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GENETIC AND ENVIRONMENTAL FACTORS CONTROLLING ACRYLAMIDE FORMATION IN WHEAT PRODUCTS

by

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ABSTRACT

Acrylamide forms from free asparagine and reducing sugars during cooking and products derived from the grain of cereals, including wheat and rye, contribute a large proportion of total dietary intake. In this study, free amino acid concentrations were measured in the grain of wheat varieties Spark and Rialto and four doubled haploid lines from a Spark × Rialto mapping population. The parental and doubled haploid lines had differing levels of total free amino acids and free asparagine in the grain, with one line (SR3) consistently being lower than either parent for both of these factors. Sulphur deprivation led to huge increases in the concentrations of free asparagine and glutamine, and statistical analysis showed clear separation of the grain samples as a result of treatment (environment, E) and genotype (G), and provided evidence of G × E interactions. Low grain sulphur and high free asparagine concentration were closely associated with increased risk of acrylamide formation. G, E and G × E effects were also evident in grain from six varieties of wheat grown at field locations around the UK in 2006 and 2007. Free amino acid and sugar concentrations were also measured in the grain of a range of rye varieties grown at locations in Hungary, France, Poland and the United Kingdom and harvested in 2005, 2006 and 2007. The data showed free asparagine concentration to be the main determinant of acrylamide formation in heated rye flour, as it is in wheat. Free asparagine concentration was shown to be under genetic, environmental and integrated (G × E) control. The same was true for glucose, whereas maltose and fructose were affected mainly by environmental factors while sucrose was largely under genetic control. Free asparagine concentration was closely associated with bran yield, while sugar concentration was associated with low Hagberg falling number. Rye grain was found to contain much higher concentrations of free proline than wheat grain and less acrylamide formed per unit of asparagine in rye than wheat flour. Matrix-assisted laser desorption/ionization mass spectrometric (MALDI-MS) imaging was used to visualize free asparagine distribution in wheat grain. In grain produced under full nutrition, free asparagine was localized mainly in the embryo (bran fraction) while in grain grown in the absence of sulphur it accumulated throughout the grain, notably at high levels in the endosperm (white flour fraction). Field trials with 130 doubled haploid lines and their parent varieties, Spark and Rialto, were

carried out over three years and significant progress was made towards the identification of quantitative trait loci (QTL) controlling asparagine accumulation. The data indicate that progress in reducing the risk of acrylamide formation in processed wheat and rye products could be made immediately through the selection and cultivation of low free asparagine varieties and that further genetically-driven improvements should be achievable. Environmental factors, including agronomy, are also important, and even moderate sulphur deprivation should be avoided in wheat.

1 INTRODUCTION

The discovery of acrylamide in 2002 (Tareke *et al*., 2002) in high carbohydrate, mainly plant-derived foods that undergo high-temperature cooking (frying, baking and roasting) was something of a shock for the food production and processing industries. Acrylamide (Fig. 1) affects the nervous system and fertility, causes cancer in rodents and is regarded as probably cancer-causing in humans (Friedman, 2003). Levels of over 1000 parts per billion (ppb) have been found in a number of foods, including those derived from wheat, potatoes, rye, coffee and cocoa (Friedman, 2003). Although this may seem alarming, it should be remembered that foods are complex mixtures and not all of their constituents can be expected to be 'healthy'. What is more, the presence of acrylamide in food is a recent discovery but not a new risk: acrylamide must have been present in the diet since mankind first began cooking foods. Nevertheless, it is sensible that food producers and processors do whatever they can to reduce acrylamide formation in their products.

The position adopted by most regulators around the world, including the Food Standards Agency (FSA) of the United Kingdom, is that they wish to see dietary levels of acrylamide reduced. The European Food Safety Authority is expected to issue guideline levels this year but so far has not gone as far as setting regulatory limits, although most member states favour that approach. The reaction of consumers, retailers and processors to the issuing of guidance levels is difficult to predict. To date, there has been no indication of consumers avoiding acrylamide-containing foods, despite intermittent national media coverage of the issue. However, some food companies have developed products made from alternative raw materials (rice, for example) in place of relatively high risk raw materials from crops such as potato and wheat. This should be a concern for UK farmers and highlights the need to develop varieties of UK crops with low acrylamide risk.

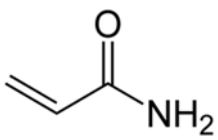

Figure 1 Chemical structure of acrylamide.

1.1 Acrylamide formation

The major route for acrylamide formation is a reaction called the Maillard reaction (named after a French chemist who first described it in the early 20th Century). The Maillard reaction is in fact a complex series of interactions between sugars and amino acids that occur at high temperature (>120 ºC). Many of the products of the reaction are desirable, contributing to colour, flavour and aroma. However, when the amino acid that participates is asparagine, acrylamide is formed (Mottram *et al*., 2002, Stadler *et al*., 2002). The precursors for acrylamide formation are therefore free asparagine (that is asparagine that is not incorporated into protein) and sugars.

1.2 Dietary intake of acrylamide

For adults, estimates of dietary intake of acrylamide range from approximately 0.3 to 0.6 µg per kg of body weight per day (Mucci and Wilson, 2008) and the average intake for children and teenagers is higher. The contribution of cereal products to this intake varies from country to country according to dietary preferences, but in the USA, for example, cereal products make up 33 % of the total and the situation in the UK (for which data is not available) is likely to be similar. Levels of acrylamide in bread are typically less than 100 ppb, but this is offset by the amount that is consumed, and bread accounts for approximately 10 % of dietary intake in the USA. Some wheat-derived breakfast cereals, biscuits and snacks also cause concern, depending on the processing methods used to produce them. The levels in rye-based crispbreads are generally even higher and, coincidentally, some of the first studies on dietary acrylamide intake were undertaken in Sweden, where *per capita* consumption of rye crispbreads is particularly high. Acrylamide levels in some rye crispbreads were found to exceed, by a considerable margin, 1000 µg per kg (parts per billion) and rye crispbreads were estimated to account for 6 % of the total intake. Crispbread manufacturers responded rapidly and some have reduced the acrylamide levels in their products significantly through changes in baking processes (details of these and other methods for reducing acrylamide formation can be found in the 'Acrylamide Toolbox' produced by the Confederation of the Food and Drinks Industries of the European Union (CIAA):

http://www.ciaa.eu/asp/documents/brochures_form.asp?doc_id=65).

The efforts of the food industry to reduce acrylamide formation in their products would be facilitated if the levels of the precursors for acrylamide formation were lower in raw materials. A previous study has shown free asparagine concentration to be the key parameter controlling acrylamide formation in wheat products and factors affecting asparagine accumulation in wheat grain were, therefore, the focus of this project. During the course of the project an opportunity arose to analyse rye samples provided by the EU FP6 HEALTHGRAIN programme, to augment the wheat study.

1.3 Genes involved in asparagine biosynthesis and degradation

Asparagine, aspartate, glutamine and glutamate are nitrogen (N)-transport amino acids; in other words they are used by plants to transfer nitrogen from the source organs (roots and leaves) to sink tissues (e.g. grain) and to build up reserves during periods of nitrogen availability. Asparagine is relatively inert and therefore particularly suited to the role of a nitrogen transport and storage compound. The main enzymes involved in the primary assimilation of inorganic nitrogen from the soil and re-assimilation (secondary assimilation) are glutamine synthetase (GS – 6.3.1.2), glutamate dehydrogenase (GDH), aspartate aminotransferase (AspAT – 2.6.1.1) and asparagine synthetase (AS – 6.3.5.4) (Buchanan, 2000) (Fig. 2).

Asparagine metabolism involves the initial assimilation of ammonia to produce glutamine with the enzymes glutamine synthetase and glutamate synthase (Lea and Miflin, 2003). Glutamine and aspartate then react to form asparagine, under catalysis by asparagine synthetase. Asparagine synthetase also catalyses the adenosine triphosphate-dependent reaction between glutamine and aspartate to generate glutamate. In asparagine catabolism, the enzyme asparaginase is important for hydrolysis of asparagine to aspartate and ammonia.

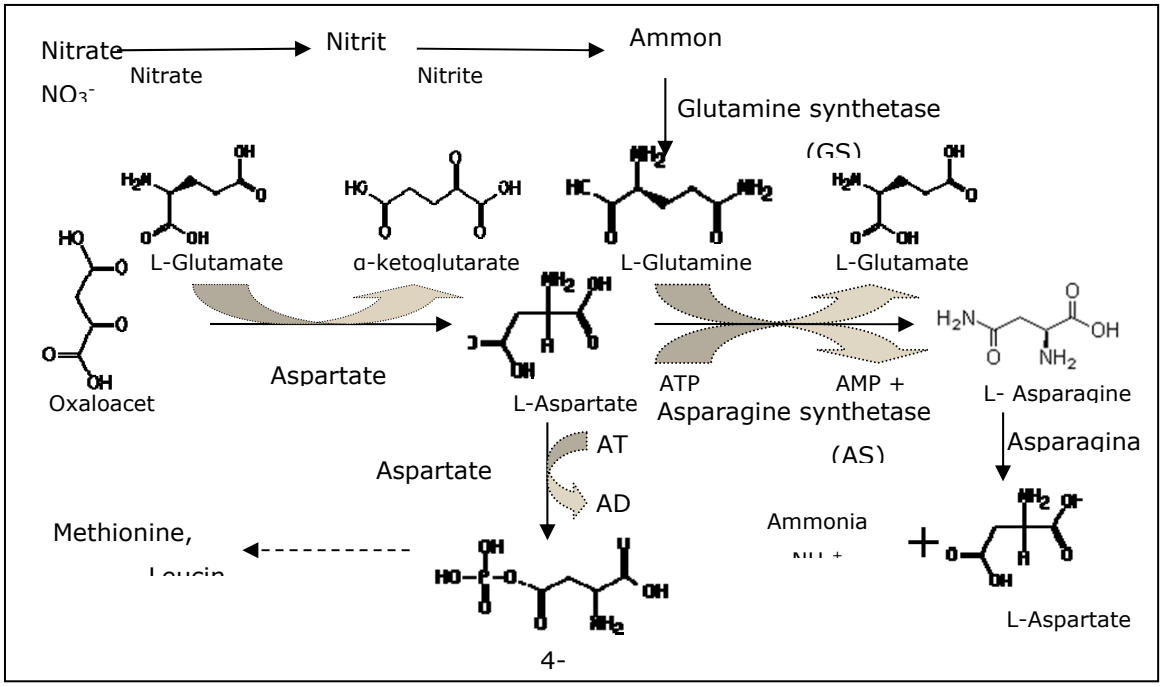

Figure 2 Asparagine metabolism and catabolism

A key hypothesis underpinning this project is that elucidating and understanding the regulation of this pathway and the expression patterns of the genes encoding these key enzymes will enable the manipulation of levels of free asparagine that accumulate in the grain and, consequently, the levels of acrylamide that form in wheat products during processing. It is important to find out the main pathway of synthesis of asparagine and whether free asparagine accumulates in the endosperm, the aleurone layer, maternal seed coat or in the embryo.

2 AIMS OF THE PROJECT

The original aims of this project were to identify genetic and environmental factors controlling free asparagine accumulation in wheat grain and therefore acrylamide formation in wheat products. During the course of the project its scope was extended to include a study on rye. Specific objectives were:

- To identify differences between wheat genotypes (G) with respect to asparagines and sugar accumulation, the effect of environmental conditions (E), and G × E interactions.

- To perform a similar study on rye using samples provided by the EU FP6 HEALTHGRAIN programme.

- Molecular cloning of the genes that encode the key enzymes of asparagine accumulation and breakdown in wheat

- To determine the locations for the biosynthesis and accumulation of asparagine, other amino acids and sugars

- To develop tools for plant breeders to reduce the levels of free asparagine in wheat grain

3 MATERIALS AND METHODS

3.1 Doubled haploid wheat lines

A mapping population of doubled haploid lines produced from a cross between elite UK wheat (*Triticum aestivum*) varieties Spark and Rialto was kindly provided by John Snape of the John Innes Centre, Norwich, UK (Snape *et al*., 2007). Four lines, SR3, SR41, SR107 and SR7, were selected for study because analyses of material grown for a separate experiment showed a range of free asparagine concentrations in their grain (Peter Shewry and Claudia Underwood, Rothamsted Research, unpublished data). The doubled haploid and parental lines were grown in a glasshouse in pots containing compost (Rothamsted mixture), arranged in a randomised block design. Day temperature was maintained at 18 ºC and night temperature at 14 ºC; supplementary lighting was used to provide the plants with a 16-hour day. Grain was collected at maturity and 5 g samples were milled to fine, wholemeal flour in a ball mill.

3.2 Growth of doubled haploid and parental wheat lines with and without sulphur feeding

The four doubled haploid lines and the parents, Spark and Rialto, were also grown in pots in a glasshouse with or without sulphur (S+ and S-, respectively) in a split-plot design. The pots contained vermiculite and the plants were therefore reliant on supplied minerals. S+ plants were watered with medium containing sufficient amounts of potassium, phosphate, calcium, magnesium,

sodium, iron, nitrate and sulphate ions (1 mM $MgSO_4$), while S- plants were watered with the same medium lacking the sulphate. Grain was harvested at maturity and milled in a ball mill.

3.3 Grain from six different elite wheat varieties grown at different locations around the United Kingdom in 2006 and 2007

Grain from six elite UK wheat varieties, Solstice, Malacca, Robigus, Einstein, Xi19 and Claire, which had been harvested in 2006 and 2007, was kindly provided by HGCA. Each variety had been grown at six different sites in the UK. The grain was milled to fine, wholemeal flour in a ball mill.

3.4 Rye grain samples

Samples of rye grain were provided by the EU FP6 HEALTHGRAIN diversity programme. Eleven old and modern varieties/populations had been grown at Martonvasar, Hungary, in 2005. Five selected varieties were then grown at Martonvasar in 2006 and 2007, and at sites in the United Kingdom (Nickersons, Suffolk, UK), Poland (Danko Plant Breeders Ltd, Choryn) and France (INRA, Clermont-Ferrand) in 2007. Wholemeal flour was produced from all of the samples by milling in a ball mill.

3.5 Concentrations of Free Amino Acids

Amino acids were extracted from fine, wholemeal flour. Flour samples (0.5 g) were 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 minutes. 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 (i.e. converted to a molecule more readily analysed) using the EZ-Faast amino acid derivatization technique for gas chromatography and mass spectrometry (GC-MS). GC-MS analysis of the samples was carried out using an Agilent 5975 system (Agilent, Palo Alto, CA) in electron impact mode.

3.6 Measurements of total grain nitrogen and sulphur

Measurements of total grain nitrogen and sulphur were made by the Analytical Unit of the Soil Science Department, Rothamsted Research. Total grain nitrogen was determined by the 'Dumas' digestion method using a LECO CNS 2000 Combustion Analyser. Total sulphur concentration was determined using an Accuris Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) (Applied Research Laboratories, Vallaire, Ecublens, Switzerland; supplied by Thermo Optek, Crawley, United Kingdom) after digesting the samples with a mixture of HNO_3 and $HClO_4$.

3.7 Production and Analysis of Acrylamide

Acrylamide formation was measured by Stephen Elmore at the University of Reading. Flour samples (0.5 g) in unsealed glass ampoules (1 mL capacity) were heated for 20 min at 180 °C. Acrylamide was extracted from these samples with 25 % (v/v) aqueous methanol and converted to the dibromo-derivative for analysis by GC-MS. Labeled $^{13}C_3$-acrylamide was used as the internal standard.

3.8 Analysis of Sugars by Ion Chromatography

Analyses were performed using methods adapted from Elmore *et al*. (2008). Each flour sample (0.200 ± 0.005 g) was weighed into a 14 mL screw-top bottle. Aqueous methanol (10 mL; 50 % vol/vol) containing 100 mg/L lactose was added to the bottle and the sample was stirred for 15 min at room temperature. After holding for a further 15 min, 1.5 mL of supernatant was removed from the bottle and centrifuged at 7200 *g* for 15 min. An aliquot (500 µL) of the centrifuged supernatant was diluted ten-fold in water and 2 mL of the diluted extract were then filtered through a 0.2 µm syringe filter.

The extracts were analyzed using a Dionex ion chromatography system with a 250 × 4 mm Carbopac™ PA1 column (Dionex Corporation, Sunnyvale, CA), operated using Chromeleon™ software. The ion 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. The eluant was 260 mM NaOH at an initial flow rate of 1.2 mL/min; at 3.5 min the flow rate was increased to 1.5 mL/min for the

remainder of the run, the run ending at 6.5 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. Glucose, maltose, fructose and sucrose standards (Sigma-Aldrich and Fluka) were used for quantification. Each sample was extracted and analyzed in triplicate, i.e. three technical replicates for each of three random samples of flour for each combination of site, year and variety were used. The means of technical replicates were taken for statistical analysis.

3.9 Statistical Analyses

Statistical analyses were performed by or under the guidance of Dr Stephen Powers, Biomathematics and Bioinformatics Department, Rothamsted Research. The GenStat® statistical system (GenStat®, 2007, 10th Edition, © Lawes Agricultural Trust (Rothamsted Research), VSN International Ltd., UK) was used for residual maximum likelihood (REML) analyses, canonical variate analyses (CVA) and principal component analyses (PCA) of the data. Pearson's correlation coefficient (r) was calculated between the mean acrylamide, asparagine and sugars (fructose, glucose, sucrose, maltose and total sugars) data. A regression analysis of position and parallelism was used to consider the linear relationship between acrylamide and asparagine for samples of rye and wheat together, having calculated the means of these variates across replicates.

3.10 Molecular cloning and nucleotide sequence analysis

DNA products encoding glutamine synthetase (GS) (isoforms GS1a, GS1b and GS1c), asparagine synthetase, aspartate kinase and asparaginase were amplified from wheat grain total RNA by the polymerase chain reaction (PCR). For the GS isoforms, oligonucleotide primers were designed from gene database entries with the following accession numbers: DQ124209, DQ124210, DQ124211, respectively.

Table 1 Oligonucleotide primers for Glutamine synthetase.

GS1a 5′	CCC AGT CAG CCG GAG CCG GAT
GS1a 3′	TGG CCA CCC ACC AAA TCC AAC
GS1b 5′	ACC CGC CTT CCT TCC TCC GCG
GS1b 3′	GAC AAG CGT CGT GCG TGG TAC
GS1c 5′	ATG GCG CTC CTC ACC GAT CTC
GS1c 3′	CTC CAG TGG CCA CCA CCA AAC

Asparaginase (ASPase) primers were based on barley (*Hordeum vulgare*) asparaginase gene complete cds with accession number AF308474.

Table 2 Oligonucleotide primers for Asparaginase

ASPase 5′	ATG GCG CGC TGG GCC ATT GCC
ASPase 3'	TCA CTC CCA GAT GCC GAC CTC

For aspartate kinase (AK), primers were generated from rice (*Oryza sativa*) (Japonica cultivar-group) mRNA, accession number NM_001051806. Aspartate kinase has three published loci: Os01g70300; Os03g63330 and Os07g20544.

Table 3 Oligonucleotide primers for Aspartate kinase

ASPK 5′	AGC TCC CCT CTC CCT CTC TCA
ASPK 3′	GCG AGG AAC TTG TGA CGC ATG

Asparagine synthetase (AS) primers were designed from wheat glutamine-dependent asparagine synthetase gene (ASN1) mRNA with accession No: AY621539.

Table 4 Oligonucleotide primers for Asparagine synthetase

ASYNT 5′	TGT TGC CGT CGA TCC AGG AAA
ASYNT 3′	GAG ATT GGC AAG CAG GAC AGG

RNA was extracted from grain using the CTAB method or from other tissues using Trizol and cDNA was synthesised by reverse transcription with SuperScript™ II Reverse Transcriptase (Invitrogen) and non-specific Oligo dT $_{(12-18)}$ (500 µg/mL) primers. PCR amplification of specific products was carried out after temperature optimization for each primer set. The PCR parameters were: initial denaturation at 95 ºC for 2 min; 34 cycles of denaturation for 1 min at 95 ºC, annealing with a temperature gradient from 50.2 to 64.9 ºC for 1 min, and extension at 72 ºC for 2 to 3 min; final extension for 10 min at 72 ºC; hold at 22 ºC after the end of the reaction. The optimal annealing temperature for AS, GS and ASPase was 64.9 ºC, and for AK it was 55.9 ºC. PCR products were separated by electrophoresis on 0.8 to 1% gels and those larger than 800 bp were purified with Roche Applied Science - High Pure PCR Product Purification Kit, following the manufacturer's instructions.

The enzyme used for PCR was high fidelity Pfu (Promega Ref No: M7741) for AS, GS and ASPase, and Phusion High-Fidelity DNA Polymerase (Finnzymes F-530S, 100U) for ASPK. PCR products were poly-A tailed for ligation into pGEM-T® Easy Vectors. After ligation, plasmids were transformed into XL10-Gold Ultracompetent *Escherichia Coli* (*E. Coli*) cells. Plasmid DNA purification was performed using the QIAprep system (QIAGEN). Confirmation of the identity of the cloned DNA fragment was obtained by restriction digestion and nucleotide sequence analysis using standard protocols.

3.11 Matrix-assisted laser desorption/ionization mass spectrometric imaging (MALDI-MS) IMAGING of asparagine distribution in wheat grain

MALDI imaging was undertaken at the University of Sheffield in collaboration with Professor Mike Burrell (Burrell *et al*., 2007). Developing wheat ears were tagged when anthesis started and developing seeds harvested at specific time points: 7, 14, 21 and 28 days post anthesis. The developing seeds were flash-frozen in liquid nitrogen and stored at -80 °C. For sectioning, seed were embedded in ice and then cryo-dissected with a Leyca CM1900 cryostat, with the sample at -17°C and the knife at -20°C. Sections were attached to double-sided

adhesive carbon tape and freeze-dried at -40 ºC in a freeze drier. Pictures of the sections were taken by using a Leica Stereomicroscope (Leyca Microsystems, Wetzlar, Germany) and SPOT camera (RT-KE Slider 7.4X Diagnostic instruments, Sterling Lights, USA). Sections were then coated with 25mg mL^{-1} α-CHCA in methanol containing 0.1% (v/v) trifluoroacetic acid.

MALDI-MS spectra were acquired with an Applied Biosystems/MDS Sciex hybrid quadrapole time-of-flight (Q-Star Pulsar-i) mass spectrometer, fitted with an orthogonal MALDI ion source and an Nd : YAC laser. The instrument conditions were repetition rate 1000Hz, laser energy for CHCA – 20% (2.3μJ), and analysis time of 5s per position. At a resolution of 200 μm it took approximately 1h and 20 min to run one section. After analyses the masses of interest were normalised against the appropriate peak before the intensity was plotted.

3.12 Quantitative trait loci (QTL) analyses

A mapping population of 130 wheat doubled haploid lines obtained from a Spark x Rialto cross were used for QTL analyses. Plant material was grown under glass and in the field at the Rothamsted farm at Woburn in a split-plot design for 2007-2008, 2008-2009 and 2009-2010 under two treatments of sulphur-sufficient and -deficient conditions. There was one replication for 2007-2008 and 2008-2009 and two replications for 2009-2010. The experiment was also designed with replication of the parents (11 per variety) to clarify the background variation. From each plot, 20 heads were randomly harvested. The samples were bench dried and threshed, measured and milled for amino acid and total S and N analyses. The analyses of free amino acids were performed by GC-MS as above. The design of experiments for 2008, 2009 and 2010 are presented in Figures 3, 4 and 5.

63	77	53	29	95	8	**S**	62	112	81	21	88	128	41	**R**	122	43	129	105
S	45	102	104	**R**	33	121	10	**S**	110	113	103	**R**	120	106	2	**S**	50	127
11	20	30	64	107	118	68	59	73	116	57	91	111	25	117	26	130	87	4
46	28	**R**	85	65	100	**S**	5	109	124	**R**	99	119	37	**S**	19	27	98	**R**
44	79	39	13	115	125	36	18	86	47	92	51	15	84	114	72	108	97	96
R	93	6	52	**S**	23	24	48	**R**	34	67	61	**S**	42	82	78	**R**	55	75
9	60	80	94	3	56	83	1	12	89	7	17	35	76	49	90	123	58	101
32	71	**S**	14	126	54	**R**	40	22	38	**S**	69	66	70	**R**	74	31	16	**S**

Figure 3 Design for sulphur experiment 2007-2008 using 130 doubled haploid lines with the two parents.

Description of Design: Row and column design with check-plots. All plots were split for sulphur application. Check-plots were located strategically on alternate rows (except in the first two rows) and columns for both the parents. Results from these plots, employing the row-column framework, can be considered to account for spatial trend as necessary.

Treatments: 130 doubled haploid lines of wheat as numbered above with the parents, Spark (S) and Rialto (R), all with and without sulphur augmentation. Sulphur augmentation is indicated by a shaded half-plot.

Replication: 1 replicate for each line by sulphur combination; 11 replicates of each parent by sulphur combination.

R	14	7	98	**S**	87	30	99	**R**	8	78	140	**S**	28	33	48	**R**	97	144
62	41	24	85	117	58	76	47	101	5	26	123	35	60	127	142	65	19	45
39	63	**S**	94	129	16	**R**	90	21	131	**S**	103	92	66	**R**	20	77	44	**S**
9	73	4	51	67	118	116	1	106	18	72	143	56	55	15	53	11	120	59
S	10	46	31	**R**	40	110	111	**S**	133	83	100	**R**	64	25	79	**S**	57	3
112	115	105	130	75	12	107	74	49	81	61	34	102	138	37	124	134	109	13
84	89	**R**	136	2	137	**S**	36	122	119	**R**	71	141	126	**S**	38	6	43	**R**
22	104	80	29	**S**	54	68	95	23	70	17	132	**R**	50	93	108	69	128	96

Figure 4 Design for sulphur experiment, 2008-2009, using 130 doubled haploid lines with the two parents, Spark and Rialto.

BL I	1	2	3	4	5	6	7	8
1	110	37	119	3	S	4	81	5
2	R	7	26	112	59	68	85	101
3	130	16	S	63	90	14	47	65
4	48	82	87	1	66	126	R	79
5	20	67	117	S	9	72	43	54
6	103	6	70	107	R	8	88	23
7	S	62	31	46	10	114	11	R
8	71	99	R	29	121	77	129	105
9	58	57	73	33	83	38	S	95
10	106	104	30	R	102	80	69	100
11	115	56	25	92	S	75	127	55
12	R	98	15	89	24	120	36	S
13	84	128	S	22	111	40	91	78
14	51	49	97	28	109	118	R	74
15	124	93	52	S	45	19	12	53
16	21	50	32	13	R	76	41	44
17	S	35	96	2	34	64	18	R
18	116	17	R	113	94	122	60	108
19	61	27	125	39	123	42	S	86
20	5	R	42	92	117	6	100	22
21	8	123	93	28	9	R	17	21
22	110	88	127	S	14	37	24	120

BL II	9	10	11	12	13	14	15	16
1	119	70	R	14	50	60	4	110
2	S	86	29	53	51	81	102	20
3	91	63	77	55	S	116	105	8
4	40	123	115	99	2	108	R	130
5	9	R	37	19	46	103	44	17
6	41	45	S	16	3	62	28	15
7	R	79	76	112	120	58	106	S
8	89	1	78	12	R	32	82	59
9	61	35	13	10	5	48	S	90
10	21	S	97	36	124	94	54	85
11	87	26	R	125	47	104	93	129
12	S	127	30	122	121	109	111	R
13	57	96	84	126	S	98	52	100
14	42	67	69	88	73	68	R	31
15	56	R	18	117	39	38	6	92
16	83	128	S	66	64	114	25	74
17	R	49	22	113	33	27	23	S
18	75	24	101	95	R	118	43	71
19	72	80	7	34	107	65	S	11
20	S	50	34	5	83	94	1	36
21	41	16	85	3	S	28	125	21
22	91	S	114	22	18	93	35	R

23	112	41	129	116	57	40	79	**S**
24	1	61	12	128	118	2	**R**	69
25	58	**S**	27	107	53	96	108	73
26	121	103	104	87	67	**S**	66	4
27	64	23	54	**R**	63	84	71	81
28	**S**	20	90	78	62	91	19	**R**
29	95	31	99	49	115	114	**S**	18
30	26	**R**	3	59	48	56	10	101
31	39	83	80	11	72	**R**	35	34
32	130	29	60	**S**	113	98	89	124
33	**R**	125	94	111	97	33	106	**S**
34	122	45	68	44	46	102	**R**	109
35	74	**S**	52	50	76	77	86	13
36	51	38	55	15	85	**S**	119	43
37	105	70	82	**R**	32	65	7	126
38	**S**	25	36	47	16	75	30	**R**

23	92	10	**R**	20	117	52	13	100
24	43	58	55	113	116	103	**R**	105
25	**R**	40	56	90	9	75	79	86
26	30	61	53	77	**R**	130	23	29
27	62	**R**	97	110	118	121	60	**S**
28	112	69	**S**	65	38	68	67	72
29	126	128	17	7	27	99	**S**	15
30	**S**	70	107	54	31	119	95	115
31	11	24	51	88	**S**	49	46	44
32	109	**S**	101	6	80	26	98	**R**
33	39	47	**R**	127	120	96	71	78
34	32	33	4	82	73	48	**R**	37
35	**R**	111	2	81	64	57	25	8
36	63	124	74	89	**R**	129	59	42
37	102	12	19	104	76	45	66	**S**
38	123	108	**S**	106	87	84	14	122

Figure 5 Woburn 2010 Sulphur Experiment using 130 doubled haploid lines with parents Spark and Rialto.

Description of Design: Split-plot in 2 blocks, with Sulphur treatment (+ or -) on whole plots and lines (130 + parents) on split plots.
There are 11 replicates of each parent in each whole plot to allow a check on the spatial trends in sulphur across the design.
Note: If plot dimensions are 1m by 3m, the planted area would be 38m by 48m.

4 RESULTS

4.1 Free amino acid concentrations in the grain of wheat varieties Spark and Rialto and four doubled haploid lines from a Spark × Rialto mapping population

A mapping population of doubled haploid lines produced from a cross between elite United Kingdom wheat (*Triticum aestivum*) varieties Spark and Rialto (Srinivasachary *et al*., 2008) was kindly provided by Professor John Snape at the John Innes Centre. Four doubled haploid lines, SR3, SR41, SR107 and SR7, were selected because they had previously been shown to have differing concentrations of free asparagine in their grain (unpublished data). Plants of the four doubled haploid lines and the two parental varieties, Spark and Rialto, were grown under glass in compost and grain was harvested at maturity and milled to a fine (wholemeal) flour. Free amino acid concentrations in the flour were determined by GC-MS and analysis of variance (ANOVA) applied to the data for each individual amino acid. The means on the $\log_e$ scale and their back-transforms are presented in Table 5.

The major contributors to the free amino acid pool were asparagine, aspartate and glutamate and all three showed significant differences ($p < 0.05$) in concentration between the lines. Of the two parents, Rialto contained a higher overall concentration of free amino acids (10.71 compared with 9.92 mmol per kg) but less free asparagine than Spark (2.45 compared with 2.71 mmol per kg), a slightly lower concentration of free glutamate (1.71 compared with 1.81 mmol per kg) and a higher concentration of free aspartate (2.81 compared with 2.43 mmol per kg). In other words the ratios of free asparagine and to a lesser extent glutamate to aspartate differed between the two varieties. In three of the four doubled haploid lines, SR3, SR7 and SR107, these ratios were shifted even further, with free asparagine levels being lower than in either parent (1.68, 2.13 and 2.33 mmol per kg for SR3, SR7 and SR107, respectively), and free aspartate levels being higher (3.26, 3.48 and 2.86 mmol per kg). SR3 and SR7 also differed from the parental lines in having a smaller total pool of free amino acids (8.73 and 9.42 mmol per kg, respectively). The decreases in free asparagine concentration compared with Rialto (the parent variety with the lower asparagine concentration) were 31 % for SR3 and 13 % for SR7.

Table 5 Means of free amino acid concentrations (mmol per kg) in fine flour from grain of wheat varieties Spark and Rialto (n = 9) and in doubled haploid lines grown under glass in compost with $\log_e$ data means in parenthesis. GABA = γ-Aminobutyrate; s.e.d. = standard error of difference between $\log_e$ data means; l.s.d. = least significant difference between $\log_e$ data means (5 % level, comparisons made on the $\log_e$ scale); NS = Not significant at 5 %. The s.e.d. and l.s.d. are on 10 degrees of freedom. Reproduced with permission of the American Chemical Society (Curtis *et al*., 2009).

Amino acid	SR3	SR7	SR41	SR107	Rialto	Spark	s.e.d	l.s.d	Significance (%)
Alanine	0.48 (-0.74)	0.46 (-0.78)	0.66 (-0.42)	0.54 (-0.61)	0.63 (-0.47)	0.57 (-0.55)	0.08	0.19	0.70
Asparagine	1.68 (0.52)	2.13 (0.76)	3.23 (1.17)	2.33 (0.84)	2.45 (0.89)	2.71 (0.10)	0.15	0.33	2.20
Aspartate	3.26 (1.18)	3.48 (1.25)	3.95 (1.37)	2.86 (1.05)	2.81 (1.03)	2.43 (0.89)	0.12	0.28	3.30
GABA	0.20 (-1.61)	0.17 (-1.75)	0.24 (-1.43)	0.27 (-1.32)	0.31 (-1.18)	0.23 (-1.49)	0.15	0.35	4.70
Glutamate	1.19 (0.17)	1.48 (0.39)	1.88 (0.63)	1.86 (0.62)	1.71 (0.54)	1.81 (0.59)	0.13	0.28	3.00
Glutamine	0.06 (-2.74)	0.07 (-2.67)	0.17 (-1.78)	0.13 (-2.06)	0.18 (-1.73)	0.19 (-1.67)	0.39	0.86	NS
Glycine	0.21 (-1.56)	0.21 (-1.58)	0.28 (-1.27)	0.28 (-1.26)	0.28 (-1.28)	0.26 (-1.34)	0.09	0.20	1.30
Histidine	0.06 (-2.79)	0.06 (-2.89)	0.04 (-3.32)	0.07 (-2.64)	0.08 (-2.61)	0.08 (-2.55)	0.34	0.75	NS
Isoleucine	0.11 (-2.20)	0.08 (-2.50)	0.11 (-2.19)	0.10 (-2.29)	0.15 (-1.87)	0.11 (-2.22)	0.14	0.31	2.40
Leucine	0.15 (-1.88)	0.14 (-1.98)	0.17 (-1.80)	0.17 (-1.76)	0.21 (-1.56)	0.16 (-1.83)	0.12	0.27	NS
Lysine	0.15 (-1.93)	0.16 (-1.83)	0.19 (-1.66)	0.20 (-1.60)	0.23 (-1.47)	0.16 (-1.83)	0.13	0.30	5.00
Methionine	0.04 (-3.25)	0.09 (-2.40)	0.13 (-2.06)	0.08 (-2.57)	0.09 (-2.43)	0.04 (-3.34)	0.66	1.46	NS
Ornithine	0.01 (-4.47)	0.01 (-4.23)	0.03 (-3.57)	0.02 (-3.80)	0.03 (-3.63)	0.02 (-3.85)	0.22	0.49	0.01
Phenylalanine	0.07 (-2.72)	0.07 (-2.59)	0.07 (-2.62)	0.07 (-2.64)	0.09 (-2.35)	0.08 (-2.52)	0.15	0.34	NS

Proline	0.18	0.14	0.28	0.23	0.38	0.19	0.21	0.46	0.70
	(-1.74)	(-1.99)	(-1.26)	(-1.47)	(-0.98)	(-1.64)			
Serine	0.17	0.11	0.28	0.30	0.19	0.27	0.32	0.71	NS
	(-1.74)	(-2.23)	(-1.27)	(-1.21)	(-1.65)	(-1.31)			
Threonine	0.08	0.09	0.11	0.10	0.11	0.09	0.16	0.36	NS
	(-2.58)	(-2.42)	(-2.17)	(-2.34)	(-2.21)	(-2.38)			
Tryptophan	0.37	0.25	0.39	0.45	0.40	0.19	0.25	0.55	4.00
	(-0.98)	(-1.37)	(-0.95)	(-0.79)	(-0.92)	(-1.66)			
Tyrosine	0.03	0.03	0.04	0.03	0.05	0.03	0.10	0.22	0.03
	(-3.39)	(-3.40)	(-3.32)	(-3.50)	(-3.09)	(-3.44)			
Valine	0.23	0.19	0.27	0.25	0.34	0.30	0.10	0.22	0.20
	(-1.49)	(-1.67)	(-1.29)	(-1.38)	(-1.09)	(-1.21)			
TOTAL	8.73	9.42	12.52	10.34	10.71	9.92			

Canonical variate analysis (CVA) was applied to the data to show the separation of the genotypes graphically and to confirm which amino acids differed most. This statistical analysis highlighted the separation of the two parents from each other and of SR3, SR7 and SR41 from either parent, while SR107 was shown to be similar to Spark (Figure 6). The major contributors to the discrimination between genotypes were free asparagine, aspartate, glycine and valine.

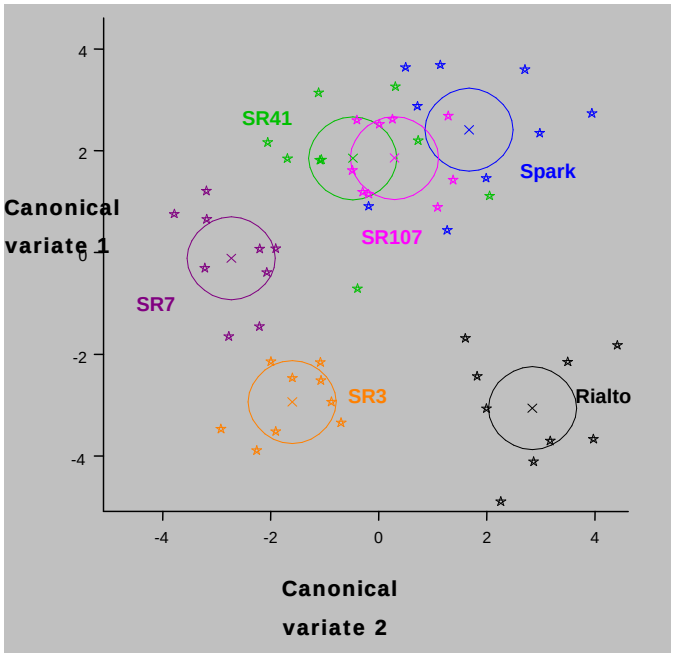

Figure 6 Canonical variate (CV) analysis plot of data on free amino acid concentrations in grain of wheat varieties Spark and Rialto and four doubled haploid lines, SR3, SR7, SR41 and SR107, from a Spark × Rialto mapping

population, grown under glass in compost. The plot shows the CV scores (stars) and means (crosses); 95 % confidence circles are shown around each mean. Reproduced with permission of the American Chemical Society from Curtis *et al*., 2009.

4.2 Effect of sulphur deprivation on free amino acid levels in wheat grain

Sulphur deprivation has been shown previously to cause a dramatic increase in free asparagine accumulation in grain of pot- and field- grown wheat (Muttucumaru *et al*., 2006; Granvogl *et al*., 2007) and a sulphur feeding experiment was performed with the parental varieties, Spark and Rialto, and the four doubled haploid lines. The aim was to provide evidence for the genetic regulation of free asparagine accumulation under severe sulphur deficiency and to determine whether varieties and lines that can be considered as relatively low in acrylamide risk under normal (S+) conditions are also low risk when grown under sulphur-deficient (S-) conditions.

The plants were grown in vermiculite, with or without sulphur feeding (S+ and S-, respectively). S deprivation caused yellowing of the leaves, with reduced chlorophyll content, decreased tiller number (by up to 50 %), slow growth, decreased height at maturity (by up to 50 %), late anthesis and decreased grain yield. The top of the ear was light yellow to white and deteriorated earlier than any other part of the plant. Grain was harvested at maturity, milled and analysed. The concentrations of free amino acids are presented in Table 6.

Table 6 Free amino acid concentrations (mmol per kg) in fine flour from grain of wheat varieties Spark and Rialto and in doubled haploid lines grown under glass in vermiculite either with or without sulphur (S+ and S-, respectively). Means (*n* = 5) are given in bold, standard errors in normal type. Reproduced with permission of the American Chemical Society from Curtis *et al*., 2009.

Amino acid	**SR3**		**SR7**		**SR41**		**SR107**		**Rialto**		**Spark**	
	S+	S-	S+	S-	S+	S-	S+	S-	S+	S-	S+	S-
Alanine	**0.70**	**3.15**	**0.99**	**6.28**	**0.89**	**6.88**	**1.00**	**5.62**	**1.04**	**6.35**	**0.85**	**7.15**
	0.05	0.25	0.04	0.33	0.05	0.53	0.23	0.77	0.12	0.60	0.05	0.85
Asparagine	**2.05**	**25.8**	**3.21**	**45.4**	**3.09**	**38.5**	**2.29**	**51.5**	**3.27**	**39.0**	**2.54**	**62.0**
	0.14	**7**	0.26	**6**	0.25	**1**	0.35	**7**	0.37	**1**	0.20	**2**
		2.09		3.63		3.44		6.75		5.04		6.77
Aspartate	**3.78**	**13.0**	**3.24**	**15.4**	**5.15**	**13.0**	**3.03**	**16.6**	**3.55**	**14.9**	**2.58**	**16.6**
	0.20	**8**	0.18	**9**	0.35	**9**	0.20	**0**	0.19	**3**	0.45	**8**
		0.95		0.62		0.46		0.55		0.88		0.92
Glutamine	**0.11**	**8.22**	**0.49**	**22.8**	**0.17**	**28.0**	**0.37**	**18.6**	**0.36**	**14.0**	**0.37**	**36.5**
	0.02	1.54	0.08	**0**	0.07	**8**	0.18	**2**	0.09	**9**	0.09	**3**
				2.19		4.08		5.40		2.94		5.11
GABA	**0.28**	**0.52**	**0.50**	**0.79**	**0.31**	**0.91**	**0.43**	**0.73**	**0.58**	**1.02**	**0.47**	**0.73**
	0.03	0.06	0.07	0.05	0.04	0.05	0.08	0.09	0.04	0.11	0.15	0.08
Glutamate	**1.70**	**4.87**	**1.73**	**8.52**	**2.07**	**7.36**	**1.72**	**7.74**	**1.91**	**8.11**	**1.60**	**10.7**
	0.11	0.35	0.20	0.55	0.15	0.78	0.19	0.52	0.16	0.84	0.18	**5**
												1.37
Glycine	**0.25**	**1.09**	**0.36**	**2.40**	**0.24**	**2.08**	**0.31**	**1.66**	**0.34**	**1.84**	**0.31**	**2.46**
	0.01	0.09	0.02	0.23	0.01	0.20	0.07	0.27	0.03	0.17	0.04	0.27
Histidine	**0.12**	**0.25**	**0.09**	**0.36**	**0.10**	**0.41**	**0.10**	**0.35**	**0.13**	**0.35**	**0.16**	**0.70**
	0.03	0.07	0.01	0.07	0.01	0.03	0.04	0.09	0.03	0.08	0.08	0.13
Isoleucine	**0.13**	**0.44**	**0.17**	**0.62**	**0.12**	**0.66**	**0.20**	**0.66**	**0.20**	**0.47**	**0.15**	**0.83**
	0.01	0.04	0.01	0.02	0.01	0.06	0.06	0.10	0.03	0.03	0.01	0.15
Leucine	**0.24**	**0.57**	**0.30**	**0.82**	**0.20**	**0.88**	**0.30**	**0.74**	**0.34**	**0.71**	**0.22**	**0.99**
	0.01	0.03	0.01	0.04	0.02	0.07	0.08	0.09	0.03	0.06	0.01	0.14
Lysine	**0.17**	**0.93**	**0.30**	**2.04**	**0.26**	**1.71**	**0.23**	**1.79**	**0.29**	**1.83**	**0.18**	**2.60**
	0.02	0.08	0.01	0.19	0.03	0.14	0.04	0.32	0.03	0.20	0.03	0.39
Methionine	**0.07**	**0.41**	**0.12**	**0.46**	**0.20**	**0.44**	**0.19**	**0.40**	**0.12**	**0.43**	**0.08**	**0.51**
	0.06	0.03	0.05	0.02	0.06	0.02	0.06	0.10	0.05	0.02	0.02	0.03
Ornithine	**0.02**	**0.18**	**0.03**	**0.65**	**0.03**	**0.47**	**0.03**	**0.41**	**0.04**	**0.49**	**0.03**	**0.55**
	0.00	0.02	0.01	0.05	0.00	0.06	0.00	0.06	0.02	0.06	0.01	0.09
Phenylala-	**0.09**	**0.19**	**0.12**	**0.22**	**0.11**	**0.24**	**0.14**	**0.22**	**0.15**	**0.24**	**0.10**	**0.28**
nine	0.00	0.01	0.01	0.01	0.01	0.01	0.04	0.02	0.01	0.02	0.00	0.02
Proline	**0.50**	**1.80**	**1.51**	**3.39**	**0.55**	**3.26**	**1.36**	**2.22**	**1.71**	**4.01**	**0.64**	**2.44**
	0.11	0.22	0.12	0.27	0.15	0.46	0.64	0.45	0.45	0.31	0.15	0.15
Serine	**0.21**	**1.40**	**0.45**	**4.49**	**0.21**	**4.43**	**0.37**	**3.01**	**0.39**	**4.07**	**0.21**	**4.36**
	0.04	0.12	0.06	0.36	0.03	0.53	0.11	0.63	0.12	0.37	0.06	0.56
Threonine	**0.06**	**0.46**	**0.12**	**1.22**	**0.12**	**1.16**	**0.12**	**1.03**	**0.13**	**1.00**	**0.09**	**1.30**

	0.03	0.07	0.00	0.08	0.02	0.11	0.03	0.16	0.02	0.09	0.02	0.12
Tryptoph-an	**0.21** 0.04	**0.18** 0.02	**0.14** 0.01	**0.14** 0.01	**0.24** 0.03	**0.22** 0.01	**0.21** 0.03	**0.27** 0.02	**0.21** 0.05	**0.21** 0.02	**0.12** 0.03	**0.18** 0.01
Tyrosine	**0.05** 0.00	**0.10** 0.01	**0.05** 0.00	**0.14** 0.01	**0.04** 0.00	**0.15** 0.01	**0.06** 0.02	**0.14** 0.01	**0.06** 0.00	**0.13** 0.00	**0.05** 0.01	**0.15** 0.02
Valine	**0.36** 0.02	**1.34** 0.12	**0.49** 0.04	**2.28** 0.13	**0.33** 0.04	**2.32** 0.21	**0.41** 0.09	**2.07** 0.32	**0.58** 0.07	**1.92** 0.16	**0.40** 0.03	**2.92** 0.36
TOTAL	**11.10**	**65.05**	**14.41**	**118.57**	**14.43**	**113.26**	**12.87**	**115.85**	**15.40**	**101.21**	**11.15**	**154.13**

In the sulphur-deprived plants, free amino acid concentrations were greatly increased, mainly as a result of a huge accumulation of free asparagine and glutamine, with asparagine being by far the most abundant free amino acid. Highest levels of all were measured in the grain of the parent variety Spark, which had an asparagine concentration of 62.02 mmol per kg and a total free amino acid pool of 154.13 mmol per kg, compared with 39.01 mmol per kg asparagine and 101.21 mmol per kg total free amino acids in Rialto. Once again line SR3 had the lowest concentration of total free amino acids (65.05 mmol per kg) and asparagine (25.87 mmol per kg).

CVA was applied to the data (Fig. 7): the major contributors to this discrimination were free asparagine, valine, alanine, glycine and phenylalanine. The analysis illustrated the very clear separation of the data based on treatment (environment, E) and genotype (G). There was also a strong G × E interaction, illustrated by the fact that the genotypes were in different positions relative to each other under the different treatments. With respect to free asparagine concentrations, the lines fell in to two groups: In the S- grain of Rialto, SR3, SR7 and SR41, the free asparagine concentrations were 12- to 14-fold higher than in the corresponding S+ grain, while Spark and SR107 showed much larger increases of 23- and 24-fold in response to sulphur deprivation. This indicates clearly that there is genetic control of free asparagine accumulation and that genetic effects interact with environmental effects such as sulphur deprivation.

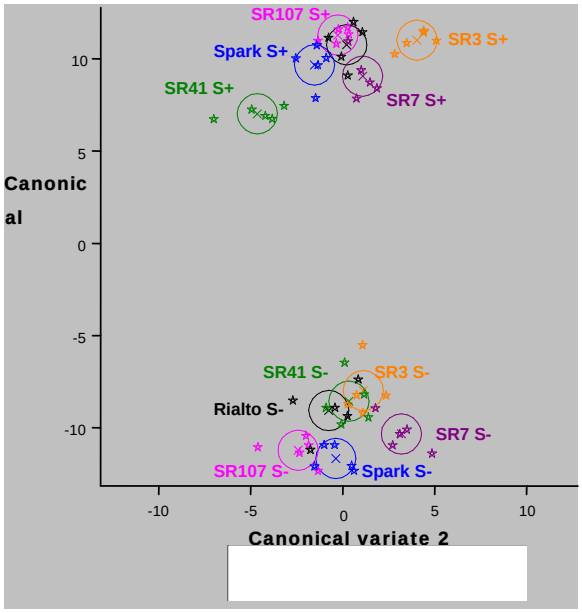

Figure 7 Canonical variate (CV) analysis plot of data on free amino acid concentrations in grain of wheat varieties Spark and Rialto and four doubled haploid lines, SR3, SR7, SR41 and SR107, from a Spark × Rialto mapping population, grown with or without sulphur (S+ and S-). The plots show the CV scores (stars) and means (crosses); 95 % confidence circles are shown around each mean. Reproduced with permission of the American Chemical Society from Curtis *et al*., 2009.

4.3 Effect of harvest year, variety and location on the free amino acid concentration of wheat grown in the United Kingdom

Free amino acid levels were measured in grain from six different wheat varieties, Claire, Einstein, Malacca, Robigus, Solstice and Xi19, grown at six different locations around the United Kingdom and harvested in 2006 and 2007. The results are given in Table 7. The concentrations of free asparagine in the grain samples varied widely and are shown graphically in Figure 8. Variety (genotype, G) location and year of harvest (environment, E) all affected the concentration. Robigus had the highest average concentration across the sites and years of 2.59 mmol per kg, while Einstein had the lowest overall average concentration of 1.89 mmol per kg, a difference of over 30 %. Grain from wheat grown at the sites in Lincolnshire and Kent generally contained higher concentrations of asparagine than grain from wheat grown at the other sites. G × E interactions were evident, with Claire and Einstein, for example, showing little effect of location on free asparagine concentration while the other varieties contained much higher concentrations of free asparagine when grown on the Lincolnshire

and Kent sites. The free asparagine concentrations were higher in grain harvested in 2007 (mean 2.37 mmol per kg) than in grain harvested in 2006 (mean 2.21 mmol per kg). CV analysis showed glutamate, glycine, alanine, asparagine aspartic acid, asparagine and phenylalanine to be contributing most to the variance.

Table 7 Free asparagine concentrations in Claire, Einstein, Malacca, Robigus, Solstice and Xi19 harvested in 2006 and 2007 at six sites in the United Kingdom; all concentrations (mmol per kg fresh weight) are given as means (bold) with standard error (normal print). Reproduced with permission of the American Chemical Society from Curtis *et al*., 2009.

Site	**1. NIAB site, Harper Adams college, Shropshire**		**2. NIAB site, Stoke, Hampshire**		**3. NIAB site, Ivychurch, Kent**		**4. BSPB site, Nickersons, Quigenham, Norfolk**		**5. Scottish Agricultural College, East Lothian**		**6. The Arable Group site, Caythorpe, Lincolnshire**		**Average**
Harvest year	**2006**	**2007**	**2006**	**2007**	**2006**	**2007**	**2006**	**2007**	**2006**	**2007**	**2006**	**2007**	**06**
													07
Variety													
Claire	**2.37**	**2.38**	**1.79**	**2.68**	**2.50**	**2.47**	**2.20**	**2.06**	**0.82**	**2.04**	**1.92**	**2.31**	**2.00**
	0.12	0.22	0.20	0.55	0.15	0.04	0.05	0.06	0.04	0.12	0.09	0.39	**2.32**
Xi19	**4.14**	**1.99**	**1.67**	**1.67**	**2.77**	**2.36**	**2.72**	**2.07**	**0.70**	**1.65**	**3.00**	**4.06**	**2.50**
	0.28	0.03	0.06	0.04	0.33	0.06	0.01	0.02	0.05	0.04	0.05	0.16	**2.30**
Einstein	**2.43**	**2.03**	**1.57**	**1.67**	**2.43**	**2.79**	**1.82**	**1.87**	**0.67**	**1.60**	**1.87**	**1.87**	**1.80**
	0.06	0.09	0.04	0.05	0.03	0.09	0.07	0.03	0.02	0.01	0.02	0.03	**1.97**
Malacca	**2.79**	**2.46**	**1.72**	**1.92**	**2.09**	**3.14**	**2.16**	**2.08**	**0.68**	**1.65**	**1.55**	**3.87**	**1.83**
	0.08	0.02	0.05	0.03	0.02	0.04	0.08	0.31	0.03	0.21	0.09	0.06	**2.52**
Robigus	**3.51**	**2.81**	**3.55**	**1.91**	**4.46**	**3.09**	**2.33**	**1.88**	**0.67**	**1.88**	**2.58**	**3.79**	**2.85**
	0.18	0.03	0.04	0.05	0.11	0.12	0.07	0.05	0.10	0.15	0.18	0.02	**2.56**
Solstice	**3.45**	**1.44**	**2.56**	**1.65**	**3.34**	**3.27**	**1.77**	**2.05**	**0.70**	**2.81**	**2.15**	**3.92**	**2.33**
	0.09	0.06	0.17	0.16	0.21	0.20	0.06	0.04	0.09	0.03	0.09	0.15	**2.52**
Average	**3.12**		**2.14**		**2.93**		**2.17**		**0.71**		**2.18**		**2.21**
		2.19		**1.92**		**2.85**		**2.00**		**1.94**		**3.30**	**2.37**

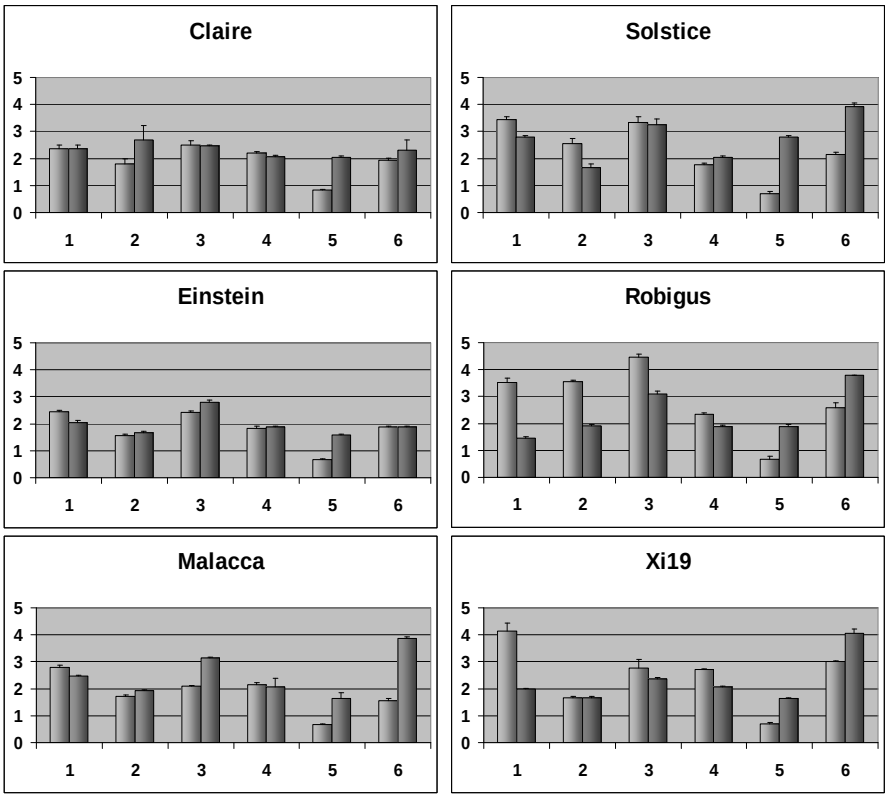

Figure 8 Concentration of free asparagine (mmol per kg) in six wheat varieties grown at six different locations (shown as 1-6, Table 7) in the United Kingdom in 2006 ( ) and 2007 ( ). Means and standard errors are shown in each case. Reproduced with permission of the American Chemical Society from Curtis *et al*., 2009.

4.4 Effect of variety on free amino acid and sugar concentrations in the grain of rye

Grain samples were obtained from eleven varieties of rye that had been grown at Martonvasar, Hungary, in 2005, as part of the HEALTHGRAIN programme (Ward et al., 2008, Rakszegi et al., 2008). The varieties chosen included old and modern types. The older populations were Portugaise-3 and Portugaise-6 from Poland, and Haute Loire, Grandrieu, and Queyras from France. These are no longer commercially available and are relatively heterogeneous compared with modern varieties. The modern varieties were Nikita and Rekrut from Germany and Warko from Poland. The other three varieties were Amilo and Dankowskie-Zlote from Poland and Lovaszpatonai-1 from Hungary, which are older but still commercially available.

Although the term variety is used in this report, it should be noted that rye is an open-pollinating species and varieties are genetically heterogeneous, particularly the older ones.

Free amino acid concentrations in the flour of all varieties were determined and the data were subjected to residual maximum likelihood (REML) analyses and CVA. REML analysis showed significant differences between the varieties and CVA was used to show the separation of the varieties graphically (Figure 9) and to identify which amino acids were responsible for the separation: these were alanine, asparagine, aspartate, glycine, proline, threonine and valine. The concentrations of these amino acids are given in Table 8.

Table 8 Means (n = 3) (in bold) and natural log (log_e) of the means (normal print) of free alanine, asparagine, aspartate, glycine, proline, threonine and valine concentrations (mmol per kg) in fine flour from 11 varieties of rye harvested in Hungary in 2005. Reproduced with permission of the American Chemical Society from Curtis *et al*., 2010.

	Amilo	**Dansk. Zlote**	**Haute Loire**	**Nikita**	**Recruit**	**Portugaise-3**	**Portugaise-6**	**Queryaras**	**Warko**	**Grandrieu**	**Lovaszpatonai-1**	**sed (72df)**	**lsd**	**p values**
Ala	**0.85**	**1.65**	**1.33**	**1.36**	**1.33**	**1.52**	**1.39**	**1.13**	**1.19**	**1.53**	**1.22**	0.09	0.18	<0.001
	-0.16	0.39	0.28	0.30	0.28	0.42	0.33	0.12	0.17	0.43	0.20			
Asn	**3.91**	**6.05**	**7.39**	**6.84**	**5.85**	**8.01**	**6.57**	**5.21**	**4.61**	**8.16**	**6.78**	0.09	0.18	<0.001
	1.35	1.77	2.00	1.92	1.76	2.08	1.87	1.65	1.51	2.10	1.91			
Asp	**2.75**	**4.19**	**4.37**	**3.97**	**4.12**	**5.08**	**4.52**	**3.90**	**4.16**	**5.45**	**6.27**	0.12	0.24	<0.001
	1.01	1.42	1.46	1.38	1.41	1.62	1.49	1.33	1.42	1.69	1.82			
Gly	**0.29**	**1.18**	**0.56**	**0.39**	**0.38**	**0.54**	**0.49**	**0.38**	**0.36**	**0.54**	**0.39**	0.18	0.36	<0.001
	-1.22	-0.38	-0.59	-0.95	-0.96	-0.61	-0.70	-0.98	-1.03	-0.62	-0.94			
Pro	**0.69**	**1.06**	**1.48**	**0.49**	**0.46**	**2.57**	**2.46**	**0.50**	**0.50**	**2.05**	**0.71**	0.10	0.20	<0.001
	-0.37	-0.08	0.39	-0.72	-0.77	0.94	0.90	-0.69	-0.69	0.72	-0.35			
Thr	**0.22**	**0.78**	**0.44**	**0.26**	**0.33**	**0.66**	**0.37**	**0.25**	**0.24**	**0.37**	**0.28**	0.24	0.48	<0.001
	-1.54	-1.02	-0.84	-1.35	-1.12	-0.43	-1.07	-1.43	-1.46	-1.00	-1.28			
Val	**0.36**	**1.04**	**0.57**	**0.43**	**0.48**	**0.70**	**0.55**	**0.36**	**0.37**	**0.58**	**0.41**	0.17	0.35	<0.001
	-1.03	-0.46	-0.57	-0.84	-0.74	-0.36	-0.60	-1.03	-1.00	-0.54	-0.89			
Total														
	15.39	**30.55**	**24.67**	**20.79**	**19.69**	**29.13**	**24.39**	**17.35**	**18.25**	**27.55**	**22.57**			

sed = standard error of difference between log_e data means, lsd = least significant difference between log data means (5 % level, comparisons made on the log_e scale).

The modern German varieties Nikita and Rekrut grouped together in the CVA while Warko grouped with the old varieties (Fig. 9). In general the asparagine concentrations in the older populations were higher than in the modern varieties, with the exception of Amilo, one of the older Polish varieties, which had the lowest asparagine concentration. The data showed clearly that there is genetic control of the concentration of free asparagine and other amino acids in rye.

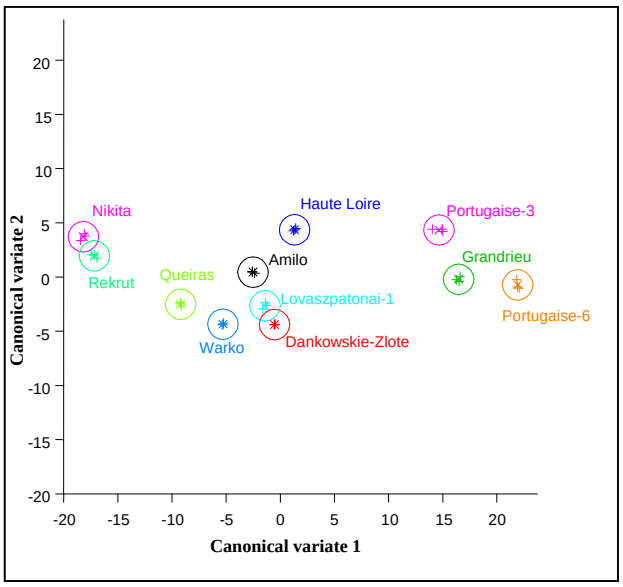

Figure 9 Canonical variate analysis (CVA) plot of data on free amino acid concentrations in grain of eleven rye varieties harvested in Hungary in 2005. CV scores (+), means (×) and 95 % confidence circles are shown. Reproduced with permission of the American Chemical Society from Curtis *et al*., 2010.

Grain sugar concentrations are given in Table 9. The reducing sugars, glucose, fructose and maltose, were analysed because they are major participants in the Maillard reaction. Sucrose was also analysed because it is the most abundant sugar in cereal grain and because it can take part in the later stages of the Maillard reaction after thermal and pH degradation.

Table 9 Means (n = 3) of sucrose, fructose, maltose and glucose concentrations (mmol per kg fresh weight) in flour from 11 rye varieties harvested in Hungary in 2005. Predicted mean natural log ($\log_e$) values from the REML analyses for glucose are given in parentheses, along with the p-value for the combined effect of country, year, and variety for this sugar (varieties were not significantly different (NS), $p > 0.05$, for the other sugars).

	Fructose	Maltose	Sucrose	Glucose	Total
Amilo	0.57	0.62	22.81	1.48 (0.38)	24.00
Dankowskie-Zlote	0.31	1.40	25.86	0.78 (-0.27)	27.57
Grandrieu	0.51	0.98	30.88	1.40 (0.33)	32.37
Haute Loire	0.89	0.70	38.75	1.66 (0.50)	40.34
Lovanspatonai-1	0.81	0.72	26.70	1.16 (0.08)	28.23
Nikita	0.60	0.57	29.51	0.86 (-0.18)	30.68
Portugaise3	1.40	1.66	38.85	3.05 (1.09)	41.91
Portugaise6	1.47	1.90	38.57	2.33 (0.80)	41.94
Queyras	1.26	1.00	26.34	1.19 (0.04)	28.60
Rekrut	1.02	0.73	26.87	1.16 (0.07)	28.62
Warko	0.26	0.70	26.88	0.71 (-0.36)	27.84
sed	NS	NS	NS	0.23	
lsd				0.46	
p-value				<0.001	

CVA of the data (Fig. 10) showed similar groupings to those based on the analysis of free amino acids, separating old varieties from new and distinguishing between the varieties within each group. In particular, the older varieties had higher levels of sucrose in comparison to the new varieties, ranging from 30.9 mmol/kg for Grandrieu to 38.8 mmol/kg for Portugiase 3 compared with 26.9 mmol/kg (Recrut) and 29.51 mmol/kg (Nikita) for the new varieties. Amilo had the lowest concentration of sucrose at 22.8 mmol/kg.

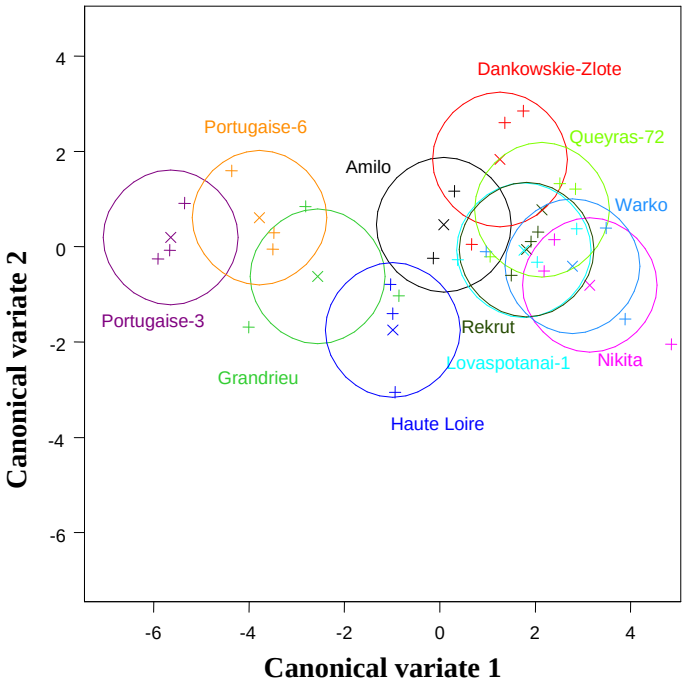

Figure 10 Canonical variate analysis (CVA) plot of data on sugar concentrations in grain of eleven rye (*Secale cereale*) varieties harvested in Hungary in 2005. CV scores (+), means (×) and 95 % confidence circles are shown. Reproduced with permission of the American Chemical Society from Curtis *et al*., 2010.

4.5 Effects of location, variety and harvest year on the concentrations of free amino acids and sugars in rye grain

Additional grain samples were obtained from varieties Amilo, Dankowskie-Zlote, Haute Loire, Nikita and Rekrut that had been grown at the same location in Hungary and harvested in 2006 and 2007. The same varieties were also grown at locations in the United Kingdom, Poland and France and harvested in 2007. All of the samples were analysed together to establish the effects of variety (genotype, G), location and harvest year (environment, E) on free amino acid concentrations in rye grain. CVA was applied to the data to show separation of the samples graphically and to indicate the significance of particular amino acids in this separation (Fig. 11). The analysis separated the samples firstly into five groupings and secondly between those from the UK and those from the other countries. The latter probably resulted from climatic differences between the

continent and the UK. The main contributors to the variation in the free amino acid levels between samples were alanine, asparagine, aspartate, glutamate, glutamine, proline and threonine, with significant p values for the interaction between country, year and variety (genotype) effects (<0.001 for asparagine, glutamine and glutamate and 0,003 for aspartate).

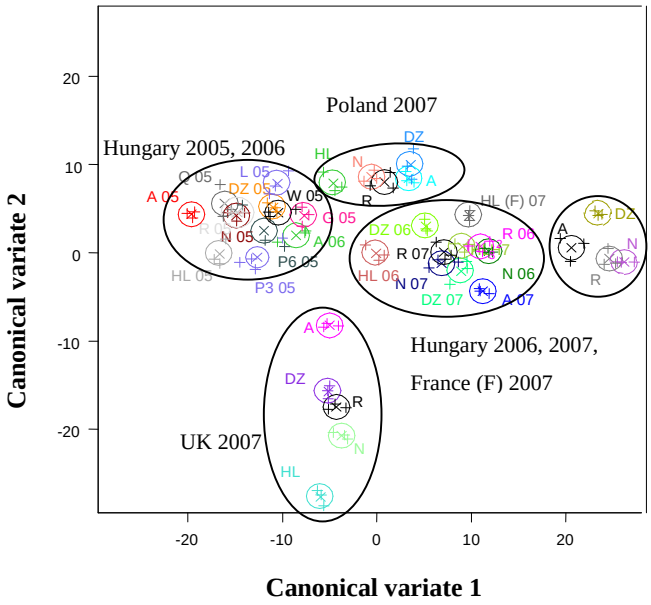

Figure 11 Canonical variate analysis (CVA) plot of free amino concentrations in rye grain, showing the CV scores (+) (with corresponding codes in parenthesis) for combinations of variety (Haute Loire (H), Rekrut (R), Nikita (N), Dankowskie-Zlote (DZ), Amilo (A), Queyras (Q), Warko (W), Lovaszpatonai-1 (L), Grandrieu (G), Portugaise-3 (P3) and Portugaise-6 (P6)), location (indicated) and year (2005, 2006 and 2007, indicated as 05, 06 and 07). Also shown are the corresponding CV means (×) and 95% confidence circles around each mean. Reproduced with permission of the American Chemical Society from Curtis *et al*., 2010.

In this data it was possible to observe effects of G, E and the interaction between the two (G × E) on free asparagine concentrations. For example, the levels of free amino acids in the samples from the same location in Hungary in the three harvest years ranged from 14.8 – 26.3 mmol/kg in 2005; 14.6 – 33.3 mmol/kg in 2006 and 36.8 – 50.5 mmol/kg in 2007. In the case of Amilo the concentration of free amino acids in 2005 was 14.8 mmol/kg while in 2007 it was 42.7 mmol/kg, almost three times higher. Amilo had the lowest concentration of asparagine in 2005 and the second highest in 2007.

The other varieties also showed differences between 2005, 2006 and 2007, with Recrut, for example, showing low levels in 2005 and the highest levels in 2007. Dankowskie Zlote, on the other hand, had the highest asparagine levels in 2006, while Nikita had the lowest asparagine levels in 2005 but higher levels in 2006 and 2007.

The concentration of glucose, fructose, maltose and sucrose in the samples are present in Table 10. Of these sugars, only glucose was affected significantly by the location, harvest year and variety (genotype), and $\log_e$ of means for glucose alone are included in the table. REML analyses showed that maltose and fructose were affected significantly by the environmental factors (location and harvest year) (Table 11), but not by genotype, while sucrose concentrations were affected by genotype (variety) and the environment (Tables 11 and 12) but not by the interaction between the two.

Table 10 Means of Sugar Concentrations (mmol/kg) in Flour from Five Rye (*Secale cereale*) Varieties Grown at Locations in Hungary in 2006 and 2007 and in France, Poland and the United Kingdom in 2007. Natural log (log_e) from REML analyses of glucose concentrations are in parentheses; p values for country by variety by year interaction are given. Reproduced with permission of the American Chemical Society from Curtis *et al*., 2010.

Country	Year	Variety	Fructose	Maltose	Sucrose	Glucose	Total
Hungary	2006	Amilo	0.61	0.74	26.81	0.64 (-0.46)	28.16
		D-Zlote	1.34	1.24	38.5	2.09 (0.71)	41.08
		Haute Loire	0.99	2.16	38.89	2.92 (1.06)	42.04
		Nikita	1.25	2.08	36.96	2.16 (0.76)	40.29
		Rekrut	0.96	1.22	40.18	2.13 (0.75)	42.36
Hungary	2007	Amilo	0.96	2.56	44.15	2.68 (0.99)	47.67
		D-Zlote	1.01	1.26	41.85	2.31(0.83)	44.12
		Haute Loire	0.88	2.17	44.72	2.35 (0.85	47.77
		Nikita	1.43	2.34	37.94	2.17 0.72)	41.71
		Rekrut	0.69	2.45	41.97	1.73 (0.49)	45.11
France	2007	Amilo	1.85	0.72	34.19	3.31 (1.16)	36.76
		D-Zlote	1.29	0.66	34.66	3.33 (1.13)	36.61
		Haute Loire	1.77	1.04	44.94	3.80 (1.32)	47.75
		Nikita	1.71	15.33	36.76	4.38 (1.44)	53.8
		Rekrut	1.99	0.9	36.12	4.10 (1.35)	39.01
Poland	2007	Amilo	1.21	1.43	37.06	1.77 (0.50)	39.7
		D-Zlote	1.15	1.34	38.52	1.75 (0.54)	41.01
		Haute Loire	0.92	21.05	41.01	2.06 (0.72)	62.98
		Nikita	0.71	1.71	40.27	1.75 (0.56)	42.69
		Rekrut	1.29	1.49	38.96	1.95 (0.66)	41.74
United Kingdom	2007	Amilo	1.16	5.4	36.59	5.29 (1.66)	43.15
		D-Zlote	2.34	7.89	40.45	15.96 (2.77)	50.68
		Haute Loire	7.02	11.05	49.52	33.43 (3.51)	67.59
		Nikita	4.67	9.9	36.91	21.95 (3.09)	51.48
		Rekrut	2.92	7.22	36.19	14.54 (2.67)	46.33
		sed				0.23	
		lsd	N/S[bc]	N/Sb	N/S[a]	0.46	
		p values				<0.001	

Table 11 Predicted means from canonical variate analysis for fructose, sucrose and maltose concentrations in rye grain harvested from locations in Hungary in 2005, 2006 and 2007 and in France, Poland and the United Kingdom (UK) in 2007. p values for country by year interaction <0.001. Reproduced with permission of the American Chemical Society from Curtis *et al*., 2010.

	Fructose			Sucrose			Maltose		
	2005	2006	2007	2005	2006	2007	2005	2006	2007
	-	-							
Hungary	0.71	0.0041	-0.05	3.32	3.58	3.74	-0.22	0.29	0.48
France			0.39			3.55			0.11
Poland			0.03			3.67			0.67
UK			1.16			3.68			2.05
sed	0.28			0.11			0.28		
lsd	0.55			0.22			0.56		

Table 12 Predicted means from canonical variate analysis for sucrose concentrations in the grain of eleven rye varieties. Reproduced with permission of the American Chemical Society from Curtis *et al*., 2010.

Variety	Predicted means
Amilo	-0.14
Dankowskie-Zlote	-0.08
Grandrieu	-0.68
Haute Loire	0.31
Lovanspotanai-1	0.13
Nikita	0.19
Portugaise-3	0.95
Portugaise-6	1.02
Queyras	0.38
Rekruit	0.09
Warko	-0.67
sed	0.49
Lsd	0.97
p values	≤ 0.05

sed = standard error of difference between $\log_e$ data means; lsd = least significant difference between log data means (5 % level, comparisons made on the $\log_e$ scale)

4.6 Relationship between free amino acids, sugars and grain properties of rye

The grain properties (thousand kernel weight, test weight, flour protein, grain protein, Hagberg falling number, and yields of flour and bran on milling) of the grain samples were determined. Principle component analysis (PCA) was applied because there were only single samples for the 35 combinations of country, year and variety. The analysis discriminated firstly between the old and new varieties on the basis of flour protein, grain protein, thousand kernel weight and test weight. Secondly it discriminated between the varieties within the 'old' and 'new' groups on the basis of bran yield, Hagberg falling number and test weight. The effect of Hagberg falling number was notable because a falling number under 100 is indicative of high concentrations of sugars due to starch breakdown caused by α-amylase activity, resulting usually from pre-harvest sprouting. Portugiase-3, Portugiase-6, Grandrieu and Haute Loire had the lowest falling numbers and these varieties also had the highest levels of free proline and asparagine. Another comparison could be made between the bran and flour yield from rye varieties grown in the United Kingdom and France. The grain samples from France had higher bran and lower flour yield in comparison to samples from the same varieties grown in the UK. They had also higher free asparagine concentration. This is consistent with the bran having a higher concentration of free asparagine than the flour, as has been reported for wheat (Shewry et al., 2009).

4.7 Correlation between grain sulphur and acrylamide formation

A selection of grain samples were used to analyse a wide range of free asparagine concentration and how this affects acrylamide formation in wheat and rye grain. The samples were milled and flours were heated for 20 min at 180 °C to form acrylamide. A wide range of concentrations of acrylamide was measured in the heated wheat flour (Table 13), from 690 µg/kg (parts per billion; ppb) to 16840 µg/kg, a difference of more than 24-fold. There was a close correlation between asparagine and acrylamide formation, as has been reported by Muttucumaru *et al*., 2006, but the best fitting model was a curve, possibly indicating that another factor became limiting at very high asparagine concentrations (Fig. 12). The data also revealed a strong negative correlation between grain sulphur content and acrylamide formation (Fig. 13).

Table 13 Concentration of acrylamide formed in fine wheat flour after heating at 180 ºC for 20 minutes. Samples of grain showing a range of free asparagine concentrations were selected for analysis. Means (*n* = 5 for asparagine, *n* = 3 for acrylamide) are given in bold, standard errors in normal type.

Genotype and sample	Sulphur regime	Free asparagine (mmol per kg)	Acrylamide (µg per kg)
Spark Glasshouse	-	**62.02** 6.77	**16190** 870
SR107 Glasshouse	-	**51.57** 6.75	**16840** 170
SR7 Glasshouse	-	**45.46** 3.63	**15970** 370
Rialto Glasshouse	-	**39.01** 5.04	**16630** 120
SR41 Glasshouse	-	**38.51** 3.44	**14280** 140
SR3 Glasshouse	-	**25.87** 2.09	**12700** 690
Robigus Field site 3, 2006	+	**4.46** 0.11	**3120** 170
Rialto Glasshouse	+	**3.27** 0.37	**3010** 60
Claire Field site 3, 2006	+	**2.50** 0.15	**2220** 50
SR3 Glasshouse	+	**2.05** 0.14	**1900** 50
Claire Field site 5, 2006	+	**0.82** 0.04	**880** 50
Solstice Field site 5, 2006	+	**0.70** 0.09	**830** 20
Einstein Field site 5, 2006	+	**0.67** 0.02	**690** 10

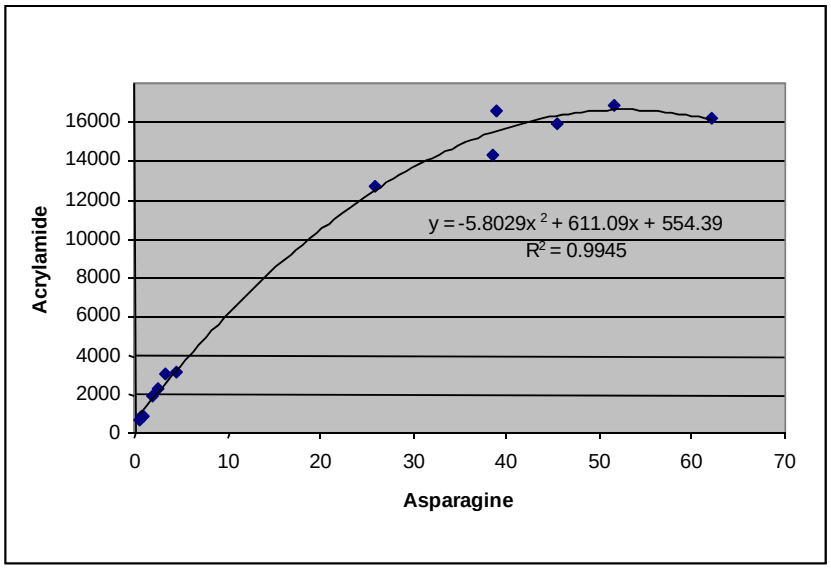

Figure 12 Free asparagine concentration (mmol per kg) in wheat grain plotted against acrylamide formed in wheat flour (µg per kg).

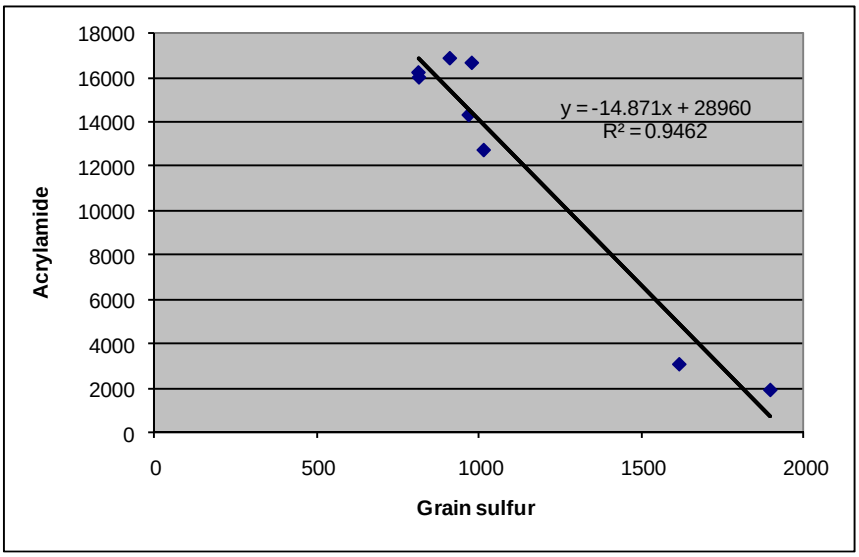

Figure 13 Total grain sulphur (mg per kg) plotted against acrylamide formed in heated flour (µg per kg).

A close correlation was also found between asparagine concentration and acrylamide formation in rye flour (Table 14). However, when it was plotted alongside the wheat data (Figure 14), the fitted lines (slopes and intercepts) for the two sets of data were separate ($p<1$). More acrylamide accumulated per unit of asparagine concentration in wheat (697 µg/kg per unit of asparagine concentration (mmol/kg)) compared with rye (180 µg/kg per unit of asparagine concentration (mmol/kg)). Rye also differed from wheat in that asparagine accumulation was much less sensitive to sulphur availability. No rye samples were found to have low grain sulphur content, despite the fact that some had been produced on soils with very low sulphur levels, probably reflecting rye's greater ability to scavenge scarce nutrients. This meant that no correlation could be established between grain sulphur and acrylamide risk.

Table 14 Acrylamide (µg per kg) formed in fine rye flour after heating at 180°C for 20 min. Samples with a range of free asparagine concentrations (mmol/kg) were selected for analysis.

Rye samples

Variety	Harvest Year and Country of Cultivation	Acrylamide	Asparagine
Amilo	2006, Hungary	954	3.63
Amilo	2007, UK	1354	5.37
D-Zlote	2005, Hungary	1266	6.05
D-Zlote	2007, France	2196	11.18
D-Zlote	2006, Hungary	2108	9.67
Haute Loire	2006, Hungary	2402	10.80
Haute Loire	2007, France	2845	13.53
Haute Loire	2007, Hungary	2947	15.14
Nikita	2007, France	2322	9.04
Portugaise-3	2005,Hungary	2232	8.01
Warko	2005, Hungary	1209	4.61

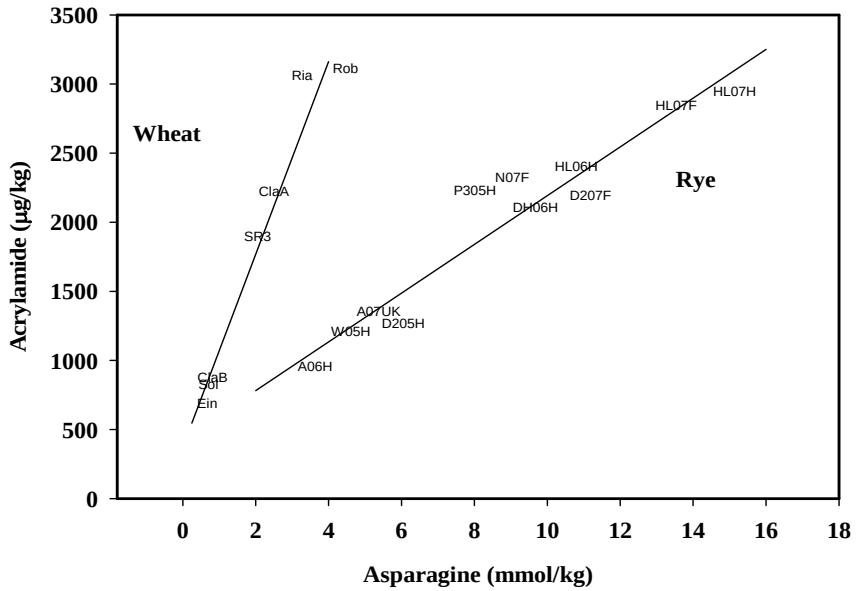

Figure 14 Free asparagine concentration (mmol/kg) in wheat and rye grain plotted against acrylamide formed in heated flour (µg/kg).

4.8 Matrix-assisted laser desorption/ionization mass spectrometric (MALDI-MS) imaging of asparagine distribution in wheat grain

MALDI-MS imaging of asparagine and other free amino acid distribution in wheat grain was undertaken at the University of Sheffield in collaboration with Professor Mike Burrell. This technique allowed the visualization of the spatial distribution of asparagine within thin grain sections. Data profiles were obtained using Analyst QS software and transformed into three separate file formats to be used for analyses of multiple data sets, one of which was a mass spectrometric image (img) file. These files were imported into the Bio Map program and used to generate images of wheat grain based on the spectra that had been obtained from the mass spectrometer. Each image was derived from detected mass spectra peaks for each metabolite plotted against the matrix peaks.

Figures 15 shows images of asparagine distribution in sections of developing wheat grain (14 days post-anthesis) obtained using the Bio Map program. This is a widely used program in medical research, where it is used to plot metabolite distribution in tissue samples. The peaks were plotted against 132.00 amu peaks, corresponding to the mono-isotopic mass of asparagine. The peak at 132.00 amu was normalised against the 172.000 amu peak of the matrix α-CHCA covering the sample (detected in each spectrum) and plotted again against 132.00 amu. The image shows the highest asparagine concentration in red (1.00) and lowest concentration in dark blue (0).

Grain was analysed from wheat (cv. Spark) plants grown in vermiculite under sulphur-sufficient (S+) and sulphur-deficient (S-) conditions, or in compost. In grain sections from plants grown in compost or with S feeding, asparagine was localised predominantly in the embryo (Fig. 15 A-D), whereas in the grain from plants grown in S-deprivation conditions, asparagine was distributed more widely and in higher concentrations, particularly in the endosperm (Fig. 15, E-F).

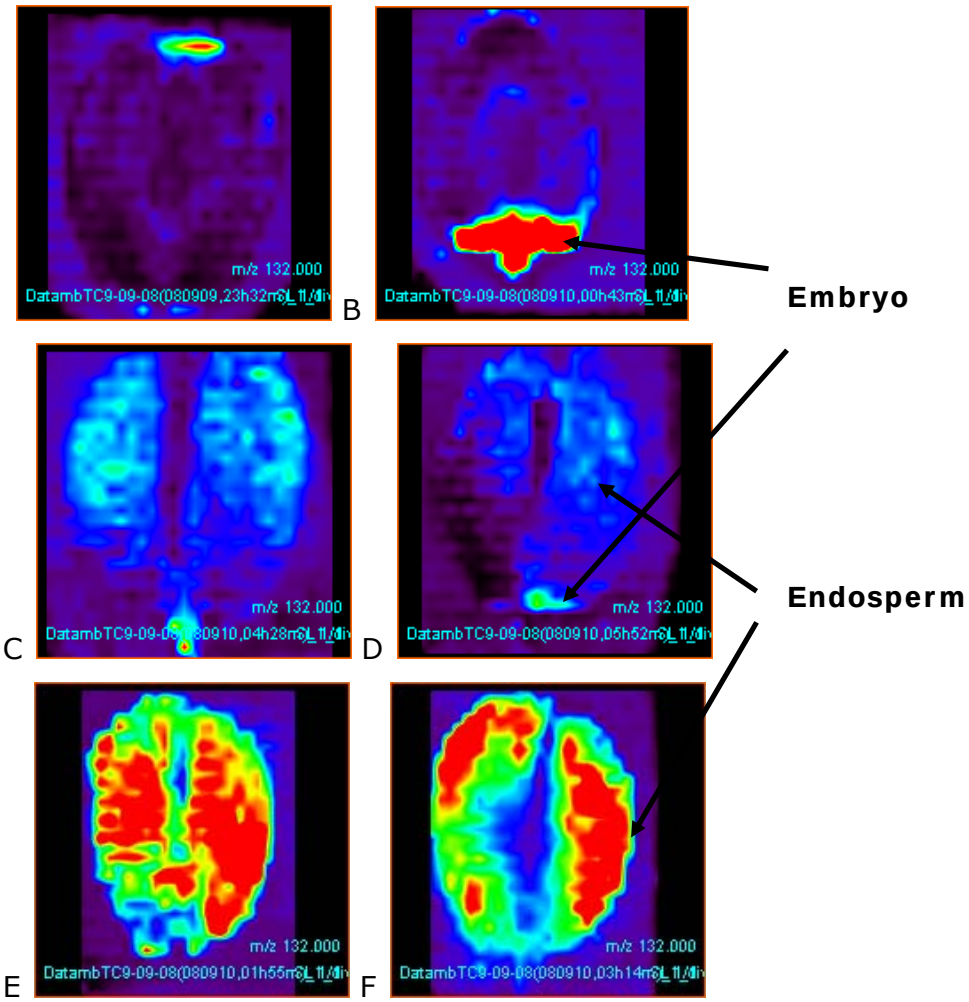

Figure 15 Bio Map images of wheat (cv Spark) grain showing the distribution of free asparagine. A and B are from sulphur-fed plants, C and D from plants growing in compost, and E and F from sulphur-deprived plants.

4.9 Identification of quantitative trait loci (QTL) that affect asparagine accumulation in wheat grain

A key target of the project was to begin the process of identifying QTL that affect asparagine accumulation in wheat grain, in other words regions of the wheat genome that contain a gene or genes that control, at least in part, asparagine synthesis, utilisation or degradation. All amino acid data from the 130 doubled haploid lines in the Spark × Rialto

mapping population and the parent varieties were analysed by GC-MS in a single replication for 2007-2008 to establish if there were significant differences between the lines and if there was enough variation for QTL analyses. The data were analysed by principle component analyses (Fig. 16).

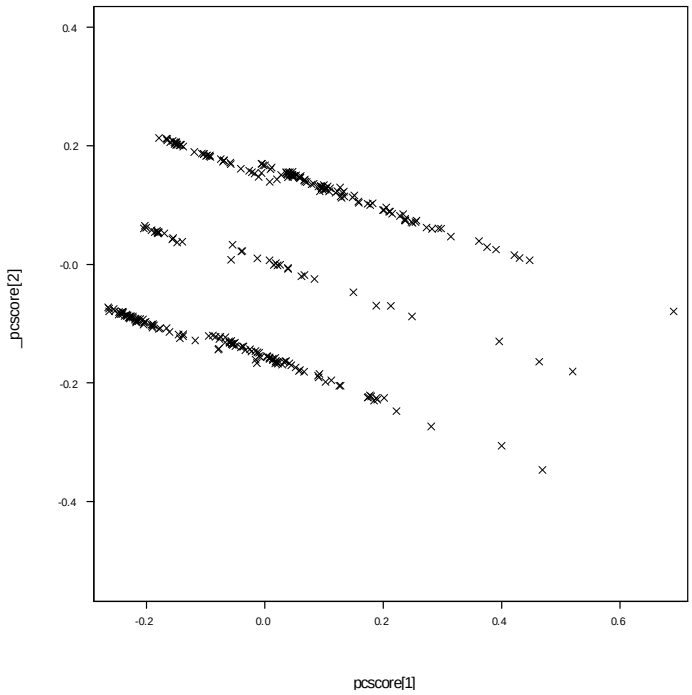

Figure 16 Principle component analyses of 130 doubled haploid lines and parent varieties Spark and Rialto grown at the Rothamsted farm, Woburn, 2007-2008. All free amino acids were analysed together.

The analysis showed that the DH lines could be divided into three separate groups. The top group in Figure 15 are a-type, the bottom group are b-type (with a rye translocation) and the middle group could be a- or b-type. This is consistent with the presence of QTL. Separation within the groups is due to an environmental factor, in this case sulphur availability.

This data provided confidence that QTL could be identified and another field experiment was conducted in 2008-2009, in which all 130 doubled haploid lines and the parent varieties were analysed under sulphur-deficient and -sufficient conditions. The analyses of

free amino acids were performed in triplicate and are being analysed further. A third field trial was initiated in 2009 for harvest and analysis in 2010 to complete the QTL analyses.

5 DISCUSSION AND CONCLUSIONS

Effects of environment (E), genotype (G) and the interaction between the two (G x E) on free amino acid and reducing sugar concentrations were confirmed in wheat and rye flour (Curtis *et al*., 2009; 2010,). It was shown that there is a major contribution of variety (genotype) to the amino acid concentrations in both species. Free asparagine concentration was shown to be the main determinant of acrylamide risk in both species.

In the wheat analyses, the genotype effect was demonstrated clearly in experiments where closely related doubled haploid lines were grown in carefully controlled conditions and showed significant differences between each other (Curtis *et al*., 2009). The lowest observed asparagine concentration from all evaluated wheat samples was doubled haploid line SR3. This line has been shown to have very good bread making qualities (Wan *et al*., 2009), therefore could potentially be used in breeding for low acrylamide wheat varieties, or developed directly for testing for suitability for commercial use.

Significant differences were also seen in the free asparagine concentrations in the grain of different rye varieties. The environmental effects, however, were also significant, with both location of cultivation and year of harvest affecting the accumulation of asparagine and other free amino acids (Curtis et al., 2010).

Another important result from the research was the confirmation of massive accumulation of free asparagine in wheat grain in response to sulphur deficiency (Halford et al., 2007, Muttucumaru et al., 2008, Curtis et al., 2009). The study showed for the first time the inverse correlation between the sulphur content of the grain and acrylamide formation. We propose that wheat grain with less than 1000 µg/kg sulphur is not suitable for uses in which there is a high risk of acrylamide formation, such as the production of biscuits and some breakfast cereals. This is particularly pertinant given that the European Commission is considering adopting 1000 parts per billion as a guideline maximum level for acrylamide content in foods (a guideline level already adopted in Germany); in our study low sulphur

wheat grain could produce far higher levels of acrylamide than that. Even moderate sulphur deficiency should be avoided, making the natural distribution of sulphur in fields and the even application of sulphur fertiliser to all parts of the field, including the margins, significant.

Although not studied here, nitrogen has been shown to have the opposite effect on wheat to sulphur; in other words increasing nitrogen application exacerbates the acrylamide problem by increasing the concentrations of free amino acids. Nitrogen is essential, of course, to maintain grain yield and quality, but there may be scope for reducing application of nitrogen at certain stages of development to avoid high asparagine concentrations at harvest. This requires further study. What is clear is that it is critical to avoid sulphur deficiency when nitrogen is applied because when the plant is unable to incorporate nitrogen into protein, as is the case when sulphur is not available, it stores the nitrogen as free asparagine. Further research is required to establish the optimum ratio of nitrogen to sulphur.

From our results showing the curved correlation between asparagine and acrylamide formation we could extract an equation helping to predict the amount of acrylamide that could be formed from the amount of asparagine:

$$\mathbf{y = -6.305x^2 + 648.21x + 2;\ (R^2 = 0.9918),}$$

where **y** (in µg per kg) is the acrylamide level potentially formed in the heated flour and **x** is free asparagine level in the flour (mmol/kg).

It should be noted that the method used in this study gives an indication of the maximum acrylamide-forming potential of the flour, not what would form during processing for food production. Further analyses would have to be undertaken to derive equations relating the concentration of free asparagine, other amino acids and possibly sugars to acrylamide formation during different processes, such as bread, biscuit, snack and breakfast cereal production.

For the grain analysed in the study, the relationship between asparagine and acrylamide was best represented as a curve, probably indicating that other factors than asparagine were limiting at very high asparagine concentrations. This is not inconsistent with previous studies in which the relationship was best represented by a straight line because the range of asparagine concentrations in the grain used in this study was much greater than that studied previously (Muttucumaru et al., 2008, Elmore et al., 2008, Muttucumaru et al., 2006).

Rye did not respond in the same way as wheat to sulphur deficiency in that grain sulphur concentration was not related to the availability of sulphur in the soil, suggesting that rye plants are able to acquire enough sulphur even if the soil levels are very low. Furthermore, grain sulphur did not correlate with the concentration of free amino acids, including asparagine, or with acrylamide formation, although it should be noted that rye's tolerance of low soil sulphur meant that the range of grain sulphur levels that was available was much narrower than for wheat.

Another difference between rye and wheat was the relatively high concentration of proline present in the rye samples. This may be a specific property of rye; like asparagine, proline is known to accumulate in plants in response to stress, but the rye studied here was not deliberately stressed. Proline has been shown to reduce the levels of acrylamide in model systems and that may explain the lower level of acrylamide formation per unit of asparagine concentration in rye samples compared with wheat.

The concentrations of reducing sugars (fructose, glucose and maltose) plus sucrose were investigated in rye and significance effects were detected in all four of them. The influence of environment (E), variety (G) and the interaction between them (G x E) was significant for glucose, while for sucrose the main factor was variety and maltose, fructose and sucrose were all affected by variety by year interactions. This is an important result because varieties with low sugars in one environment may have high sugars in another year and/or at another location due to complex interaction between variety and environment.

Correlations were established between amino acid concentrations, sugar and grain properties. Total free asparagine in the grain was related to bran yield, the concentration being higher in grain with a higher proportion of bran on milling. Analyses of milling fractions in wheat have shown that the bran fractions contain higher concentrations of asparagine than white flour fractions (Shewry et al.) and the data reported here indicate that the same is likely to be true for rye.

The results from the MALDI MS imaging could be significant for the early detection of asparagine accumulation in the endosperm (and therefore the white flour fraction) as a result of sulphur deficiency or other stress. The technique was able to detect asparagine accumulation as early as 10 days post anthesis. That would provide enough time for a farmer to take remedial action, for example by applying sulphur fertiliser.

The application of the technology in this way would require standardisation of the protocols and cost-effectiveness but it has the potential to provide a reliable and fast semi-quantitative method for early detection of unacceptable levels of free asparagine accumulation in the grain. The method could also be used for analysis of metabolite distribution *in situ*, which is critical for further understanding of the metabolic pathways involving asparagine, other amino acids and sugars. The sensitivity of the technology to detect not only end metabolites but also metabolic intermediates could make it an invaluable tool for metabolic research.

6 CONCLUSIONS

- Acrylamide formation correlates closely with free asparagine concentration in wheat and rye; equations have been developed to enable acrylamide potential to be determined from asparagine concentration.
- There is a massive accumulation of asparagine in wheat grain in response to sulphur deficiency. This does not occur in rye.
- There is an inverse correlation between grain sulphur content and acrylamide formation in wheat.
- It is essential to avoid even moderate sulphur deficiency in wheat; even distribution of sulphur fertiliser, including to field margins, is important.

- Nitrogen exacerbates acrylamide risk: there is a need to establish the optimum nitrogen: sulphur ratio to protect yield and quality while minimising the risk of acrylamide formation.
- Rye and wheat differ in their response to sulphur deficiency and in the amount of acrylamide that is formed per unit of asparagine concentration, rye being significantly lower than wheat.
- MALDI-mass spectrometry imaging is potentially a reliable, semi-quantitative method for early detection of unacceptable levels of asparagine accumulation and an invaluable tool for detailed metabolic research of amino acid metabolism.
- Reductions in acrylamide formation in wheat and rye products could be achieved by using low free asparagine varieties that are already available. Further reductions are likely to be achievable by plant breeding.
- A very low asparagine wheat line (SR3) was identified and could be used in breeding programmes or developed for commercial use.

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