

REVIEW

Reducing the acrylamide-forming potential of wheat

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Acrylamide, asparagine, bread, food safety, free amino acids, processing contaminants, reducing sugars, sulfur fertilization, wheat.

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Abstract

Acrylamide is a Class 2a carcinogen that was discovered in a variety of popular foods, including baked cereal products, in 2002. The predominant route for its formation is from free asparagine and reducing sugars in the Maillard reaction, with free asparagine concentration being the main determinant of acrylamide-forming potential in cereal products. The European Commission set “indicative” levels for acrylamide in food in 2011 and 2013, and is currently reviewing its options for further measures. Agronomic and genetic approaches to reducing the acrylamide-forming potential of wheat include the evaluation of existing varieties for low asparagine accumulation in the grain, ensuring adequate sulfur fertilization in relation to nitrogen supply, developing an understanding of the genetic control of asparagine metabolism, and identifying quantitative trait loci or molecular markers for low asparagine accumulation in the grain. Asparagine concentration in grain is affected by environmental factors (E), genetic factors (G), and interactions between the two ($G \times E$). This paper reviews the continuing efforts being made to reduce the acrylamide-forming potential of wheat, and to increase awareness of the issue among wheat breeders, farmers, and the food industry.

Acrylamide

Acrylamide is a neurotoxin and probable (Class 2a) carcinogen in humans (Schulze and Siegers 2004; Taeymans et al. 2005). According to the opinion on threshold of toxicological concern (Dybing and Sanner 2003), the no-observed-effect level (NOEL) is 0.2 mg/kg body weight (bw). The lower limit of this range is called the benchmark dose lower confidence limit (BMDL) (Parzefall 2008); a BMDL of 0.17 mg/kg bw/day has been set for tumor-inducing effects, and 0.43 mg/kg bw/day for neurological effects (http://www.efsa.europa.eu/sites/default/files/corporate_publications/files/acrylamide150604.pdf). The European Food Safety Authority (EFSA) Expert Panel on Contaminants in the Food Chain (EFSA 2015b) stated that the margins of exposure for acrylamide indicate a concern for neoplastic effects based on animal evidence (EFSA Panel on Contaminants in the Food Chain [EFSA 2015b]). The European Commission had already issued “indicative” levels for the presence of acrylamide in food in 2011, and revised them downward for many products in 2013 (European Commission 2013). Furthermore, in response to the CONTAM report, the

Commission is currently reviewing additional options for risk management measures.

Acrylamide Formation

Acrylamide forms during cooking and processing at temperatures above 120°C, usually during the processes of frying, roasting, and baking, and levels higher than 2000 ppb have been reported in some foods produced from high-sugar and free asparagine-containing raw materials. It is formed during the Maillard reaction, a series of nonenzymatic reactions between free (nonprotein) amino acids and reducing sugars when all desirable flavors are formed. The Maillard reaction occurs in three stages: during the first stage, free amino acids and other amino groups react with reducing sugars to form carbonyl compounds (Mottram et al. 2002; Stadler et al. 2002; Stadler 2005). The reaction is initiated by the condensation of the carbonyl (C=O) group of a reducing sugar with the amino group, producing a Schiff base. If the sugar is an aldose, the Schiff base cyclises to give an *N*-substituted aldosylamine, such as glucosylamine from glucose.

Acid-catalyzed rearrangement of the aldosylamine gives a 1,2-enaminol, which is in equilibrium with its ketotautomer, an *N*-substituted 1-amino-2-deoxyketose: these are known as Amadori rearrangement products. Ketoses, such as fructose, give related Heyns rearrangement products by similar pathways.

In the second stage of the reaction, the Amadori and Heyns rearrangement products undergo enolization, deamination, dehydration, and fragmentation, giving rise to sugar dehydration and fragmentation products containing one or more carbonyl groups, including heterocyclic furfurals, furanones, and pyranones. These carbonyl compounds can undergo condensation reactions with amino groups and other components present at this stage of the Maillard reaction, resulting in the formation of many different flavor compounds (International Agency For Research On and International Agency For Research On 1994). An important reaction of carbonyl compounds is Strecker degradation: the deamination and decarboxylation of an amino acid to give an aldehyde in which the α -carbon of the amino acid is converted to an aldehyde group, the reaction also yielding an α -aminoketone. Acrylamide is formed in a Strecker-type reaction involving sugar-derived carbonyl compounds and asparagine (Zyzak et al. 2003; Mottram 2007; Koehler et al. 2008).

During the third and final stage of the Maillard reaction, a thermally induced reaction between the carbonyl compounds, amino acids, and their degradation derivatives produces the desired flavor compounds and melanoidin pigments (Mottram et al. 2002).

Acrylamide in Food

The following food categories are the main contributors to acrylamide exposure in the diet (European Food Safety Authority 2011, EFSA 2015a,b): potato crisps, French fries, bread, breakfast cereals, biscuits, other cereal-based snacks, battered fried foods, popcorn, coffee, ginger bread, and chocolate. Recently, it was found that there is also acrylamide in processed olives (Casado and Montano 2008; Javier Casado et al. 2010, 2013, 2014). Acrylamide formation depends on the levels of its precursors (free asparagine and reducing sugars) in the raw material and the processing methods that are used, and the concentrations of free asparagine and reducing sugars are affected by specific variety, location, and management of the crop (Curtis 2010; Curtis et al. 2010a,b, Curtis et al. 2009).

Risk Assessment by International Authorities

The latest report from EFSA's Expert Panel on Contaminants in the Food Chain (EFSA) described the

risk characterization for neoplastic effects using as a reference point the BMDL of 0.17 mg/kg bw/day of acrylamide in food, although it considered the neurological, reproductive, and developmental effects of acrylamide not to be a concern at the current levels of dietary exposure (EFSA 2015a). "Since MOEs calculated are substantially lower than the value of 10,000, the CONTAM Panel concluded that, although the available human studies have not demonstrated acrylamide to be a human carcinogen, the MOEs across survey and age groups indicate a concern with respect to neoplastic effects" (Food Standard Agency 2012). The Food and Agriculture Organisation of the United Nations and the World Health Organisation (FAO/WHO) Joint Expert Committee of Food Additives (JECFA) has also concluded that the presence of acrylamide in the human diet is a concern (World Health Organization 2006).

EFSA monitors data supplied by EU Member States on acrylamide in food, and the figures for cereal-based products from 2007 to 2012 are given in Table 1. In 2011 and 2013, the European Commission set "indicative" levels for acrylamide in different food categories (European Commission 2013), based on the results of this screening exercise, and these are also shown in Table 1. Indicative levels are not maximum levels or an indication of safety or lack of it, rather they are the levels that the Commission believes the food industry should be able to achieve, based on the screening data.

Coverage of Acrylamide in the Media

The coverage of the acrylamide issue in the press has not reflected the efforts of the food industry to mitigate the acrylamide issue and to decrease the levels of acrylamide found in food.

Baby food is of greatest interest because a baby's body weight is lower than that of adults. The European Commission therefore set a relatively low indicative level for cereal-based baby foods of 100 ppb in 2011 and reduced it to 50 ppb in 2013. Table 1 shows that the maximum level of acrylamide found in cereal-based baby foods has been well above the 50 ppb mark throughout the screening period. It is important to note that NOELs for babies will be considerably lower than for an adult because of the difference in body weight. For a body weight of, for example, 12 months old baby of 7.1 kg (growth chart), the NOEL would be 1.42 mg, whereas for a 15-g portion of breakfast cereal (even based on the maximum level of 2072 $\mu\text{g/kg}$ in Table 1), the acrylamide content would be 31.08 μg . Even if no other acrylamide-containing food were eaten in that day, the MOE would be approximately 40, not the 10,000 favored by EFSA.

Table 1. Acrylamide concentrations measured in cereal-based foods in Europe from 2007 to 2012 (EFSA 2015a,b) and indicative levels set by the European Commission (European Food Safety Authority 2011).

Food type	Acrylamide levels ($\mu\text{g}/\text{kg}$)										Indicative levels ($\mu\text{g}/\text{kg}$)	
	Mean					Max						
	2007	2008	2009	2010/2011	2012	2007	2008	2009	2010/2011	2012	2011	2013
<i>Biscuits</i>												
Crackers	291–292	203–206	195–208	275	333	1526	1042	1320	473	1062	500	500
Infant	197–204	98–110	88–108	110	86	2300	1200	430	598	470	250	200
Unspecified	299–303	213–223	128–140	625	289	4200	1940	2640	1574	5849		500
Wafers	206–210	251–254	244–246	154	389	1378	2353	725	154	1300		500
<i>Bread</i>												
Bread crisp	221–226	229–231	219–223	197	249	2430	1538	860	326	1863	150	450
Bread soft	54–68	31–46	27–37	15	30	910	528	364	37	425		80
Unspecified	172–190	45–231	54–76	14		2565	86	1460	51			150
Breakfast cereals	130–150	140–156	132–142	149	138	1600	2072	1435	325	1290	400	200–400
Baby food	48–69	35–51	55–70	18	51	353	660	710	68	578	100	50

Bold values are the indicative levels recognized by the EU Commission.

Industry and Research Outcomes

Significant efforts have been made by the food industry in recent years toward reducing levels of acrylamide in its products. Approaches include selecting varieties which contain low levels of acrylamide precursors (free asparagine and reducing sugars); removing precursors before processing (Friedman 2003, 2005; Howie et al. 2006, 2007; Friedman and Levin 2008); using the enzyme asparaginase to hydrolyze asparagine to aspartic acid and ammonia prior to cooking or processing; controlling processing conditions such as pH, temperature, time, processing, and storage atmosphere to minimize acrylamide formation; and adding food ingredients that have been reported to inhibit acrylamide formation, such as amino acids, antioxidants, nonreducing carbohydrates, garlic compounds, protein, and metal salts. For the reduction of acrylamide formation in biscuits on an industrial scale the following measures were published by Graf et al.: replacement of ammonium hydrogen carbonate by sodium hydrogen carbonate which reduced the acrylamide content by about 70%; use of a sucrose solution instead of inverted sugar syrup (glucose and fructose) had a similar effect (sucrose will participate in the Maillard reaction, but only if it is first hydrolyzed through enzymatic, thermal, or acid-catalyzed reaction [Vleeschouwer et al. 2009]), while the addition of extra tartaric acid reduced the acrylamide content by approximately 30%. These results showed that mitigation on an industrial scale, based on the optimization of baking agent, reducing sugars, and organic acid, is feasible (Graf et al. 2006). The overall reduction presented in this paper was from 3200 ppb ($\mu\text{g}/\text{kg}$) acrylamide to 960 ppb ($\mu\text{g}/\text{kg}$) (this is 70%).

Methods for reducing acrylamide formation have been compiled and reviewed in the “Acrylamide Toolbox” produced by Food Drink Europe (2014). The Toolbox consists of four pillars: agronomy, recipe, processing, and final preparation. The agronomic advice concerns the amount of reducing sugars and free asparagine in the raw material; recipe refers to basic formulae, ingredients, and product form; processing deals with thermal input and moisture, the addition of asparaginase, pretreatments, finished product color, texture, and flavor; while the term final preparation includes instruction and consumer guidance.

The main objective for the food industry is to continue working to reduce acrylamide levels to as low as reasonably achievable (the ALARA principle). Table 1 shows that the mean level of acrylamide in foods such as wafers, breakfast cereals, and baby foods has decreased substantially between 2007 and 2012, whereas some other food categories only show modest results. This is because many of the acrylamide mitigation tools are food system specific and show large variations in effectiveness across food categories. An example would be pretreatment with the enzyme asparaginase. This works well in foods that have an “aqueous” preparation step, but does not work well in foods that are produced with limited moisture content. Therefore, the food industry has a continuing need for the development of acrylamide mitigation tools that are more universal in nature and can be applied across food categories and in the home. Research on producing wheat with a low concentration of free asparagine in the grain targets this need. It is critical to continue with research in this direction in order to have tools and concepts in place for the future. The challenges facing the food industry require more detailed research and understanding of

acrylamide precursors in different elite wheat varieties in order to identify agronomic, environmental, or genetic factors likely to influence acrylamide formation in the end product (Curtis 2010).

Acrylamide Level Uptake Due to Cereal Products

Table 2 shows the acrylamide intake due to cereal-based products in various European countries (Curtis and Halford 2014, EFSA 2015a,b). In France, Germany, and Sweden, a major contributor to dietary acrylamide intake is bread, while in the United Kingdom, the contribution of bread and cereal products overall is lower. This reflects differences in dietary preferences rather than the acrylamide levels in the products between the four countries, with more fried potato products being consumed in the United Kingdom. Similarly, muesli and crisp bread are high contributors in Sweden because of the popularity of those foods in that country.

Bread contains relatively low levels of acrylamide, but due to its large consumption it is a main contributor to total dietary intake. It is important to note that some bread is consumed as toast and acrylamide levels in the product before toasting are a lot lower in comparison to the levels in the toasted bread. For example, Granby et al. reported that levels of $<5 \mu\text{g/kg}$ in a soft bread slice rose to 11–161 $\mu\text{g/kg}$ in a toasted slice of bread depending on the coloration (Granby et al. 2008). This highlights the problem of how foods are cooked in the home, and the need to educate consumers on ways to reduce acrylamide formation, something to which consumers in the United Kingdom at least have so far been unreceptive.

In the United Kingdom, the contribution of acrylamide from cereal products is highest in bread, followed by biscuits and breakfast cereals, but these products are not consumed every single day and consumer preferences are changing anyway as alternative foods become more readily available and popular. Detailed annual statistics on family food and drink purchases, for example, demonstrate a trend of declining white bread consumption. Standard unsliced white bread sales, for example, have

decreased by 15% since 2011 and 9% since 2013, while white bread, soft grain, sliced and unsliced, sales have decreased by 156% since 2011, although they have levelled out from 2013. On the other hand, the consumption of total other products such as takeaway bread in pre-prepared sandwiches from takeaway outlets has increased by 38% since 2011 and 7% since 2013. Additionally, consumption of takeaway breads has increased 15% since 2011 and a little decline of 5% since 2013 (<https://www.gov.uk/government/statistical-data-sets/family-food-datasets>).

Asparagine as a Major Precursor of Acrylamide Formation in Food: Factors Affecting Asparagine Accumulation

Free asparagine is the main amino compound precursor of acrylamide formation in food, as confirmed by studies using isotopically labeled asparagine which showed that the three C atoms and single N atom of acrylamide were all derived from asparagine (Zyzak et al. 2003). In addition, asparagine concentration has been shown to be the main limiting factor for acrylamide formation in wheat (Muttucumaru et al. 2008) and rye (Curtis et al. 2010a; Postles et al. 2013).

Asparagine is one of the main amino acids involved in nitrogen accumulation and transport in plants, together with glutamine (Lea et al. 2007). It is the major transport compound in the xylem from the root to the leaves and in the phloem from the leaves to the developing seeds in a range of plants (Lea et al. 2007), although this is not the case for potato (Muttucumaru et al. 2014) and has not been established for wheat. Asparagine is relatively inert and therefore particularly suited to the role of a nitrogen transport and storage compound. Asparagine is also one of the building blocks of wheat and rye seed proteins. It is present in γ -gliadin and secalin (1.3–1.47%), α -gliadin and secalin (2.54–2.68%), ω -gliadin and secalin (0.75–0.77%), and low-molecular-weight glutenin subunits (LMW subunits) (0.7–1.08%), but not in high-molecular-weight subunits (HMW subunits) (Khan 2007). It is one of the nonessential amino acids in the diet.

Table 2. Contribution of cereal products (%) to dietary acrylamide intake for adults in selected European countries (Curtis and Halford 2014).

Country	Food group					Total
	Biscuits	Crisp bread	Bread	Breakfast cereal	Muesli	
France	7.6	5.3	25.7	1.3	1.0	40.9
Germany	6.1	4.0	32.0	1.2	2.1	45.4
Sweden	5.0	9.7	11.9	1.5	13.1	41.2
United Kingdom	6.3	2.0	15.0	5.0	3.6	31.9

Efforts to Reduce Acrylamide-Forming Potential

Efforts to reduce free asparagine accumulation in wheat grain have involved the following strategies:

1. Identification of existing varieties with low grain asparagine concentrations.
2. Identification of genotypes with low grain asparagine concentration that are not current commercial varieties but could be incorporated into breeding programmes.
3. Development of a comprehensive understanding of asparagine metabolism (including the use of mathematical modeling).
4. Elucidation of genetic (G) and environmental (E) factors (including crop management) that affect asparagine accumulation in the grain.
5. Understanding of the relationship between asparagine concentration, total grain sulfur and nitrogen, and acrylamide formation.
6. Identification of quantitative trait loci (QTL), genes, and markers for use by plant breeders to produce very low acrylamide varieties.

Genetic Differences Between Wheat Varieties

Comparison of four doubled haploid (DH) lines and Spark and Rialto parental lines

To establish the differences between doubled haploid (DH) lines from a Spark \times Rialto mapping population provided by the John Innes Centre Wheat Genetics Group, Spark and Rialto parental lines plus SR3 (a low asparagine DH line), SR41 (a high free asparagine DH line), and SR7 together with SR107 (both intermediary asparagine DH lines) were grown under controlled conditions in a glass-house (Curtis et al. 2009). Free amino acids were extracted, derivatized, and analyzed by gas chromatography mass spectrometry (GC-MS). Statistical analysis showed the lines differed significantly in concentration of free asparagine, aspartic acid, glycine, and valine. The main contributors to the total free amino acid pool were asparagine, aspartic acid, and glutamic acid, and the difference between SR3 as a low free asparagine genotype and SR41, the most abundant free asparagine genotype, was the ratio between aspartic acid and asparagine, and the concentration of the total free amino acid pool. In SR3, the concentration of the total free amino acid pool was 8.5 mmol/kg, whereas in SR41 it was 12.5 mmol/kg, while the concentration of free asparagine in SR3 was 1.68 mmol/kg and aspartic acid was 3.26 mmol/kg. In contrast, in SR41, the concentration of free asparagine was 3.23 mmol/kg and aspartic

acid was 3.95 mmol/kg. This implied that the contrast between the DH lines could possibly be explained by differences in asparagine synthetase activity, resulting from changes in gene expression, protein turnover, or enzyme activity (Curtis et al. 2009).

Varietal differences were also observed in additional studies by Corol et al. (2016), who analyzed 150 bread wheat varieties grown at a single site in 2005. The varieties were separated by asparagine content into low asparagine 0.32–0.43 mg/g dry matter (dm) (correlates to 2.42–3.25 mmol/kg) (cvs Chinese Spring, Palesio, Blasco, Mv-Emese, Bilancia, Granbel, Soissons, Nomade, Valoris, and Alba) and high asparagine 1.50–1.56 mg/g dm (correlates to 11.3534–11.8076 mmol/kg) (cvs Fleischmann, Spark, Kirkpinar 79). Varieties with asparagine contents of 1.28–1.40 mg/g dm were Mexique 50, Renan, Bankuti 1201, Alanasskaja, while Kirac 66, Qualital and Blue/A had 1.10–1.25 mg/g dm. In our analyses (Curtis et al. 2009), cv Spark had a concentration of free asparagine in our tested samples between 2.54 mmol/kg (under a sulfur-sufficient regime) and 62.02 mmol/kg (under a sulfur-deficient regime) compared to that used by Corol et al., where cv Spark had a free asparagine content of 11.35 mmol/kg and was classified as a high asparagine variety. That level of free asparagine suggests that the sample was obtained from wheat that was grown with insufficient sulfur, or there were other stresses preventing it from achieving its usual level of asparagine (2.54 mmol/kg).

Structure and Expression of the Asparagine Synthase Gene Family of Wheat

Identification of the sites of synthesis and accumulation of free asparagine in the grain under sulfur-deficient and sulfur-sufficient conditions is an important objective. There is evidence that free asparagine is predominantly accumulated in the embryo and aleurone layer under sulfur sufficiency, but accumulates at high levels in the endosperm under sulfur-deficient conditions (Shewry et al. in press). This is reflected to some extent in the expression of asparagine synthetase genes (Gao et al. 2016). There are four asparagine synthetase genes in wheat, *TaASN1-4* (Gao et al. 2016), although *TaASN4* has only been identified in genome data and has not been analyzed in detail. Of the other three, the expression of *TaASN2* in the embryo during mid-development dwarfs the expression of any of the genes in any other tissue, although it is also expressed at relatively high levels in the endosperm, even when the wheat is well supplied with sulfur. Indeed, *TaASN1* appears to be the most responsive to both sulfur deficiency and nitrogen

Table 3. Asparagine synthetase genes in different plant species.

Gene name	Species	Comment	References
<i>AS</i> (cDNA clone)	<i>Asparagus officinalis</i>	The study showed that AS plays different roles to asparagine synthetase genes studied in other plants and is induced in harvested asparagus spears in response to carbohydrate stress	Davies and King (1993)
<i>ASN1</i>	<i>Arabidopsis thaliana</i>	The study provided experimental confirmation that phytochrome plays a role in the transmission of light signals to repress accumulation of <i>Arabidopsis ASN1</i> mRNA Light and metabolic control of amide amino acid biosynthesis was demonstrated Light was shown to repress the synthesis of asparagine, which therefore accumulates only in the tissues of dark-adapted plants	Lam et al. (1994)
<i>ASN1</i> , <i>ASN2</i> and <i>ASN3</i>	<i>Arabidopsis thaliana</i>	<i>ASN1</i> gene expression was shown to be mainly in the stem, leaves, and flowers. <i>ASN1</i> and <i>ASN2</i> showed reciprocal regulation in response to light: levels of <i>ASN2</i> mRNA (extremely low in dark) were rapidly increased in a light treatment, whereas <i>ASN1</i> expression was repressed by light. The levels of <i>ASN1</i> and <i>ASN2</i> mRNA were also affected by organic nitrogen in the form of glutamate, glutamine, and asparagine	Lam et al. (1998a)
<i>AS</i>	Maize (<i>Zea mays</i>)	An exogenous supply of metabolizable sugars downregulated gene expression, while nonmetabolizable sugars induced gene expression. Effects of nitrogen metabolite supply and stress conditions indicated that gene expression might be under metabolic control in maize root tips	Chevalier et al. (1996)
<i>AS</i>	Rice (<i>Oryza sativa</i>)	Immunoblotting revealed a high content of asparagine synthetase protein in the leaf sheath at the second position from the fully expanded top leaf and in grains at the middle stage of ripening. Accumulation of mRNA for AS was also observed in these organs. During the ripening of the spikelets, the AS protein contents increased during the first 21 days after flowering, then declined rapidly	Nakano et al. (2000)
<i>ZmAsnS1</i> , <i>ZmAsnS</i> , <i>ZmAsnS3</i> , and <i>ZmAsnS4</i>	Maize (<i>Zea mays</i>)	Four asparagine synthetase genes, <i>TaASN1–4</i> , were identified and shown to be differentially expressed The asparagine synthetase enzymes were shown to be kinetically distinct	Todd et al. (2008) Duff et al. (2011)
<i>TaASN1</i> and <i>TaASN2</i>	Wheat (<i>Triticum aestivum</i>)	Salinity and osmotic stress caused rapid accumulation of <i>TaASN1</i> transcript in both shoots and roots. The expression levels of <i>TaASN2</i> were different from <i>TaASN1</i> as the levels were only increased by addition of abscisic acid (ABA) at 24 h exposure and there was no response under osmotic or salinity stress	Wang et al. (2005)
<i>DIN6</i> (dark-inducible-6 = asparagine synthetase (<i>ASN1</i>))	<i>Arabidopsis thaliana</i>	The expression of asparagine synthetase gene is induced 256 ± 1.2 -fold by sucrose nonfermenting-1-related protein kinase (SnRK) 1.1 <i>DIN6</i> is glutamine-dependent asparagine synthetase (<i>ASN1</i>)	Baena-Gonzalez et al. (2007)
<i>TaASN1–4</i>	Wheat (<i>Triticum aestivum</i>)	The expression of three genes, <i>TaASN1–3</i> , was studied in different tissues and in response to nitrogen and sulfur supply. The expression of <i>TaASN2</i> in the embryo and endosperm during mid to late grain development was the highest of any of the genes in any tissue, but <i>TaASN1</i> showed more response to nitrogen feeding and sulfur deficiency. <i>TaASN4</i> was identified from recent genome data but was not studied in detail	Gao et al. (2016)
<i>HvASN1–5</i>	<i>Hordeum vulgare</i>	Five ASN genes were sequenced and characterized in this paper and were shown to be differentially expressed. The paper only discusses <i>HvASN1</i> , <i>HvASN3</i> , <i>HvASN4</i> , and <i>HvASN5</i> . The <i>HvASN1</i> , <i>HvASN3</i> , and <i>HvASN5</i> were repressed by aging and low-nitrate conditions. All four genes were induced by leaf senescence	Avila-Ospina et al. (2015)

supply (Byrne et al. 2012; Gao et al. 2016). There is evidence that the sulfur response of *TaASN1* involves the protein kinase, general control nonderepressible-2 (GCN2) (Byrne et al. 2012), and the role of GCN2 and

a putative regulatory element (Gao et al. 2016) that is identical to the N-motif or GCN4-like regulatory motif of storage protein genes but this requires further investigation. There are also four asparagine synthetase genes

in maize (*Zea mays*) and barley (*Hordeum vulgare*) and, similarly wheat genes, these too are differentially expressed (Todd et al. 2008). The kinetic parameters of the enzymes encoded by three of the maize genes also show significant differences (Duff et al. 2011). To conclude, asparagine synthetase genes and enzymes are main factors defining asparagine accumulation and acrylamide formation in wheat grain.

The fact that wheat, barley, and maize all have a complement of four asparagine synthetase genes suggests that this may be typical of the cereals. Other species have also been shown to have multiple asparagine synthetase genes. The first to be characterized at the molecular level were AS1 and AS2 from pea (*Pisum sativum*) (Goruzzi 1990; Tsai and Coruzzi 1990, 1991). They were shown to encode proteins of 66.3 and 65.6 kDa, respectively, with 50–55% amino acid sequence identity with human asparagine synthetase and highly conserved glutamine binding sites (Met-Cys-Gly-Ile) (Goruzzi 1990). Tsai et al. (1990) showed that both AS1 and AS2 exist as single copies in peas and northern analyses revealed that both are dark induced, particularly in mature plants. Subsequently, three genes, ASN1, ASN2, and ASN3, were identified in Arabidopsis (Lam et al. 1994) and shown to be differentially expressed tissue specifically and in response to stress stimuli, light, and sucrose (Lam et al. 1998b). Light, for example, represses expression of ASN1 in a phytochrome-dependent manner, whereas expression of ASN2 is extremely low in the dark but rapidly induced by light treatment. The expression of both ASN1 and ASN2 is also affected by the supply of organic nitrogen in the form of glutamate, glutamine, or asparagine (43). Details of these and other asparagine synthetase studies are given in Table 3.

Sulfur Content in the Grain, Asparagine Accumulation, and Acrylamide Formation

Reaction to sulfur deficiency of DH lines

Analyses showed that the lowest free asparagine line was SR3, but it still demonstrated a large and significant increase of free asparagine content in response to sulfur deficiency: 26 mmol/kg under sulfur-deficient conditions compared with 1.6 mmol/kg under sulfur-sufficient conditions. Spark on the other hand had the most dramatic increase in free asparagine, from 2.8 mmol/kg to over 61 mmol/kg (Fig. 1). After canonical variate analysis (CVA), the main contributors in canonical variate 1 (CV1) were again asparagine, alanine, and aspartic acid, and in CV2 were alanine, glycine, and phenylalanine (data not shown) (Curtis et al. 2009).

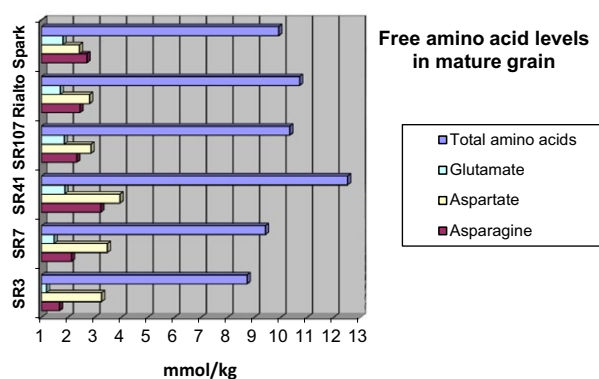


Figure 1. Free asparagine (red), glutamate (light blue), and aspartic acid (yellow), and total amino acid concentration levels (blue) in mature grain of wheat varieties Spark and Rialto and four DH lines from a Spark × Rialto mapping population. Concentrations are in mmol/kg (Curtis et al. 2009).

Relationship between asparagine concentration and acrylamide formation in whole grain flour samples from wheat

To establish the relationship between asparagine concentration and acrylamide formation in wholegrain wheat matrixes, whole wheat grain was milled and 0.5 g analyzed for total amino acid. Fractions of the samples were then baked dry and analyzed for acrylamide, the correlation calculated from these data showed a range of possible asparagine concentrations, and quadratic correlation between asparagine and acrylamide levels gave $R^2 = 0.9945$. Most of the samples analyzed that were grown under sulfur-sufficient conditions were in the range between 1 and 3.5 mmol/kg (Fig. 2). The highest levels of asparagine and therefore increased acrylamide-forming potential were the samples grown under acute sulfur-deficient conditions (Fig. 2). Asparagine concentration was closely linked with

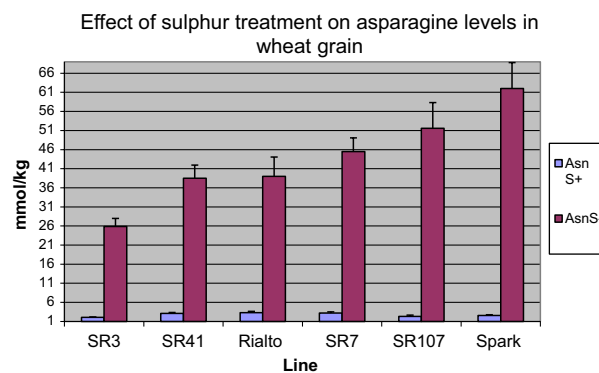


Figure 2. Asparagine concentration (mmol/kg) under sulfur-sufficient (blue Asn S+) and sulfur-deficient (red Asn S-) treatments of Spark and Rialto parental varieties and four DH lines: SR3, SR41, SR7, and SR107.

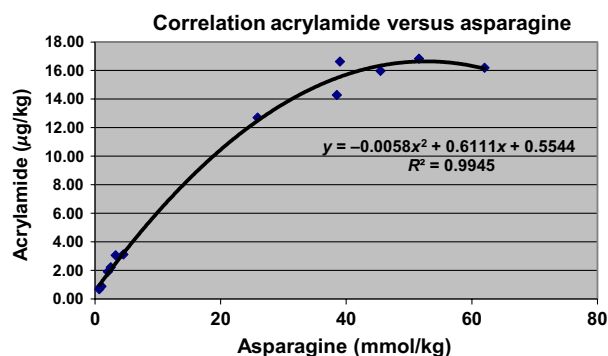


Figure 3. Free asparagine concentration (mmol/kg) in wheat (*Triticum aestivum*) grain plotted against acrylamide formed in heated flour (Curtis et al. 2009).

acrylamide formation (Fig. 3). For example, if the wheat grain contains 1.9 mmol/kg asparagine, the acrylamide-forming potential will be 1.69 µg/kg (0.042 µmol/kg), which is well below the indicative levels set by the European Commission (Curtis et al. 2009).

The formula predicting the acrylamide concentration based on the asparagine content is as follows: $y = -0.0058x^2 + 0.6111x + 0.5544$.

Relationship between acrylamide and sulfur in wheat grain

To establish a correlation between acrylamide content and sulfur content in the grain, the same samples with already measured asparagine and acrylamide contents were analyzed for total sulfur and nitrogen content. Acrylamide levels measured in samples grown under sulfur-sufficient, sulfur-deficient, and control conditions were negatively correlated with sulfur content in the grain (Fig. 4). The samples with the highest concentration of acrylamide were the samples with the least sulfur in the grain. For example,

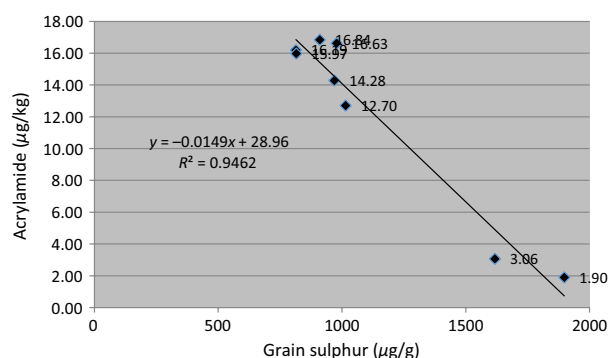


Figure 4. Total grain sulfur (mg/g [ppm]) plotted against acrylamide formed in heated flour (µg/kg [ppb]) (Curtis et al. 2009).

samples with 16.84 µg/kg acrylamide had 800 µg/g sulfur. This is a very important finding as it would suggest that the higher the sulfur in the grain, the lower the acrylamide formed in the grain will be (Fig. 4). However, there will be a level when the addition of more sulfur does not result in a change in the level of asparagine. As asparagine is needed for germination the level is unlikely to ever fall to zero (Curtis et al. 2009).

Previously, the high levels of acrylamide 2600 and 5200 µg/kg were measured in wheat flour of sulfur-deprived treatment in comparison to 600–900 µg/kg acrylamide content of normal levels of sulfate-fertilized wheat (Muttucumaru et al. 2006). Independent research of wheat cultivar “Star” in Germany was tested to determine the impact of sulfur fertilization on asparagine accumulation and acrylamide formation levels in the flour. In sufficient sulfur-fertilized wheat, the free asparagine was determined at 0.03–0.4 g/kg and significantly higher 3.9–5.7 g/kg in sulfur-deficient wheat sample. The high levels of acrylamide and 3-aminoproionamide (40–76 mg/kg) were also observed in the low-sulfur samples (1.7–3.1 mg/kg) (Granvogl et al. 2007). Additional evidences for elevated levels of acrylamide in response to higher asparagine were presented in 2008 in paper by Elmore et al., where acrylamide was up to six times higher in sulfur-deprived wheat flour in comparison to the wheat flour obtained from sulfur-sufficient wheat grain (Elmore et al. 2008).

Conclusions From This Study

It was concluded that the most important factor controlling asparagine concentration was sulfur deficiency, although there were also significant effects of G, E, and G × E (Curtis et al. 2009). Therefore, a key aspect of acrylamide mitigation would be the avoidance of sulfur deficiency during wheat cultivation. Even small proportions of sulfur-deficient grain would lead to a large increase in acrylamide formation during baking. Screening existing varieties for low asparagine accumulation and further improvement by plant breeding could also be part of the solution.

Grain Storage Protein and Sulfur Content in Wheat

Prolamins, known as grain storage proteins, play a major role in the distribution and accumulation of sulfur and nitrogen in the grain. A high level of available nitrogen results in an increased proportion of prolamin storage proteins. If no additional sulfur is provided, then the proportion of sulfur-poor prolamins and HMW prolamins increases and the proportion of sulfur-rich prolamins decreases (Shewry et al. 2001). The wheat grain consists of two predominant types of grain storage proteins: monomeric gliadins and

polymeric glutenins, comprising 60–80% of the total protein content of the mature grain (Shewry et al. 2001). Gliadins are classified into ω 1, 2-, ω 5-, α/β -, and γ -gliadins, and the glutenins into high-molecular-weight glutenin subunits (HMW-GS) and low-molecular weight glutenin subunits (LMW-GS). Low sulfur-containing proteins are also called sulfur-poor proteins: HMW-GS, some LMW-GS, and ω 1, 2-, ω 5-gliadins are called sulfur-poor because they differ in their content of the sulfur-containing amino acids cysteine and methionine in their poly amino acids chains (Shewry et al. 1997). To understand the mechanisms of sulfur regulation of storage protein gene expression Dai et al. investigated different treatments of sulfur and nitrogen availability (Dai et al. 2015). In their results they reported that the sulfur-deficient grain contained 28% less total sulfur than the control grain. The kinetic analyses also showed that sulfur-rich and sulfur-poor proteins accumulated at different rates depending on the nitrogen and sulfur supply, the most important results being that under sulfur-deficient conditions the levels of S-poor grain storage proteins were greater per grain at maturity (Dai et al. 2015). Another interesting observation was that by adding additional sulfur midway through grain filling the mass per grain increased, ultimately relieving the sulfur deficiency. After the nutritional shift the mass of sulfur-rich grain storage proteins increased gradually (Dai et al. 2015).

One additional finding by Dai et al. was that the expression of sulfur-poor genes was tightly regulated at the transcriptional level. They also confirmed that nitrogen and sulfur availability affected the free amino acid pools. When sulfur was added at the mid grain-filling stage, the effects of sulfur deficiency were reversed and levels of asparagine, aspartate, lysine, arginine, tyrosine, leucine, and valine quickly decreased, whereas the levels of glutathione (GSH) and glutathione disulfide (GSSG) increased to the levels observed under normal nitrogen and sulfur conditions (Dai et al. 2015).

The timing of regulatory events in response to N and S supply is also very important. Dai et al. also found that the increase in most amino acids in response to sulfur supply started midway through grain filling (~600°C dpa) thus indicating the critical point in grain response to sulfur deficiency leading to more efficient application of sulfur fertilizer in the field. It was also shown that S supply at 490°C days after a thesis can also efficiently mitigate S-deficiency (Dai et al. 2015).

Effects of sulfur fertilization on acrylamide-forming potential of wheat

The effect of sulfur fertilization on the acrylamide-forming potential in wheat was measured in a group of samples provided by Prof Steve McGrath (Rothamsted Research)

and grown at Woburn (UK), Rosemaund (Hereford), and Frostender (Suffolk) (UK) in 2010 and 2011 under five sulfur treatments at 0, 12.5, 25, 50, and 75 kg/ha SO_3 . Analysis of the levels of free amino acids and acrylamide gave a recommended application rate of 50 kg per hectare SO_3 . This is at the upper end of the recommended application rate given in the Fertilizer Manual (RB209) for wheat used for the biscuit and breakfast cereal market. These data were published in HGCA information sheet on sulfur for cereals and oilseed rape (<http://cereals.ahdb.org.uk/media/357116/is28-sulphur-for-cereals-and-oilseed-rape.pdf>).

Advice to Farmers, Breeders, and Food Processors

The data suggest that there are significant differences between wheat varieties with respect to their acrylamide-forming potential, the limiting factor being the level of free asparagine concentration in the grain. This is the parameter on which varietal selection should be based and a trait that should be incorporated into breeding programmes.

The identification of the QTL for free asparagine concentration has so far been unsuccessful but may be possible with different mapping populations. One potential target for breeders could be the *TaANS2* gene.

E factors have a significant effect on free asparagine accumulation in wheat, on their own and in combination with varietal differences ($G \times E$). It is therefore important that varieties are tested over a range of environmental conditions.

Management of wheat production is an important additional factor. Sulfur deficiency in particular causes a massive accumulation of free asparagine in wheat grain which should be avoided: an application rate of 50 kg/ha SO_3 is recommended, more if the soil is already sulfur deficient.

The use of nitrogen fertilizer increases the free asparagine and total free amino acid concentrations in wheat causing a concomitant increase in the acrylamide-forming potential (Sandli et al. 1993; Kingston-Smith et al. 2006; Hoegy et al. 2013). This, however, is only the case when nitrogen fertilization is in much greater proportion than sulfur. Nitrogen fertilizer is required to maintain the yield and quality of the crop, but excessive application should be avoided. Ensuring that other minerals are available to the crop may mitigate the effect of excessive nitrogen. Some or all of these points may apply to other cereals.

Conclusions

The acrylamide issue is a difficult problem facing the food industry in Europe and worldwide.

Avoidance of sulfur deficiency is essential: even small amounts of sulfur-deficient grain entering the food chain should be avoided. The application of 50 kg/ha of SO₃ equivalent (20 kg/ha of sulfur) is currently advisable depending on the soil type.

Plant breeders must take on board this issue or risk losing market share to those who do. However, many of the compounds which make a food product appealing to the consumer are formed by similar pathways to acrylamide, so changes in free amino acid concentrations may affect desirable tastes and aromas.

There are significant health benefits associated with eating cereal (particularly wholegrain) products and these must be retained while acrylamide levels are decreased.

Acrylamide is a good news story! It is new knowledge, not a new risk, and its discovery should enable food to be made safer. However, managing how this issue is covered by the media and therefore perceived by the public is important: facts should not be misinterpreted, misunderstood, or overexaggerated.

Conflict of Interest

Tanya Curtis is currently supported by a consortium of companies from the wheat supply chain.

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