Effects of Plant Sulfur Nutrition on Acrylamide and Aroma Compounds in Cooked Wheat

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Wheat flour from plants deficient in sulfur has been shown to contain substantially higher levels of free amino acids, particularly asparagine and glutamine, than flour from wheat grown where sulfur nutrition was sufficient. Elevated levels of asparagine resulted in acrylamide levels up to 6 times higher in sulfur-deprived wheat flour, compared with sulfur-sufficient wheat flour, for three varieties of winter wheat. The volatile compounds from flour, heated at 180 °C for 20 min, have been compared for these three varieties of wheat grown with and without sulfur fertilizer. Approximately 50 compounds were quantified in the headspace extracts of the heated flour; over 30 compounds were affected by sulfur fertilization, and 15 compounds were affected by variety. Unsaturated aldehydes formed from aldol condensations, Strecker aldehydes, alkylpyrazines, and low molecular weight alkylfurans were found at higher concentrations in the sulfur-deficient flour, whereas low molecular weight pyrroles and thiophenes and sugar breakdown products were found at higher concentrations in the sulfur-sufficient flour. The reasons for these differences and the relationship between acrylamide formation and aroma volatile formation are discussed.

KEYWORDS: Wheat; agronomy; sulfate fertilizer; amino acids; sugars; volatile compounds

INTRODUCTION

Sulfur deficiency in agricultural crops grown in the United Kingdom and many other regions of the world is an increasing problem. A reduction in industrial pollution has resulted in a decrease in sulfur dioxide emissions, leading to less deposition of sulfur from the air. Furthermore, changes in fertilizer application practices have also been a contributing factor (*1*). The degree of sulfur deficiency is dependent on location, soil type, and plant species grown. For example, clay soils retain sulfur more readily than sandy soils, and crops in remote areas will suffer more than those grown near industrial activity.

Wheat flour from plants grown under sulfur-deficient conditions has been shown to be much higher in free amino acids than flour from plants sufficient in sulfur (*2, 3*). Of particular importance was the effect of sulfur deficiency on levels of free asparagine, which is the main precursor of the suspect carcinogen acrylamide. Asparagine was present at levels up to 30 times higher in sulfur-deficient wheat, and when wheat flour was heated at 180 °C for 20 min, levels of acrylamide were up to 6 times higher in the sulfur-deficient flour, compared with its sulfur-sufficient equivalent. Many of the other free amino acids were also affected by a lack of sulfur; in particular, glutamine, serine, lysine, and ornithine were all present in the variety Malacca at levels >20 times higher in the sulfur-deficient wheat, compared with the sulfur-sufficient samples. In fact, sulfur deficiency increased levels of glutamine in varieties Malacca and Solstice by a factor of 100.

It would seem likely that the differences in free amino acids caused by sulfur deficiency would affect the volatile composition of cooked wheat flour, with implications for the flavor of wheat-containing baked goods. Addition of free amino acids to foods as a means of acrylamide mitigation has been studied by several workers (*4–8*), and differences in the free amino acid composition of potato, caused by the addition of glycine, led to changes in the aroma volatile profile of heated potato cakes (*8*).

In this work we have heated wheat flour at 180 °C for 20 min to determine how its volatile composition is affected by sulfur deprivation and to examine the relationship between acrylamide formation and aroma formation. The three varieties of winter wheat studied, grown with or without added sulfur, were Malacca (premium breadmaking), Claire (biscuit), and Solstice (standard breadmaking). The precursor compositions of these flour samples and the effect of their compositions on acrylamide formation in heated wheat flour have been previously reported (*2*).

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MATERIALS AND METHODS

Chemicals. 1,2-Dichlorobenzene (99%), iodomethane (99.5%), iodoethane (99%), 1-iodo-2-methylpropane (97%), (S)-(+)-1-bromo-2-methylbutane (99%), 1-bromo-3-methylbutane (96%), 1-butyl-3-methylimidazolium hexafluorophosphate (96%), deuterated chloroform (100% D), dibutylamine (99.5%), toluene (99%), pyrrole (98%), 1-methylpyrrole (99%), 3-methylpyrrole (Sigma grade), 2-pyrrolecarboxaldehyde (98%), trimethylpyrazine (99%), 2-ethyl-5(6)-methylpyrazine (98%), dimethyl disulfide (Sigma grade), dimethyl trisulfide (98%), 2-methylthiophene (97%), 2-thiophenecarboxaldehyde (98%), 3-thiophenecarboxaldehyde (98%), 2-methylfuran (99%), 2,5-dimethylfuran (99%), 2-furancarboxaldehyde (99%), 3-furancarboxaldehyde (97%), formaldehyde solution (37% in water), acetaldehyde (99.5%), 2-methylpropanal (99%), 2-methylpropenal (95%), 2-butanone (99%), (E)-2-butenal (99%) pentanal (97%), 2-heptanone (98%), (E)-2-heptenal (98%), octanal (99%), nonanal (95%), benzaldehyde (99.9%), 2-methylbutyric acid (98%), 2,3-butanedione (97%), 2,3-pentanedione (97%), nitrobenzene (99.5%), methyltriphenylphosphonium bromide (98%), and sodium borohydride powder (98.5%) were all purchased from the Sigma-Aldrich Co. Ltd. (St Louis, MO); methylpyrazine (99%), 2,5-dimethylpyrazine (99%), 2,6-dimethylpyrazine (98%), ethylpyrazine (98%), 2-ethyl-3-methylpyrazine (99%), tetramethylpyrazine (98%), 2-ethyl-3,5(6)-dimethylpyrazine (99.5%), 3-methylbutanal (98%), and 3-methylfuran (98%) were all purchased from Fisher Scientific U.K. Ltd. (Loughborough, U.K.); trimethylpyrazine (99%), 2,3-diethylpyrazine (98%), butanal (99%), 2-methylbutanal (95%), thiophene (99%), 2-methyl-2-butenal (97%), hexanal (98%), heptanal (97%), and 2-pentylfuran (98%) were all purchased from Alfa Aesar (Heysham, U.K.); 2-ethylfuran was purchased from Oxford Chemicals Ltd. (Hartlepool, U.K.). Solvents used were methanol, *n*-pentane, and diethyl ether (all of AnalaR grade) from Merck Ltd. (Poole, U.K.).

Growing of Wheat. 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 (2). Half of the pots for each variety, designated S+, were watered with medium containing sufficient amounts of potassium, phosphate, calcium, magnesium, sodium, iron, nitrate, and sulfate ions (1 mM $MgSO_4$), and half were watered with the same medium lacking the sulfate (designated S−). After harvesting, the whole grains were milled, using a ball mill from Glen Creston Limited (Twickenham, U.K.), and the resulting flour was vacuum packed.

Preparation of Cooked Wheat Samples for Flavor Analysis. Wholemeal flour samples (0.50 g) in unsealed glass ampules (1 mL capacity) were heated for 20 min at 180 °C. Copper wire was tied around the necks of the filled ampules, and the ampules were then heated by being suspended by the wire from the ceiling of a GC oven. Three replicates were prepared for each flour sample, and the volatile composition of the entire cooked sample was analyzed.

Although the cooking conditions used were relatively intense, very low yields of the sulfur-deprived samples (as low as 15 g for the Malacca S− wheat) meant that very little sample was available for volatile analysis, requiring the need to cook at a high temperature to obtain sufficient volatiles for comparison of treatments. Samples cooked at 160 °C for 20 min contained insufficient volatile material for an effective comparison of treatments (Elmore, unpublished data).

Analysis of Volatiles. Aroma isolates were collected on Tenax TA (9). The cooked flour was transferred to a 250 mL conical flask with a screw-thread neck, and 100 mL of HPLC-grade water was added. Nitrogen, at 40 mL min^{-1}, flowed over the cooked flour sample, which was held at 35 °C, for 45 min, sweeping volatiles onto a glass-lined, stainless steel trap (105 mm × 3 mm i.d.) containing 85 mg of Tenax TA (Scientific Glass Engineering Ltd., Milton Keynes, U.K.). A standard (100 ng of 1,2-dichlorobenzene in 1 μL of methanol) was added to the trap at the end of the collection, and excess solvent and any water retained on the trap were removed by purging the trap with nitrogen at 100 mL min^{-1} for 10 min.

All analyses were performed on a Hewlett-Packard 5972 mass spectrometer, fitted with an HP5890 series II gas chromatograph and a G1034C Chemstation. A CHIS injection port (Scientific Glass Engineering Ltd.) was used to thermally desorb the volatiles from the

Tenax trap onto the front of a Factor Four VF-5 ms fused silica capillary column (60 m × 0.25 mm i.d., 0.25 μm film thickness; Varian, Oxford, U.K.). The front of the column was made into a coil of five loops, which were cooled in solid carbon dioxide, contained within a 250 mL beaker, during the desorption period of 5 min, while the oven was held at 40 °C. After desorption, the temperature of the oven was held at 40 °C for a further 2 min before being raised at 4 °C min^{-1} to 280 °C. Helium at 20 psi was used as the carrier gas, resulting in a flow of 1.2 mL min^{-1} at 40 °C. A series of *n*-alkanes (C_5–C_{25}) in diethyl ether was analyzed, under the same conditions, to obtain linear retention index (LRI) values for the cooked wheat aroma components.

The mass spectrometer operated in electron impact mode with an electron energy of 70 eV and an emission current of 35 μA. The mass spectrometer scanned from *m/z* 29 to 400 at 1.9 scans/s. Compounds were identified by first comparing their mass spectra with those contained in the NIST/EPA/NIH Mass Spectral Database or in previously published literature. In all cases, identities were confirmed by comparison of mass spectra and LRI values with those of authentic standards. Approximate quantities of the volatiles in the headspace were estimated by comparison of their peak areas with that of the 1,2-dichlorobenzene internal standard, obtained from the total ion chromatograms, using a response factor of 1.

An aroma extract of heated Solstice S− flour was also analyzed, using an HP-Innowax column (60 m × 0.25 mm × 0.25 μm; Agilent Technologies, Inc., Santa Clara, CA), to provide additional retention time data, for compound identification.

Synthesis of 2-Methylpyrrole. 2-Methylpyrrole was synthesized by refluxing 2-pyrrolecarboxaldehyde with sodium borohydride in 2-propanol (10). The product was distilled with admixed 2-propanol at 40 °C and 40 mmHg. The solvent was then removed from the product by fractional distillation, to leave 2-methylpyrrole of sufficient purity to prepare 1-alkyl-2-methylpyrroles.

Synthesis of 1-Alkylpyrroles. N-Substituted pyrroles were prepared from 1*H*-pyrroles and alkyl halides by heating in an ionic solvent with powdered potassium hydroxide (11). 1,2-Dimethylpyrrole was prepared from 2-methylpyrrole and iodomethane; 1,3-dimethylpyrrole was prepared from 3-methylpyrrole and iodomethane; 1-ethylpyrrole was prepared from pyrrole and iodoethane, 1-ethyl-2-methylpyrrole was prepared from 2-methylpyrrole and iodoethane; 1-ethyl-3-methylpyrrole was prepared from 3-methylpyrrole and iodoethane; 1-(2-methylpropyl)pyrrole was prepared from pyrrole and 1-iodo-2-methylpropane; 1-(2-methylbutyl)pyrrole was prepared from pyrrole and 1-bromo-2-methylbutane; and 1-(3-methylbutyl)pyrrole was prepared from pyrrole and 1-bromo-3-methylbutane. In all cases 1-butyl-3-methylimidazolium hexafluorophosphate was used as the ionic solvent. All reaction mixtures were heated for 2 h; those containing iodomethane and iodoethane were heated at 40 °C, whereas the others were heated at 80 °C.

Synthesis of Vinylfurans and Vinylthiophenes. Vinylthiophenes and vinylfurans were synthesized from their corresponding formyl-thiophenes and formylfurans, which were heated in nitrobenzene at 90 °C with methyltriphenylphosphonium bromide and potassium carbonate (12). The products were diluted in diethyl ether and analyzed by GC-MS.

Synthesis of α,β-Unsaturated Aldehydes. 2-Isopropylpropenal was prepared from 3-methylbutanal and 37% formaldehyde solution, using 2-methylbutyric acid and dibutylamine as catalysts (13). Formation of the product was monitored by solid phase microextraction (SPME). 2-Isopropylpropenal was extracted from the headspace above the reaction mixture, using SPME with a 75 μm Carboxen−PDMS fiber (Supelco, Bellefonte, PA). The needle of the SPME syringe was injected through a septum in a sidearm of the reaction vessel; the fiber was suspended inside the vessel for a few seconds, before being retracted. The contents of the fiber were analyzed by GC-MS, as described previously (14).

Base-catalyzed aldol condensation of aldehydes was used to prepare 2-isopropyl-2-butenal and 5-methyl-2-hexenal (15). 3-Methylbutanal and acetaldehyde were combined with 5% sodium hydroxide in a 100 mL two-necked round-bottom flask with condenser; the contents of the flask were mixed using an ultrasonic bath, which contained ice. The ice was allowed to melt, and the reaction continued for 24 h. Both

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2-isopropyl-2-butenal and 5-methyl-2-hexenal were formed as reaction products; their formation was monitored by SPME followed by GC-MS.

Although 2-isopropyl-2-butenal and 5-methyl-2-hexenal possessed very different mass spectra, which could be related to their chemical structures, it was necessary to separate these compounds from the reaction mixture to provide unequivocal confirmation of their identities by nuclear magnetic resonance spectroscopy (NMR). The reaction mixture was neutralized with hydrochloric acid and diluted to give a 50% aqueous suspension, which was steam-distilled with stirring. Fractions were collected, and those high in the desired products were combined, extracted in redistilled diethyl ether, and frozen to remove any residual water.

Flash Chromatography. 2-Isopropylbutenal and 5-methyl-2-hexenal were separated using a flash chromatography column (30 cm × 1.4 cm i.d.; Sigma-Aldrich, St. Louis, MO) containing silica gel 60 (40−63 μm for column chromatography; Merck Chemicals Ltd., Nottingham, U.K.), using *n*-pentane/diethyl ether (90:10, 150 mL) as the eluting solvent. Chromatography was performed under nitrogen pressure at a flow rate of 5 mL/min. Fractions were analyzed by GC-MS, and those fractions in which one of the compounds of interest was present in high purity were combined. The two 2-alkenal solutions were then concentrated to dryness using a stream of high-purity nitrogen, before being redissolved in deuterated chloroform (CDCl3; 2 mL). The deuterated chloroform solutions were then concentrated to 0.5 mL, again using a stream of nitrogen, to remove any residual ether and pentane.

Nuclear Magnetic Resonance Spectroscopy (NMR). A Bruker AMX 400 instrument with a 400 MHz operating frequency and a 9.4 T magnet was used to obtain ^{1}H NMR spectra of the purified 2-alkenals. Each 2-alkenal in CDCl3 was placed in a 5 mm tube inside a 5 mm probe. One hundred and sixty scans with 6 s repetition were measured.

Physicochemical Data from Synthesized Compounds. Seventeen compounds were synthesized to confirm their presence or absence in the heated flour extracts. The mass spectra and LRIs for those compounds are listed in **Table 1**, when appropriate.

As 2-isopropyl-2-butenal and 5-methyl-2-hexenal were prepared by the same reaction, they were separated and analyzed by NMR to unequivocally identify them. An NMR spectrum has not been previously reported for either compound. For the sake of clarity, long-distance coupling data are not presented.

5-Methyl-2-hexenal was isolated with a purity of 95%, of which 95% was the same isomer as that found in the heated wheat. The ^{1}H NMR spectrum (δ in ppm) was δ 0.96 (d, *J* = 6.8 Hz, 6H, (CH3)2) 1.83 (m, *J* = 6.8 Hz, 1H, (CH3)2CH), 2.23 (dd, *J* = 7.0 Hz, *J* = 7.0 Hz, 2H, (CH3)2CHCH2), 6.11 (dd, *J* = 6.7 Hz, *J* = 15.6 Hz, 1H, CH=CHCH=O) 6.83 (dd, *J* = 7.4 Hz, *J* = 15.6 Hz, 1H, CH=CHCH=O), 9.51 (d, *J* = 7.9 Hz, 1H, CH=O). The high coupling constant of 15.6 Hz between the protons in the double bond showed that (*E*)-5-methyl-2-hexenal was the geometric isomer formed in the cooked wheat.

2-Isopropyl-2-butenal was isolated with a purity of 85%, of which 75% was the same isomer as that found in the heated wheat. The ^{1}H NMR spectrum was δ 1.18 (d, *J* = 7.1 Hz, 6H, CH3CH=C[CH(CH3)2]CH=O) 2.00 (d, *J* = 7.1 Hz, 3H, CH3CH=C), 2.92 (m, *J* = 7.1 Hz, 1H, [CH(CH3)2]), 6.45 (quad, *J* = 7.1 Hz, 1H, CH3CH=C), 9.32 (s, 1H, CH=O). Using published chemical shift data (*16*), it was calculated that the geometric configuration of the isomer found in wheat was (*E*)-2-isopropyl-2-butenal; that is, the isopropyl and methyl groups were on the same side of the double bond. In addition, the 2D plot from a NOESY experiment showed that the proton on the double bond was interacting with the proton on the aldehyde, showing that both of these protons were on the same side of the double bond.

Statistical Analysis. XLStat 2006 (Addinsoft, Paris, France) was used to perform two-way analysis of variance on the data.

RESULTS AND DISCUSSION

Effect of Sulfur Deprivation on the Precursors of Acrylamide and Flavor in Wheat Flour. Acrylamide is formed when asparagine is heated with reducing sugars, and, to a lesser extent, with sucrose (*17*). In wheat, asparagine is regarded as the

Table 1. Mass Spectra and Linear Retention Indices of Synthesized Compounds

compound	$LRI_{VF5}{}^{a}$	$LRI_{Wax}{}^{b}$	mass spectral data, *m/z* (relative intensity)^{c}
3-vinylfuran	728	1109	**94**, 65 (40), 39 (36), 40 (20), 66 (17), 93 (9), 63 (8), 38 (8), 95 (6), 68 (4), 62 (4), 51 (4), 37 (4), 50 (3), 29 (3)
2-vinylfuran	728	1079	**94**, 65 (53), 66 (31), 39 (26), 40 (14), 63 (8), 38 (7), 95 (7), 62 (5), 27 (4), 51 (3), 37 (3), 50 (3), 55 (2), 61 (2)
2-isopropyl-2-propenal	733	1002	41, 55 (72), 69 (61), 39 (60), **98** (54), 27 (44), 29 (36), 83 (33), 53 (26), 97 (22), 43 (16), 42 (15), 56 (15), 70 (14), 51 (13), 50 (10)
1-ethylpyrrole	816	1188	NIST^{d}
1,3-dimethylpyrrole	840	1217	94, **95** (71), 42 (15), 53 (13), 39 (8), 41 (7), 93 (7), 27 (6), 51 (6), 52 (6), 67 (6), 78 (5), 96 (5), 50 (4), 65 (4)
2-methylpyrrole	845	1553	NIST
3-methylpyrrole	849	1563	NIST
1,2-dimethylpyrrole	869	1281	94, **95** (67), 42 (15), 53 (15), 39 (12), 93 (10), 27 (8), 41 (7), 51 (6), 15 (5), 52 (5), 67 (5), 78 (5), 96 (5)
(*E*)-2-isopropyl-2-butenal	878	1192	**112**, 41 (64), 55 (60), 97 (52), 83 (49), 69 (37), 39 (29), 43 (29), 27 (23), 79 (20), 29 (17), 67 (15), 53 (12), 77 (10), 111 (8), 113 (8)
2-vinylthiophene	902	1306	NIST
1-ethyl-3-methylpyrrole	911	1261	**109**, 108 (67), 94 (64), 80 (44), 53 (21), 27 (12), 93 (11), 39 (9), 41 (9), 51 (8), 110 (8), 28 (7), 52 (7), 54 (7), 78 (7)
3-vinylthiophene	916	1349	**110**, 109 (38), 84 (24), 66 (21), 39 (18), 45 (17), 65 (10), 58 (9), 111 (9), 51 (6), 50 (5), 69 (5), 38 (4), 63 (4), 77 (4), 112 (4)
(*E*)-5-methyl-2-hexenal	922	1271	70, 41 (52), 43 (49), 69 (41), 39 (25), 27 (23), 56 (16), 94 (16), 97 (16), 42 (14), 29 (12), 55 (11), 68 (6), 79 (6), 81 (6), **112** (1)
1-ethyl-2-methylpyrrole	927	1298	**109**, 108 (79), 94 (66), 80 (66), 53 (29), 27 (24), 39 (20), 41 (13), 78 (12), 67 (12), 42 (12), 28 (12), 93 (11), 81 (10), 54 (10)
1-(2-methylpropyl)pyrrole	951	1265	80, **123** (76), 81 (53), 53 (25), 68 (22), 41 (20), 67 (16), 39 (15), 27 (12), 29 (7), 78 (7), 122 (7), 124 (7), 51 (6), 54 (6)
1-(2-methylbutyl)pyrrole	1059	1374	81, 80 (68), **137** (66), 53 (18), 41 (16), 68 (14), 67 (12), 39 (11), 27 (9), 138 (7), 136 (7), 82 (6), 54 (6), 29 (6)
1-(3-methylbutyl)pyrrole	1062	1395	81, **137** (43), 80 (41), 53 (14), 41 (13), 39 (10), 27 (8), 54 (8), 67 (7), 82 (6), 68 (5), 43 (4), 55 (4), 94 (4), 138 (4)

^{a} Linear retention index on VF5-ms column. ^{b} Linear retention index on an HP-Innowax column. ^{c} First number is the base peak; molecular ion in bold type. ^{d} Spectrum is present in the NIST/NIH/EPA database (2005).

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Table 2. Free Asparagine, Total Free Amino Acids (Excluding Arginine), and Total Sugars (Glucose, Fructose, Maltose, and Sucrose) in Flour from Three Varieties of Wheat, Fertilized with (S+) or without (S−) Sulfur [Adapted from Muttucumaru et al. (*2*)]

wheat variety	free asparagine (mmol kg^{-1})	total free amino acids (mmol kg^{-1})	total sugars (mmol kg^{-1})
Solstice S+	4.54	13.2	32.9
Solstice S−	75.4	151	31.1
Claire S+	4.12	16.0	31.8
Claire S−	47.9	86.3	32.7
Malacca S+	5.20	16.9	34.4
Malacca S−	153	274	33.0

Table 3. Relative Amounts of Free Amino Acids in Flour Grown without Sulfur, Compared to Flour Grown with Sulfur [Adapted from Muttucumaru et al. (*2*)]

	wheat variety		
amino acid	Solstice	Claire	Malacca
asparagine	16.8	10.6	29.4
glutamine	112	35.0	160
tryptophan	0.83	0.57	1.11
alanine	4.84	2.65	9.51
glycine	5.43	3.47	14.9
valine	4.88	2.64	11.7
leucine	3.47	1.70	7.60
isoleucine	2.51	1.33	7.81
threonine	9.78	4.21	17.9
serine	17.1	6.87	43.3
proline	8.16	3.56	9.44
aspartic acid	3.94	1.42	2.80
methionine	2.25	1.94	5.89
glutamic acid	3.32	1.82	3.27
phenylalanine	2.05	1.20	2.43
ornithine	13.1	7.00	21.1
lysine	9.71	4.48	21.5
histidine	5.21	1.16	8.91
tyrosine	3.04	2.07	4.21
total free amino acids	8.40	2.87	10.2

Table 4. Relative Amounts of Sugars in Flour Grown without Sulfur, Compared to Flour Grown with Sulfur [Adapted from Muttucumaru et al. (*2*)]

	variety		
sugar	Solstice	Claire	Malacca
maltose	0.74	0.95	0.87
sucrose	0.96	1.05	0.96
fructose	1.48	1.06	1.15
glucose	1.25	1.05	1.22
total sugars	0.95	1.03	0.96

glucose increased in sulfur-deficient flour. Sucrose was unaffected by sulfur levels, and maltose was lower in the S− flour, which meant that total sugar levels were not affected by sulfur feeding (**Table 4**).

Effect of Sulfur Deprivation on Acrylamide Formation in Cooked Wheat. Acrylamide levels in cooked wheat flour were up to 6 times higher in S− wheat, compared to S+ wheat (*2*) at 180 °C (**Table 5**), largely due to the >10-fold increases in asparagine caused by sulfur deprivation. **Figure 1** shows that conversion of asparagine to acrylamide occurred to a greater extent in S+ wheat, probably because the measured amount of sugar relative to free amino acid was far higher in S+ wheat (2.0−2.5), compared with S− wheat (0.12−0.38). Although we have reported levels of monosaccharides, disaccharides, and free amino acids in wheat flour, wheat also contains higher molecular weight reducing sugars, such as maltotriose (*20*), and low molecular weight peptides, such as glutathione (*21*), both of which can participate in the Maillard reaction (*22, 23*). However, levels of these compounds are relatively low.

Effect of Sulfur Deprivation on the Volatile Compounds in Cooked Wheat. Approximately 50 compounds were present in at least one of the headspace extracts of the heated flour at a concentration of 5 μg kg^{-1} or higher (**Table 5**). Thirty-two compounds (or groups of compounds—individual pyrazines were often poorly resolved) were affected by sulfur treatment, and 15 compounds were affected by variety.

Different groups of compounds were associated with the different treatments. All of the quantified alkylpyrazines, Strecker aldehydes (2-methylpropanal, 2-methylbutanal, and 3-methylbutanal), and aldol condensation products [2-isopropyl-2-propenal, (*E*)-2-isopropyl-2-butenal, and (*E*)-5-methyl-2-hexenal] were at higher concentrations in the cooked wheat flour from S− plants, along with four alkylfurans (2-methyl-, 3-methyl-, 2-ethyl-, and 2-vinylfuran). In the cooked wheat flour from the S+ plants many of the alkylpyrroles and all of the thiophenes were present at higher levels, as were the sugar breakdown products 2,3-butanedione, 2,3-pentanedione, and 2-furancarboxaldehyde and the methionine breakdown product dimethyl trisulfide.

Volatile Compounds That Increase in Sulfur-Deprived Wheat Flour upon Heating. Aldehydes are formed at an early stage in the Maillard reaction, from the oxidation of α-amino acids with α-dicarbonyls, a process known as the Strecker reaction (*24*). The aldehydes formed, which are widely reported in cooked foods, are known as Strecker aldehydes and include 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal, formed from valine, isoleucine, and leucine, respectively. The α-dicarbonyls are usually derived from sugar breakdown. Hence, unless sugars become limiting, the amounts of Strecker aldehydes and aldol condensation products are likely to increase when amino acid levels increase. Hence, 2-methylpropanal, 2-methylbutanal,

limiting precursor, when acrylamide is formed on heating; that is, levels of sugars in wheat are higher than those of asparagine. For example, asparagine levels from 49 to 250 mg kg^{-1} (0.37−1.9 mmol kg^{-1}) were reported in nine varieties of European wheat (*18*), whereas reducing sugar levels in the same samples varied from 3900 to 9300 mg kg^{-1} (21.7−70.5 mmol kg^{-1}, expressed as glucose). Furthermore, when these flours were made into doughs for baking, fermentation reduced levels of asparagine and increased reducing sugar levels.

Although **Table 2** does not include data for arginine, it shows that levels of sugars in S+ wheat were higher than levels of free amino acids [arginine was present in commercial Claire wholemeal wheat flour at approximately 0.75 mmol kg^{-1} (*19*)]. However, in the S− wheat, levels of both free amino acids and free asparagine were far higher than those of sugars, for all three varieties.

Table 3 shows the relative amount of each amino acid in each S− wheat flour variety, compared to its amount in the sulfur-sufficient equivalent. Every amino acid, apart from tryptophan, was at a higher concentration in the S− wheat flour, and in all of the flours, in particular, Malacca, concentrations of many of the free amino acids were at least 5 times higher than in the S+ wheat flour. Changes in sugar concentrations due to sulfur feeding were much smaller, and only fructose and

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Table 5. Effect of Sulfur Fertilization of Wheat on the Headspace Volatile Compounds[a] and the Acrylamide Content of Flour Cooked for 20 min at 180 °C

LRI[b]	compound	Solstice		Claire		Malacca		SE	effect of sulfur[c]	effect of variety[c]
		S−	S+	S−	S+	S−	S+			
559	2-methylpropanal	457[d]	202	301	260	479	206	33.9	**	NS
570	2-methylpropenal	5.30	4.33	6.05	6.10	17.3	4.16	1.38	NS	NS
595	2,3-butanedione (diacetyl)	6.85	15.2	7.27	7.15	5.22	8.38	1.01	*	NS
600	butanal	8.08	9.44	12.3	9.70	9.12	10.1	0.74	NS	NS
602	2-butanone	37.9	37.9	45.8	29.9	99.8	31.9	9.90	NS	NS
605	2-methylfuran	81.5	57.0	60.6	54.1	102	62.7	5.47	*	NS
613	3-methylfuran	15.1	8.88	12.0	9.9	17.5	12.2	0.86	**	*
654	(E)-2-butenal	10.0	7.57	15.2	19.6	5.94	14.0	1.26	NS	**
657	3-methylbutanal	646	271	662	404	1120	302	73.4	***	NS
668	2-methylbutanal	775	605	609	699	1240	501	64.7	*	NS
672	thiophene	30.2	60.3	19.6	25.5	13.7	66.2	5.59	**	NS
701	2,3-pentanedione	5.68	9.34	7.83	7.98	4.76	6.85	0.52	*	NS
703	2-ethylfuran	9.64	6.35	9.25	7.90	19.7	6.42	1.26	**	NS
703	pentanal	19.5	36.3	48.9	69.8	31.3	37.3	4.83	*	**
708	2,5-dimethylfuran	5.29	5.14	5.45	4.18	8.70	4.35	0.50	NS	NS
724	2-vinylfuran	82.6	7.20	40.8	7.97	218	8.60	18.7	***	*
735	2-isopropylpropenal	4.64	1.53	4.33	1.96	8.97	1.92	0.65	***	*
740	1-methylpyrrole	24.1	53.3	41.3	74.0	22.2	56.3	5.04	***	*
746	2-methyl-2-butenal	4.38	2.42	3.56	1.69	6.33	2.39	0.43	***	*
747	dimethyl disulfide	26.0	25.8	33.4	38.5	21.6	21.9	2.60	NS	NS
756	pyrrole	61.1	70.1	93.3	109	62.5	85.6	5.34	NS	**
770	toluene	11.0	9.83	11.6	8.08	21.8	11.4	1.26	*	*
774	2-methylthiophene	2.64	8.35	2.83	2.86	1.80	6.57	0.66	**	NS
805	hexanal	59.5	108	134	174	87.3	95.7	11.5	NS	**
816	1-ethylpyrrole	27.7	19.2	30.9	39.8	37.6	28.6	2.19	NS	NS
833	methylpyrazine	19.2	16.9	24.1	16.7	21.8	13.8	1.13	**	NS
839	1,3-dimethylpyrrole	2.14	4.67	4.65	7.42	1.02	5.60	0.57	***	**
841	furfural	11.4	21.2	19.1	22.0	9.66	25.0	1.63	**	NS
852	3-methylpyrrole	3.58	4.90	5.47	6.42	2.81	5.57	0.37	**	*
878	(E)-2-isopropyl-2-butenal	5.70	0.78	3.68	0.92	8.56	1.23	0.72	***	*
894	2-heptanone	9.25	9.64	12.9	7.35	18.3	8.77	1.00	**	NS
907	heptanal	7.50	14.1	13.4	13.4	11.2	10.2	0.84	NS	NS
911	1-ethyl-3-methylpyrrole	4.57	6.55	9.79	13.8	2.69	9.72	1.02	**	**
912	3-vinylthiophene	2.6	5.4	1.9	3.5	0.8	6.2	0.54	**	NS
922	5-methyl-2-hexenal	3.94	0.54	3.62	1.18	6.43	1.05	0.51	***	*
923	2,5(and 2,6)-dimethylpyrazine	16.2	8.64	13.3	6.06	24.8	7.49	1.73	***	NS
925	ethylpyrazine	10.2	8.13	11.3	9.03	8.82	7.48	0.48	*	NS
952	1-(2-methylpropyl) pyrrole	24.3	25.5	23.8	21.5	28.9	17.1	1.65	NS	NS
965	(E)-2-heptenal	2.43	5.56	2.92	6.50	2.48	4.30	0.45	***	NS
976	benzaldehyde	5.02	8.53	7.58	8.72	8.10	7.93	0.53	NS	NS
981	dimethyl trisulfide	7.19	11.3	8.58	12.7	4.64	9.43	1.03	*	NS
994	2-pentylfuran	46.1	73.2	60.3	37.4	65.7	59.3	7.01	NS	NS
1007	2-ethyl-6-methyl pyrazine	10.5	4.91	6.66	4.25	9.33	5.31	0.70	**	NS
1009	octanal	6.32	12.4	8.70	8.67	9.76	8.91	0.69	NS	NS
1011	2-ethyl-5-methyl pyrazine, 2-ethyl-3-methylpyrazine + trimethylpyrazine	10.1	5.65	8.88	5.29	11.0	5.79	0.63	***	NS
1060	1-(2-methylbutyl)pyrrole	8.98	13.9	6.81	11.0	13.0	9.20	0.89	NS	NS
1063	1-(3-methylbutyl)pyrrole	11.8	12.8	9.20	11.7	21.4	10.2	1.23	NS	NS
1085	MW 136 pyrazines	15.0	5.46	8.28	6.77	23.1	7.10	1.84	**	NS
1110	nonanal	24.5	45.3	26.1	28.5	30.6	28.4	2.49	NS	NS
	total volatiles	2690	1970	2520	2350	4010	1870	196	NS	**
	acrylamide (μg kg⁻¹)[e]	7860	1320	6920	1960	7830	2460	697	***	NS

[a] All compounds were identified by comparison of LRI and mass spectra with those of authentic compounds. [b] Linear retention index on VF5-ms column. [c] Probability that there is a difference between means for sulfur feeding or variety; NS, no significant difference between means ($p > 0.05$); *, significant at the 5% level; **, significant at the 1% level; ***, significant at the 0.1% level. [d] Approximate concentrations in headspace relative to 100 ng of 1,2-dichlorobenzene standard, expressed as ng per g of flour. Values quoted are the means of triplicate analyses. [e] Data taken from Muttucumaru et al. (2).

and 3-methylbutanal were all present at higher levels in the heated S− wheat flour.

Aldol condensation can occur between two Strecker aldehydes to give α,β-unsaturated aldehydes. Three compounds that showed large increases in heated sulfur-deprived wheat flour were discovered to be aldol condensation products. 2-Isopropyl-2-propenal was formed from the condensation of formaldehyde (from glycine) with 3-methylbutanal, whereas (E)-2-isopropyl-2-butenal and (E)-5-methyl-2-hexenal were formed from the condensation of acetaldehyde (from alanine) with 3-methylbutanal. (E)-2-Isopropyl-2-butenal and (E)-5-methyl-2-hexenal

have both been reported in potato chips (25, 26); (E)-2-isopropyl-2-butenal has also been reported in fried chicken (27). 2-Isopropyl-2-propenal does not appear to have been previously reported in food. All three aldol condensation products showed large increases in concentration in the sulfur-deprived wheat, up to 7-fold for 2-isopropyl-2-butenal and 5-methyl-2-hexenal in sulfur-deprived Solstice flour. Only 2-vinylfuran showed a greater effect of treatment.

The Strecker reaction not only leads to the formation of a Strecker aldehyde, it also results in the formation of an α-aminoketone (24). Two α-aminoketones can condense to yield
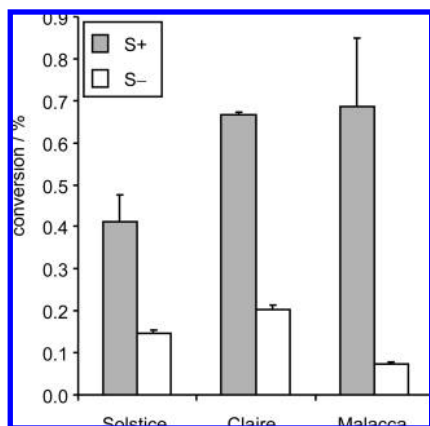
Figure 1. Percentage conversion of asparagine to acrylamide at 180 °C in three varieties of wheat flour grown with (S+) or without (S−) sulfur fertilization.

an alkylpyrazine, which would explain why, when there is an increase in free amino acids, as in S− wheat, there is also an increase in alkylpyrazine formation.

2-Vinylfuran was present in the sulfur-deficient wheat at concentrations up to 25 times higher than in sulfur-sufficient wheat. In S− Malacca flour, in fact, 2-vinylfuran was the compound reported at the fourth highest amount. This compound has been reported in several heated foods but not as a major component.

Volatile Compounds That Increase in Sulfur-Sufficient Wheat Flour upon Heating. As there was a molar excess of sugars compared to amino acids in the S+ wheat, sugar breakdown products are more likely to form, as there will be fewer free amino acids and amino acid breakdown products available to react with them. Hence, furfural, 2,3-butanedione, and 2,3-pentanedione were present at higher concentrations in the heated S+ flour than in the S− flour.

Nine pyrroles were identified in the heated flours, four of which were at higher concentrations in the S+ wheat. In fact, 1,3-dimethylpyrrole was present at >5 times higher levels in heated S+ Malacca flour than in S− flour. Many of the alkylpyrroles reported in this paper have been identified in roasted coffee (28, 29). Amino acids, such as glycine, alanine, leucine, and isoleucine, may decompose on heating to yield amines, which may then react with sugar degradation products to give alkylpyrroles, with the amino acid providing the alkyl group in the N-position (29), although it is not clear why such a reaction favors methyl substitution in the 3-position rather than the 2-position. It is also unclear why levels of some of these pyrroles are higher in heated S+ flour.

As alkylpyrazines are generally more widely reported in Maillard reaction systems, it is possible that they are more readily formed than alkylpyrroles. In the S− flour, the higher concentrations of free amino acids may favor alkylpyrazine formation, at the expense of alkylpyrrole formation. The issue is complicated further by increases of serine and threonine in S− wheat. These hydroxyamino acids can form pyrazines and pyrroles in the absence of sugars (30, 31).

The sulfur amino acids cysteine and cystine could not be determined in wheat flour and, for all flours, free methionine levels were higher in the S− wheat. Even so, of the five quantified sulfur-containing volatile compounds in the heated wheat flour, four were present at higher concentrations in S+ flour, and dimethyl disulfide was unaffected by treatment. 3-Vinylthiophene, thiophene, and 2-methylthiophene have all been reported in cysteine/glucose reaction mixtures (32) and

were all present at higher levels in S+ wheat than in S− wheat. Of particular interest was 3-vinylthiophene, which was found at an 8 times higher concentration in S+, compared to S−, flour from Malacca wheat. This compound has not been widely reported in foods. It has been reported in roasted coffee (33) and may also have been wrongly identified as 2-vinylthiophene in extruded wheat and maize flour (34, 35), based on the LRI values presented in those papers.

It is not clear why there is such a difference in the levels of sulfur-containing volatile compounds between the two treatments. Possibly, the formation of sulfur-containing compounds in S− wheat was reduced, due to a lack of suitable sugar fragments. Instead, sulfur reacted elsewhere, with carbonyls from lipids perhaps, to give compounds that could not be extracted and analyzed by GC-MS. As levels of protein cysteine and methionine have been shown to be 2−3 times higher in S+ wheat, compared to S− wheat (36), sulfur-containing volatile compounds in cooked S+ wheat may be formed as a result of the release of hydrogen sulfide and methanethiol from protein cysteine and methionine on heating.

Correlations between Aroma Compounds and Acrylamide. Assuming a linear relationship passing through the origin, the formation of (E)-5-methyl-2-hexenal ($r^2 = 0.789$) showed the highest positive correlation with acrylamide formation in cooked wheat flour, whereas 1-methylpyrrole showed the highest negative correlation ($r^2 = 0.794$). However, the highest positive correlation with acrylamide formation was shown by the formation of a coeluting group of MW 122 pyrazines, containing 2-ethyl-3-methylpyrazine, 2-ethyl-5-methylpyrazine, and trimethylpyrazine. The relationship between the headspace concentration of these pyrazines and acrylamide concentration can be expressed by

$$\text{MW 122 pyrazines} = 0.792 \times [\text{acrylamide}] + 4.05$$

where acrylamide concentration was in mg kg^{-1} and the concentration of MW 122 pyrazines was based on their chromatographic peak area (expressed as ng g^{-1} of flour), compared to that of 100 ng of 1,2-dichlorobenzene ($r^2 = 0.954$). This relationship suggested that formation of these pyrazines will occur to a reasonable extent before acrylamide formation starts to occur. Low (37) showed that the formation of acrylamide and MW 122 pyrazines followed similar curves at 180 °C in potato cakes. These two sets of results suggest that MW 122 alkylpyrazines, being relatively easy to extract, may be a useful marker for acrylamide formation in cooked foods.

Effect of Existing Acrylamide Mitigation Strategies on Flavor. Relationships between amounts of sugars, asparagine, and other free amino acids are important in both acrylamide and aroma formation. The sulfur agronomy treatments compared in this work are extreme, and the effects of variations in conventional agricultural practice are likely to be smaller. What is clear, though, is that acrylamide mitigation strategies that cause large changes in the free amino acid composition are likely to lead to significant effects in aroma volatile compositions and, hence, flavor. It seems clear that acrylamide mitigation strategies involving manipulation of precursors should aim to reduce asparagine only, without affecting levels of other free amino acids or sugars present in the food. Otherwise, there is likely to be a profound impact on flavor quality.

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J. Agric. Food Chem., Vol. 56, No. 15, 2008 **6179**

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