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Formation of High Levels of Acrylamide during the Processing of Flour Derived from Sulfate-Deprived Wheat

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When wheat was grown under conditions of severe sulfate depletion, dramatic increases in the concentration of free asparagine were found in the grain of up to 30 times as compared to samples receiving the normal levels of sulfate fertilizer. The effect was observed both in plants grown in pots, where the levels of nutrients were carefully controlled, and in plants grown in field trials on soil with poor levels of natural nutrients where sulfate fertilizer was applied at levels from 0 to 40 kg sulfur/Ha. Many of the other free amino acids were present at higher levels in the sulfate-deprived wheat, but the levels of free glutamine showed increases similar to those observed for asparagine. In baked cereal products, asparagine is the precursor of the suspect carcinogen acrylamide, and when flours from the sulfate-deprived wheat were heated at 160 °C for 20 min, levels of acrylamide between 2600 and 5200 μg/kg were found as compared to 600−900 μg/kg in wheat grown with normal levels of sulfate fertilization.

KEYWORDS: Acrylamide; wheat; asparagine; agronomy; sulfate fertilizer

INTRODUCTION

Acrylamide was first reported in cooked foods in 2002 (*1*), causing considerable disquiet within the food industry and regulatory authorities. The International Agency for Research on Cancer has classified acrylamide as probably carcinogenic to humans, and carcinogenic action in rodents has been described; acrylamide also has neurological and reproductive effects (*2*). Foods with the highest levels of acrylamide are carbohydrate-rich and cooked at high temperatures, for example, by frying, roasting, or baking. They include foods derived from wheat, maize, other cereals, and potato, as well as coffee.

Shortly after acrylamide was first reported in cooked foods, the thermal degradation of free asparagine in the presence of sugars in the Maillard reaction was proposed as the major route for acrylamide formation (*3*, *4*). Labeling experiments confirmed that the carbon skeleton of acrylamide and the nitrogen of the amide group derived from asparagine (*4*, *5*). The Maillard reaction is also responsible for the generation of desirable flavors and colors in heated food, and, therefore, acrylamide levels are affected by the same factors that influence flavor and color formation during heating. These include reactant concentrations (i.e., the reducing sugar and free amino acid content of food), time−temperature conditions during processing, moisture levels, pH, and the presence of additives. Reactant levels are influenced not only by the type of food but also by cultivar, soil conditions, harvesting times, and storage conditions of the raw food.

Proposals for lowering acrylamide levels in food include reducing cooking times and temperatures (*6*−*8*), lowering the pH (*6*, *9*, *10*), as well as using raw materials with low sugar or asparagine content (*11*−*14*). The use of asparaginase to convert asparagine to aspartic acid has been shown to reduce acrylamide levels in processed foods (*5*), although the enzyme has not yet been approved for food use. While reduction of cooking time and temperature or lowering of pH may reduce acrylamide by limiting the extent to which the Maillard reaction occurs, this is likely to reduce color and flavor development as well. Similarly pre-washes of raw materials, especially potatoes, may reduce the reactants available for the Maillard reaction, but this will also reduce color and flavor alongside acrylamide. In potatoes, the ratio of reducing sugars to free amino acids is relatively low as compared to cereals, and the sugar concentration may be a limiting factor in acrylamide formation. This is not the case with cereals where the molar concentrations of sugars are much higher than the free amino acids, and, therefore, changes in natural free asparagine levels in cereals will have a direct impact on the acrylamide-forming potential. Both genetic factors and agronomy may be expected to affect asparagine accumulation, although neither area has received much attention to date.

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Severe sulfate deficiency is known to increase the levels of free asparagine in barley grain (*15*), but this has not previously been linked with acrylamide formation. It also causes a decrease in the synthesis of the major seed storage proteins, the prolamins (*16*). The inference is that the decrease in storage protein synthesis while plenty of nitrogen is available leads to the accumulation of glutamine and asparagine. Conversion of glutamine to asparagine may be advantageous because asparagine has a higher N:C ratio, its accumulation therefore tying up less carbon, and because asparagine is less metabolically active. Free asparagine can therefore be viewed as a nitrogen "sink". The leaves of wheat plants grown under sulfate-deficient conditions have been shown to accumulate high levels of asparagine (*17*), but the grain from these plants was not analyzed.

In this paper, we report the effect of sulfate deficiency during the growing of wheat on the levels of free asparagine and other amino acids in the grain and show how this influenced the levels of acrylamide formed during heating of flour derived from the grain.

MATERIALS AND METHODS

Reagents and chemicals were purchased from normal laboratory suppliers and were of analytical grade. $^{13}C_3$-Acrylamide (1 mg/mL in methanol) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA).

Growing of Wheat in Pots. Three varieties of winter wheat, Malacca (premium breadmaking), Claire (biscuit), and Solstice (standard breadmaking), were grown in a glasshouse in pots containing vermiculite, which does not retain nutrients. There were six plants per pot and 20 pots per variety. Feeding was started 3 weeks after potting: one-half of the pots for each variety were watered with medium containing sufficient amounts of potassium, phosphate, calcium, magnesium, sodium, iron, nitrate, and sulfate ions (1 mM $MgSO_4$), and one-half were watered with the same medium lacking the sulfate. Seed was harvested at maturity, approximately 20−21 weeks after planting, and pooled for each variety and treatment. The seeds were milled and vacuum packed.

Field-Grown Wheat. Samples from a sulfur response trial in the 2001−02 season were kindly provided by Fangjie Zhao of Rothamsted Research. Winter wheat (cv Hereward) was grown on a sandy loam soil on the Rothamsted Research farm at Woburn, Bedfordshire, UK. This soil has very poor nutrient retention and is severely sulfate deficient (extractable soil sulfate concentrations range from 0.5 to 1.8 mg S/kg) (*18*). Treatments included 0, 10, and 40 kg sulfur per hectare. Sulfate was applied as gypsum (calcium sulfate dihydrate) at the tillering stage in March 2002. Nitrogen was applied at 200 kg N per hectare to all treatments as ammonium nitrate. Grain was harvested at crop maturity, dried at 80 °C for 24 h, and ground with a stainless steel centrifugal mill to pass a 0.5 mm sieve. Standard methods were used to determine total sulfur and nitrogen content of the grain (*19*).

Determination of Free Amino Acids. Flour samples (0.500 ± 0.005 g) were weighed into a 14 mL screw-top bottle. Hydrochloric acid (10 mL, 0.01 M) was added to the vial, and the sample was stirred for 15 min at room temperature. An aliquot of supernatant (2 mL) was centrifuged at 7200*g* for 15 min, and 100 μL of the centrifuged supernatant was then derivatized using the EZ-Faast amino acid derivatization technique for GC−MS (Phenomenex, Torrance, CA) as described previously (*20*).

GC−MS analysis of the derivatized samples was carried out using an Agilent 5975 system (Agilent, Palo Alto, CA) in electron impact mode. An aliquot of the derivatized amino acid solution (1 μL) was injected at 280 °C in split mode (40:1) onto a Zebron ZB-AAA capillary column (10 m × 0.25 mm; 0.25 μm film thickness). The oven temperature was held at 110 °C for 1 min and then increased at 30 °C/min to 310 °C. The transfer line and ion source were maintained at 320 and 230 °C, respectively; carrier gas flow rate was kept constant throughout the run at 1.5 mL/min. Three analyses were performed for each sample.

Analysis of Sugars. Each flour sample (0.200 ± 0.005 g) was weighed into a 14 mL screw-top bottle. Aqueous methanol (50%, 10 μL) containing 100 mg/L trehalose was added to the bottle, and the sample was stirred for 15 min at room temperature. After a further 15 min, 1.5 mL of supernatant was removed from the bottle and centrifuged at 7200*g* for 15 min. Five hundred microliters of the centrifuged supernatant was diluted 10-fold in water; 2 mL of the diluted extract was then filtered through a 0.2 μm syringe filter.

The extracts were analyzed on a Dionex high performance anion exchange chromatography system using a 250 × 4 mm Carbopac PA1 column with pulsed amperometric detection (Dionex Corp., Sunnyvale, CA) and controlled by Chromeleon software. The chromatography system consisted of an AS50 autosampler, LC25 column oven, GS50 pumps, and an ED50 pulsed amperometric detector, running in internal amperometric mode. Injection volume was 25 μL. A gradient program was set up using 200 mM NaOH (solvent A) and water at a flow rate of 1 mL/min; 50% solvent A, held for 10 min and then increased to 100% at 40 min. The column was then washed for 8 min with 500 mM sodium acetate in 125 mM NaOH and re-equilibrated with 50% solvent A for 7 min. The waveform of the pulsed amperometric detector was: 400 ms at 0.1 V, 20 ms at −2.0 V, 10 ms at 0.6 V, and 60 ms at −0.15 V. Standards of glucose, maltose, fructose, and sucrose were used for quantification. Each sample was extracted and analyzed in triplicate.

Production and Analysis of Acrylamide. Samples (0.5 g) in unsealed glass ampoules (1 mL capacity) were heated for 20 min, at either 160 or 180 °C. Copper wire was tied around the necks of the filled ampoules, and the ampoules were then heated by being suspended by the wire from the ceiling of a GC oven. Acrylamide was extracted from these samples with 25% aqueous methanol and converted to the dibromo-derivative, prior to analysis by GC−MS, using the method of Castle et al. (*21*), with the modifications described by Elmore et al. (*20*). Labeled $^{13}C_3$-acrylamide was used as the internal standard.

The brominated extracts (2 μL) were injected onto the Agilent 5975 GC−MS system in pulsed splitless mode at 250 °C, the splitter opening after 0.5 min. The helium carrier gas pressure was 21 psi in pulsed mode, falling to 9.6 psi for the rest of the run. A DB-17 MS capillary column was used (30 m × 0.25 mm i.d., 0.15 μm film thickness; Agilent). The oven temperature was 85 °C for 1 min, rising at 8 °C/min to 200 °C, then 30 °C/min to 280 °C for 10 min. The transfer line was held at 280 °C and the ion source at 180 °C. The mass spectrometer was operated in electron impact mode with selected ion monitoring. Two ions were used to monitor brominated $^{13}C_3$-acrylamide (*m*/*z* 153 and 155), and another two ions were used for brominated acrylamide (*m*/*z* 150, 152). The ion *m*/*z* 155 was used to quantify brominated $^{13}C_3$-acrylamide, and the ion *m*/*z* 150 was used to quantify brominated acrylamide. Each sample was prepared and analyzed in triplicate.

RESULTS AND DISCUSSION

Three varieties of winter wheat, Malacca, Claire, and Solstice, were grown in pots and watered with medium containing sufficient amounts of the normal nutrients with the exception of sulfate, which was added to only one-half of the pots. The wholegrain flour from the plants showed only small differences in the free amino acid contents between varieties grown with all nutrients (**Table 1**). However, the level of free asparagine in the flour prepared from the grain of all three cultivars rose dramatically in response to sulfate deprivation (**Table 1**), in the case of Malacca by almost 30-fold from 5 to 153 mmol/kg. Overall, the levels of most free amino acids were higher in the sulfate-deprived wheat, but the only other amino acid to increase very substantially was glutamine, with Malacca again showing the biggest change, from 0.43 to 69 mmol/kg.

We also obtained samples of wheat flour, which had been grown in the 2001−2002 season on a very sandy soil that suffers high nutrient leaching. Plots were treated with three levels of sulfate fertilizer, 0, 10, and 40 kg S/Ha, but the same level of nitrate (200 kg N/Ha). Results similar to those from the pot-

Table 1. Free Amino Acids (mmol per kg wet wt) in Flour from Three Varieties of Wheat Samples, Grown in Pots on Vermiculite with or without Added Magnesium Sulfate[a]

	without sulfate			with sulfate		
amino acid	Solstice	Malacca	Claire	Solstice	Malacca	Claire
Asn	75.4 (3.80)	153 (1.25)	47.9 (1.14)	4.54 (0.50)	5.20 (1.10)	4.12 (0.26)
Gln	40.9 (3.92)	69.1 (4.35)	15.7 (1.43)	0.37 (0.04)	0.43 (0.09)	0.41 (0.09)
Try	0.06 (0.01)	0.12 (0.00)	0.06 (0.00)	0.07 (0.00)	0.11 (0.02)	0.10 (0.01)
Ala	4.08 (0.13)	7.66 (0.18)	2.30 (0.03)	0.85 (0.11)	0.81 (0.09)	0.79 (0.03)
Gly	1.22 (0.02)	3.27 (0.13)	0.74 (0.02)	0.23 (0.03)	0.22 (0.02)	0.19 (0.01)
Val	1.60 (0.09)	2.69 (0.05)	0.66 (0.02)	0.33 (0.04)	0.23 (0.04)	0.23 (0.00)
Leu	0.54 (0.05)	1.09 (0.03)	0.28 (0.01)	0.16 (0.04)	0.14 (0.03)	0.15 (0.01)
Ile	0.38 (0.02)	0.81 (0.03)	0.18 (0.01)	0.15 (0.03)	0.10 (0.03)	0.13 (0.01)
Thr	0.55 (0.01)	1.05 (0.07)	0.29 (0.01)	0.06 (0.00)	0.06 (0.02)	0.06 (0.01)
Ser	1.74 (0.05)	3.55 (0.37)	0.64 (0.06)	0.10 (0.01)	0.08 (0.01)	0.08 (0.02)
Pro	3.10 (0.13)	5.27 (0.25)	1.50 (0.13)	0.39 (0.06)	0.56 (0.12)	0.38 (0.05)
Asp	13.3 (0.24)	15.8 (0.31)	9.86 (0.56)	3.41 (0.15)	5.67 (0.68)	6.28 (0.08)
Met	0.11 (0.01)	0.20 (0.03)	0.14 (0.10)	0.05 (0.02)	0.03 (0.01)	0.06 (0.00)
Glu	7.50 (0.91)	9.36 (1.36)	5.50 (0.62)	2.29 (0.56)	2.88 (0.46)	2.74 (0.16)
Phe	0.26 (0.01)	0.55 (0.04)	0.23 (0.01)	0.13 (0.03)	0.23 (0.03)	0.17 (0.01)
Lys	0.34 (0.02)	0.89 (0.03)	0.15 (0.01)	0.04 (0.00)	0.04 (0.01)	0.03 (0.01)
His	0.06 (0.02)	0.19 (0.09)	0.06 (0.01)	0.01 (0.00)	0.02 (0.02)	0.04 (0.02)
Tyr	0.05 (0.00)	0.11 (0.01)	0.04 (0.01)	0.02 (0.00)	0.03 (0.00)	0.02 (0.01)

[a] Values are means of three replicates with standard deviations in parentheses.

Table 2. Free Amino Acids (mmol per kg wet wt) in Five Samples of Flour from Wheat (var. Hereward) Field-Grown with or without Added Sulfate Fertilizer[a]

	sulfate added (kg S per Ha)				
amino acid	0	0	10	40	40
Asn	75.7 (2.62)	55.7 (1.77)	7.80 (0.29)	4.43 (0.11)	3.07 (0.23)
Gln	12.1 (0.27)	7.43 (0.62)	0.58 (0.10)	0.51 (0.08)	0.30 (0.06)
Try	0.40 (0.00)	0.42 (0.03)	0.87 (0.01)	1.25 (0.13)	1.06 (0.09)
Ala	5.27 (0.21)	3.77 (0.08)	0.99 (0.15)	0.84 (0.07)	0.72 (0.04)
Gly	2.08 (0.01)	1.61 (0.03)	0.40 (0.02)	0.32 (0.03)	0.27 (0.02)
Val	2.19 (0.07)	1.65 (0.06)	0.28 (0.04)	0.21 (0.02)	0.17 (0.03)
Leu	0.93 (0.01)	0.74 (0.03)	0.22 (0.05)	0.14 (0.01)	0.11 (0.01)
Ile	0.67 (0.08)	0.49 (0.19)	0.13 (0.02)	0.08 (0.01)	0.07 (0.02)
Thr	1.29 (0.05)	0.90 (0.04)	0.18 (0.02)	0.13 (0.02)	0.09 (0.02)
Ser	4.06 (0.04)	2.71 (0.09)	0.32 (0.03)	0.22 (0.05)	0.17 (0.04)
Pro	6.20 (0.15)	5.26 (0.06)	0.90 (0.01)	0.52 (0.02)	0.41 (0.04)
Asp	14.0 (0.29)	13.1 (0.22)	5.46 (0.33)	2.94 (0.07)	2.95 (0.23)
Met	0.24 (0.21)	0.09 (0.03)	0.17 (0.10)	0.04 (0.02)	0.38 (0.61)
Glu	7.86 (0.68)	5.90 (0.45)	1.39 (0.10)	1.23 (0.09)	0.88 (0.03)
Phe	0.38 (0.01)	0.33 (0.01)	0.15 (0.03)	0.11 (0.00)	0.07 (0.01)
Lys	1.02 (0.11)	0.60 (0.07)	0.14 (0.01)	0.11 (0.01)	0.09 (0.01)
His	0.13 (0.07)	0.08 (0.03)	0.04 (0.01)	0.02 (0.01)	0.03 (0.01)
Tyr	0.36 (0.03)	0.30 (0.01)	0.22 (0.02)	0.19 (0.01)	0.19 (0.01)

[a] Values are means of three replicates with standard deviations in parentheses.

grown wheat were obtained for the free amino acid contents (**Table 2**), with the grain from the plots with no added sulfate giving an average asparagine content of 66 mmol/kg as compared to 3.7 mmol/kg in the grain from wheat grown on soil with 40 kg/Ha S addition. It is notable that even the grain from wheat grown with the addition of 10 kg/Ha S had twice as much free asparagine as that from wheat grown with 40 kg/Ha S. Analysis of the grain from the three different levels of fertilizer treatment showed mean total sulfur contents of 865, 1170, and 1314 mg/kg for 0, 10, and 40 kg/Ha sulfate, respectively, while the total nitrogen contents were 2.04%, 1.97%, and 1.86%. These data clearly show sulfate deprivation in samples grown with zero and 10 kg/Ha sulfate addition.

Although there were some variations in the sugar (glucose, fructose, sucrose, maltose) concentrations in the flours, there was no clear relationship between sulfate addition and sugar (**Table 3**). For the grain from the plants supplied with adequate sulfate, the reducing sugars were in molar excess as compared to the free amino acids; however, in the samples from sulfate-deprived plants, this was no longer the case.

The amounts of acrylamide formed on heating flour from the sulfate-deprived grain were dramatically higher than in the material from plants, which were grown with an adequate supply of sulfate (**Table 4**). Some of the trials produced only small amounts of flour, and, to ensure reproducible heating conditions, samples were heated in glass ampoules in a GC oven. This method gave consistent results with low coefficients of variation for replicate determinations. The levels in the samples from plants grown with adequate sulfate from both the pots and the field trials heated at 160 °C were consistent with well-cooked wheat products (*20*) although higher than levels found in most commercial wheat products. Previous work has shown that with prolonged heating acrylamide reaches a maximum (*20*). However, this was not attained in the present samples heated at 160 °C, because higher quantities of acrylamide were found on heating at 180 °C. The greatly increased concentration of

Table 3. Sugars (mmol per kg wet wt) in Flour from Wheat Grown with or without the Addition of Sulfate[a]

variety	sulfate added	glucose	fructose	sucrose	maltose
		Pot-Grown			
Solstice	0	2.30 (0.04)	1.01 (0.06)	24.53 (0.55)	5.19 (0.25)
Malacca	0	2.01 (0.03)	0.85 (0.03)	23.83 (0.55)	6.04 (0.13)
Claire	0	1.85 (0.06)	0.88 (0.05)	24.74 (0.72)	3.62 (0.37)
Solstice	1 mM[b]	1.89 (0.12)	0.88 (0.15)	25.65 (0.62)	5.95 (0.24)
Malacca	1 mM[b]	1.91 (0.13)	0.80 (0.05)	22.77 (0.83)	6.35 (0.38)
Claire	1 mM[b]	1.49 (0.02)	0.60 (0.01)	25.89 (1.69)	4.92 (0.23)
		Field-Grown			
Hereward	0	2.15 (0.11)	0.77 (0.12)	24.3 (0.48)	2.30 (0.06)
Hereward	0	2.38 (0.17)	0.71 (0.02)	21.6 (0.37)	1.93 (0.06)
Hereward	10 kg S/Ha[c]	1.97 (0.10)	1.40 (0.05)	23.8 (0.12)	1.67 (0.09)
Hereward	40 kg S/Ha[c]	2.67 (0.46)	1.88 (0.41)	24.3 (0.64)	1.68 (0.10)
Hereward	40 kg S/Ha[c]	7.00 (0.77)	1.90 (0.04)	19.5 (1.85)	3.93 (0.41)

[a] Values are the means of three replicates with standard deviations shown in parentheses. [b] In feedwater. [c] As $CaSO_4 \cdot 2H_2O$.

Table 4. Acrylamide (μg/kg) Formed in Flour from Wheat, Grown with or without the Addition of Sulfate, and Heated at 160 or 180 °C for 20 min[a]

		acrylamide formed on heating	
variety	sulfate added	160 °C	180 °C
		Pot-Grown	
Solstice	0	3759 (121)	7858 (638)
Malacca	0	5198 (384)	7832 (636)
Claire	0	2616 (54)	6921 (491)
Solstice	1 mM[b]	679 (44)	1316 (77)
Malacca	1 mM[b]	934 (121)	2461 (333)
Claire	1 mM[b]	836 (54)	1956 (121)
		Field-Grown	
Hereward	0	5286 (164)	na[c]
Hereward	0	3976 (76)	na
Hereward	10 kg S/Ha[d]	1163 (34)	na
Hereward	40 kg S/Ha[d]	723 (15)	na
Hereward	40 kg S/Ha[d]	741 (22)	na

[a] Values are the means of three replicates with standard deviations shown in parentheses. [b] In feedwater. [c] Not analyzed. [d] As $CaSO_4 \cdot 2H_2O$.

asparagine in grain from the sulfate-deprived wheat resulted in increases of acrylamide; for the pot-grown wheat, the average increase across the three varieties heated at 160 °C was 4.7-fold, while for the field-grown wheat the increase was 6.3-fold. The fact that acrylamide levels did not rise in proportion to the increase in asparagine may be due to the reaction becoming limiting in sugar concentration when the free asparagine attained the extremely high levels present in the sulfate-deprived wheat.

Sulfur nutrition in the UK was of little practical concern until the latter part of the last century when it became apparent that increasing areas of land used for crop production were becoming sulfur-deficient. This was probably because of a decrease in the use of sulfur-containing fertilizers, such as ammonium sulfate and superphosphate (a mixture of dihydrogen phosphate and hydrated calcium sulfate), increased crop yields resulting in the removal of minerals from the soil, and, ironically, reduced atmospheric deposition due to a switch away from coal as a fuel to low sulfur or sulfur-free alternatives such as natural gas (*22*). In the UK, the Home-Grown Cereals Authority (www.hgca.com) estimates that 23% of the United Kingdom is currently at high risk of sulfur deficiency for cereal cultivation. Sulfur deficiency in cereals has been reported in many countries including northern and western Europe, Australia, and New Zealand; therefore, the implications of the data presented in this paper could be widespread (*23*, *24*).

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