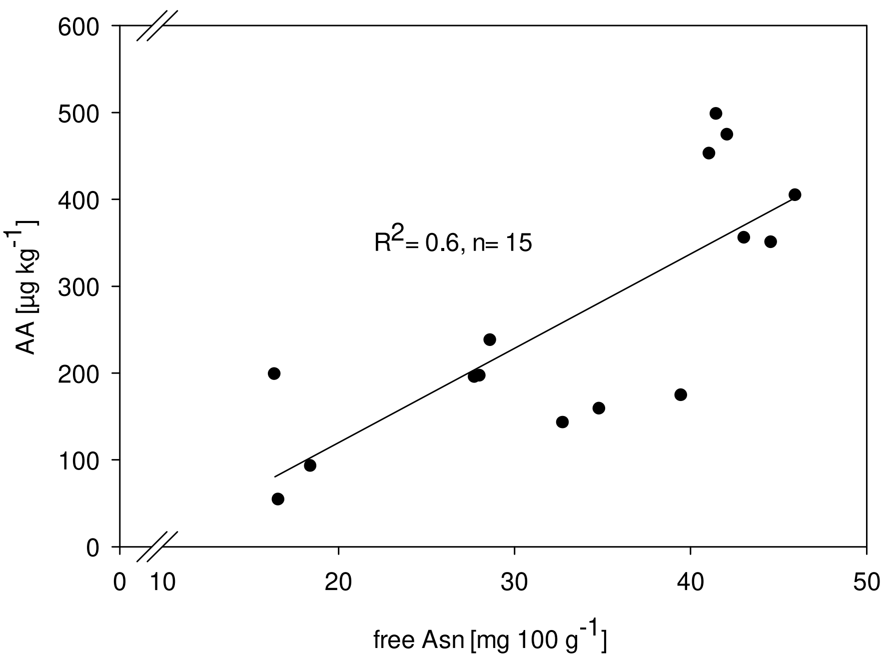


Figure 5: Correlation of free Asn [mg 100 g^{-1}] in flour and AA [µg kg^{-1}] in crust of baked breads of analyzed einkorn and emmer samples.

However, comparing the obtained AA content with benchmark levels recommended by the commission regulation of the EU (2017/2158) most of the analyzed AA concentrations exceeded almost 5-fold the recommended benchmark level of 100 µg kg^{-1} (Annex IV of the regulation for soft bread, category b: soft bread other than wheat-based bread).
That clearly shows that products based on ancient cereal species einkorn and emmer may contribute to a high AA exposure when consumed daily. This is contrary to the supposed healthy image of those cereals. Nevertheless, it remains unclear if the benchmark level refers to the whole bread or just the crust.

Returning to free Asn level in cereals, it was obvious that ancient grains include a high amount of free Asn compared to modern wheat or spelt. The concentrations found in grains even topped the well-studied Asn content found in rye. The question is, where do they come from? Different possible theories might be taken into account:

1. Grain composition incl. protein structure/composition
2. N efficiency and transferring ability of N from plant tissues to grain
3. Grain size and shape of cereals

Crude protein level of einkorn and emmer in this trial was generally very high ranging from 12 to 18.2% for einkorn and 12.4 to 21.4% for emmer. In contrast sedimentation value (describes the protein quality by swelling ability) was very poor as emmer reached an average of 11.4 ml (highest 19.0 ml) while einkorn reached the absolutely lowest value with a mean of 6.7 ml (highest was 8 ml). These very low contents allow the assumption that protein composition seems to be different from cereals synthesising a high sedimentation value like modern soft wheat. This was also shown in different studies, which described the low protein quality of ancient cereal species (Miedaner & Longin, 2012, Longing et al., 2016). Furthermore, gliadins were accumulated to a higher amount than glutenin in einkorn and emmer (Geisslitz et al., 2019). Thus, inferring from the analyzed trials and research studies, protein content and protein compositions seem to be highly involved in the Asn accumulation potential when comparing different cereal species. Hence, Table 3 depicts the cereals mostly used for bread making, describing their characteristics regarding quality traits.

Table 3: Common bread cereals and selected quality traits.

Cereal species	crude protein amount	sedimentation value	free Asn	bread making ability*
wheat	medium-high	high	medium	high
spelt	medium-high	medium	low-medium	medium
einkorn	very high	low	very high	low
emmer	very high	low	high	low
rye	low	low	very high	low

*refers to high volume bread

Especially for emmer, einkorn and rye it seems quite clear that baking quality in terms of low sedimentation values accompanied by a low bread making ability lead to high Asn accumulation in grain. Wheat in contrast, contains less free Asn and simultaneously has high baking ability, which is most likely due to high protein quality including glutenin.

For spelt, the lowest levels of free Asn were reported in paper 2 and 5 included in this thesis. It can be suggested that the medium sedimentation value accompanied by a medium level of crude protein and medium bread making ability led to such low free Asn levels.

Taking a closer look at grain composition, Curties et al. (2014) analyzed the effect of sulfur deficiency on Asn accumulation in grains of wheat and rye. The level of free Asn elevated highly in wheat flour, most likely due to the effect on sulfur-rich proteins like glutenin. Such proteins contain plenty of the amino acid cysteine and methionine. Both amino acids include sulfur molecules. In case of sulfur loss, the synthesis of such amino acids might stop leading to either other proteins, e.g. gliadin, or the accumulation of other amino acids, e.g. free Asn. Halford et al. (2015) observed an increase of Asn-synthetase gene expression under sulfur deficiency of wheat. Additionally, they reported the enzyme protein kinase might be involved in Asn-synthetase gene expression. Thus, it seems possible that this enzyme is connected to Asn-synthetase and protein storage.

Interestingly, Curtis et al. (2014) did not analyze increased free Asn levels within rye if sulfur was in deficit. As rye contains a very low level of glutenin and higher amounts of the prolamin secalin (Richter, 1998) the varying reactions concerning sulfur supply on amino acid accumulation can be explained by the divergent protein composition.

Concerning einkorn and emmer, although both ancient cereal species reached a very high crude protein amount it seems that different protein proportions and structures led to an accumulation of amino acids like free Asn in the grain. Curtis et al. (2018) stated that Asn formation and decomposition are associated with the amino acid and N metabolism in plants. Thus, it seems obvious that free Asn accumulation is affected by protein structure. Moreover, Claus et al. (2006a) observed that the effect of higher protein levels is accompanied by increasing levels of amino acids. Such findings correspond well with Shewry et al. (2013), who reported elevated protein levels under a raised N supply. In this regard, Godfrey et al. (2010) recognized that a higher protein concentration is mostly caused by a higher level of the storage protein gliadin. Also, Garg et al. (2005) reported various protein fractions comparing several cereal species. Thus, the high levels of free Asn in einkorn and emmer can be explained by higher total protein amounts but the type of protein differs from that of modern wheat. After all, the relation between free Asn, amino acids and crude protein composition must be taken into account when analyzing the differences in the range of free Asn between cereal species.

However, as breeding continues, new varieties of ancient cereals can deliver a better baking quality. Geisslitz et al. (2019) analyzed several einkorn and emmer genotypes and found some genotypes with a much better protein composition leading to a better baking performance. It seems quite interesting to screen new einkorn and emmer cultivars for their level of free Asn. Especially, as it was shown by Longin et al. (2016) that the known relation between grain yield and crude protein (more grain yield less crude protein and the other way around) could not be found in einkorn. Thus, breeding new varieties can deliver both a good grain yield and baking quality, and at the same time lower levels of free Asn.

Nevertheless, grain composition can also be highly affected by weather conditions and crop management strategies as both have a tremendous impact on gene expression of Asn-synthesis as well as protein-synthesis enzymes (Halford, et al., 2015, Raffan & Halford et al., 2018, Shewry et al., 2018). High temperatures during grain filling can influence starch accumulation negatively while protein content especially of the prolamin gliadin fraction increased (Gooding et al., 2003). This emphasizes that climate conditions can change grain composition.

Nevertheless, also N efficiency of cereal species and cultivars including a divergent N transferring ability during senescence seems to play a crucial role in accumulating free Asn in grains. This has already been explained in chapter 3.1. “Impact of harvest timing on free asparagine formation in cereals”.

A new realisation concerning free Asn accumulation seems to be grain size. Einkorn and rye have a very high free Asn accumulation potential and at the same time a very small grain size if compared to the most common cereal species like spelt, wheat, oat and maize. Such effects should also be taken into account. As plant density, grains per spike, tillering ability may influence grain size, such issues must also be considered not only for species but for cultivars, too. Such effects have already been discussed in the chapter “Paper 4”.

Finally, as cereals differ in their grain size, shape and form including the amount and thickness of the aleurone layer the distribution of grain components may differ. Shewry et al. (2009) reported a higher level of free Asn in bran compared to white flour. Hence, in relation to grain surface smaller grains may contain more bran leading to elevated free Asn levels.

Concluding remarks

By contributing a high level of free Asn in flour as well as AA in baked bread it is without doubt that food products based on ancient cereal species einkorn and emmer can become a real health-threatening issue.

Nevertheless, as breeding may generate new varieties, which deliver a good baking performance accompanied by a sufficient grain yield, free Asn accumulation potential should also be addressed simultaneously.

However, after revealing that cereals differ in their grain free Asn accumulation potential, upcoming studies should focus on grain size, N efficiency, N transferring ability and protein composition concerning free Asn concentration.

3.3 Pseudocereals amaranth and quinoa

Amaranth and quinoa are so called superfoods as they offer a high content of health beneficial vitamins, fatty acids, or essential amino acids like lysine and minerals (Janssen et al., 2017). Plus, they are gluten free and thus important for people who suffer from gluten intolerance or sensitivity (Janssen et al., 2017). Thus, during the last years grains of amaranth and quinoa have gained high attention and their consumptions is continuously growing.

Both ancient species have been cropped since around 3,500 to 4,000 B.C. (Aufhammer, 2000). They originated mostly from Middle to South America. Major cultivation takes place in Peru, Bolivia, Ecuador, China and Russia (Aufhammer, 2000). Nevertheless, during the last years cultivation in Germany has grown to some extent. As grain yields are low compared to wheat and cropping might be challenging, the need to sell at higher prices forces the cultivation under organic farming, where higher prices seem to be more accepted.

As they provide starch rich raw material, similar to cereals, amaranth and quinoa are also called pseudocereals. Further, their crude protein levels are very high reaching 12 to 21% (Palombini et al., 2013, Präger et al., 2018) and the processing conditions concerning heat treatments of those grains are similar to common cereals. Especially the reported high level of protein content similar to those found within ancient cereal species einkorn and the high concentrations of amino acids, strongly lead to expected high values of free Asn, the main precursor of AA. Further, Gamel et al., (2004) observed during seed germination elevated aspartic acid by decreasing prolamins. Amino acid screening of quinoa and amaranth (Palombini et al., 2013, Präger et al., 2018) showed that aspartic acid and glutamic acid were highest in non-essential amino acids. As both are highly related to free Asn, pseudocereals might contribute to AA exposure when consuming these foods.

Food products based on amaranth and quinoa are mainly popped, toasted or roasted grains. Those grains are parts of breakfast cereals and candy bars or they can be bought only as popped grains.

Studies concerning level of AA in food products are not available to date. Graeff et al. (2008) investigated heated flours of amaranth and quinoa genotypes for their AA formation potential. Highest AA amount was found for quinoa genotypes reaching a maximum of 990 ng 100 g^{-1} (mean 613 ng 100 g^{-1}) while amaranth genotypes reached a maximum of 492 ng 100 g^{-1} (mean 450 ng 100 g^{-1}). A clear genotype effect was obvious within quinoa while only a small effect of amaranth genotypes was reported without being significant. The formed mean AA levels comparing both ancient species were significantly different.
These observed AA contents in heated flours might presume a health relation as the Commission regulation of the EU announced in 2017 benchmark levels, which should not be exceeded. Within the regulation in Annex IV for breakfast cereals (bran products and whole grain cereals, gun puffed grain) a benchmark level of 300 µg kg^{-1} is fixed. Hence, both pseudocereals surpassed this benchmark. Especially the heated quinoa flours reached AA amounts three times as high. Since heated flours are normally not used as food product, analyzing heated (popped) amaranth and quinoa grains seems highly essential to estimate AA exposure.
For this purpose, amaranth and quinoa grain samples from the same field trials reported by Graeff et al. (2008) were heat treated (popped) and analyzed for their AA formation. Additionally, free Asn concentration and reducing sugars content in flour was measured.

Material and methods

For material and methods concerning sample material, site description and cultivation practices see Graeff et al. (2008). In addition to the six amaranth and three quinoa samples, two amaranth and one quinoa grain sample were selected from an organic market. Free Asn in flours were analyzed as described in the chapter “Paper 2” of this thesis. Analyzes of the amount of reducing sugars was performed using the method of Luff Schoorl (Matissek et al., 1992). Concerning AA formation, determination was done by heating grains of each genotype. Grains were distributed on a metal plate and then put into an oven. Oven temperature was 415 to 425 °C while the metal plate provided a heat temperature of 190 °C. Grains were heat treated for 10 seconds until they popped. After cooling, the popped grains were analyzed for AA as described in the chapter “Paper 2”.

Results

Concentration of free Asn and reducing sugars in flours are presented in Table 4. Quinoa samples reached very little free Asn concentrations. The minimum was 0.54 mg 100 g^{-1} while maximum level was reached by genotype Q1 with 1.88 mg 100 g^{-1}. Compared to cereals (Weber et al., 2007, Stockmann et al., 2018b & 2019a) the values are approximately 60-fold lower. Nevertheless, a difference of 70% was observed within individual quinoa samples. But even the highest Asn content of 1.88 mg 100 g^{-1}, meant a very low amount. Thus, it seems that free Asn in quinoa offers the possibility to form low AA levels during heat processing. Indeed, amaranth samples showed around four times more Asn but in contrast to “normal” cereals, the mean of 4.4 mg free Asn 100 g^{-1} can still be considered as very low. Hence, the expected AA formation for amaranth seems to be low, too.

Table 4: Concentration of free Asn and reducing sugars of amaranth and quinoa samples. Different letters next to numbers within each trait indicate significant differences (α≤0.05, Tukey Test).

Amaranth samples	**free Asn [mg 100 g^{-1}]**	**reducing sugars [mg 100 g^{-1}]**
A1	7.13 g	1234 ab
A2	3.92 cde	1141 ab
A3	3.50 bc	1552 b
Pastewny	4.66 f	989 ab
Bärenkrafft	3.16 a	1293 ab
Amar	4.68 f	1068 ab
A7	4.24 ef	1465 b
A8	4.12 def	569 a
Quinoa samples		
Q1	1.88 c	1707 a
407	0.92 ab	3647 ab
Faro	1.06 b	3305 b
Tango	0.54 a	4620 c

Reducing sugars (Table 4) were highest for quinoa reaching around 3000 mg 100 g^{-1} while amaranth reached a level which was around 30 % lower (mean: 1160 mg 100g^{-1}). However, reducing sugars were much higher than free Asn leading to the expectation that they will not affect AA formation. In cereals free Asn is known to be the main precursor for AA formation thus the same can be supposed for pseudocereals. If free Asn reacts with reducing sugars to form AA, less free Asn is available for a reaction with reducing sugars. This is confirmed by the analyzed regression of reducing sugars and AA, which was poor (R^2= 0.16).

The low free Asn amount for both ancient plant species, and especially for quinoa samples indicated that free Asn makes up a smaller part in grain compared to sugar. This is in contrast to cereals where Asn is highly needed for uptake, transport and storage of N (Lea et al. 2007). However, the minor role of free Asn might be explained by N supply. While cereals need around 180 to 220 kg N ha^{-1} to deliver high yields and quality ingredients like proteins, amaranth and quinoa are only fertilized by around 80 to 90 kg N ha^{-1}. A higher N level only increases plant biomass (stems & leaves) and may cause lodging while the grain amount is only slightly increased (Schulte et al., 2005). Such differences in N application rates may lead to a smaller relevance of free Asn in grain metabolism in general. Moreover, N use efficiency was shown to decrease by raised N fertilisation for amaranth and was not affected for quinoa (Schulte et al., 2005). This indicates that even if most of the fertilized N was taken up, only small amounts were transferred to grain, finally leading to a lower harvest index e.g. of amaranth. Further, both pseudocereals originated from Middle or South America, where day length is different from Germany. These species are only slightly adopted to German daylength conditions, which leads to various reactions concerning photoperiodism. In consequence, maturation of amaranth and quinoa takes longer, leading to less senescence and plants might still be green during grain harvest. This might also lead to lower translocation rates of free Asn from plant biomass to grain.

Further, grains of amaranth and quinoa have a much smaller size (TKW: amaranth ≈ 0.1 g 1000 grains^{-1}, quinoa ≈ 2 g 1000 grans^{-1}) than cereals (TKW: ≈ 30-45 g 1000 grans^{-1}), which might implicate diverse grain compositions. As already described in chapter 3.2 (einkorn and emmer) also grain aleuronic layer might be smaller leading to less Asn.
Finally, amaranth and quinoa proteins mostly consist of albumin and globulin, while in cereals prolamin and glutelin are the major proteins (Janssen et al., 2016). Such differences should additionally be regarded in terms of low Asn concentrations in pseudocereal grains.
Determination of AA are depicted in Figure 6. Realising that free Asn of amaranth was around 75% higher than the determined Asn level in quinoa a similar distribution can be seen for AA.
Comparing both species AA level of amaranth heat-treated grains reached a mean of 145 µg kg^{-1} and by this AA level was significantly higher compared to quinoa samples where a mean of 63 µg kg^{-1} was analyzed.
In quinoa samples AA ranged from 8 to 109 µg kg^{-1} and from 76 to 227 µg kg^{-1} in amaranth. A high impact of genotype was obvious in both plant species, as shown in Figure 6. Interestingly, from very low free Asn concentrations partially high AA levels were formed during heating. However, comparing them with the current benchmark levels of the Commission Regulation of the EU, where 300 µg kg^{-1} is the fixed amount for bran products and whole grain cereals (e.g. gun puffed grains), all samples are below this amount.

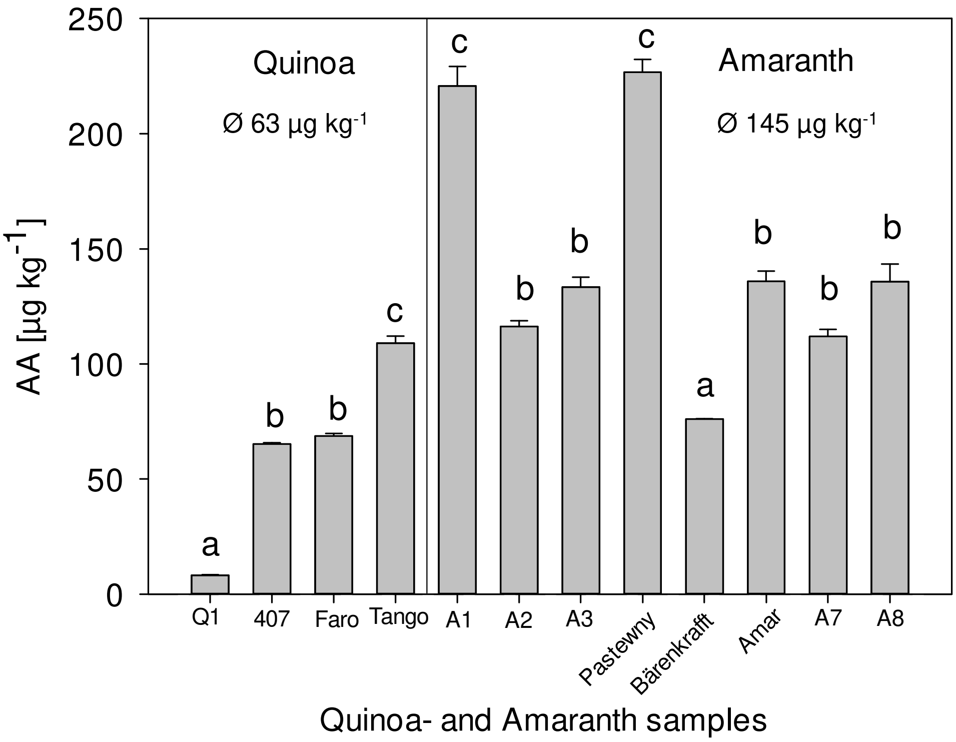


Figure 6: AA level of popped quinoa and amaranth grains. Different letters within each species indicates significant differences (α≤0.05, t-Test).

Nevertheless, although the AA contents were under the benchmark level it was a surprise, that such low free Asn concentrations partially led to a high AA formation.
Compared to cereals einkorn and emmer (chapter 3.2) the factor between free Asn in flour and AA level in bread or popped grains was much higher (see Table 5). Taking the average

of both species (*poaceae vs. amaranthaceae*) the pseudocereals almost reached a 6-fold higher AA formation from the present free Asn.

Table 5: Calculated factor between free Asn concentration in flour and formed AA in bread or heated grains of quinoa, amaranth, einkorn and emmer

species	factor between free Asn and AA formation
quinoa	57
amaranth	32
einkorn	10
emmer	6

Such differences might lead to the assumption that either AA formation by free Asn of pseudocereals is much more efficient compared to cereals, or other compounds except free Asn might contribute to AA formation. Analyzing the regression of free Asn and AA indicated a close relation between free Asn and formed AA by R^2 = 0.58** (Figure 7). Nevertheless, alternative formation pathways of AA have to be considered too. Especially after separating quinoa from amaranth a close negative relation was found with R^2= 0.9* for quinoa samples, while regression analyzes for amaranth showed an R^2 of 0.59*. Thus, other AA formation pathways have to be taken into account.

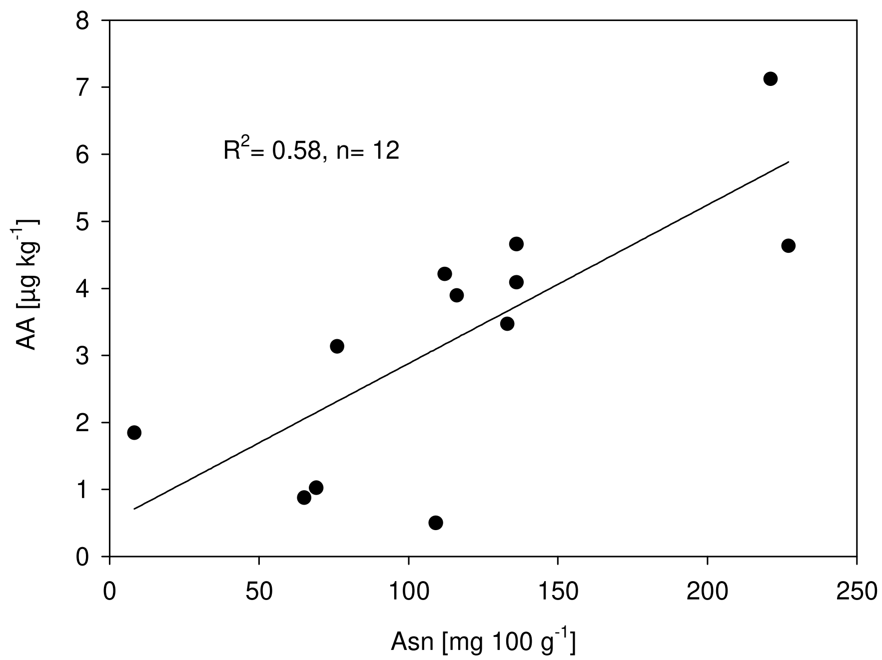


Figure 7: Relation between free Asn [mg 100 g^{-1} DM] in flour and AA [µg kg^{-1} DM] in heat treated (popped) grains of amaranth and quinoa samples.

Amrein et al. (2007) discovered substantial concentrations of AA in black canned olives, dried fruits and almonds. Keeping in mind that such foods highly differ from cereals, alternative formation pathways are obvious. In this context main differences compared to cereals are:

- free Asn content is lower,
- heating temperature during food processing is lower,
- and partialy high-water contents occur.

Casado & Montano (2008) reported no relation between known precursors and AA formation in black olives. The authors assumed that reducing sugars and amino acids are no AA precursors in olives as both occurred in small amounts and no correlation with AA was found. Nevertheless, they found up to 1578 µg AA kg^{-1} in black ripe olives while only a heat treatment of 121° C was applied. Similar findings were observed by Becalsci et al. (2011), who reported the formation of AA in prune products by applying temperatures lower than 100°C. In contrast to the study of Casado & Montano (2008), they attributed the AA formation in prune products to the presence of Asn and sugars in the raw material before heating.

However, since fatty acids and oxidised lipids were reported as being integrated into AA formation (Zamora & Hidalgo, 2008, Capuano et al., 2010), additional precursors or formation pathways seem evident. Therefore, the higher AA levels found in amaranth and quinoa (contrary to the small free Asn amount) seems to be explainable by the high content of fatty acids.

Moreover, Claus et al. (2006b) reported a pyrolytic AA formation from purified wheat gluten. The presence of amino acids was reported by Yaylayan et al. (2004), who found that amino acids, which can produce acrylic acid, might play a role in AA formation. As the amino acid content in both amaranth and quinoa are present in higher amounts, their integration into AA formation must be supposed.

Further, Yaylayan et al. (2004) reported that certain amino acids can generate pyruvic acid, which can be converted into acrylic acid and form AA by reaction with ammonia. Such amino acids are serine and cysteine. This corresponds well with the findings of Thanapornpoonpong (2004), which reported that amaranth and quinoa grains can include higher levels of sulfur containing amino acids like cysteine. Thus, AA formation of pseudocereals could have been forced by higher contents of sulfur-rich amino acids.

However, one thing might have affected the AA formation presented in Figure 6. For analysing free Asn white flours were used while AA analyzes were done on heated whole grain material. As free Asn in cereals is highly located in the aleuronic layer the small Asn contents might be a consequence of removing parts of the aleuronic layer. This might also explain the higher AA formation by low free Asn levels.

Nevertheless, a study of Salazar et al. (2012) revealed the mitigating effect of amaranth protein on AA formation in foods. Isolated amaranth protein reduced AA in cookies by 89% and in tortilla chips by 51 to 62%. Hence, AA formation during cooking may be influenced by proteins in both directions, increase or decrease. Similar findings were reported for buckwheat by Jing et al. (2019). Extracts from Tartary buckwheat seeds reduced AA in bread by up to 23.5%. As buckwheat belongs to the pseudocereals, those ancient plant species seems to have a natural lower AA formation potential.

Concluding remarks

As AA amount was below the postulated benchmark level of the EU, at this moment both ancient species do not seem to be a heath-threatening issue. Accompanied by high-value

compounds like vitamins, essential amino acids e.g. lysine and the absence of gluten, foods from pseudocereals seem rather to contribute to a healthy constitution.
However, if amaranth and quinoa become more and more popular, breeding varieties adopted to German climate conditions including a raised N fertilisation might need to be forced. In consequence, grain compositions may change including the relation of ingredients and thus AA could be an issue in future. Moreover, AA occurance depends on the prepared food product. Hence, there should be a continuous investigation of heated food products derived out of pseudocereals.

3.4 Addressing main future research needs

Concluding remarks

At the beginning of this thesis, research on agronomic strategies to influence the level of free Asn as the major precursor for AA within cereals had just started. Thus, this dissertation focussed on revealing the impact of the cropping system, nutrient supply, row distance, seed density, cereal species and cultivars grown organically on free Asn, which is considered as the major precursor of AA.

In this study the following main conclusions could be drawn:

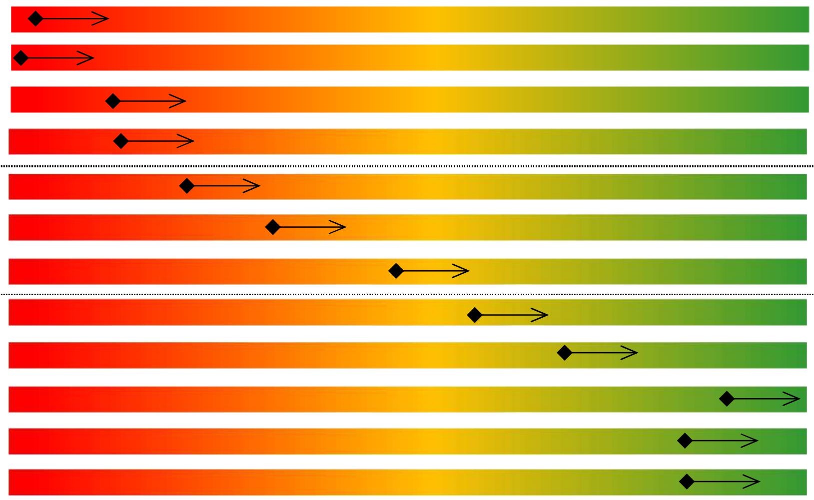
- Raising the implementation of organic farming applications will lead to safer food products originating from cereals. This fits well to the current movement in food product development, e.g. in Bavaria, which is trying to highly boost organic farming until 2020.
- The current decree of the German government to lower the overall nitrogen fertilization to an amount of 170 kg ha^{-1} within the framework of the fertilizer ordinance of 2017 forces a well-balanced nitrogen fertilization in winter wheat cultivation, independent of the chosen cropping system. This will lead to lower Asn levels while no negative effect on baking quality could be determined.
- Adjustment of sulfur fertilizing strategies concerning amount and S-type to lower free Asn level will not be necessary, if soils are sufficiently supplied with sulfur.
- Ensuring the requirements of the baking industry concerning baking quality, larger row distances can be a beneficial strategy in low input farming systems to raise crude protein while free Asn is not affected.
- As yield components e.g. grains per spike and free Asn, showed a close relation, grain size may have an effect on Asn concentration in grain. This provides new insights into Asn synthesis during grain development and offers the opportunity to predict free Asn formation without expensive and time-consuming chemical analyzes.
- Organically grown cereals (species & cultivars) differ a lot even under low nitrogen input. Thus, Asn seems to be highly affected by species and cultivars rather than by nitrogen supply. Plus, grain structure seems to influence free Asn synthesis. Compared to wheat, rye and ancient grains like einkorn included the highest free Asn concentrations and simultaneously had the smallest grain size. Effects on free Asn can also be caused by different protein compositions as well as amino acids of these species.

- As heritability was partially high, there is a strong need to implement free Asn in cereal breeding programs. Especially within spelt, Asn seems to be related more strongly to genotype than to environmental conditions.
- A well-timed grain harvest and especially avoiding a delayed harvest, seems to be suitable to lower free Asn as i) soluble N-fractions will not be transferred into grain, and ii) the risk of pre-harvest sprouting is lowered.

Summing up the results and conclusions gained within the several field trials and based on a thorough literature review, agronomic strategies have a huge potential to successfully reduce Asn in raw material of cereals. In addition, different processing steps alongside crop management chain have to be considered. The combination of several agronomic strategies seems to be the best approach to lower free Asn and thus AA in final food products. Finally, a prototype of a cropping system aiming at low free Asn amounts can be designed. Table 6 tries to rank the relevance of agronomic strategies affecting free Asn.

Table 6: Expected impact of different strategies, sorted by impact (red=high impact, yellow=medium impact, green=low impact).

Influencing factors	**Impact on free Asn**
Year	
Species and cultivars	
Cropping system	
Nutrient supply	
Plant protection	
Harvest timing	
Post-harvest treatments	
Location	
Row distance	
Seed density	
Crop rotation	
Soil preparation	



The most attention should be paid to the impact of year (weather conditions), species and cultivars, the cropping system and the nutrient supply. Of minor importance seem to be row distance, seed density, crop rotation and soil preparation, while medium impact is expected considering plant protection, harvest timing and post-harvest treatments.
Finally, **Best Practice Cropping Applications,** given to farmers to lower free Asn most efficiently, should include the following advices:

1. adopt a well selected crop rotation that represses weeds and diseases
2. use well prepared soils that provides best starting conditions for plant growth
3. apply a balanced nutrient supply by nitrogen and sulfur
4. choose species that show a low Asn accumulation in grain, e. g. spelt
5. choose cultivars naturally low in free Asn and less susceptible to variable climate conditions as well as plant diseases

6. increase row distance to gain better nutrient supply maybe combined with a lower seed density
7. use plant protection means (if possible)
8. switch to organic farming conditions (if possible)
9. avoide growth stress conditions in general (if possible)
10. avoide a delayed harvest (if weather conditions are uncertain an earlier harvest should be chosen)
11. avoide post-harvest storing conditions that affect grain quality

The application of the strategies above will lead to lower amounts of AA precursor free Asn in raw material. As a holistic approach promises a maximum effect in suppressing AA, merging agronomic strategies and food processing applications will finally lower AA to the possible minimum.

Future research outlook

After gathering almost every possible agronomic management strategy in a thourough literature review, and after the evaluation of the field trials carried out within the framework of this thesis, one central key element could be identified with the most potential to change free Asn amount. In almost each published study regarding crop management methods, including this thesis, cereal species and cultivars contributed highly to different free Asn formations in grains. Of course, other agronomic practices like nutrient supply, row distance, cropping system and harvest timing have more or less the capacity to influence free Asn formation, but the impact of species and/or cultivar has been determined extensively. Thus, the central and most crucial question that should be addressed in future is:

Why do species and cultivars differ in their Asn synthesis and grain accumulation potential?

A similar impact on free Asn was observed in regard to the year, as in most of the studies climate conditions in terms of rainfall, temperature and stress conditions highly affect free Asn level. As weather conditions hardly can be changed, selecting cultivars with a low Asn accumulation potential regardless of climate conditions seems to be the best fitting strategy.

Curtis et al. (2014 & 2016) reported the high relevance of cultivar selection in cereals for free Asn reduction. They announced several minimization strategies, which were mostly related to cultivar selection. Most relevant, concerning cereal species and cultivars, seems to be understanding the whole Asn metabolism in cereal plants and the interaction of environment*genotype. The identification and control of the genes highly involved in Asn synthesis promises an immense impact on the regulation of free Asn accumulation in cereal grains.

Here, Rapp et al. (2018) identified gene QTL, explaining to some extent the Asn synthesis ability of different wheat cultivars. This might lead to promising applications in breeding programs. Nevertheless, gaining more knowledge about controlling the expression of Asn syntheses genes will be a big step ahead in controlling Asn formation in cereals. A further step will be to aim for better understanding of N efficiency and N translocation ability (from

plant tissues to grain) of species and cultivars leading to diverse grain Asn accumulations. Barbottin et al. (2005) found several wheat genotypes which were able to maintain N remobilization efficiency under high disease pressure. Such genotypes seem to be able to maintain protein synthesis under this condition without accumulating free Asn.
Thus, an initial strategy in regard to Asn formation, should be based on the selection of existing cultivars, which provide an inert reaction towards climate conditions, pest attack, disease pressure, special site conditions, soil properties and nutrient supply. Here, heritability will be a major factor. Hence, breeding programs should permanently implement free Asn as breeding trait.
In summary, answering the central question, **why do cereal species and cultivars differ,** the following main factors have to be taken into account:

Main characteristics influencing free Asn synthesis and accumulation in grain:

- Protein composition, - quality, and- amount (including involved amino acids)
- Nitrogen translocation ability from plant tissue to grain
- Nitrogen efficiency
- Asn-synthesis gene expression and its influence by nutrient supply and stress conditions (e.g. salt, disease pressure)

Gaining deeper insight into each single factor including the crosslink between these factors will be the major task in future to predict Asn formation more precisely.

As it was presented that protein and amino acid composition might influence free Asn accumulation in grains, protein metabolism must also be considered. It seems that a high N supply may enhance the accumulation of free Asn in grain when at the same time no sufficient protein synthesis is established. Thus, special breeding programs, partially within the framework of organic farming, should focus on wheat cultivars providing a good baking performance needing only few nutrients.
Especially N efficiency of plants will be a central issue as the upcoming National Emission Ceilings Directive (NEC) of the EU forces the lowering of ammonia emissions, which will include consequences for the national fertilization ordinance. Additionally, if nutrient uptake efficiency is increased it lowers the risk of nitrate leaching.
Hence, cereal breeding programs having N efficiency in mind should simultaneously include free Asn as an important breeding aim. This also includes the implementation of free Asn in cereal recommendation lists for cultivars, like the German Descriptive Variety Lists of the Federal Plant Variety Office (Bundessortenamt).

Main emphasizes should be given in controlling Asn synthesis gene expression. “How can genes switch on and off?”, seems an immensely interesting issue for upcoming studies. This includes also biotechnology approaches, which mostly regulate gene expression. Raffan & Halford (2019), reported in their review of some already available genetically modified potato varieties, where the influence of Asn-synthetase expression by RNA interference, led to a decrease in AA formation of French fries. Thus, genome editing techniques seem to be very promising. Indeed, for cereals such items have not been reported up to now.

However, if pathways of Asn synthesis and gene expression metabolism are revealed, caution is advised to not affect plant growth, plant physiology in terms of grain yield, or grain quality traits, simultaneously.

As a consequence of all revealed knowledge during this thesis, a **Best Practice Cropping Tool** should be established that finally leads to a **Prediction Model** to estimate the appearance of free Asn in cereal grain.

Mathematic model approach:

Free Asn =	∑	(potential of species/cultivar) + (crop management applications e.g. N & S-fertilization, plant protection) + (harvest timing) + (climate conditions) + error

The starting point should be a cereal species/cultivar that naturally provides a low free Asn formation potential. This potential can be further lowered by agronomic strategies like fertilization, row distance, cropping systems and plant protection.
The presented prediction tool is just the starting point in order to determine how free Asn can be predicted mathematically. This model must be refined towards different cereal species and cultivars. Additionally, the model should be adapted to different food groups as potatoes, olives, sweet potatoes, pseudocereals and cereals highly differ in their agronomic management.

4 Summary

Around 500 years ago Paracelsus stated “All things are poison, and nothing is without poison, the dosage alone makes it so a thing is not a poison.”

The citation of Paracelsus is believed to be true for most substances, but in the case of acrylamide (AA) it is not certain as just one molecule can cause cancer, for instance. Thus, strengthening all means to reduce AA in food products to the maximum possible extend is of highest priority.
Consequently, in 2017 the Commission Regulation of the EU announced regulation that limits AA and postulates mitigation strategies if AA surpasses certain levels. This shows that at present AA is seriously regarded as a harmful substance in foods, which can cause cancer among other harmfull effects. Thus, the food industry faces the big challenge to lower AA accordingly.
The relation between food and AA was first noted in 2002. Afterwards, the most important relations of AA formation, were postulated. Especially starch rich foods based on cereals and potatoes indicated a high AA content due to the fact, that AA is formed within the Maillard reaction during heating where reducing sugars and the amino acid asparagine (Asn) react in a non-enzymatic browning reaction.
To date a lot of studies have revealed the impact of, for instance, baking agents, fermentation time, additives and enzymes on final AA content. Nevertheless, such treatments may fail, as negative effects on rheological and sensory properties or quality and taste of the final products occur or their use is merely too expensive. Additionally, since 2011 AA levels have fluctuated from year to year, which shows that raw material such as cereal flours seems to have a high impact. Therefore, the production of raw material with a lower amount of precursors would be an important step. However, lowering precursors of AA in the raw material necessitates suitable agronomic strategies to grow cereals and cultivars low, particularly in free Asn. Hence, the major goal of this thesis was to investigate.

1. Which role does the management system play, as organic vs. conventional farming systems differ highly in the implemented cropping strategies?
2. What is the best nitrogen fertilization strategy comparing organic vs. conventionally cropping systems to lower free Asn in cereals?
3. Is there an impact of sulfur fertilization concerning sulfur amount and sulfur type on free Asn?
4. Can strategies like expanding row distance and lowering seed density in low-input farming systems positively influence baking quality while simultaneously keeping free Asn amounts as low as possible?
5. For organically grown cereals no information was available concerning the level of free Asn. Thus, the question came up in which content organically grown cereal species and cultivars including ancient grains like einkorn and emmer differ in free Asn.
6. Should the trait Asn be implemented in breeding programs as heritability is high?
7. Is there an impact of harvest timing on free Asn formation?

Simultaneously yield, yield components and quality traits were analyzed, too. After establishing and analyzing several field trials the following main results were obtained:

- The cropping system had a significant impact on grain yield, the level of free Asn and quality parameters. Across all species, free Asn contents in the flour were 26% lower under organic conditions compared to conventional farming. Wheat cultivars, in particular, showed that a maximum reduction of 50% in free Asn content was possible, if produced organically. Spelt and rye were minor affected as only in single years organically grown cultivars had up to 33% lower free Asn contents. (**paper 1+2**)
- Nitrogen (N) fertilization significantly influenced grain yield and baking quality in both cropping systems. In contrast, up to a certain N fertilizer amount free Asn was only affected to a minor extend. In particular, within the organic farming samples, no significantly higher amounts of free Asn were determined even if N fertilizer was raised or the N form was changed. A late N fertilization within the conventional cropping system increased crude protein content, while no clear effect was found on free Asn. Furthermore, neither type nor amount of sulfur fertilization influenced free Asn significantly. However, also cultivars influenced the free Asn amount significantly as cultivar Capo showed the lowest AA formation potential at a N supply of 180 kg N ha^{-1} while at the same time reaching a crude protein content > 15% (conventional) and > 12% (organic). Thus, minimizing free Asn by adjusting N treatments should not necessarily affect baking quality. Finally, it was proven that free Asn contents in wheat varied widely both within cultivars and between cropping systems (**paper 3**).
- Above all, increasing row distance can raise quality traits in case of protein and sedimentation value. Seed density was highly related to grain yield and test weight. Most importantly, free Asn was only affected to a minor extend by both treatments. Thus, larger row distances can be recommended to raise baking quality in organic farming systems without simultaneously affecting free Asn.

  As the number of grains per spike seems highly related to free Asn accumulation (R^2=0.72), this provides new insight on Asn synthesis during grain development and offers the opportunity to predict free Asn formation without expensive and time-consuming chemical analyzes.

  In contrast Asn and protein content did not show any relation, while if crude protein increased AA formation in heated flour decreased. (**paper 4**)
- The impact of organically grown species and cultivars in combination with marginal N supply on free Asn was clearly shown. A reduction potential of 85% was reached if rye was replaced by spelt. Surprisingly, the ancient species einkorn and emmer reached a very high free Asn content similar to rye. For the trait free Asn a high heritability was found for wheat and spelt concerning locations, while for years, heritability was low for wheat but high for spelt and rye. Concerning cereals cropped under organic conditions, the relation between free Asn and AA formation had never been investigated. Across species and years, a close relation was found shown by a R^2 of 0.69. Hence, free Asn can serve as an indicator for AA formation. (**paper 5**)
- Additionally, it was proven that harvest timing affects free Asn levels. In this context a delayed harvest can increase Asn significantly while shifting harvest 1-2 weeks earlier decreased Asn by up to 60% depending on cereal species and cropping system.

Out of the results the following main **conclusions** could be drawn:

- Cropping systems have a high impact on free Asn formation as organic farming methods lead to a significantly lower level.
- A well-balanced nutrient fertilization is necessary to not raise free Asn due to an insufficient protein synthesis.
- Special crop management applications like row distance ensures reaching a sufficient baking quality especially under low input farming systems while Asn will not be affected.
- N efficiency has to be taken into account in future in regard to N translocation rates in cereals to estimate grain free Asn accumulation effects.
- Cereal grain size may lead to differences on Asn accumulation.
- It seems highly important to illuminate why species and cultivars differ, in particular, under low nitrogen supply.
- In this context protein- and amino acid composition and the ratio between protein and soluble N fractions have to be investigated leading to reveal differences in free Asn accumulation concerning cereal species and cultivars.
- There is a strong need to implement free Asn as a quality trait in future cereal breeding programs.

Within the general **discussion** it was revealed that a well-timed grain harvest, and especially avoiding a delayed harvest seems quite suitable to lower free Asn, since soluble N-fractions will not be transferred into grain and the danger of pre-harvest sprouting is diminished. The investigation of the ancient cereal species einkorn and emmer highly suggests that heated food products of such species can become a real issue threatening health.
The pseudocereals amaranth and quinoa seem to contribute much more to a healthy constitution as high value ingredients are present in high concentrations while AA formation potential seems low. At the end of the discussion the major outcomes of the thesis stating agronomic measurements which can be transferred into practice were presented. This was followed by a future research outlook which focussed on the most promising agronomic strategy to lower free Asn in the long-term.
Nevertheless, it has been crucial, especially during the discussion, to think outside the “normal cereal” box in order to create crosslinks and deductions between different issues. This can help to better understand physiologic processes in plants and may lead to clarify pathways of Asn synthesis heading to more efficient agronomic strategies.

Summing up, implementing agronomic strategies to lower the amount of free Asn seems to be a promising approach. However, the strategies differ in their implication to lower free Asn. Therefore, a prediction tool similar to, for instance, the risk of fusarium, that classifies the impact of agronomic strategies on free Asn and finally leads to cropping and cultivar recommendations for farmers, should be implemented.
The overall future in agronomic minimization strategies can be seen in answering the question: “Why do species and cultivars differ in their Asn synthesis and grain accumulation potential”? Solving this riddle will provide a deep inside view and full understanding of the dynamics of Asn formation and accumulation in grain.

Nevertheless, a combined approach of agronomic and food technology strategies is needed and seems to be the most promising step to lower AA in food products to a maximum extend.

5 Zusammenfassung

Vor rund 500 Jahren postulierte Paracelsus „Alle Dinge sind Gift, und nichts ist ohne Gift. Allein die Dosis macht, daß ein Ding kein Gift ist".

Dieses Zitat gilt sicher für viele Substanzen, für Acrylamid (AA) jedoch ist sie in Frage zu stellen, da die Gefahr besteht, dass schon ein einziges Molekül Krebs auslösen könnte. Daher ist es wichtig AA in Nahrungsmitteln (NM) so niedrig wie möglich zu halten.
Das AA in NM mittlerweile ernsthaft als gesundheitsschädlich eingestuft wird, zeigt eine Verordnung der EU-Kommission aus dem Jahr 2017. Diese Verordnung limitiert AA in NM und fordert Vermeidungsstrategien, wenn die festgesetzten Richtwerte überschritten werden. Daher sieht sich die NM-Industrie mit großen Herausforderungen konfrontiert.
Das AA in NM vorkommt, wurde erstmals im Jahr 2002 bewiesen. Danach wurden schnell die wichtigsten Zusammenhänge erkannt. Am stärksten betroffen sind NM die auf Getreide- oder Kartoffelbasis hergestellt werden. Dabei wird AA innerhalb der Maillard Reaktion, der nichtenzymatischen Bräunung, während der Erhitzung von stärkereichen NM gebildet. Hierbei reagieren reduzierende Zucker und die Aminosäure Asparagin (Asn).
Bis heute wurden viele Studien durchgeführt, die zeigen, dass viele Eingriffsmöglichkeiten innerhalb der NM Produktion vorhanden sind, AA zu senken (u.a. Höhe der Temperatur, Länge der Hitzeeinwirkung, Austausch von Backtriebmitteln, Verlängerung der Teigstandzeit, Einsatz von Zusatzstoffen und Enzyme).
Solche Maßnahmen sind jedoch teilweise begrenzt, da unerwünschte rheologische und sensorische Auswirkungen auf die Qualität und den Geschmack der hergestellten NM auftreten können. Außerdem sind einige Maßnahmen nur bedingt umsetzbar, weil sie schlicht zu teuer zur Umsetzung im Großindustriellen Maßstab sind. Ferner hat sich gezeigt, dass seit 2011 die AA Gehalte von NM schwanken und sogar wieder angestiegen sind. Dies zeigt, dass vor allem der Rohstoff (z. B. Mehl) entscheidend ist.
Somit wäre ein wichtiger Schritt schon vor der NM Herstellung, auf dem Feld, Rohstoffe zu produzieren, die geringe Gehalte an AA Vorstufen aufzeigen.
Jedoch erfordert die Reduzierung von freiem Asn, der Hauptvorstufe von AA in Getreide, beim Anbau geeignete ackerbauliche Maßnahmen, um Getreide zu produzieren, die geringe Mengen an Asn bereitstellen.
Somit war das Hauptziel der vorliegenden Arbeit zu untersuchen:

1. Welche Rolle das Produktionssystem (ökologisch vs. konventionell) spielt?
2. Welche Stickstoff Düngestrategie sollte beim Getreideanbau favorisiert werden, im Vergleich der Produktionssysteme?
3. Hat die Schwefeldüngung einen entscheidenden Einfluss, hinsichtlich der eingesetzten Schwefelmenge und der Art des Schwefeldüngers, auf die Bildung von Asn?
4. Können sich ackerbauliche Maßnahmen, wie die Erweiterung der Reihenabstände und eine reduzierte Saatmenge in sog. „Low Input" Systemen steigernd auf die Backeigenschaften von Mehlen auswirken ohne aber den Gehalt an freiem Asn zu erhöhen?
5. Welche Rolle spielen Getreidearten und Sorten inklusive der Urgetreidearten Einkorn und Emmer im ökologischen Landbau hinsichtlich der Asn-Gehalte im Korn?

6. Sollte das Merkmal Asn als Züchtungsziel eingeführt werden, wenn sich eine hohe genetische Fixierung bestätigt, also der Asn-Gehalt vorwiegend sortengebunden ist?
7. Hat die Variierung im Erntezeitpunkt einen signifikanten Einfluss auf die Asn Akkumulation im Korngut?

Neben dem Asn-Gehalt wurden simultan der Kornertrag, die Ertragsparameter und die Qualitätsmerkmale erfasst, um Aussagen zur Vermarktungsfähigkeit zu treffen.

Nach der Auswertung der Feldversuche wurden folgende **Ergebnisse** erhalten:

- Das Produktionssystem hatte einen signifikanten Einfluss auf den Asn-Gehalt.
  Im Mittel über die Getreidearten lag der Asn-Gehalt bei den ökologisch erzeugten Proben 26 % niedriger verglichen zu den konventionellen. Besonders prägnant war der Unterschied bei Weizen, mit bis zu 50 % weniger Asn im ökologisch angebauten Sortiment. Bei Dinkel und Roggen war dieser Effekt mit maximal 33 % geringer ausgeprägt und außerdem nur in einzelnen Jahren erreicht. (**Paper 1+2**)
- Die Stickstoffdüngung wirkte sich signifikant auf den Kornertrag und die Backqualität in beiden Produktionssystemen aus. Im Gegensatz dazu zeigten sich bis zu einer Stickstoffdüngung von 180 kg N ha^{-1} keine signifikanten Unterschiede im Asn-Gehalt zur ungedüngten Variante. Vor allem im ökologischen Produktionssystem war eine Steigerung im Stickstoffniveau ebenso wie die applizierte Düngerform (Horngries oder Gülle) nicht ausschlaggebend für erhöhte Asn-Mengen. Spätdüngemaßnahmen im konventionellen Anbau erhöhten den Proteingehalt signifikant. Auf den Asn-Gehalt hatte dies keine eindeutige Auswirkung. Hinsichtlich einer zusätzlichen Schwefeldüngung, egal wie hoch und egal welche Schwefel-Düngerform, zeigten sich keine signifikanten Effekte auf den Asn-Gehalt. Sehr großen Einfluss hatte die Weizensorte. Die Sorte Capo erzielte die geringsten Asn-Gehalte bei einer N-Düngung von 180 kg N ha^{-1} jedoch hervorragende Rohproteingehalte von > 15 % (konventionell bewirtschaftet) und > 12 % (ökologisch angebaut). Somit scheint die Reduzierung von Asn über geeignete Stickstoff Düngestrategien nicht auf Kosten der Backqualität zu gehen. Letztlich was klar ersichtlich, dass das freie Asn stark zwischen Weizensorten und den Produktionssystemen variierte. (**Paper 3**)
- Weite Reihenabstände bei Winterweizen können die Backqualität bzgl. Den Rohprotein und den Sedimentationswert unter ökologischen Anbaubedingungen positiv beeinflussen. Die Saatdichte war signifikant verknüpft mit höheren Kornerträgen und Hektolitergewichten. Hinsichtlich des Gehalts an Asn war der Effekt beider Maßnahmen gering. Somit können größere Reihenabstände empfohlen werden, die Backqualität in ökologischen Anbausystemen zu steigern ohne den Asn-Gehalt zu beeinflussen.
  Der Asn-Gehalt stieg deutlich mit der Anzahl der Getreidekörner pro Ähre an (R^2=0.72). Dies eröffnet neue Einblicke in die Asn Synthese, während der Kornreifung und ermöglicht die einfache Vorhersage des Asn Gehalts ohne aufwendige und teure Laboranalysen. Dahingegen wurde zwischen dem Asn-Gehalt und dem Rohprotein keine Beziehung gefunden. Ein deutlicher Zusammenhang wurde zwischen dem AA Gehalt und dem Rohproteingehalt gefunden. Wenn der Rohproteingehalt steigt sinkt dahingegen die AA Bildung in erhitzen Mehlen. (**Paper 4**)

- Der Gehalt an Asn variierte signifikant zwischen ökologisch erzeugten Getreidearten und Sorten bei sehr marginaler Stickstoffversorgung. Wenn Roggen mit Dinkel ersetzt würde ergibt sich eine Reduktion im Asn-Gehalt von 85 %. Überraschenderweise erreichten die Urgetreidearten Einkorn und Emmer sehr hohe Asn-Mengen. Vor allem Einkorn lag in Höhen, die sonst nur bei Roggen gemessen wurden.
  Die Heritabilität, also, dass das Merkmal Asn mit der Sorte verknüpft ist, war im Vergleich der Standorte sehr hoch für Weizen und Dinkel. Im Jahreseinfluss war die Heritabilität niedrig für Weizen aber hoch für Dinkel und Roggen. Für ökologisch erzeugte Getreidearten und Sorten war der Zusammenhang zwischen Asn und AA bisher noch nicht untersucht wurden. Die analysierte Regression zeigt deutlich (R^2=0,69), dass Asn als Indikator für die AA Bildung dienen kann. (**Paper 5**)
- Der Erntezeitpunkt hatte einen signifikanten Einfluss auf die Asn-Bildung. Eine Vorverlegung der Kornernte um ein bis zwei Wochen führte, in Abhängigkeit des Produktionssystems und der Getreideart, zu einer Abnahme im Asn-Gehalt von bis zu 60 %.

Aus den Ergebnissen konnten folgende **Schlussfolgerungen** gezogen werden:

- Anbausysteme haben einen signifikanten Einfluss auf den Asn-Gehalt. Getreide die unter den Maßnahmen des ökologischen Landbaus angebaut werden, bilden signifikant weniger Asn.
- Die Reduzierung von Asn über eine ausgewogene Nährstoffversorgung scheint bei Winterweizen, unabhängig vom Anbausystem, aussichtsreich, bei gleichzeitiger Sicherstellung einer ausreichenden Backqualität.
- Erweiterte Reihenabstände im Winterweizenanbau können zur Absicherung der Backqualität im ökologischen Landbau empfohlen werden ohne den Asn-Gehalt zu erhöhen.
- Es schein, dass die Korngröße Einfluss auf die Asn-Akkumulation im Korn, im Vergleich von Getreidearten, hat.
- Es ist sehr wichtig zu untersuchen, warum Getreidearten und Sorten auch bei sehr marginaler Stickstoffversorgung deutlich im Asn-Gehalt divergieren. Dabei scheinen die Protein- und Aminosäurezusammensetzung sowie das Verhältnis von Protein zu löslichen N-Verbindungen im Korn von großer Relevanz zu sein.
- Es ist unabdingbar, das Merkmal Asn in Züchtungsprogramme als beschreibendes Qualitätsmerkmal aufzunehmen.

Innerhalb der „Generellen **Diskussion**“ wurde aufgezeigt, dass eine vorverlegte Kornernte sehr gut geeignet scheint, den Asn-Gehalt zu senken. Dadurch kann vor allem ein deutlicher Anstieg im Asn-Gehalt, durch die Gefahr von Auswuchs, vorgebeugt werden. Die in Einkorn und Emmer gefundenen hohen Asn-Gehalte, die sich auch in einer ehrhöhten AA-Bildung in Brot wiederspiegeln, legen es nahe Produkte dieser Urgetreidearten nur in geringen Mengen zu verzehren. Wohingegen, basierend auf den analysierten geringen AA- und Asn–Mengen bei Amaranth und Quinoa, diese Pseudogetreidearten eher gesundheitsförderlich sind.

Am Ende der Diskussion wurde aufgezeigt, welche Maßnahmen in die landwirtschaftliche Praxis überführt werden sollten, um die Bildung von Asn beim Anbau zu minimieren. Zukünftig scheinen weitere agronomische Strategien wichtig, um Asn kontinuierlich zu senken.
Dabei ist der Blick über den "Getreide" Tellerrand wichtig, um Querverbindungen und Ableitungen zwischen verschiedenen Themen herzustellen. Dies kann helfen die physiologischen Abläufe bezogen auf die Asn-Synthese und Akkumulation bei Getreide besser zu verstehen, was schlussendlich zu effizienteren pflanzenbaulichen Strategien einer Minimierung von Asn führen kann.

Zusammenfassend können agronomische Strategien einen wesentlichen Beitrag leisten, um die AA-Vorstufe freies Asn deutlich zu minimieren. Da sich die Strategien in ihrer Effizienz unterscheiden, wäre es wichtig, ein pflanzenbauliches Vorhersage-Model zu entwickeln, das unter Einbezug einer Gewichtung der Maßnahmen Asn-Gehalte schon im Vorfeld abschätzt. In der Weiterentwicklung sollten daraus zusätzliche Empfehlungen für die landwirtschaftliche Praxis abgeleitet werden.
Die Zukunft der agronomischen Minimierungsstrategien liegt in der Beantwortung der zentralen Frage: „Warum unterscheiden sich Getreidearten und Sorten im Asn-Bildungs- und Akkumulierungspotenzial im Korn?“ Ist diese Frage gelöst sollten sich weitaus effizientere Maßnahmen ergeben Asn im Getreidekorn zu senken. In der vorliegenden Arbeit wurden Erklärungen angedeutet und weitere Puzzleteile zur Senkung von Asn beigetragen. Jedoch ist ein weitaus detaillierterer Ansatz essentiell.
Um AA jedoch auf ein Minimum zu senken, ist ein ganzheitlicher Ansatz nötig, der die agronomischen Strategien und die Anwendungen bei der NM-Herstellung als Synergie versteht.

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Appendix

Next to the main chapters (paper 1 to paper 5) of the thesis the following listed publications were originated during the research work and were part of scientific conferences in terms of oral or visual presentations including a conference publication or were published in local magazines.

Cereals

- Stockmann, F. (2011). Einfluss pflanzenbaulicher Maßnahmen bei Getreide zur Minimierung von Acrylamidvorstufen im Korngut. Landinfo 1, 55-57.
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Affidavit

pursuant to Sec. 8(2) of the University of Hohenheim's doctoral degree regulations for Dr.sc.agr.

1. I hereby declare that I independently completed the doctoral thesis submitted on the topic

Agronomic Strategies to Reduce Potential Precursors of Acrylamide Formation in Cereals

2. I only used the sources and aids documented and only made use of permissible assistance by third parties. In particular, I properly documented any contents which I used - either by directly quoting or paraphasing - from other works.
3. I did not accept any assistance from a commercial doctoral agency or consulting firm.
4. I am aware of the meaning of this affidavit and the criminal penalties of an incorrect or incomplete affidavit.

I hereby confirm the correctness of the above declaration. I hereby affirm in lieu of oath that I have, to the best of my knowledge, declared nothing but the truth and have not omitted any information.

Feldkirchen, 20.11.2019

……………………………………………………………………………………………......

(Place, date) (Signature)

Affidavit
Information

The University of Hohenheim requires an affidavit declaring that the academic work was done independently in order to credibly claim that the doctoral candidate independently completed the academic work.

Because the legislative authorities place particular importance on affidavits, and because affidavits can have serious consequences, the legislative authorities have placed criminal penalties on the issuance of a false affidavit. In the case of wilful (that is, with the knowledge of the person issuing the affidavit) issuance of a false affidavit, the criminal penalty includes a term of imprisonment for up to three years or a fine.

A negligent issuance (that is, an issuance although you should have known that the affidavit was false) is punishable by a term of imprisonment for up to one year or a fine.

The respective regulations can be found in Sec. 156 StGB (Criminal Code) (false affidavit) and in Sec. 161 StGB (negligent false oath, negligent false affidavit).

Sec. 156 StGB: False Affidavit

Issuing a false affidavit to an authority body responsible for accepting affidavits or perjury under reference to such an affidavit shall be punishable with a term of imprisonment up to three years or with a fine.

Sec. 161 StGB: Negligent False Oath, Negligent False Affidavit:

Subsection 1: If one of the actions described in Secs. 154 and 156 is done negligently, the action shall be punishable by a term of imprisonment of up to one year or a fine.
Subsection 2: Impunity shall apply if the perpetrator corrects the false information in a timely manner. The regulations in Sec. 158 (2) and (3) apply mutatis mutandis.

The German original version of this affidavit is solely valid; all other versions are merely informative.

I have taken note of the information on the affidavit.

……………………………………………………………………………………………………......

(Place, date) (Signature)

Acknowledgements / Danksagungen

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Curriculum vitae

Personal data

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Born 14th of January 1973 in Leipzig

Married, three children

Education

2007 – 2020	Doctorate at the Faculty of Agricultural Sciences, Institute of Crop Sciences, University of Hohenheim
2005 – 2007	Degree: Master of Science (M.Sc. Agr.) University of Hohenheim, Stuttgart-Hohenheim
2002 – 2005	Degree: Oecotrophologie Dipl.-oec.-troph. (FH), University of applied Sciences Niederrhein, Mönchengladbach
2001 – 2002	Vocational diploma, Upper vocational school Triesdorf
1995 – 1996	Civilian service
1990 – 1994	Education to a cook, Hotel Geiger, Hopfen am See
1979 – 1989	General education, Allgemeinbildende polytechnische Oberschule, Leipzig

Scholarship

2008 – 2009	Scholarship holder in the doctoral study program Agricultural Sciences, University of Hohenheim

Professional career and internships

2015 – today	Project manager subjected to biogas and mobility at C.A.R.M.E.N. e. V., Straubing
2011 – 2015	Scientific assistant at the Technology and Support Centre (TFZ) of Renewable Resources, Straubing
07 – 09/2004 & 02 – 03/2005	Internship at the Technology and Transfer-Centre (ttz), Department: Sensory Laboratory, Bremerhaven
1994 – 2001	Employed as cook