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Effect of sulphur fertilisation on the acrylamide-forming potential of wheat

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CONTENTS

1. Abstract

Acrylamide is a contaminant that forms through the Maillard reaction from free asparagine and reducing sugars such as glucose, fructose and maltose during high-temperature cooking and processing. Acrylamide is a Class 2a carcinogen and also affects the nervous system and fertility at high doses. The FAO/WHO Expert Committee on Food Additives has recommended that dietary exposure should be reduced and the European Food Safety Authority issued 'indicative' levels for acrylamide in food in early 2011. Wheat products are major contributors to acrylamide in UK and European diets and the development of best agronomic practice to keep wheat's acrylamide-forming potential as low as reasonably achievable is important.

Free asparagine concentration is the main determinant of acrylamide-forming potential in wheat grain and accumulates to very high levels if wheat is grown under conditions of sulphur deficiency. This makes sulphur availability the most important factor affecting the acrylamide-forming potential of wheat grain. The aim of the present project was to provide data on acrylamide formation in flour from grain samples produced from six field trials in which different levels of sulphur had been applied. The trials comprised four different varieties of winter wheat, grown at three different locations over three harvest years, with five different levels of sulphur fertilisation. Free amino acid concentrations were measured by gas chromatography-mass spectrometry and acrylamide in heated flour was measured by liquid chromatography and tandem mass spectrometry. The data showed a clear and significant effect of sulphur application in reducing the acrylamide-forming potential of wheat in five of the six trials. The exception was a trial at Woburn, Bedfordshire in 2011/12, a year in which heavy rainfall in spring and early summer, followed by a dry mid to late summer, may have affected the outcome of the experiment.

The most appropriate level of sulphur application was deemed to be that which gave a significant benefit compared with no sulphur application, with no further significant benefit with higher levels of application. This ranged from 12.5 kg SO_3 per hectare to 50 kg SO_3 per hectare (corresponding to 5 to 20 kg of sulphur per hectare) in the five trials in which sulphur application was effective. However, application at the 50 kg SO_3/ha rate led to significantly less acrylamide formation compared with the 25 kg SO_3/ha rate in two of the trials. Given the necessity of preventing free asparagine accumulation in all conditions, it is therefore recommended that sulphur-containing fertiliser be applied at a rate of 50 kg SO_3/ha (20 kg sulphur/ha). This is the top of the range of rates of sulphur fertiliser recommended for wheat in the Fertiliser Manual (RB209).

2. Introduction

Acrylamide is a processing contaminant that was discovered in cooked foods in 2002 (Tareke *et al*., 2002). It is a Class 2a carcinogen, which means that it is considered to be 'probably cancer-causing' in humans. It also affects the nervous system and fertility at high doses (Friedman, 2003). As a result, the FAO/WHO Expert Committee on Food Additives has recommended that dietary exposure should be reduced and the European Commission issued 'indicative' levels for acrylamide in food in early 2011 (European Food Safety Authority, 2012). Cereal, potato and coffee products are the major sources of dietary acrylamide in UK and European diets. Since 2002, much has been learnt about the mechanisms involved in acrylamide formation, and methods have been developed to reduce its presence in foods through the modification of cooking and processing conditions. These have been compiled in a 'Toolbox' produced by Food Drink Europe: (http://www.fooddrinkeurope.eu/uploads/publications_documents/Toolboxfinal260911.pdf). However, these methods are only applicable in some products, and the food industry also remains vulnerable to fluctuations in the acrylamide-forming potential of its raw materials, so is pressing its supply chain to do more to address the problem. The development of best agronomic practice to ensure that acrylamide-forming potential is kept as low as reasonably achievable is one way in which producers can respond.

The primary route by which acrylamide forms is the Maillard reaction (Mottram *et al*., 2002; Stadler *et al.*, 2002) in which amino groups, principally those of free amino acids, react with reducing sugars to produce a plethora of compounds. The Maillard reaction only occurs at the high temperatures associated with baking, frying and roasting, and only when the last stage of the reaction involves asparagine does it produce acrylamide. However, the reaction is also responsible for many of the compounds that impart the colours, flavours and aromas that consumers expect in baked, fried and roasted foods and that define particular products and brands, making the situation more difficult for processors to address.

The identification of genetic and environmental factors that affect acrylamide precursor content has been the objective of several studies performed on wheat, rye and potato (reviewed by Halford *et al*., 2012). These studies have shown that the concentration of free asparagine correlates closely with the acrylamide-forming potential in both wheat (Figure 1) and rye (Curtis *et al*., 2009; 2010; Granvogl *et al.*, 2007; Muttucumaru *et al.*, 2006; Postles *et al.*, 2013). A LINK project on 'Genetic improvement of wheat to reduce the potential for acrylamide formation during processing', involving Rothamsted Research, HGCA and a consortium of companies and organisations from the wheat supply chain, began in 2011 and will run until 2015. The studies on wheat have also shown that the free asparagine concentration in the grain can vary over an

extremely wide range (Figure 1) and is affected by environmental conditions (E), genetic factors (G) and the interaction between the two (G × E). The most important environmental factor is sulphur (S) deficiency, which can cause an increase of up to thirty-fold in free asparagine concentration (Figure 2) (Curtis *et al*., 2009; Granvogl *et al*., 2007; Muttucumaru *et al*., 2006), with a concomitant effect on acrylamide-forming potential. Sulphur deficiency also affects the distribution of free asparagine in the grain (Shewry *et al*., 2009), causing greatly increased accumulation in the endosperm (white flour fraction). Nitrogen (N) has a lesser effect on free asparagine concentration, in the opposite direction, with free asparagine increasing by about 150 % when wheat is grown with an application rate of 180 kg/ha N compared with 0 kg/ha N, with application in early spring having the most effect (Martinek *et al*., 2009).

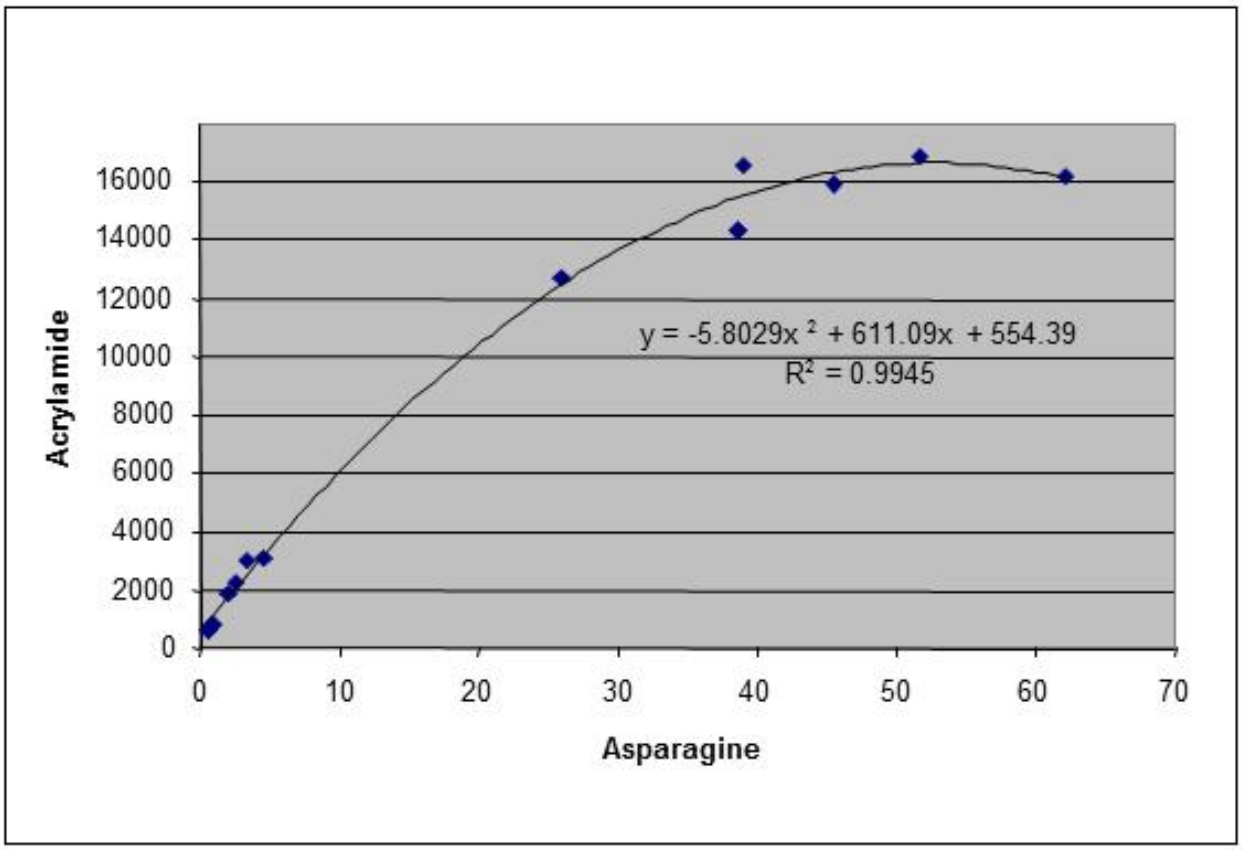

Figure 1. Free asparagine concentration (mmol per kg) in wheat grain plotted against acrylamide formed in heated flour (µg per kg) (parts per billion (ppb)) (data re-plotted from Curtis *et al*., 2009)

Zhao *et al.* (1999) showed that sulphur deficiency also limits wheat storage protein accumulation, with wheat grown in S-deficient soil favourably accumulating S-poor storage proteins at the expense of S-rich proteins. Indeed, in that study, S was shown to be more important for bread-making quality than N. This confirmed a much earlier study (Shewry *et al*., 1983) that found barley plants to have reduced ability to synthesise S-rich hordeins when

starved of sulphur and to accumulate free asparagine instead. These studies have contributed to the development of the hypothesis that free asparagine accumulates in plants as an alternative store of N when N is plentiful but protein synthesis is inhibited (Lea *et al*., 2007).

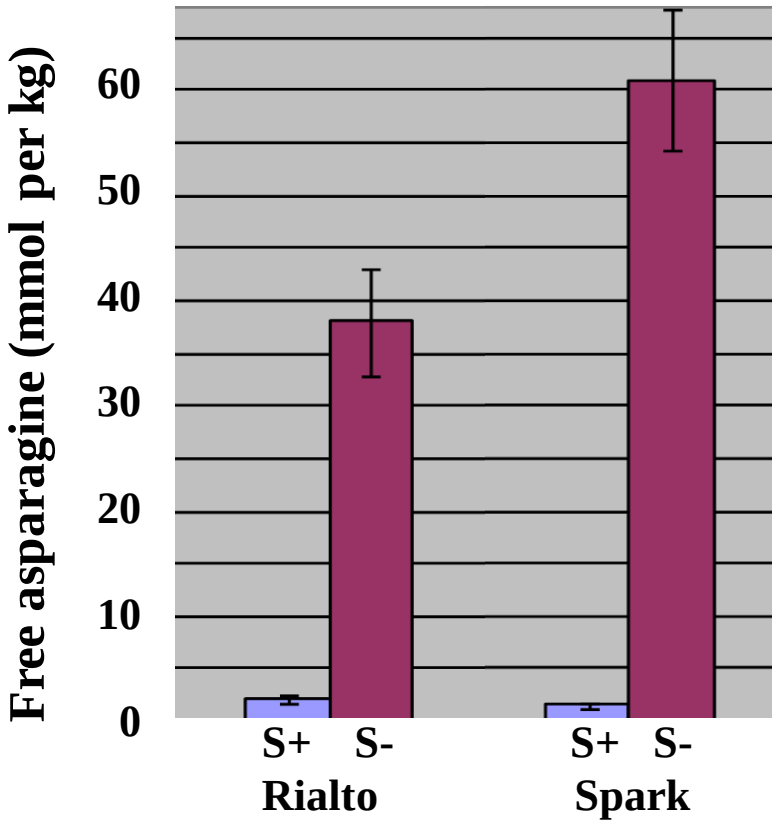

Figure 2. Free asparagine concentration (mmol per kg) in wheat cv. Rialto and Spark grain grown in pots with sulphur supplied (S+) or withheld (S-) (data re-plotted from Curtis *et al.*, 2009)

This makes sulphur deficiency the most important factor affecting acrylamide-forming potential in wheat grain and food industry representatives on the steering committee of the 'Genetic improvement of wheat to reduce the potential for acrylamide formation during processing' LINK project requested that HGCA's topic sheet on sulphur (topic sheet 54) be updated in the light of the acrylamide issue. The aim of the current project was to provide more data to inform this process by measuring amino acid levels and acrylamide-forming potential in wheat grain that had been produced with five different levels of sulphur fertilisation.

Specific objectives were:

- To measure free amino acid concentrations in wheat grain: cvs. Alchemy and Viscount from 2009–2010; cvs. Panorama and Oakley from 2010-2011; cvs. Viscount and Oakley

from 2011–2012. The grain had been produced under five different levels of sulphur fertilisation at three different locations over the three harvest years

- To measure acrylamide formation in flour produced from the different grain samples after heating for 20 minutes at 170 °C
- To inform the drafting of a new HGCA information sheet on sulphur application to wheat in response to the challenge of reducing the acrylamide-forming potential of wheat grain to as low as reasonably achievable (ALARA)

3. Materials and methods

3.1. Flour samples

Flour samples were available from randomised block field trials with four blocks, carried out at three sites, Brockhampton in Herefordshire, Frostenden in Suffolk and Woburn in Bedfordshire, over 2009/10, 2010/11 and 2011/12 (Sagoo *et al.*, 2013), extracting data for four varieties (Table 1).

Table 1. Details of field trials that provided grain for the study

Year	Site	Soil texture	Variety
2009/10	Brockhampton (Herefordshire)	Sandy loam	Alchemy
2010/11	Brockhampton (Herefordshire)	Sandy loam	Panorama
2009/10	Frostenden (Suffolk)	Sandy loam/loamy sand	Viscount
2011/12	Frostenden (Suffolk)	Sandy loam/loamy sand	Viscount
2010/11	Woburn (Bedfordshire)	Sandy loam	Oakley
2011/12	Woburn (Bedfordshire)	Sandy loam	Oakley

In each trial, sulphur (S) had been applied at five different levels as potassium sulphate (46 % SO_3; 54 % K_2O) in the early spring at similar timing to the first N application. The five S levels were 0, 12.5, 25, 50 and 75 kg SO_3 per hectare (corresponding to 0, 5, 10, 20 and 30 kg S per hectare). N and P fertilisers were applied at rates recommended in the Fertiliser Manual (2011). From each plot, a grain sample was taken and two technical replicates from this were used for amino acid and acrylamide analyses. The samples were already milled to fine, wholemeal flour.

3.2. Amino acid analyses

The flour samples were analysed for free amino acid concentration according to the method described by Curtis *et al*. (2009) and Muttucumaru *et al.* (2006). Briefly, a sample (0.5 g) was weighed into 14 mL screw-top bottles. HCl (10 mL, 0.01 M) was added to the vial and the sample was stirred for 15 min at room temperature then allowed to stand for a further 15 min. An aliquot (1.5 mL) was removed and centrifuged at 7200 *g* for 15 min; an aliquot (100 µL) of the supernatant was then derivatized using the EZ-Faast amino acid derivatisation technique for

gas chromatography and mass spectrometry (GC-MS) (Phenomenex, Torrance, CA), as described previously (Elmore *et al*., 2005). The EZ-Faast® procedure involves a simple solid phase extraction (SPE) step, followed by a rapid derivatisation reaction conducted in aqueous phase at room temperature.

GC-MS analysis of the derivatized samples was carried out using an Agilent 6890 GC coupled to a 5975 MS (Agilent, Palo Alto, CA) operating 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 °C and 230 °C, respectively; carrier gas flow rate was kept constant throughout the run at 1.5 mL/min. One analysis was performed for each sample.

Analyses of the data were performed using the Agilent 5975 system data analyser. Calibrations were prepared using standards at 5, 10, 20 and 50 nmol for all the amino acids and separate calibration curves were calculated for each amino acid. The standards were run before, in the middle and at the end of each set of analyses. New calibrations were calculated each time the column was changed due to changes in the retention times.

3.3. Acrylamide measurements

Flour samples (1.0 g) were heated for 20 min at 170 °C in unsealed glass vials (14 mL capacity) and analysed by the analytical laboratory at PepsiCo Europe, Beaumont Park, UK. Acrylamide was extracted with 10 mL water containing acrylamide-^{13}C as an internal standard. The solution was then purified by solid phase extraction with a proprietary sorbent phase followed by analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The method used was compatible with the Comité Européen de Normalisation (European Committee for Standardisation) (CEN) standard method.

3.4. Statistical analyses

The data were natural log (to base *e*) transformed to ensure a Normal distribution and constant variance across the S levels. Analysis of variance (ANOVA) was then applied to the data from each site by year combination separately, accounting for the blocks and testing the effect of S for acrylamide and for each amino acid, total free amino acids and the ratio of free asparagine to total free amino acids. The significance (F-tests) of the linear and nonlinear trend for each variable with respect to S was also tested. Following the ANOVA, when the effect of S was significant ($p < 0.05$, F-test), the means were compared using the standard error of the

difference between them (SED) on 12 degrees of freedom (df) (the residual degrees of freedom) by way of a least significant difference (LSD) at the 5% level of significance. To consider an overall effect of each level of S, acrylamide was then averaged over the six site by year by variety combinations shown in Table1.

4. Results

The complete data sets for free amino acid concentrations and acrylamide formation (back-transformed means, following analysis of variance (ANOVA)) are given in Appendix 1, with a separate table for each year by site by variety combination. The means on the log (to base *e*) scale, plus the standard error of the difference (SED) and the least significant difference (LSD) at the 5 % level for acrylamide and each amino acid, plus the p-values for the effect of S are given in Appendix 2, again with separate tables for each year by site by variety combination.

Acrylamide formation was measured in flour that had been heated at 170 °C for 20 minutes. This method results in high levels of acrylamide formation, providing a good, consistent indication of acrylamide-forming potential in different raw materials, and has been used in several previous studies of wheat and rye (Curtis *et al*., 2009; 2010; Granvogl *et al.*, 2007; Muttucumaru *et al.*, 2006; Postles *et al.*, 2013).

The acrylamide concentrations (back-transformed means) are shown graphically in Figure 3. There was a lot of variation between the different site by year by variety combinations, but the unbalance of these combinations, with site by year being confounded with variety, meant that no statistically robust conclusions could be drawn about the individual effects of variety, site or year. However, there was a significant ($p < 0.05$, F-test) effect of S, and nonlinear ($p \leq 0.056$, F-test) decrease in acrylamide with S for each of the site by year by variety combinations except for Oakley at Woburn in 2012, where acrylamide formation in heated flour from all of the grain samples was between 338 and 389 µg/kg. 2012 was a highly unusual year and a difficult one for wheat production. The crop in that year was poorly established and underwent a dry, cold winter period followed by heavy rainfall in the spring and early summer. The contrast with the same variety grown at the same site in the previous year, when acrylamide formation ranged from 7296 µg/kg in the 0 kg/ha sample to 737 µg/kg in the 75 kg/ha sample, was dramatic. This begs the question; why was acrylamide formation not high in all of the 2012 samples, rather than low. Part of the explanation could be that wet weather delayed the main N dressing until June 2012 and it is likely that this was too late for the crop to utilise it, and that N became the prime factor affecting the crop (Sagoo *et al.*, 2013).

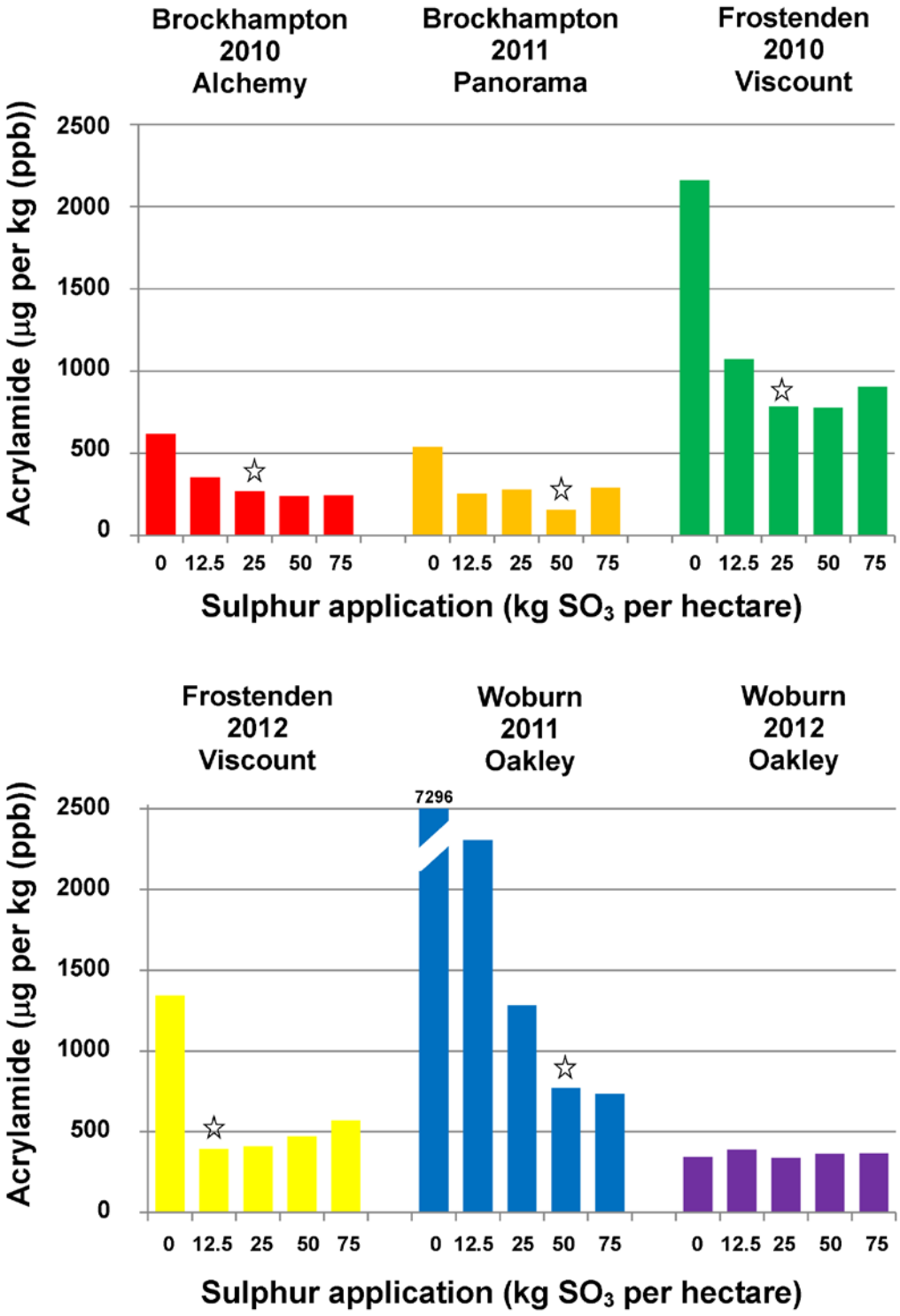

Figure 3. Acrylamide formed in wholemeal flour heated for 20 minutes at 170 °C. The flour was prepared from grain samples of four varieties of wheat (Alchemy, Viscount, Oakley and Panorama) produced at three different sites (Brockhampton, Frostenden and Woburn) over three years (2009/10, 2010/11 and 2011/12) with five different levels of sulphur application, as indicated. The star symbol indicates the lowest amount of sulphur application that gave a significant ($p < 0.05$, LSD) reduction in acrylamide formation compared with the 0 kg SO_3 sample, with no further significant ($p < 0.05$, LSD) reduction with higher SO_3 application.

The Woburn, Oakley, 2010/11 samples showed the highest acrylamide levels of all the samples and the clearest effect of sulphur. The level of acrylamide that formed in flour from the 50 kg/ha sample was 771 µg/kg and, although this was slightly higher than the figure for the 75 kg/ha sample, the difference was not significant (comparing the means on the log scale in Appendix 2 using the least significant difference (LSD) at the 5 % level given in the table). The 50 kg/ha level has therefore been denoted with a star in the graph of this data set in Figure 3 to show the level of SO_3 application that gave a significant ($p < 0.05$, LSD) reduction in acrylamide formation compared with 0 kg/ha SO_3, and above which no further statistically significant reduction was obtained. The corresponding levels for the other trials are also indicated, with 12.5 kg/ha for cv. Viscount at Frostenden in 2011/12 being the lowest. Note again that 2011/12 was an unusual growing season.

The overall means for acrylamide formation in flour for the different levels of SO_3 application over all of the site, variety and year combinations are shown graphically in Figure 4. Note that statistical analysis of these means was not performed because of the imbalance of the data as a whole, with no common variety across the site by year combinations to assess robustly the overall underlying variation. The graph shows the optimum level of application to be 50 kg SO_3/ha (equivalent to 20 kg S/ha), with a slight increase in acrylamide formation resulting from the higher level of 75 kg SO_3/ha. However, the differences between the samples from the wheat treated with 25, 50 or 75 kg/ha SO_3 (10, 20 or 30 kg S/ha) were relatively small compared with the differences between the 0, 12.5 and 25 kg SO_3/ha (0, 5 and 10 kg S/ha) samples.

5. Discussion

This short project took advantage of the availability of grain samples that had been produced in six field trials to assess the effect of sulphur (S) application on wheat (Sagoo *et al*., 2013). This cut costs and enabled the analysis to be performed very quickly. Although the grain had come from different combinations of three sites, four varieties and three years, meaning that analysis of variance (ANOVA) over the whole dataset was not possible, the information provided evidence for the effect of S application over a range of environments. Data from the six individual trials were subjected to ANOVA and a clear effect of sulphur application in reducing free asparagine concentration in the grain and acrylamide formation in heated flour was evident in five of the six trials. The exception was at Woburn in 2011/12, a year that saw very heavy rainfall in spring, followed by a dry summer. The trial at Frostenden in the same year showed a reduction in acrylamide formation between the 0 kg SO_3/ha sample and the 12.5 kg SO_3/ha sample but no further reduction with higher levels of SO_3 application. In the remaining four trials, the level of SO_3 application that was sufficient to give a significant ($p < 0.05$, LSD)

reduction compared with no sulphur application but with no further significant ($p < 0.05$, LSD) reduction at a higher level was either 25 or 50 kg/ha (equivalent to 10 or 20 kg S/ha).

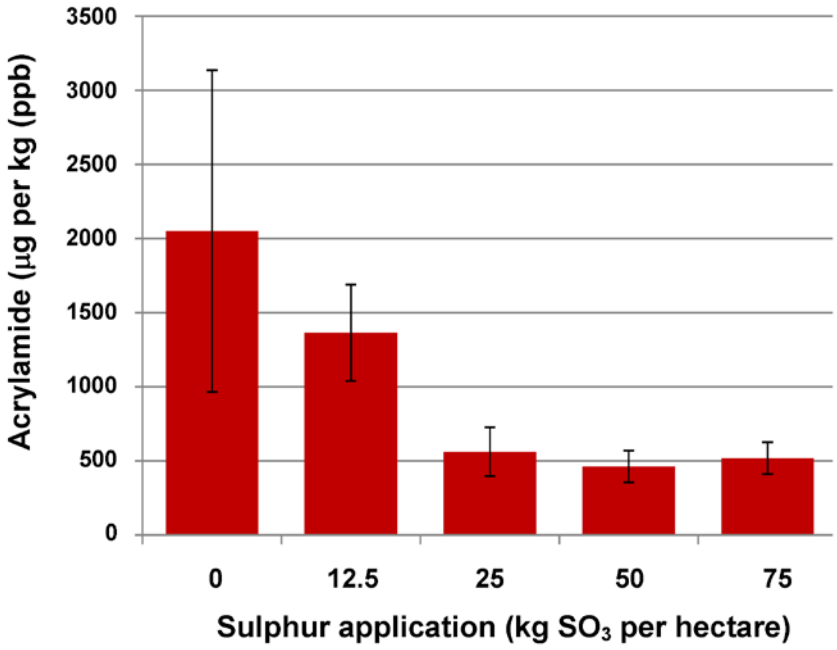

Figure 4. Acrylamide formation averaged over four varieties of wheat (Alchemy, Viscount, Oakley and Panorama) produced at three different sites (Brockhampton, Frostenden and Woburn) over three years (2009/10, 2010/11 and 2011/12) with standard errors (*n* = 6) for five levels of applied S (SO_3). Samples were wholemeal flour heated for 20 minutes at 170 °C.

The means for the five different S levels over the six trials were calculated and showed the optimum level of SO_3 application to be 50 kg/ha. This is equivalent to the top of the current recommendation for S fertiliser for wheat cultivation (Fertiliser Manual, 2011). Amounts of S fertiliser that are applied to wheat have been surveyed for a number of years and since 2001 the average application has been 50 kg/ha (+/- 10 %). The Manual recommends 25–50 kg/ha SO_3 for wheat in its 2011 list of errata. The higher end of this range of S fertiliser is thought to be necessary for high yielding wheat, and particularly to ensure protein quality of breadmaking varieties (Zhao *et al.*, 1999), and it appears that the industry has acted on this recommendation and has applied S at this average rate for at least the last 10 years. Overall, the benefit of applying 50 kg/ha of SO_3 compared with 25 kg/ha on the acrylamide concentration was modest. However, at Woburn in 2010/11 with cv. Oakley, acrylamide formation in the 25 kg SO_3/ha sample was 1284 μg/kg, whereas it was only 771 μg/kg in the 50 kg SO_3/ha sample, a reduction of 40 %. Acrylamide formation in the samples from the Brockhampton trial of cv. Panorama in 2010/11 was lower. However, acrylamide formed to 280 μg/kg in the 25 kg SO_3/ha sample whereas it was only 158 μg/kg in the 50 kg SO_3/ha sample, a reduction of 44 %. Given

the necessity of preventing free asparagine accumulation in all conditions, without the benefit of hindsight, it is therefore recommended that sulphur-containing fertiliser be applied at a rate of 50 kg SO_3/ha (20 kg S/ha). This rate of sulphur application will keep acrylamide-forming potential as low as reasonably achievable, and should be used regardless of yield and other quality issues. As discussed above, this appears to be in line with current practice, although the average values that are published do not give detailed information about the spread of actual applications around this average, and it could be that some farmers are applying much more or much less; the latter having potential consequences for acrylamide formation. It is also worth noting that some food products with a relatively high acrylamide risk, such as breakfast cereals, do not require wheat with the high quality protein content that is preferred for breadmaking. The results of this study show that it is important that sulphur be applied at a rate of 50 kg SO_3/ha to wheat destined for these products as well as wheat being grown for bread production.

Soils at the sites used in this study were light in texture (two sandy loams and one sandy loam/loamy sand; Table 1). S deficiency is more likely to occur on these soils and the available S may be lower under these conditions than on other, heavier soils. What is not known is how the amount of acrylamide may respond to different S doses on other soils, where there is either no apparent S deficiency or a lower risk of S deficiency. However, the recommendations for S fertiliser use (Fertiliser Manual, 2011) do not vary according to soil texture, and so it appears sensible to apply 50 kg/ha SO_3 wherever S deficiency in wheat is thought to be a risk.

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Appendix 1.

Free amino acid concentrations (mmol/kg) and acrylamide formed on heating (μg/kg) (ppb) in flour samples, given separately for each site/year/variety combination. The figures are back-transformed means from ANOVA (Appendix 2).

AAB = α-Amino butyric acid; Asn = Asparagine

Brockhampton 2010 Alchemy

	SO_3	**Back-transformed means**			
	0	**12.5**	**25**	**50**	**75**
Acrylamide	*620*	*354*	*271*	*240*	*246*
Alanine	0.947	0.593	0.580	0.565	0.536
Glycine	0.351	0.220	0.207	0.199	0.213
AAB	0.009	0.005	0.005	0.006	0.006
Valine	0.240	0.108	0.103	0.100	0.097
Leucine	0.144	0.094	0.090	0.093	0.085
Isoleucine	0.018	0.014	0.011	0.014	0.012
Threonine	0.163	0.076	0.070	0.070	0.067
Serine	0.239	0.066	0.089	0.057	0.079
Proline	0.805	0.222	0.172	0.143	0.122
Asparagine	*9.269*	*2.736*	*2.029*	*1.747*	*1.574*
Aspartate	3.461	1.730	1.686	1.516	1.545
Methionine	0.032	0.012	0.013	0.020	0.012
Glutamine	0.368	0.300	0.231	0.249	0.223
Glutamate	1.217	0.803	0.775	0.769	0.758
Phenylalanine	0.086	0.064	0.063	0.062	0.057
Ornithine	0.006	0.002	0.002	0.002	0.002
Lysine	0.056	0.029	0.028	0.027	0.026
Histidine	0.039	0.025	0.032	0.041	0.022
Tyrosine	0.035	0.024	0.024	0.024	0.025
Tryptophan	0.327	0.530	0.528	0.508	0.578
Cysteine	0.420	0.419	0.420	0.419	0.419
Total aa	21.436	10.275	9.301	8.832	8.609
Asn/Total	0.432	0.266	0.218	0.198	0.183

Brockhampton 2011 Panorama

	SO_3	Back-transformed means			
	0	**12.5**	**25**	**50**	**75**
Acrylamide	*540*	*255*	*280*	*158*	*293*
Alanine	1.213	1.017	0.976	0.981	1.077
Glycine	0.399	0.345	0.334	0.344	0.378
AAB	0.009	0.009	0.007	0.008	0.008
Valine	0.252	0.201	0.195	0.200	0.237
Leucine	0.203	0.158	0.135	0.142	0.147
Isoleucine	0.043	0.024	0.024	0.016	0.030
Threonine	0.140	0.104	0.106	0.104	0.113
Serine	0.219	0.153	0.173	0.154	0.186
Proline	0.424	0.294	0.336	0.330	0.356
Asparagine	*5.445*	*3.260*	*3.565*	*3.464*	*4.073*
Aspartate	6.297	4.993	5.223	5.320	5.309
Methionine	0.033	0.061	0.033	0.052	0.034
Glutamine	0.302	0.239	0.239	0.209	0.229
Glutamate	1.214	0.885	0.909	0.825	0.967
Phenylalanine	0.153	0.122	0.107	0.114	0.115
Ornithine	0.005	0.003	0.003	0.003	0.003
Lysine	0.039	0.031	0.031	0.030	0.034
Histidine	0.051	0.041	0.046	0.030	0.074
Tyrosine	0.034	0.030	0.025	0.028	0.032
Tryptophan	0.215	0.346	0.218	0.286	0.283
Cysteine	0.421	0.420	0.419	0.420	0.422
TOTAL aa	19.335	14.903	15.315	15.250	16.608
Asn/Total	0.282	0.219	0.233	0.227	0.245

Frostenden 2010 Viscount

	SO_3	Back-transformed means			
	0	**12.5**	**25**	**50**	**75**
Acrylamide	*2160*	*1073*	*786*	*777*	*904*
Alanine	2.129	1.798	2.039	2.150	1.996
Glycine	0.736	0.588	0.655	0.711	0.656
AAB	0.019	0.016	0.015	0.017	0.015
Valine	0.695	0.618	0.730	0.826	0.730
Leucine	0.446	0.463	0.543	0.632	0.546
Isoleucine	0.273	0.288	0.346	0.385	0.341
Threonine	0.437	0.347	0.397	0.457	0.415
Serine	0.812	0.643	0.748	0.919	0.792
Proline	5.735	4.515	4.460	4.526	3.940
Asparagine	*16.566*	*6.827*	*4.526*	*4.420*	*4.684*
Aspartate	7.858	4.563	3.373	3.523	3.362
Methionine	0.090	0.054	0.065	0.079	0.070
Glutamine	0.784	0.592	0.544	0.599	0.522
Glutamate	2.006	1.546	1.509	1.939	1.672
Phenylalanine	0.488	0.551	0.614	0.721	0.586
Ornithine	0.011	0.005	0.005	0.005	0.003
Lysine	0.106	0.066	0.065	0.077	0.067
Histidine	0.142	0.103	0.125	0.146	0.120
Tyrosine	0.234	0.227	0.252	0.290	0.251
Tryptophan	0.244	0.418	0.420	0.479	0.500
Cysteine	0.431	0.435	0.446	0.453	0.454
TOTAL aa	45.225	27.375	24.206	25.646	24.067
Asn/Total	0.366	0.249	0.187	0.172	0.195

Frostenden 2012 Viscount

	SO_3	Back-transformed means			
	0	**12.5**	**25**	**50**	**75**
Acrylamide	*1343*	*395*	*410*	*473*	*570*
Alanine	2.906	1.993	1.924	2.071	2.050
Glycine	1.016	0.676	0.662	0.645	0.644
AAB	0.018	0.007	0.007	0.007	0.007
Valine	0.496	0.247	0.249	0.224	0.233
Leucine	0.266	0.147	0.139	0.138	0.141
Isoleucine	0.107	0.019	0.039	0.014	0.016
Threonine	0.335	0.133	0.126	0.119	0.130
Serine	0.589	0.177	0.167	0.155	0.193
Proline	2.450	0.965	0.978	0.888	0.916
Asparagine	*9.738*	*2.813*	*2.982*	*2.851*	*2.620*
Aspartate	8.704	4.365	4.275	4.187	3.891
Methionine	0.053	0.033	0.016	0.027	0.039
Glutamine	0.401	0.189	0.215	0.213	0.210
Glutamate	1.654	0.654	0.705	0.642	0.664
Phenylalanine	0.191	0.115	0.118	0.110	0.107
Ornithine	0.009	0.003	0.004	0.004	0.003
Lysine	0.077	0.038	0.041	0.041	0.042
Histidine	0.075	0.048	0.060	0.056	0.050
Tyrosine	0.097	0.059	0.059	0.059	0.058
Tryptophan	0.213	0.416	0.448	0.455	0.478
Cysteine	0.420	0.419	0.419	0.419	0.418
TOTAL aa	32.137	15.683	15.839	15.503	15.056
Asn/Total	0.303	0.179	0.188	0.184	0.174

Woburn 2011 Oakley

	SO_3	Back-transformed means			
	0	**12.5**	**25**	**50**	**75**
Acrylamide	*7296*	*2307*	*1284*	*771*	*737*
Alanine	8.727	2.255	1.422	0.947	1.118
Glycine	3.880	0.824	0.441	0.249	0.302
AAB	0.085	0.022	0.013	0.008	0.009
Valine	2.400	0.633	0.353	0.175	0.218
Leucine	0.889	0.291	0.184	0.126	0.161
Isoleucine	0.586	0.113	0.060	0.016	0.022
Threonine	1.789	0.441	0.233	0.118	0.131
Serine	6.306	1.161	0.504	0.200	0.247
Proline	10.335	3.243	1.813	0.982	1.130
Asparagine	*87.303*	*20.882*	*11.276*	*5.238*	*6.515*
Aspartate	23.088	12.088	9.042	6.234	5.647
Methionine	0.121	0.130	0.112	0.062	0.102
Glutamine	1.806	0.697	0.452	0.264	0.303
Glutamate	8.446	2.674	1.726	1.226	1.240
Phenylalanine	0.394	0.177	0.129	0.109	0.127
Ornithine	0.073	0.015	0.008	0.005	0.005
Lysine	0.468	0.111	0.059	0.039	0.045
Histidine	0.179	0.073	0.042	0.039	0.040
Tyrosine	0.227	0.088	0.056	0.039	0.044
Tryptophan	0.101	0.194	0.245	0.215	0.171
Cysteine	0.445	0.432	0.421	0.420	0.420
TOTAL aa	160.067	49.378	30.931	19.102	20.363
Asn/Total	0.545	0.423	0.365	0.274	0.320

Woburn 2012 Oakley

	SO_3	Back-transformed means			
	0	**12.5**	**25**	**50**	**75**
Acrylamide	*344*	*389*	*338*	*365*	*366*
Alanine	1.451	1.307	1.361	1.409	1.290
Glycine	0.330	0.309	0.317	0.322	0.312
AAB	0.006	0.006	0.006	0.006	0.005
Valine	0.166	0.155	0.145	0.156	0.139
Leucine	0.094	0.057	0.077	0.082	0.079
Isoleucine	0.012	0.024	0.012	0.012	0.017
Threonine	0.109	0.099	0.098	0.098	0.093
Serine	0.192	0.178	0.172	0.174	0.180
Proline	0.736	0.695	0.668	0.702	0.624
Asparagine	*1.454*	*1.424*	*1.364*	*1.266*	*1.339*
Aspartate	2.092	2.023	1.974	1.653	1.903
Methionine	0.020	0.019	0.028	0.015	0.015
Glutamine	0.285	0.248	0.225	0.243	0.272
Glutamate	0.920	0.889	0.913	0.765	0.858
Phenylalanine	0.074	0.066	0.065	0.069	0.066
Ornithine	0.004	0.003	0.003	0.003	0.003
Lysine	0.025	0.023	0.023	0.021	0.023
Histidine	0.042	0.048	0.040	0.049	0.053
Tyrosine	0.034	0.031	0.028	0.030	0.028
Tryptophan	0.124	0.089	0.120	0.096	0.069
Cysteine	0.419	0.418	0.419	0.418	0.418
TOTAL aa	10.732	10.243	10.191	9.685	9.948
Asn/Total	0.135	0.139	0.134	0.131	0.135

Appendix 2

ANOVA

Free amino acid concentrations (mmol/kg) and acrylamide formed on heating (μg/kg) (ppb) results for each site by year by variety combination are shown below, testing the effect of S, and linear and non-linear trend in acrylamide concentrations with respect to SO_3 supplied. Means are given on the log (to base *e*) scale. The standard error of the difference (SED) between means, least significant difference (LSD) at the 5 % level and p-values are given for each amino acid and for acrylamide. P-values in bold are those indicating significance ($p < 0.05$, F-test), excepting those for significant ($p < 0.05$, F-test) nonlinear effects with respect to S without corresponding significant ($p < 0.05$, F-test) linear effect.

AAB = α-Amino butyric acid; Asn = Asparagine

Brockhampton 2010 Alchemy

	SO_3	Means (log-scale)				SO_3 effect p-value	SED (12 df)	LSD	Trend with SO_3 p-value Linear	Non-linear
	0	**12.5**	**25**	**50**	**75**					
Acrylamide	*6.430*	*5.870*	*5.603*	*5.479*	*5.507*	***0.037***	*0.2935*	*0.6394*	***0.010***	*0.056*
Alanine	-0.055	-0.522	-0.545	-0.571	-0.623	**0.006**	0.1303	0.2839	**0.003**	**0.023**
Glycine	-1.047	-1.515	-1.574	-1.615	-1.548	**0.006**	0.1334	0.2906	**0.008**	**0.006**
AAB	-4.673	-5.232	-5.268	-5.118	-5.167	**0.031**	0.1736	0.3782	0.101	0.032
Valine	-1.426	-2.222	-2.273	-2.306	-2.328	**0.006**	0.2170	0.4728	**0.005**	**0.013**
Leucine	-1.939	-2.366	-2.407	-2.377	-2.471	**0.031**	0.1529	0.3331	**0.016**	0.082
Isoleucine	-3.997	-4.263	-4.545	-4.246	-4.388	0.646	0.3579	0.7797	0.464	0.430
Threonine	-1.815	-2.577	-2.661	-2.663	-2.705	**0.004**	0.2025	0.4411	**0.004**	**0.011**
Serine	-1.432	-2.716	-2.424	-2.859	-2.544	0.053	0.4461	0.9720	0.062	0.042
Proline	-0.217	-1.503	-1.763	-1.947	-2.106	**0.001**	0.3324	0.7242	**<0.001**	**0.008**
Asparagine	*2.227*	*1.006*	*0.708*	*0.558*	*0.453*	***0.001***	*0.3350*	*0.7299*	***<0.001***	***0.008***
Aspartate	1.242	0.548	0.523	0.416	0.435	**0.015**	0.2220	0.4837	**0.009**	0.026
Methionine	-3.458	-4.442	-4.307	-3.904	-4.465	0.370	0.5620	1.2245	0.325	0.622
Glutamine	-0.999	-1.205	-1.466	-1.390	-1.502	**0.004**	0.1128	0.2458	**0.001**	**0.047**
Glutamate	0.196	-0.220	-0.255	-0.262	-0.277	**0.010**	0.1223	0.2664	**0.008**	**0.019**
Phenylalanine	-2.452	-2.743	-2.768	-2.779	-2.859	0.087	0.1364	0.2971	**0.025**	0.202
Ornithine	-5.149	-6.077	-6.128	-6.128	-6.215	**0.004**	0.2392	0.5211	**0.004**	**0.014**
Lysine	-2.891	-3.539	-3.589	-3.599	-3.642	**0.006**	0.1793	0.3906	**0.005**	**0.016**
Histidine	-3.240	-3.677	-3.439	-3.189	-3.826	0.051	0.2162	0.4711	0.161	0.254
Tyrosine	-3.341	-3.714	-3.749	-3.745	-3.679	0.080	0.1479	0.3223	0.109	0.033
Tryptophan	-1.119	-0.636	-0.639	-0.677	-0.548	**0.018**	0.1500	0.3269	**0.013**	0.086
Cysteine	-0.867	-0.870	-0.868	-0.869	-0.870	0.642	0.0025	0.0055	0.431	0.767
Total aa	3.065	2.330	2.230	2.178	2.153	**0.004**	0.2051	0.4468	**0.002**	**0.011**
Asn/Total	-0.838	-1.323	-1.522	-1.620	-1.699	**<0.001**	0.1383	0.3013	**<0.001**	**0.007**

Brockhampton 2011 Panorama

	SO_3	Means (log-scale)				SO_3 effect p-value	SED (12 df)	LSD	Trend with SO_3 p-value	
	0	**12.5**	**25**	**50**	**75**				**Linear**	**Non-linear**
Acrylamide	*6.291*	*5.541*	*5.635*	*5.060*	*5.679*	***0.004***	*0.2326*	*0.5069*	***0.016***	***0.002***
Alanine	0.193	0.017	-0.024	-0.019	0.074	0.057	0.0723	0.1575	0.264	0.008
Glycine	-0.919	-1.064	-1.097	-1.067	-0.973	**0.027**	0.0530	0.1154	0.770	0.003
AAB	-4.752	-4.658	-4.916	-4.830	-4.786	0.526	0.1474	0.3211	0.593	0.405
Valine	-1.377	-1.605	-1.634	-1.611	-1.438	0.100	0.1051	0.2290	0.961	0.011
Leucine	-1.592	-1.848	-2.002	-1.949	-1.916	**0.001**	0.0719	0.1566	**0.002**	**0.001**
Isoleucine	-3.137	-3.734	-3.748	-4.138	-3.512	0.235	0.4082	0.8893	0.349	0.048
Threonine	-1.963	-2.260	-2.249	-2.263	-2.180	0.055	0.1018	0.2217	0.165	0.018
Serine	-1.518	-1.878	-1.755	-1.871	-1.680	0.177	0.1539	0.3353	0.546	0.053
Proline	-0.857	-1.225	-1.091	-1.109	-1.032	0.133	0.1290	0.2810	0.626	0.077
Asparagine	*1.695*	*1.182*	*1.271*	*1.243*	*1.404*	*0.071*	*0.1711*	*0.3728*	*0.368*	*0.022*
Aspartate	1.840	1.608	1.653	1.671	1.669	0.303	0.1072	0.2335	0.380	0.191
Methionine	-3.412	-2.796	-3.403	-2.952	-3.387	0.481	0.4317	0.9406	0.862	0.398
Glutamine	-1.197	-1.433	-1.431	-1.566	-1.476	**0.009**	0.0819	0.1784	**0.005**	**0.012**
Glutamate	0.194	-0.122	-0.095	-0.193	-0.034	**0.048**	0.1151	0.2509	0.131	0.013
Phenylalanine	-1.880	-2.101	-2.236	-2.176	-2.164	**<0.001**	0.0553	0.1204	**0.001**	**<0.001**
Ornithine	-5.384	-5.904	-5.817	-5.695	-5.680	0.191	0.2071	0.4512	0.629	0.111
Lysine	-3.245	-3.461	-3.467	-3.509	-3.381	0.051	0.0823	0.1793	0.232	0.008
Histidine	-2.980	-3.195	-3.079	-3.505	-2.598	0.264	0.3819	0.8320	0.471	0.084
Tyrosine	-3.367	-3.522	-3.680	-3.569	-3.455	**0.029**	0.0843	0.1836	0.620	0.004
Tryptophan	-1.535	-1.062	-1.524	-1.251	-1.263	0.098	0.1802	0.3925	0.378	0.708
Cysteine	-0.864	-0.866	-0.870	-0.868	-0.862	0.189	0.0034	0.0073	0.463	0.030
Total aa	2.962	2.702	2.729	2.725	2.810	0.117	0.0994	0.2165	0.404	0.031
Asn/Total	-1.267	-1.520	-1.458	-1.482	-1.406	0.062	0.0801	0.1744	0.372	0.024

Frostenden 2010 Viscount

	SO_3	Means (log-scale)				SO_3 effect p-value	SED (12 df)	LSD	Trend with SO_3 p-value	
	0	**12.5**	**25**	**50**	**75**				**Linear**	**Non-linear**
Acrylamide	*7.678*	*6.979*	*6.667*	*6.655*	*6.807*	***0.043***	*0.3229*	*0.7036*	***0.032***	***0.022***
Alanine	0.756	0.586	0.713	0.766	0.691	0.899	0.1991	0.4337	0.897	0.989
Glycine	-0.306	-0.532	-0.423	-0.341	-0.422	0.832	0.2058	0.4484	0.987	0.820
AAB	-3.944	-4.160	-4.195	-4.071	-4.189	0.768	0.2227	0.4853	0.511	0.636
Valine	-0.363	-0.481	-0.314	-0.191	-0.315	0.798	0.2299	0.5010	0.486	0.665
Leucine	-0.806	-0.771	-0.611	-0.459	-0.605	0.501	0.2112	0.4602	0.191	0.295
Isoleucine	-1.297	-1.246	-1.061	-0.954	-1.077	0.564	0.2274	0.4954	0.219	0.304
Threonine	-0.828	-1.057	-0.924	-0.783	-0.879	0.854	0.2608	0.5682	0.728	0.925
Serine	-0.209	-0.442	-0.291	-0.084	-0.233	0.775	0.2770	0.6035	0.595	0.945
Proline	1.747	1.507	1.495	1.510	1.371	0.718	0.2654	0.5783	0.258	0.725
Asparagine	*2.807*	*1.921*	*1.510*	*1.486*	*1.544*	***0.027***	*0.3966*	*0.8641*	***0.013***	***0.028***
Aspartate	2.062	1.518	1.216	1.259	1.213	**0.002**	0.1833	0.3993	**0.001**	**0.008**
Methionine	-2.413	-2.911	-2.731	-2.536	-2.658	0.303	0.2299	0.5008	0.983	0.438
Glutamine	-0.243	-0.524	-0.609	-0.512	-0.650	0.443	0.2242	0.4885	0.179	0.446
Glutamate	0.696	0.436	0.412	0.662	0.514	0.608	0.2197	0.4786	0.917	0.608
Phenylalanine	-0.718	-0.596	-0.488	-0.327	-0.535	0.266	0.1664	0.3627	0.166	0.090
Ornithine	-4.552	-5.219	-5.355	-5.319	-5.767	0.172	0.4476	0.9752	**0.034**	0.530
Lysine	-2.241	-2.712	-2.736	-2.567	-2.707	0.477	0.3034	0.6610	0.357	0.370
Histidine	-1.953	-2.275	-2.076	-1.924	-2.117	0.584	0.2321	0.5056	0.932	0.955
Tyrosine	-1.451	-1.483	-1.379	-1.240	-1.382	0.729	0.1857	0.4045	0.410	0.464
Tryptophan	-1.412	-0.873	-0.867	-0.736	-0.693	**0.036**	0.2131	0.4643	**0.010**	0.094
Cysteine	-0.842	-0.833	-0.807	-0.792	-0.790	**0.014**	0.0150	0.0327	**0.001**	0.168
Total aa	3.812	3.310	3.187	3.244	3.181	0.171	0.2703	0.5890	0.080	0.144
Asn/Total	-1.004	-1.389	-1.677	-1.758	-1.637	**0.001**	0.1396	0.3043	**0.001**	**0.002**

Frostenden 2012 Viscount

	SO_3	Means (log-scale)				SO_3 effect p-value	SED (12 df)	LSD	Trend with SO_3 p-value	
	0	**12.5**	**25**	**50**	**75**				**Linear**	**Non-linear**
Acrylamide	*7.203*	*5.979*	*6.015*	*6.159*	*6.346*	***<0.001***	*0.1856*	*0.4044*	***0.020***	***<0.001***
Alanine	1.067	0.690	0.654	0.728	0.718	**0.049**	0.1309	0.2851	0.094	0.037
Glycine	0.016	-0.391	-0.412	-0.439	-0.440	**<0.001**	0.0726	0.1581	**<0.001**	**0.001**
AAB	-4.040	-4.944	-4.908	-4.995	-4.895	**0.001**	0.1756	0.3826	**0.002**	**0.001**
Valine	-0.702	-1.399	-1.392	-1.496	-1.458	**<0.001**	0.0942	0.2053	**<0.001**	**<0.001**
Leucine	-1.325	-1.919	-1.971	-1.984	-1.961	**<0.001**	0.1043	0.2273	**<0.001**	**<0.001**
Isoleucine	-2.240	-3.981	-3.235	-4.258	-4.126	**0.001**	0.3849	0.8385	**0.001**	**0.031**
Threonine	-1.095	-2.017	-2.068	-2.128	-2.040	**<0.001**	0.1255	0.2733	**<0.001**	**<0.001**
Serine	-0.530	-1.733	-1.790	-1.866	-1.646	**<0.001**	0.1775	0.3866	**<0.001**	**<0.001**
Proline	0.896	-0.036	-0.023	-0.119	-0.087	**<0.001**	0.1357	0.2956	**<0.001**	**<0.001**
Asparagine	*2.276*	*1.034*	*1.093*	*1.048*	*0.963*	***<0.001***	*0.1413*	*0.3079*	***<0.001***	***<0.001***
Aspartate	2.164	1.474	1.453	1.432	1.359	**<0.001**	0.1131	0.2464	**<0.001**	**0.001**
Methionine	-2.947	-3.409	-4.144	-3.624	-3.235	0.117	0.4186	0.9121	0.747	0.021
Glutamine	-0.915	-1.665	-1.538	-1.548	-1.559	**0.003**	0.1557	0.3392	**0.015**	**0.009**
Glutamate	0.503	-0.425	-0.350	-0.443	-0.409	**<0.001**	0.1247	0.2717	**<0.001**	**<0.001**
Phenylalanine	-1.653	-2.160	-2.136	-2.208	-2.233	**<0.001**	0.0858	0.1870	**<0.001**	**0.001**
Ornithine	-4.766	-5.731	-5.608	-5.608	-5.781	**0.002**	0.2032	0.4427	**0.003**	**0.020**
Lysine	-2.562	-3.262	-3.183	-3.203	-3.167	**<0.001**	0.0845	0.1841	**<0.001**	**<0.001**
Histidine	-2.586	-3.027	-2.821	-2.889	-3.002	**0.034**	0.1299	0.2830	**0.045**	0.349
Tyrosine	-2.329	-2.833	-2.829	-2.838	-2.844	**0.003**	0.1174	0.2559	**0.005**	**0.007**
Tryptophan	-1.546	-0.877	-0.804	-0.788	-0.738	**<0.001**	0.1012	0.2204	**<0.001**	**<0.001**
Cysteine	-0.867	-0.870	-0.869	-0.869	-0.872	0.590	0.0028	0.0061	0.191	0.934
Total aa	3.470	2.753	2.762	2.741	2.712	**<0.001**	0.0912	0.1986	**<0.001**	**<0.001**
Asn/Total	-1.194	-1.718	-1.670	-1.693	-1.749	**<0.001**	0.0627	0.1366	**<0.001**	**<0.001**

Woburn 2011 Oakley

	SO_3	Means (log-scale)				SO_3 effect p-value	SED (12 df)	LSD	Trend with SO_3 p-value	
	0	**12.5**	**25**	**50**	**75**				**Linear**	**Non-linear**
Acrylamide	*8.895*	*7.744*	*7.158*	*6.648*	*6.603*	***<0.001***	*0.2480*	*0.5403*	***<0.001***	***<0.001***
Alanine	2.166	0.813	0.352	-0.054	0.111	**<0.001**	0.1885	0.4106	**<0.001**	**<0.001**
Glycine	1.356	-0.193	-0.819	-1.390	-1.196	**<0.001**	0.2491	0.5428	**<0.001**	**<0.001**
AAB	-2.469	-3.802	-4.333	-4.880	-4.735	**<0.001**	0.2184	0.4759	**<0.001**	**<0.001**
Valine	0.876	-0.458	-1.041	-1.741	-1.524	**<0.001**	0.2421	0.5275	**<0.001**	**<0.001**
Leucine	-0.118	-1.236	-1.695	-2.073	-1.829	**<0.001**	0.1881	0.4098	**<0.001**	**<0.001**
Isoleucine	-0.535	-2.184	-2.810	-4.157	-3.833	**0.001**	0.6295	1.3715	**<0.001**	**0.011**
Threonine	0.582	-0.819	-1.459	-2.134	-2.033	**<0.001**	0.2435	0.5305	**<0.001**	**<0.001**
Serine	1.842	0.149	-0.686	-1.609	-1.398	**<0.001**	0.3247	0.7075	**<0.001**	**<0.001**
Proline	2.336	1.177	0.595	-0.018	0.122	**<0.001**	0.2225	0.4848	**<0.001**	**<0.001**
Asparagine	*4.469*	*3.039*	*2.423*	*1.656*	*1.874*	***<0.001***	*0.2808*	*0.6118*	***<0.001***	***<0.001***
Aspartate	3.139	2.492	2.202	1.830	1.731	**<0.001**	0.1116	0.2432	**<0.001**	**<0.001**
Methionine	-2.110	-2.043	-2.187	-2.773	-2.284	0.599	0.4855	1.0578	0.358	0.455
Glutamine	0.591	-0.361	-0.793	-1.334	-1.195	**<0.001**	0.1702	0.3708	**<0.001**	**<0.001**
Glutamate	2.134	0.984	0.546	0.204	0.215	**<0.001**	0.1660	0.3617	**<0.001**	**<0.001**
Phenylalanine	-0.930	-1.731	-2.045	-2.215	-2.063	**<0.001**	0.1247	0.2717	**<0.001**	**<0.001**
Ornithine	-2.613	-4.177	-4.789	-5.356	-5.242	**<0.001**	0.2664	0.5804	**<0.001**	**<0.001**
Lysine	-0.759	-2.199	-2.826	-3.235	-3.102	**<0.001**	0.2186	0.4762	**<0.001**	**<0.001**
Histidine	-1.720	-2.613	-3.175	-3.250	-3.212	**<0.001**	0.1917	0.4177	**<0.001**	**<0.001**
Tyrosine	-1.484	-2.428	-2.875	-3.253	-3.126	**<0.001**	0.1624	0.3538	**<0.001**	**<0.001**
Tryptophan	-2.290	-1.642	-1.405	-1.535	-1.769	**0.001**	0.1579	0.3441	**0.037**	**<0.001**
Cysteine	-0.809	-0.839	-0.865	-0.868	-0.868	**0.001**	0.0122	0.0265	**<0.001**	**0.006**
Total aa	5.076	3.900	3.432	2.950	3.014	**<0.001**	0.1838	0.4004	**<0.001**	**<0.001**
Asn/Total	-0.606	-0.861	-1.009	-1.294	-1.140	**<0.001**	0.1039	0.2264	**<0.001**	**0.002**

Woburn 2012 Oakley

	SO_3	Means (log-scale)				SO_3 effect p-value	SED (12 df)	LSD	Trend with SO_3 p-value	
	0	**12.5**	**25**	**50**	**75**				**Linear**	**Non-linear**
Acrylamide	*5.842*	*5.963*	*5.823*	*5.899*	*5.902*	*0.530*	*0.0857*	*0.1868*	*0.734*	*0.996*
Alanine	0.372	0.268	0.308	0.343	0.255	0.528	0.0766	0.1669	0.385	0.933
Glycine	-1.110	-1.174	-1.148	-1.133	-1.166	0.680	0.0475	0.1035	0.565	0.839
AAB	-5.109	-5.080	-5.131	-5.109	-5.391	0.643	0.2258	0.4920	0.222	0.427
Valine	-1.795	-1.861	-1.929	-1.855	-1.976	**0.022**	0.0481	0.1049	**0.009**	0.815
Leucine	-2.368	-2.866	-2.564	-2.495	-2.538	0.414	0.2513	0.5475	0.854	0.540
Isoleucine	-4.408	-3.747	-4.407	-4.426	-4.075	0.569	0.4841	1.0548	0.977	0.741
Threonine	-2.219	-2.316	-2.326	-2.324	-2.373	0.125	0.0532	0.1158	0.028	0.400
Serine	-1.651	-1.729	-1.758	-1.751	-1.715	0.591	0.0704	0.1534	0.496	0.168
Proline	-0.306	-0.363	-0.403	-0.354	-0.472	0.102	0.0561	0.1222	**0.027**	0.834
Asparagine	*0.374*	*0.353*	*0.310*	*0.235*	*0.292*	***0.012***	*0.0340*	*0.0740*	***0.005***	***0.032***
Aspartate	0.738	0.705	0.680	0.503	0.643	**0.013**	0.0577	0.1257	**0.014**	**0.034**
Methionine	-3.933	-3.952	-3.586	-4.205	-4.201	0.402	0.3425	0.7463	0.232	0.519
Glutamine	-1.254	-1.394	-1.490	-1.414	-1.304	0.506	0.1408	0.3067	0.959	0.104
Glutamate	-0.084	-0.118	-0.091	-0.268	-0.153	**0.009**	0.0452	0.0984	**0.015**	0.079
Phenylalanine	-2.601	-2.714	-2.737	-2.676	-2.723	0.298	0.0661	0.1440	0.277	0.310
Ornithine	-5.644	-5.868	-5.868	-5.817	-5.955	0.791	0.2516	0.5482	0.366	0.764
Lysine	-3.670	-3.764	-3.786	-3.856	-3.781	0.059	0.0537	0.1170	**0.042**	**0.025**
Histidine	-3.163	-3.036	-3.224	-3.009	-2.942	0.681	0.2144	0.4671	0.294	0.739
Tyrosine	-3.385	-3.469	-3.582	-3.504	-3.567	0.218	0.0871	0.1897	0.097	0.295
Tryptophan	-2.088	-2.424	-2.116	-2.339	-2.678	**0.039**	0.1817	0.3960	**0.013**	0.342
Cysteine	-0.870	-0.872	-0.869	-0.872	-0.872	0.209	0.0013	0.0027	0.179	0.877
Total aa	2.373	2.327	2.322	2.271	2.297	**0.001**	0.0179	0.0390	**<0.001**	**0.008**
Asn/Total	-1.999	-1.973	-2.011	-2.035	-2.005	0.430	0.0311	0.0677	0.336	0.435